DISSERTATION

IVERMECTIN MASS DRUG ADMINISTRATION TO HUMANS FOR MALARIA PARASITE TRANSMISSION CONTROL

Submitted by

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ABSTRACT

IVERMECTIN MASS DRUG ADMINISTRATION TO HUMANS FOR MALARIA PARASITE TRANSMISSION CONTROL

Every year, an estimated 500 million people are afflicted with malaria worldwide, killing more than one million people, most of whom are children in sub-Saharan Africa. The current malaria eradication program requires novel vector control methods to reduce the transmission of *Plasmodium*, the causative agent of malaria. These new methods must: target exophagic and exophilic *Plasmodium* vectors, integrate with current vector control efforts, be evaluated in the field in combination with other interventions, evade potential behavioral mechanisms that mosquitoes may evolve to avoid the intervention, new agents should have different modes of action, reduce the risk of physiological resistance development, and affect vector population age structure. This dissertation addresses how ivermectin mass drug administration, meets and exceeds all of these issues. Laboratory-based experiments demonstrated that ivermectin at human relevant pharmacokinetics affects *Anopheles gambiae* s.s. survivorship and that cumulative ivermectin blood meals compound mortality, blood feeding frequency, knockdown, and recovery. Field-based experiments demonstrate that ivermectin mass drug administration to humans reduces the survivorship of wild-caught *Anopheles gambiae* s.s. and probably *Anopheles arabiensis* up to six days post-administration. Most

importantly, ivermectin mass drug administration to humans was shown to reduce the proportion of field-caught *Plasmodium falciparum*-infectious *Anopheles gambiae* s.s. for at least 12 days post-treatment. Ivermectin mass drug administration could be a powerful addition to malaria eradication campaign efforts.

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TABLE OF CONTENTS

LIST OF ABREVIATIONS

ACT – Artemisinin-based combination therapy

AE – adverse event

AIC – Akaike's Information Criterion

ALB – albendazole

APOC – African program for onchocerciasis control

BMGF – the Bill and Melinda Gates Foundation

CDC – center for disease control

CDD – community-directed drug distributor

CGoVC – Consultative Group on Vector Control

Cl - chloride

CMFL – community-directed treatment with ivermectin

CNS – central nervous system

DDT – dichloro-diphenyl-trichloroethane

DEC – diethylcarbamazine

DMSO – dimethylsulfoxide

EIR – entomological inoculation rate

GABA – gamma aminobutyric acid

GluCL – glutamate gated chloride

GMAP – global malaria action plan

GMEP – global malaria eradication program

GPELF – global program to eliminate lymphatic filariasis

HBI – human blood index

HBM – health belief model

HLC – human landing catch

IRS – indoor residual spraying

ITN – insecticide-treated net

IVM – ivermectin

LC_{50} – lethal concentration that kills 50%

LF – lymphatic filariasis

LLIN – long-lasting insecticide-treated net

malERA – malaria eradication research agenda

MDA – mass drug administration

MDP – Mectizan donation program

MDSR – median daily survival rate

MEC – Mectizan expert committee

mf – microfilariae

MoH – Ministry of Health

MST – mass screening and treatment

NGDO – non-governmental developmental organization

NOC – national onchocerciasis coordinator

NOCP – national onchocerciasis control program

NOTF – national onchocerciasis task force

NTD – neglected tropical disease

OCP – onchocerciasis control program

OEPA – Onchocerciasis Elimination Program for the Americas

PBS – phosphate buffered saline

PHC – primary health care

PMI – president's malaria initiative

RAPLOA – rapid assessment procedures for loiasis

RBC – red blood cell

RBMP – Roll Back Malaria Partnership

RDT – rapid diagnostic test

REA – rapid epidemiological assessment

SAE – severe adverse event

SAS – statistical analysis software

SOx – sulfoxide

STH – soil-transmitted helminth

TDR – tropical disease research

WHO – world health organization

Chapter 1 – Literature review

I) Malaria burden

Every year, an estimated 500 million people are afflicted with malaria worldwide, killing more than one million people, most of whom are children in sub-Saharan Africa (Snow et al. 1999, Rowe et al. 2006, Hay et al. 2010a). There are now five recognized species of *Plasmodium* that cause malaria in humans (Cox-Singh and Singh 2008). *Plasmodium falciparum* is the most virulent of the *Plasmodium* species (Greenwood 2008), although *Plasmodium vivax, Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium knowlesi* are an important cause of morbidity in areas where they occur (Singh et al. 2004, Mueller et al. 2007, Price et al. 2007, Cox-Singh and Singh 2008). Malaria represents not only a major cause of morbidity and mortality but also a severe impediment to economic development and prosperity in Africa (Gallup and Sachs 2001, Sachs and Malaney 2002, Sachs 2005).

Plasmodium falciparum occurs throughout tropical and subtropical regions of Africa, Latin America, Asia, and the South Pacific (Hay et al. 2010a). Recent advancements and use of PCR-based diagnosis of human malaria infections has revealed more widespread distribution and a greater contribution of non-falciparum malaria than previously thought (Snounou et al. 1993, Rubio et al. 1999, Singh et al. 2004). *Plasmodium vivax* occurs in Asia, the Middle East, Latin America, and the South Pacific and has a range that extends beyond that of *P. falciparum* (Price et al. 2007).

Plasmodium vivax accounts for 50% of the malaria burden in South and Southeast Asia (Luxemburger et al. 1996, Zhou et al. 2005), between 70-80% in Latin America (Duarte et al. 2004), and about 5% in Africa (Mendis et al. 2001) although this may be an underestimate for the malaria burden in Africa (Ryan et al. 2006, Rosenberg 2007). *Plasmodium malariae* distribution is reported as patchy in most malaria endemic regions of the world and is most common in sub-Saharan Africa and the southwest Pacific (Mueller et al. 2007). The distribution of *P. ovale* is primarily limited to Africa although it has been reported from the Middle East, the Indian subcontinent, and parts of Southeast Asia (Mueller et al. 2007). *Plasmodium knowlesi* was only recently recognized as a significant cause of human infection (Singh et al. 2004). The distribution of *P. knowlesi* is restricted to Southeast Asia where macaques, the primary zoonotic reservoir hosts, occur (Cox-Singh and Singh 2008).

In 2007, the Bill and Melinda Gates Foundation (BMGF) declared that malaria eradication was one of their primary goals (Roberts and Enserink 2007). Unlike the previous Global Malaria Eradication Programme (GMEP) launched by the World Health Organization (WHO), research is a major emphasis of both the current eradication agenda and the Global Malaria Action Plan (GMAP) (RBMP 2008). Both the WHO and the Roll Back Malaria Partnership endorse the eradication agenda put forth by the BMGF (Greenwood 2008). There are many research aspects of the Malaria Eradication Research Agenda (malERA 2011) but this dissertation will focus on those that concern research for vector control for the reduction of malaria parasite transmission (malERA CGoVE 2011).

II) Vector control methods for *Anopheles*

Historically, in Africa, there have been three methods for vector control: indoor residual spraying (IRS) with insecticides, insecticide-treated nets (ITNs), and to a lesser extent larval control (Enayati and Hemingway 2010). Each method has its benefits and drawbacks and although they have been shown to be effective, there is a definite need for new vector control techniques that address the shortcomings of these methods (malERA CGoVC 2011).

A) Indoor Residual Spraying

Indoor Residual Spraying is the application of stable, long-lasting insecticide formulations to not only kill but more importantly reduce the daily probability of survivorship of endophilic *Anopheles* spp. Indoor residual spraying with dichloro-diphenyl-trichloroethane (DDT) and other insecticides was the mainstay of the previous WHO GMEP vector control efforts. Although the GMEP was effective in reducing the global population at risk of malaria by 50%, vector resistance to DDT and other insecticides became widespread and lead to the failure of this vector control method within a matter of years after implementation (Enayati and Hemingway 2010). A drawback to IRS is that the more intensive the efforts, the greater the exposure of mosquitoes to insecticide pressure and ultimately the more likely it is that resistance will develop in mosquitoes (Georghiou and Taylor 1977). There are several recent examples of IRS failure due to pyrethroid resistance such as that of *Anopheles gambiae* s.s. in Bioko Island, Equatorial Guinea and Benin (N'Guessan et al. 2007, Sharp et al. 2007) and *Anopheles funestus* in South Africa and Mozambique (Hargreaves et al. 2000, Brooke et

al. 2001, Casimiro et al. 2006). Targeted IRS in certain valleys in the highlands of western Kenya also affected nearby, uphill areas (Zhou et al. 2010). This method of targeted IRS may help reduce the incidence of resistance development by not exposing all mosquitoes in an area to insecticides. IRS remains the most effective vector control measure for reducing malaria morbidity and mortality (Mabaso et al. 2004). This may be due in part to the spatial repellent or contact irritancy of chemicals used for IRS on mosquitoes (Grieco et al. 2007). IRS is hampered by the need for: logistical costs and planning, trained teams of personnel, social acceptance and compliance with IRS (Mabaso et al. 2004, Enayati and Hemingway 2010).

IRS will only affect the survivorship of endophilic vectors that contact the sprayed surfaces of houses. *Anopheles nili* is endophagic but exophilic (Service 1964, Dia et al. 2003), which allows it to enter sprayed dwellings, blood feed on humans, and then exit the dwelling to rest outdoors without contacting the sprayed walls and thus render IRS mostly ineffective against this malaria vector. Behavioral resistance may also develop, whereby insecticide selection pressure leads mosquitoes that were once endophilic to become exophilic, yet still feed on humans and transmit *Plasmodium* spp.; this phenomenon has occurred with *Anopheles farauti* in the Solomon Islands (Taylor 1975). It is believed that the exophilic portion of the *An. gambiae* s.l. population led to the failure of IRS to eliminate malaria transmission during the Garki project (Molineaux and Gramiccia 1980).

B) Insecticide-treated nets

Insecticide-treated nets are used as a barrier to prevent blood feeding by malaria vectors and to kill mosquitoes that contact the surfaces of the ITNs (Curtis et al. 2003). The development of Long-Lasting Insecticide-treated Nets (LLINs) eliminated the need to re-treat ITNs with insecticides which was a significant barrier to their efficacy (Guillet et al. 2001). LLINs and ITNs have been shown to reduce vector abundance (Lines et al. 1987, Lindsay et al. 1991, Miller et al. 1991, Curtis et al. 2003, Gimnig et al. 2003) and prevent overall malaria morbidity and mortality (Lengeler 2004). Thus, ITNs have not only a personal protective effect but also an overall community-wide effect on vector population reduction (Curtis et al. 2003). ITNs are generally not as effective as IRS but they are more easily distributed and operationally less demanding than IRS (Enayati and Hemingway 2010). Issues of non-compliance and improper daily use can reduce the efficacy of ITNs (Toe et al. 2009).

Pyrethroids are the only currently licensed insecticide used on ITNs which is cause for some concern for the development of resistance (Zaim et al. 2000). In many areas *Anopheles* spp. vectors are resistant to pyrethroids but ITNs still show some efficacy, although community-wide benefits are reduced (Darriet et al. 1998, Henry et al. 2005). It is thought that pyrethroid-resistant mosquitoes are less repelled by the insecticides and thus remain in contact with the ITN longer, thereby receiving a higher dose of insecticide which still leads to a killing effect (Darriet et al. 2000). However, ITNs were ineffective in Benin where pyrethroid-resistant *An. gambiae* occurred (N'Guessan et al. 2007). Furthermore, ITNs did not reduce the human biting rate or the

mean entomological inoculation rate in an area of the Ivory Coast with pyrethroid resistant *An. gambiae* (Doannio et al. 1999)

Although ITNs are efficient against endophagic *Anopheles*, they do not directly target exophilic and exophagic *Anopheles*. Furthermore, ITNs are only effective when a person is under the net so crepuscular blood feeding *Anopheles* (i.e., *Anopheles dirus*, *An. farauti*) are not affected by ITNs (Taylor 1975, Hii et al. 1993, Trung et al. 2005). Only two field studies have demonstrated an effect of ITNs on exophagic *Anopheles* (Richards et al. 1993, Charlwood et al. 2005). It has also been shown that ITNs may shift vector host- seeking times to earlier in the evening when people will not be sleeping under an ITN as was observed for *An. farauti* in Papua New Guinea and the Solomon Islands (Taylor 1975, Charlwood and Graves 1987, Hii et al. 1995). ITN use in Kilifi, Kenya did not alter the *P. falciparum* sporozoite rate or human blood index of *An. gambiae* s.l. It was also demonstrated that ITNs decreased *An. gambiae* s.l. blood feeding indoors but outdoor-feeding increased and that vectors fed earlier in the evening (Mbogo et al. 1996). (Russell et al. 2011) found that a decade of ITN and LLIN use in Tanzania caused a blood feeding shift of *An. gambiae* s.l. and *An. funestus* from indoor-feeding to outdoor-feeding and from nocturnal-feeding to earlier in the evening. The authors conclude that although ITNs have effectively reduced indoor feeding by these vectors, alternative methods will need to be developed to target exophagic vectors.

C) Larval Control

Larval control for the prevention of malaria, although historically successful, has been largely abandoned since the advent of adulticidal chemicals (Walker and Lynch

2007). Great success with larval control has been achieved by the eradication of *An. gambiae* s.l from Brazil and Egypt (Killeen et al. 2002). These eradication efforts required extensive logistical efforts and community compliance that current public health care systems in Africa often lack. The main drawback to larval control is that it only affects the density but not the daily survivorship of adult populations which is the most important factor for *Plasmodium* transmission reduction (see Chapter 1 IV F).

Larval control can be achieved by either application of larvicidal and biocontrol agents or by permanent and temporary environmental alterations (Walker and Lynch 2007). The difficulty of larval control is that larval habitats can be extremely diverse, abundant, ephemeral or inaccessible (Keiser et al. 2004). *Anopheles gambiae* s.s. and *Anopheles arabiensis* larvae occur in a diverse variety of locations from hoofprints (Munga et al. 2007), to trash filled pits (Keating et al. 2003) and are exceedingly difficult to locate in rural environments (Gillies and de Meillon 1968). Difficulties with larval control are compounded by larval density dependence survivorship of *An. gambiae*. If *An. gambiae* larvae are removed from competition with other larvae by larvicides, then the surviving adults will be larger (Gimnig et al. 2002) and hence more fecund (Lyimo and Takken 1993) and long-lived as adults (Ameneshewa and Service 1996).

Not all habitats are amenable to larval control as exemplified by the failure of larviciding in the Gambia to reduce clinical *P. falciparum* incidence or anemia in children, 6 months to ten years of age. The failure of this study was thought to be caused by the extensive flooding that seasonally occurs along the Gambia River (Majambere et al. 2010). In Africa, the best use of larval control can be found in urban areas where the ratio of humans to vectors is higher and larval habitat is diminished by urban

development (Geissbuhler et al. 2009). As the proportion of human populations in Africa that lives in cities increases, malaria will most likely become a greater problem in urban environments and thus larval control may become more desirable (Robert et al. 2003, Keiser et al. 2004, Hay et al. 2005).

Larval control can be used to directly target exophilic populations of *Anopheles* that are not affected by ITNs or IRS. Indeed, (Geissbuhler et al. 2009) found larval control in Dar Es Salaam, Tanzania to be as effective as ITNs. This was attributed to the exophagic and crepuscular blood feeding habits of *An. arabiensis* in that location (Geissbuhler et al. 2007). Larval control has limited application for vector control but may be ecologically appropriate in certain areas, primarily urban environments, and caution should be exercised when extrapolating results from one locality to the next (Walker and Lynch 2007).

D) malERA vector control research agenda

The malERA Consultative Group on Vector Control (CGoVC) has outlined a research agenda to address the shortcomings of vector control for malaria eradication. The CGoVC specifically outlines the need for new interventions that can target exophagic and exophilic vectors and will be effective for multiple important vector species. These new interventions must integrate with current vector control efforts and be evaluated in the field in combination with other interventions to demonstrate their efficacy. New insecticides with different modes of action must be employed. Interventions must be able to evade potential behavioral mechanisms that mosquitoes may evolve to avoid the intervention. The new agents should have improved methods to

reduce the risk of physiological resistance development. New agents that can affect vector population age structure are necessary (malERA CGoVC 2011). The following dissertation will describe the development of ivermectin mass drug administration for the disruption of *Plasmodium* transmission and how this vector control method addresses the above needs outlined by the malERA CGoVC.

III) Ivermectin

A) Discovery

The bacteria, *Streptomyces avermitilis* was first isolated from a soil sample from a Japanese golf course by the Kitasato Institute in1973 (Burg et al. 1979). It has since been renamed *Streptomyces avermectinius* (Takahashi et al. 2002) although it is still published in peer-reviewed literature under its original name. A partnership between Merck, Sharpe, and Dohme, Inc. and the Kitasato Institute was formed in 1973 specifically to test novel bacterial samples for anti-nematocidal properties (Omura and Crump 2004). A tandem assay was developed whereby bacterial culture and fermentation broth was fed to mice infected with both *Eimeria muris* (Protozoan) and *Nematospiroides dubius* (Helminth), and subsequent monitoring was used to determine if the broth reduced parasite loads in the mouse (Stapley and Woodruff 1982). Of the 40,000 bacterial broths tested, only one reduced *N. dubius* parasite loads which was OS-3153, the soil sample from the Japanese golf course (Burg et al. 1979). Interestingly, *S. avermectinius* has never been found from any sample collected since its original 1973 isolation (Crump and Omura 2011).

In 1975, the active anthelmintic principle compound of *S. avermectinius* was isolated and named avermectin (Burg et al. 1979, Egerton et al. 1979, Miller et al. 1979). Avermectins are macrocyclic lactones comprised of a series of pentacyclic macrolactones attached to a disaccharide of the methylated deoxysugar L-oleandrose at C-13. Fermentation of *S. avermectinius* produces four pairs of homologous compounds named: A1a, A1b, A2a, A2b, B1a, B1b, B2a, and B2b (Albers-Schonberg et al. 1981). Various combinations of these compounds comprise the different avermectins. The A series has a 5'-methoxyl group, while the B series has a 5'-hydroxyl group, which makes the B series more active (Yoon et al. 2004, Wei et al. 2005, Pitterna et al. 2009). Each pair has a major (a) and minor (b) component. The B1 fractions, a 22,23-di-hydro B1 complex (B1a >80%, B1b < 20%), have the most effective antiparasitic properties and are generically known as ivermectin (Egerton et al. 1979).

B) Onchocerciasis

While still in development, ivermectin was tested in a horse infected with gastrointestinal nematodes and *Onchocerca cervicalis*. Ivermectin drastically reduced the microfilarial load of *O. cervicalis* in the horse (Egerton et al. 1981). Ivermectin also reduced the microfilarial load of *Onchocerca gibsoni* and *Onchocerca gutturosa* in cattle (Campbell 1991). This led Merck scientists to consider the effects of ivermectin against *Onchocerca volvulus*, the causative agent of River Blindness in humans. In 1981, Phase I pilot safety studies were performed on 32 *O. volvulus* infected humans in Dakar, Senegal (Aziz et al. 1982) and 20 West African immigrants in Paris (Coulaud et al. 1983) and both determined that ivermectin was safe for humans. Phase IV large-scale community-

based trials demonstrated that ivermectin MDA was safe and tolerable in humans, extremely potent against the circulating microfilariae (mf) of *O. volvulus*, and significantly suppressed transmission by *Simulium* spp. (Cupp et al. 1989, Remme et al. 1989, Pacque et al. 1990b, Taylor et al. 1990). Ivermectin was shown to not only kill circulating *O. volvulus* mf but to have embryostatic properties in adult females (Plaisier et al. 1995, Klager et al. 1996) that inhibited re-establishment of mf in humans for up to nine months (Awadzi et al. 1985, Awadzi et al. 1989, Duke et al. 1992, Alley et al. 1994). Furthermore, *O. volvulus* fecundity is reduced by 35% when they begin to release mf again (Plaisier et al. 1995). This leads to an overall reduction in microfilarial loads by 80 to 90% of pretreatment values (Basanez et al. 2008).

Onchocerca volvulus is a nematode parasite transmitted by several species of the genus *Simulium* (commonly known as blackflies) (Blacklock 1926). Most (99%) of the human burden of *O. volvulus* occurs in Africa, with several small foci in Latin America and Yemen (Boatin and Richards 2006). The average adult female lifespan of *O. volvulus* is 12 – 15 years and once fertilized, a single female will produce millions of mf during her lifetime (Habbema et al. 1992). Symptoms of onchocerciasis are triggered as the live mf move through dermal tissue and host immune clearance of the dead mf causes intense pruritis, altered pigmentation, atrophy and lymphadenitis (Brieger et al. 1998). When natural or chemotherapeutical mf death occurs in the eye, lesions and atrophy may occur which can cause blindness (Hall and Pearlman 1999). The pathogenesis of ocular damage is caused by the immune response due to the release of the endosymbiont bacteria *Wolbachia* upon the death of mf (Hise et al. 2003).

Prior to the development of ivermectin there were only two drugs indicated for the treatment of *O. volvulus* in humans, diethylcarbamazine (DEC) and suramin, but neither were appropriate for MDA (WHO 1987). Diethylcarbamazine was not an ideal drug because the rapid clearance of mf would trigger such a strong immune reaction in the eye that it sometimes caused treated patients to go blind (Awadzi and Gilles 1980). Suramin, although macrofilaricidal, is highly toxic to humans and must be administered by injection under strict medical attention (Awadzi et al. 1995). However, Mectizan, the human formulation of ivermectin, was ideal for the treatment of *O. volvulus* because it is very safe, has a slower clearance rate of mf from the eye of three to six months, and it does not trigger the immune response that is damaging to the eye (Campbell 1991). It is thought that the large size of the ivermectin molecule compared to DEC bars it from crossing the blood-aqueous humor barrier, thereby preventing entry to the anterior chamber of the eye which prevents inflammation caused by mf death in the eye (Dadzie et al. 1987).

Shortly after Phase I studies demonstrating the safety of ivermectin in humans and efficacy against *O. volvulus*, Merck, Sharpe and Dohme, Inc. approached the UN-based Special Programme for Research & Training in Tropical Diseases (TDR) and the Onchocerciasis Control Program (OCP). The TDR was attempting to develop an anti-filarial drug to combat onchocerciasis for the OCP. In West Africa, the OCP had been relying solely on vector control of *Simulium* spp. by aerially treating waterways where *Simulium* larvae occur with larvicides to reduce transmission of *O. volvulus* (Crump and Omura 2011). Although effective (Molyneux 1995, Boatin et al. 1997), this method was too costly to be sustainable and resistance had developed in the *S. damnosum* complex to

several larvicides (Cupp et al. 2010). Furthermore, aerial larviciding could not be performed in East and Central Africa due to the heavily forested canopies and different vector ecologies (Amazigo 2008). In 1987, ivermectin was licensed for human use and shortly thereafter Merck, Sharpe and Dohme, Inc. announced that they would donate ivermectin for the control of onchocerciasis for the duration of the elimination efforts (Collins 2004).

The African Programme for Onchocerciasis Control (APOC) was created in 1995 to replace the OCP which ended in 2002, while the Onchocerciasis Elimination Program for the Americas (OEPA) was formed in 1993. Both APOC and OEPA rely almost entirely on once or twice yearly ivermectin mass drug administration (MDA) to interrupt transmission of *O. volvulus* (Cupp et al. 2010). (Taylor and Greene 1989) determined that 150 µg/kg of ivermectin was the safest and most efficacious concentration that should be administered for MDA.

C) Lymphatic filariasis

Lymphatic filariasis (LF) is a horribly disfiguring disease that manifests itself with lymphoedema, hydrocele, and elephantiasis. LF is caused by *Wuchereria bancrofti* worldwide throughout many areas of the tropics and subtropics, *Brugia malayi* in south Asia, and *Brugia pahangi* in parts of Indonesia. These filarioid nematodes are transmitted by numerous genera of mosquitoes (Molyneux et al. 2003). Ivermectin, DEC, and albendazole were shown to be effective against *W. bancrofti* (Diallo et al. 1987, Kumaraswami et al. 1988, Ottesen et al. 1990, Richards et al. 1991, Horton 2009), and ivermectin is effective against *B. malayi* and *B. pahangi* (Campbell 1991). The

Global Programme for the Elimination of Lymphatic Filariasis (GPELF) was formed in 1998 (Molyneux and Zagaria 2002). In 1998, ivermectin was registered for the human treatment of LF and Merck pledged to donate ivermectin for the treatment of LF in all areas where it was co-endemic with onchocerciasis (Crump and Omura 2011). The GPELF relies on annual MDA of albendazole (donated by GlaxoSmithKline) in conjunction with either DEC or ivermectin to interrupt the transmission of mf parasites. In areas of Africa where *O. volvulus* is present, DEC cannot be used; therefore ivermectin and albendazole are administered by MDA (Horton et al. 2000). *W. bancrofti* and *B. malayi* adult females have a mean reproductive life span of four to six years which is the time estimated as necessary for annual MDA for the interruption of transmission (Ottesen et al. 1997), although this time period may need to be extended depending on higher baseline levels of infection and mosquito vector complexes (Kyelem et al. 2008). The GPELF campaign relies on the same, once yearly MDA method that was developed by APOC for the effective distribution of ivermectin (Molyneux et al. 2003).

D) Eradication efforts

As demonstrated with vector eradication of *Simulium neavei* in Kenya, transmission of *O. volvulus* must be interrupted for more than thirteen to seventeen years which allows for enough time for all the adult females to die off in order eliminate *O. volvulus* from a region (Roberts et al. 1967). In Latin America, the OEPA demonstrated that this time can be reduced to about seven years by twice-yearly ivermectin MDA (Cupp et al. 2010). Multiple ivermectin MDAs every three months has been shown to drastically reduce the fecundity of the adult *O. volvulus* worm (Gardon et al. 2002) which

is thought to physically tax the adult females so that their lifespan is shortened (Duke 2005). Cupp et al. (2010) strongly urge that twice-yearly or quarterly ivermectin MDA will be necessary for elimination of onchocerciasis in Africa. In Mali, it was demonstrated that twice yearly MDAs with ivermectin and albendazole at twice the concentration were more effective than a single MDA at the standard dose for interruption of LF transmission. The authors suggest that more frequent ivermectin and albendazole MDAs would reduce the amount of time necessary for LF elimination (Dembele et al. 2010).

The APOC, OEPA, and GPELF campaigns have celebrated major successes in reducing the burden of onchocerciasis in West Africa (Diawara et al. 2009) and Latin America (Cupp et al. 2010) and LF (Ottesen et al. 2008). Transmission of onchocerciasis has been eliminated in seven of thirteen foci in Latin America, and ivermectin MDA is estimated to cease in 2012 with post-treatment monitoring to continue for three years after (Sauerbrey 2008). An estimated 600,000 cases of blindness have been prevented in Africa. Furthermore, 25 million hectares of formerly abandoned fertile riverine agricultural land have been reclaimed by elimination of *O. volvulus* (Boatin and Richards 2006). Roughly 80 million people are treated annually with ivermectin with over one billion people treated to date (www.mectizan.org), and it is projected that over 200 million people will be taking the drug annually by the end of the next decade (Crump and Omura 2011). The GPELF has projected that lymphatic filariasis will be eradicated worldwide by 2020 (Molyneux et al. 2003). However, some focal areas may need to rely on vector control in addition to MDA to reach LF elimination goals which may delay eradication (Burkot et al. 2006, Bockarie et al. 2009).

E) Resistance

There have been several reports of sub-optimal treatment responses of *O. volvulus* mf with ivermectin from Ghana (Awadzi et al. 2004a, Awadzi et al. 2004b, Osei-Atweneboano et al. 2007, Osei-Atweneboana et al. 2011). In all cases, there was still a direct immediate reduction in mf observed up to one month post-ivermectin treatment, but there was an earlier repopulation of mf at three and six months post-treatment than is routinely observed. It appears as if the ability of ivermectin to cause an embryostatic effect on adult females has been diminished after ≥ 8 rounds of standard (150 μg/kg) annual treatment in Ghana (Awadzi et al. 2004a, Awadzi et al. 2004b, Osei-Atweneboana et al. 2007, Osei-Atweneboana et al. 2011). Osei-Atweneboana et al. 2011 compared the age and fecundity status of nodulectimized adult female *O. volvulus* and demonstrated that mf repopulation was not due to newly acquired infections but directly due to the unresponsiveness of adult females to ivermectin treatment.

Eng and Pritchard (2005) and Eng et al. (2006) detected changes in the β-tubulin gene from *O. volvulus* in Ghana which has been associated with ivermectin resistance in *Haemonchus contortus*, an intestinal nematode found in sheep. A limitation of these studies was that the *O. volvulus* samples came from different people in different communities (Eng and Prichard 2005, Eng et al. 2006). In Cameroon, it was found that adult females from ivermectin-naïve *O. volvulus* populations homozygous (aa) or (bb) at the β-tubulin locus were more fertile than heterozygous (ab) females (Bourguinat et al. 2006). In Cameroon, Gardon et al. (2002) administered 150 μg/kg or 800 μg/kg ivermectin by MDA quarterly or annually four years and removed (i.e., nodulectimized) adult *O. volvulus*. (Bourguinat et al. 2007) used the nodulectimized adult females from

before and four years after ivermectin treatment from the same people and compared the fecundity to heterozygosity at β-tubulin. Bourginat et al. (2007) found that *O. volvulus* populations homozygous (aa) at the β-tubulin locus were more fertile than heterozygous (ab) females after ivermectin selection and that heterozygosity increased with more frequent treatment with ivermectin. Unfortunately, none of these studies have directly compared heterozygosity at the β-tubulin locus in adult *O. volvulus* from sub-optimal responders over time.

Benzimidizole, the class of anthelmintics to which albendazole belongs, resistance in veterinary nematodes has been linked to a mutation in the β-tubulin gene at codons 167 and 200 from phenylalanine to tyrosine (Kwa et al. 1993, 1994, Elard et al. 1996, Elard and Humbert 1999). Although albendazole (see Chapter 2) and ivermectin (see Chapter 1 section IV-A) have different modes of action they both appear to impart selection pressure on the β-tubulin gene (see above). (Schwab et al. 2005) demonstrated that ivermectin and albendazole MDA for LF control may have selected for an allele change in β-tubulin at the phe-200-tyr SNP in *W. bancrofti*. The results from this study are difficult to interpret as mf of *W. bancrofti* genotyped were collected from different people, from different villages with either none, one, or two MDAs performed. Furthermore, sub-optimal responders to ivermectin and albendazole MDA for LF have not been reported in the literature.

If ivermectin resistance of *O. volvulus* or *W. bancrofti* were to occur, this would seriously threaten the progress obtained by APOC and GPELF efforts in Africa. If resistance in *O. volvulus* or *W. bancrofti* were detected, then alternative treatment with doxycycline, vector control, and nodulectomies (for *O. volvulus*) would be the only

measures currently available to contain the spread of parasite resistance (Osei-Atweneboana et al. 2007). Maintained vigilance, a better understanding of the genetic factors that cause resistance, development of rapid diagnostic assays for resistance detection, and rigorous detection of sub-optimal responders are necessary to identify resistance in *O. volvulus* and *W. bancrofti* (Prichard 2007, Churcher and Basanez 2009).

F) Safety of ivermectin in humans

1) Adverse events and severe adverse events

Ivermectin has proven to be safe in humans and very rarely are severe adverse events (SAEs) documented (De Sole et al. 1989). The adverse events (AEs) that have been reported post-ivermectin MDA are characterized primarily as mild Mazotti type reactions in response to immunological clearance of dead mf (Whitworth et al. 1988, De Sole et al. 1989, Pacque et al. 1989, Whitworth et al. 1991, Chijioke and Okonkwo 1992, Zea-Flores et al. 1992, Kipp et al. 2003). Mazotti reactions are anaphylactoid reactions that occur post chemotherapy. For ivermectin, these responses include pruritus (itching), papular rash, dermal oedema (swelling), headache, nausea, and lethargy (Mackenzie et al. 2003). (Njoo et al. 1995) attempted to correlate concentrations of ivermectin in human plasma to the severity of AEs post-ivermectin treatment but found no interaction. Adverse events are more likely to occur after ivermectin treatment when microfilarial loads of *O. volvulus* are higher (>35 mf/skin snip) (Whitworth et al. 1991, De Sole et al. 1989).

There does appear to be an increased incidence of AEs in onchocerciasis foci from East and Central Africa when compared to West Africa, specifically in regions

where *Simulium neavei* is the primary vector of *O. volvulus* (Kipp et al. 2003). This is thought to be due to actual differences in *O. volvulus* populations transmitted by members of the *S. damnosum* complex compared to *S. neavei* (Fischer et al. 1996). Humans infected with *S. neavei*-transmitted *O. volvulus* have higher microfilarial loads (some people have > 1000 microfilariae/skin snip) compared to *S. damnosum*-transmitted *O. volvulus* (typically ~ 35 mf/skin snip) (Fischer et al. 1993).

Severe adverse events have rarely been documented after ivermectin MDA with the exception of some people co-infected with *Loa loa* (Chippaux et al. 1996, Gardon et al. 1997, Boussinesq et al. 1998, Boussinesq et al. 2001, Kamgno et al. 2008). *Loa loa* is a relatively benign human nematode parasite transmitted by several *Chrysops* spp. (Diptera: Tabanidae) in foci in East and Central Africa (Duke 1955). *Loa loa* circulates in the cerebral region and is often diagnosed when the adult parasite traverses under the conjunctiva of the eye, known commonly as 'eye worm'. Passage of *L. loa* under the conjunctiva of the eye can cause congestion of the eye, itching, and photophobia. If the adult *L. loa*, travel through the anterior chamber of the eye, then vision loss can occur, albeit rare. *Loa loa* infection may also cause oedemas (swelling) and pruritus throughout the body (Boussinesq 2006). The main complication from *L. loa* occurs when a heavily-infected person is treated with ivermectin. When heavily-infected people (> 30,000 *L. loa* mf/ml of blood) ingest ivermectin, the immune response to the dying mf is so severe that it can cause SAEs characterized by confusion, lethargy, urinary incontinence, and encephalopathy, which can lead to death or chronic disability (Gardon et al. 1997, MEC 2004).

Interestingly, all ivermectin-related SAEs have been restricted to Cameroon, even though *L. loa* occurs in many other regions of Africa (Mackenzie et al. 2003, Twum-Danso 2003). This has led some investigators to suspect that there is not only an interaction between ivermectin and heavy *L. loa* clearance that leads to SAEs, but also potential genetic differences in the human populations that predispose SAEs (Bourguinat et al. 2010). Certain breeds of dogs, collies especially, may enter a coma and possibly die when treated with ivermectin (Paul et al. 1987). A 4 base pair deletion, in the MDR-1 gene has been shown to lead to a loss of function of P-glycoprotein, an important efflux pump found at the blood brain barrier. This loss of function of MDR-1 has been shown in both knockout mice (Schinkel et al. 1994, Kwei et al. 1999) and collies (Mealey et al. 2001) to be the primary cause of ivermectin toxicity in the brain. Four humans that survived ivermectin-associated SAEs in Cameroon were tested for a loss of function of MDR-1 compared to nine control ivermectin-treated humans without SAEs. No loss of function mutations were found in any of the subjects (Bourguinat et al. 2010).

Rapid assessment procedures for loiasis (RAPLOA) have been developed to determine areas of *L. loa* transmission. RAPLOA relies on assessment of the presence of eye worm (Addiss et al. 2003, Wanji et al. 2005). It has been predicted that if 40% of the people in an area have experienced sub-conjunctival migration of adult *L. loa* (i.e., 'eye worm'), then 20% of the people are microfilaremic for *L. loa* and 2% of whom would have >30,000 mf/ml blood, and therefore be at risk for an ivermectin-related SAE (Boussinesq et al. 2001, Takougang et al. 2002). The MEC (2004) concluded that ivermectin MDA should only occur in areas suspected to have *L. loa* after RAPLOA has been performed. If RAPLOA deems there to be a potential threat of ivermectin-related

SAEs, then medical staff will remain in the MDA treatment area for five days after the MDA has occurred to treat and evacuate SAE cases to treatment centers (MEC 2004). Current GPELF campaigns utilize RAPLOA methods as they expand their ivermectin MDA programs for lymphatic filariasis eradication into areas with potential *L. loa* transmission. If ivermectin MDA is ever utilized for malaria parasite transmission control, then it will also need to rely on previously developed RAPLOA methodologies to minimize ivermectin-related SAEs.

2) Ivermectin treatment of pregnant women

There are very few contraindications for the treatment of humans with ivermectin, those being that children under five years of age (under 15 kg or 90 cm), pregnant women, women one week post-partum, and acutely or chronically ill people (MEC 2004). Women of child bearing potential, who are not obviously pregnant, are screened by a series of questions concerning menstruation and other factors because pregnancy testing on a mass scale is too costly and difficult. Pacque et al. (1990b) found that this series of questions was 98% specific for detecting pregnant women with a positive predictive value of 94%. This means that some pregnant women in their first trimester will be inadvertently treated during routine ivermectin MDA. Numerous studies have monitored inadvertently or purposefully treated pregnant women and none of them detected elevated levels of *in utero* mortality (abortions or stillbirths), new born mortality, congenital malformation, birth defects, or abnormal obstetric events for ivermectin treated pregnant women (Pacque et al. 1990a, Doumbo et al. 1992, Gyapong et al. 2003, Ndyomugyenyi et al. 2008). Ndyomugyenyi et al. (2008) and Pacque et al.

(1990b) found that mean birth weight or proportion with low birth weight of children of ivermectin-treated pregnant women was not significantly different from untreated pregnant reference populations. There were no significant differences in the frequency of hospital or primary care treatment, deaths, disorders or morbidity of children born to women treated by ivermectin during pregnancy compared to the children of untreated pregnant women (Pacque et al. 1990b). The concentration of ivermectin in human breast milk during the first 24 hours post ingestion of ivermectin by lactating women to be at levels low enough to safely breast feed newborn children (Ogbuokiri et al. 1993). The contraindication (MEC 2000) of pregnant women during routine ivermectin MDA may be unnecessary in light of this evidence but will most likely remain nonetheless.

G) Delivery and cost of CDTI in Africa

1) Delivery

APOC was established in 1995 with funding support from the World Bank and other donors (Benton et al. 2002) and is expected to last until 2015 (Cupp et al. 2010). APOC oversees the onchocerciasis control efforts of 19 countries in Central and East Africa that were not part of the OCP. The goal of APOC is to establish effective and self-sustainable community-directed ivermectin MDA that is fully integrated with health services at the operational level in onchocerciasis endemic areas of all member countries. The APOC was created by a partnership between donors, NGDOs, the UN, member countries, with the WHO as the executive agency. The role of APOC is to facilitate the development of a National Onchocerciasis Task Force (NOTF) which is a combination of governmental and NGDO efforts in each member country. The NOTFs are similar to the

National Onchocerciasis Control Programs (NOCPs) established by the OCP that still function in the original OCP countries. Within each Ministry of Health (MoH) a National Onchocerciasis Coordinator (NOC) and their staff serve as secretariat to the NOTF. The NOC compiles all country data and applies for ivermectin from the Mectizan Donation Program (MDP). A major difficulty for the NOTFs is determining the number of people that need to be treated and accurate counts are needed because it takes four months from the time of request to delivery of ivermectin by the MDP (Burnham and Mebrahtu 2004).

Merck has established the Mectizan Expert Committee (MEC) within the Mectizan Donation Programme (MDP) which reviews and approves requests for Mectizan from national governments and non-governmental development organizations (NGDOs). The MEC is comprised of six independent experts in public and tropical diseases (www.mectizan.org) with outside members from the centers of disease control, WHO, and Merck (Peters and Phillips 2004). Each country's MoH submits a yearly application, which is reviewed and pre-approved by APOC, with the estimated amount of people that need to be treated and estimated number of tablets needed (Amazigo et al. 1998, Burnham and Mebrahtu 2004).

The OCP developed the Rapid Epidemiological Assessment (REA) which measures the nodule prevalence (adult *O. volvulus* females can be observed as lumps in human skin) in adult male humans to determine the community microfilarial load (CMFL). Taylor et al. (1992) demonstrated that the CMFL could be estimated by determining the percent of adult male nodule prevalence and then multiply that number by 1.5. If the CMFL was >20% then the community is a primary treatment priority for

ivermectin MDA. If the CMFL is <20% then the community is considered hypoendemic and does not have as high a priority for ivermectin MDA. The REA method was favorable over the skin snip method for detection of mf by both the communities sampled and APOC field teams (Taylor et al. 1992). REA data is then used by the NOTF and NOC to determine the initial number of people in a community that require treatment (Burnham and Mebrahtu 2004).

Two distribution methods, passive and MDA, are currently relied upon to disseminate Mectizan. Passive distribution of ivermectin utilizes clinics to store and treat individual patients as they come to the clinic. This method has proven useful in areas of low transmission that are not treated by community directed treatment with ivermectin (CDTI) but has little or no utility to prevent *O. volvulus* transmission. Originally, under the OCP, mobile teams relying heavily on assistance from NGOs were formed to distribute Mectizan from district offices. This proved effective due to direct supervision of the distribution with relatively high coverage and often times these mobile teams were the only contact remote communities had with modern medical care. Although effective, the mobile teams proved to be too costly to be sustainable; therefore the CDTI method was developed (Amazigo et al. 1998).

The CDTI method was developed based on experiences of the OCP and NGDO in the early 1990s (Burnham and Mebrahtu 2004). CDTI relies on direct community involvement in the distribution of ivermectin, selection of community drug distributors (CDDs), and decision on when to distribute the drug. This provides community ownership of the process which increases sustainability, coverage, and compliance. Two to three CDDs are appointed for every 250 people treated. It is the responsibility of the

CDD to obtain ivermectin from primary health care facilities, distribute ivermectin, and keep proper records of the number of people treated and village population sizes (Amazigo et al. 2002a). CDDs are typically unpaid volunteers, as payment for services was not found to increase total coverage (Katabarwa et al. 1999). As determined by the community, ivermectin is distributed by the CDD from a central point that everyone visits or the CDDs travel to each housing compound throughout the village. Since ivermectin is extremely safe, minimal training of CDDs by primary health care personnel is needed (Brown 1998). Primary health care workers often times oversee the MDA process in order to manage AEs, especially in areas where SAEs have occurred. PHC personnel are also expected to compile data collected by CDDs to submit to district offices (Amazigo et al. 2002a).

Initially, strict adherence to weight dosages (150 µg/kg) proved burdensome due to a lack of scales and difficulty with their transport. Height is a rapid indicator for weight which accelerated the MDA process dramatically (Alexander et al. 1993). Mectizan was originally produced in 6 mg tablets and packaged in foil blister packs. This required CDDs to remove each tablet from the foil and often times break tablets in half which slowed down the MDA process and was wasteful. This was streamlined by production of 3 mg tablets and shipment in bottles of 500 (Thylefors et al. 2008).

Mectizan is delivered to the port of countries by the MDP. From the port, Mectizan is delivered to the communities by numerous methods determined on a country by country basis. Some countries rely on a centralized governmental system (i.e., MoH) while other countries bypass centralized systems and rely on delivery by NGDOs and district medical stores. Each system has its advantages and drawbacks, countries that

bypass the MoH usually cite the inefficiency and bureaucratic bottlenecks of the MoH system. It typically takes five weeks from the time of entry at the port for Mectizan to reach the district offices (Amazigo et al. 1998). From the district offices the Mectizan is distributed to local repositories by either district level health care personnel or NGDOs. From local repositories the Mectizan is distributed by primary health care personnel or obtained directly by CDDs. NGDOs in certain countries with poor infrastructure are crucial for the distribution of ivermectin to primary health care (PHC) or CDD level (Burnham and Mebrahtu 2004). Timing and reliability is critical to maintain community interest and the coverage necessary for onchocerciasis control. Mectizan has a three year shelf-life at tropical temperatures and humidity which allows for less wastage as supplies can be used from one year to the next (Thylefors et al. 2008).

The CDTI process has been streamlined effectively and the MDP is reliable for delivery of ivermectin. Typically the breakdown in ivermectin delivery occurs at the NOTF and NOCP level, due to a lack of within country and MoH support, which places the burden of ivermectin delivery on NGDOs. NGDOs have been crucial in onchocerciasis control for the development of the CDTI process, training communities for ivermectin MDA, and are often relied upon to pick up the slack of MoH for many programmatic aspects. The Co-ordination Group for Onchocerciasis Control, known as 'The Group', work with the WHO Prevention of Blindness Program to coordinate onchocerciasis control efforts and they also work closely with the Lymphatic Filariasis Support Centre for LF efforts. 'The Group' are comprised of twelve primary NGDOs that coordinate with another >30 NGDOs that operate in Africa (Haddad 2008). NGDOs have been critical for distribution of ivermectin in conflict areas where health services are

non-existent (Hopkins et al. 2005). It was estimated that by 2002 NGDOs had supported the delivery of two-thirds of all ivermectin provided by the MDP. NGDOs have been critical in making connections with rural communities which MoHs often lack and the current success of onchocerciasis eradication efforts could not have been achieved with their past and continued contributions (Burnham and Mebrahtu 2004).

2) Cost

Merck and Co, Inc. donates millions of ivermectin tablets each year to countries that participate in APOC, OEPA, and GPELF programs. At an estimated value of $1.50 per 3 mg tablet of Mectizan and a typical adult takes four tablets at MDA, Merck incurs a substantial proportion of the economic costs of ivermectin MDA (Goldman et al. 2007). For LF eradication efforts in Burkina Faso and Tanzania it was estimated that 95 – 99% of the economic costs for ivermectin MDA were directly paid for by the pharmaceutical companies Merck and GlaxoSmithKline for ivermectin and albendazole, respectively. Since albendazole was estimated at $0.19 per 400 mg tablet, and 400 mg is the standard dose given to all MDA participants regardless of weight, it can be inferred that Merck incurred > 90% of economic costs estimated for LF MDA in Burkina Faso and Tanzania (Goldman et al. 2007). The onchocerciasis and LF control efforts would not be sustainable without the donation of ivermectin and continued support from Merck.

Merck also pays for transportation cost of ivermectin to country ports (Amazigo et al. 1998). Port authorities in most countries typically waive customs fees, but in areas where customs fees did occur, they were substantial, $0.02/tablet in OCP countries and $0.04/tablet in Nigeria in 1996. MoHs could rarely afford these up front costs which

were typically paid for by NGDOs, the WHO, or the United Nations Children's Fund (Amazigo et al. 1998). Fortunately, the APOC has since negotiated with OCP and APOC member countries to have all customs charges waived (Burnham and Mebrahtu 2004).

APOC initially provided necessary start-up costs to purchase vehicles, computers, office space for the WHO within MoHs, and other fixed costs. APOC projects that it will cover 75% of ivermectin MDA costs with 25% commitment from country governments and NGOs during the first five years of new APOC sponsored countries. During the initial five years, APOC will be progressively phased out and ivermectin MDA will be supported by individual countries, but it was realized that not all countries capabilities to sustain ivermectin MDA are the same and APOC has since extended initial support another three years to countries that require it (Burnham and Mebrahtu 2004).

The primary cost that MoHs and NGDOs are expected to manage is the physical within country delivery of ivermectin for MDA. The initial use of mobile teams was too costly to be sustainable but CDTI drastically reduced these costs (WHO 1991). In one study in Mali, the cost of treatment by CDTI was $0.06/treated person which was eight times less than mobile teams $0.50/treated person (WHO 1996). Another study in Mali found CDTI to cost an average of $0.10/treated person (Etya'ale and Resnikoff 1997). In Uganda, average distribution costs were estimated to be $0.29/treated person (Kipp et al. 1998). During the first year of ivermectin MDA in Nike and Achi, towns in Nigeria, average per person distribution costs were $0.17 and $0.13, respectively (Onwujekwe et al. 2002). The most extensive cost analysis study assessed distribution costs of MDA for LF control over several years in Burkina Faso, Ghana, Tanzania, and other countries outside onchocerciasis transmission zones (Egypt) and outside Africa (Dominican

Republic, Haiti, and the Philippines). In all cases, costs per person treated diminished after the first year as the number of people treated increased in subsequent years. The lowest reported distribution costs per person treated were $0.06 in Burkina Faso, $0.17 in Ghana, and $0.26 in Tanzania (Goldman et al. 2007). After summarizing these data it appears that the average per person treatment costs per MDA adjusted for time since inception of MDA is somewhere between $0.10 to $0.15.

Cost recovery as a 'fee-for-service' is a mechanism used in many African countries to recoup government health care costs. Although there was some initial support from APOC (Amazigo et al. 1998) this method has been dropped as it was perceived as a barrier to obtaining maximum coverage (Burnham and Mebrahtu 2004). The OCP had initially instituted cash or in kind payment for services of CDDs but this has been abandoned by APOC as studies found that it did not increase overall treatment coverage and increased MDA costs (Amazigo et al. 2002b).

Donated money is managed through a World Bank trust fund (Cupp et al. 2010) and is typically disbursed to WHO offices within MoHs in each member country (Burnham and Mebrahtu 2004). The first fifteen years of APOC operation cost was $182.5 million with $137 million provided by the World Bank and another $45.5 million coming from governments and non-governmental organizations (NGOs). Sustainability of NOTFs and NOCPs is defined as the ability of the benefits achieved by CDTI to be continued by governments and NGDOs after APOC support has been discontinued (Tarimo 2000). Progress towards sustainability has been most lacking at the national level where MoHs have failed to fund NOTFs to the amount agreed upon with APOC.

Failure of political will and government support as APOC comes to a close is the biggest threat to the success of onchocerciasis eradication in Africa (Burnham and Mebrahtu 2004).

IV) Ivermectin - effects on mosquitoes

Ivermectin has been documented to effect the survivorship or fecundity of numerous arthropod vectors of medical and veterinary diseases and pests including mites (Mounsey et al. 2008), Ixodid ticks (Drummond et al. 1981), lice (Glaziou et al. 1994), Triatomine bugs (de Azambuja et al. 1985), botflies, Calliphorids (Drummond 1985), stable flies, horn flies (Miller et al. 1986), TseTse flies (Distelmans et al. 1983), Ceratopogonids (Standfast et al. 1984), sand flies (Mascari et al. 2008), and mosquitoes (Ali and Nayar 1985, Pampiglione et al. 1985, Iakubovich et al. 1989, Tesh and Guzman 1990, Cartel et al. 1991, Focks et al. 1991, Mahmood et al. 1991, Jones et al. 1992, Gardner et al. 1993, Bockarie et al. 1999, Chandre and Hougard 1999, Foley et al. 2000, Fritz et al. 2009, Chaccour et al. 2010). Surprisingly, ivermectin at human relevant concentrations has not been shown to have any effects on various *Simulium* spp. (Boussinesq et al. 1999, Chandre and Hougard 1999).

A) Mode of action

The mode of action for ivermectin was originally believed to target γ-aminobutyric acid (GABA) gated chloride (Cl) channels in invertebrates (Duce and Scott 1983) and mammals (Pong et al. 1980). Although ivermectin does affect GABACl channels in mammals, fortunately there is little toxicity of ivermectin to most vertebrates

because GABACl channels are located in the central nervous system (CNS) where P-glycoprotein efflux pumps at the blood brain barrier prevent ivermectin from entry into the CNS (Edwards 2003). In *Caenorhabditis elegans*, it was demonstrated that a loss of function of avr-14, avr-15, and glc-1 genes which encode glutamate-gated chloride (GluCl) channels led to high-level resistance, which means that GluCl may be the more important target of ivermectin than GABACl channels (Dent et al. 2000). GluCl channels are not present in vertebrates (Cully et al. 1996). Recently, ivermectin was shown to be a partial allosteric agonist of GluCl. In *C. elegans*, ivermectin sequesters in the membrane bilayer and binds at the subunit interfaces on the periphery of the transmembrane domains next to GluCl receptors, which causes a local distortion of the GluCl receptor transitioning it from a closed, resting state to an open, activated state (Hibbs and Gouaux 2011). Once the GluCl receptor is in the open state the cell is hyperpolarized which causes flaccid paralysis of pharyngeal and somatic muscles (Cully et al. 1994, Cully et al. 1996, Kane et al. 2000). This model is complicated by the fact that GluCl and GABACl channel subunits form heteromultimeric channel receptors for ivermectin in *Drosophila melanogaster* (Ludmerer et al. 2002). It has not been determined if these GluCl and GABACl subunit heteromultimeric channel receptors occur in mosquitoes.

B) Adult survivorship

Numerous studies have been published concerning the effects of ivermectin on various mosquito species' survivorship (Pampiglione et al. 1985, Iakubovich et al. 1989, Tesh and Guzman 1990, Focks et al. 1991, Mahmood et al. 1991, Cartel et al. 1991,

Jones et al. 1992, Gardner et al. 1993, Bockarie et al. 1999, Foley et al. 2000, Fritz et al. 2009, Chaccour et al. 2010). The first report of the effect of ivermectin on mosquitoes was from Pampiglione et al. (1985) which demonstrated that the survivorship of colony raised *Anopheles stephensi*, *Aedes aegypti*, and *Culex quinquefasciatus* was reduced after feeding on ivermectin-injected mice or ivermectin soaked cotton. Although susceptibility of the mosquitoes was demonstrated, the authors did not calculate a lethal concentration that killed 50% (LC_{50}) of the mosquitoes, despite this being the standard used to compare relative susceptibility between species (Robertson et al. 2007). *Anopheles stephensi* was the most susceptible mosquito species, followed by *Ae. aegypti* and *Cx. quinquefasciatus* (Pampiglione et al. 1985).

Iakubovich et al. (1989) blood fed colonized *Ae. aegypti*, *Anopheles sacharovi* and *An. stephensi* on rabbits injected with 340 µg/kg of ivermectin. Six days post blood feed 93% of the *An. stephensi* and 79% of the *An. sacharovi* had died, but no LC_{50} values were calculated.

Tesh and Guzman (1990) blood-fed ivermectin-spiked human blood to colonized mosquitoes and determined LC_{50} values for *Ae. aegypti* Rock strain (126 ng/ml), *Aedes albopictus* Houston strain (208 ng/ml), and *Cx. quinquefasciatus* Gainesville strain (698 ng/ml). However, these LC_{50} values are too high for any relevant killing effect of ivermectin treated vertebrates as blood plasma concentrations of ivermectin in vertebrates never reach these levels (see Chapter 2).

Both, Mahmood et al. (1991) and Focks et al. (1991) again demonstrated that *Ae. aegypti* was susceptible to ivermectin. Focks et al. (1991) fed mosquitoes on rabbits injected with two different concentrations of ivermectin, although the amount of

ivermectin in the rabbit was not quantified and therefore LC_{50} values were not calculated. Mahmood et al. (1991) found that 23.5% of adult *Ae. aegypti* Rock strain fed on 100 ng/ml of ivermectin in human blood died by day 8 post blood feed.

Cartel et al. (1991) was the first to demonstrate that colonized mosquitoes that fed on humans that ingested 100 µg/kg of ivermectin had reduced survivorship. Surprisingly, *Aedes polynesiensis* that fed on ivermectin treated humans one, three and six months post-treatment had significantly reduced survivorship. This level of control has not been demonstrated for any other mosquito species since. The authors speculate that the drug may have deposited in the tissues (Cartel et al. 1991) as ivermectin is extremely lipophilic (Baraka et al. 1996). If this were the reason, then similar studies should have similar results, which is not the case. An alternative explanation for their results is that the ivermectin-treated humans were microfilaremic for *W. bancrofti*, and the immune response to the dying mf, or the bacteria released upon death of the mf, could have made the blood toxic to *Ae. polynesiensis*.

Jones et al. (1992) found that field-caught *Anopheles quadrimaculatus* blood fed on ivermectin-injected dogs from 10 - 2500 µg/kg had > 90.5% mortality within 24 hours and > 98.6% mortality within 48 hours. The concentration of ivermectin in the dogs' blood ranged from 0, 33-37, 159-186, 282-286, and 383-456 ng/ml for dogs injected with 0, 10, 500, 1000, 2500 µg/kg ivermectin respectively. This demonstrates for the first time that a field-caught *Anopheles* species is susceptible to ivermectin at potentially relevant concentrations in vertebrate blood (~35 ng/ml) (Jones et al. 1992). Follow-up studies (Gardner et al. 1993), demonstrated that dogs injected with a dose of 0, 6, 12 and 24 µg/kg, which corresponded to 0, 6 ± 1, 11 ± 2, 16 ± 7.5 ng/ml in blood was still lethal

to field-caught *An. quadrimaculatus* (3.9, 33.2, 66.9, 65.3%) but not to *Ae. albopictus* (2.5, 1.6, 5.0, 1.7%). Neither field-caught *Culex salinarius* (7.5, 15.0, 6.3, 16.6, 9.2%), nor colonized *Ae. albopictus* (6.7, 7.5, 5.0, 7.5, 8.3%) had significant mortality when fed on dogs injected with 0, 15, 30, 60, or 120 µg/kg of ivermectin.

Bockarie et al. (1999) performed a field study on *Anopheles punctulatus* survivorship at the time of ivermectin MDA for lymphatic filariasis control in Papua New Guinea. Two MDA treatment regimens were performed: 400 µg/kg ivermectin (pilot) and 400 µg/kg ivermectin with 6 mg/kg diethylcarbamazine. The pilot study aspirated mosquitoes from two houses in the control and MDA-treated village (400 µg/kg ivermectin) the next day and 38 days after MDA. Field-caught *An. punctulatus* caught the day after ivermectin MDA and held for one day had a 68.9% reduction in survivorship compared to 5.7% from the control village, while *An. punctulatus* caught 38 days post MDA had a 2.8% reduction in survivorship compared to 0% from the control village. The second experiment aspirated mosquitoes from the same nine houses in Nanaha three consecutive days before and after MDA (400 µg/kg ivermectin and 6 mg/kg diethylcarbamazine) and again 28 days later from one MDA-treated village. Within one day post aspiration, 70% of *An. punctulatus* collected within three days post-MDA died, while none of the *An. punctulatus* pre-MDA died within one day. By the ninth day post aspiration, none of *An. punctulatus* collected within three days post-MDA were alive while 67% of *An. punctulatus* caught pre-MDA survived. This study, albeit poorly designed, demonstrates that a field-caught *Anopheles* species is susceptible to ivermectin treated humans during mass drug administration. The authors also present data from monthly human biting rates of *An. punctulatus* in Nanaha. They conclude that the

significant increase in human biting rates over the three months after MDA compared to the three months prior to MDA means that the effect of ivermectin on mosquito survival and reproduction is less than 30 days. Without a control village to compare human biting rates against it is difficult to interpret these data. Furthermore, an impact of ivermectin MDA on the human biting rate, used here as an approximation of adult mosquito abundance, is less important than a reduction in the daily probability of survivorship (see Chapter 1 IV F). The dose of ivermectin was 2.67 times higher than that given during routine MDA and the second study also co-administered DEC which may have confounded results. In later work by (Alexander et al. 2003), the authors suggest that the removal of *W. bancrofti* from the human population by MDA would increase the survivorship of *An. punctulatus,* and thereby increase the likelihood of *Plasmodium* transmission. However, Alexander et al. (2003) does not present any data to support this claim. The results obtained by Bockarie et al. (1999) do not discourage the possibility that ivermectin MDA to humans could reduce mosquito survivorship in the field.

Chandre and Hougard (1999) fed *Cx. quinquefasciatus* on chickens injected with 2000 µg/kg and humans that ingested 170 µg/kg of ivermectin. Although there was a 25% reduction in survivorship of *Cx. quinquefasciatus* fed on injected chickens, there was no reduction in survivorship when *Cx. quinquefasciatus* fed on orally-treated humans.

Foley et al. (2000) fed colonized *Anopheles farauti* Sarong strain on a single human that had ingested 250 µg/kg of ivermectin up to 44 days post ingestion. Even though they demonstrated significant departure of mosquito survivorship from the "control" it is difficult to accept that these findings are accurate. Only one human was

used in this study which precludes the use of mosquitoes from the same cohort being fed on a true second human control. Laboratory conditions (which were not stated) or quality of mosquito rearing could have easily altered the survivorship of *An. farauti* fed at days 26 and 44 post ivermectin ingestion to be different from "control" mosquitoes. However, this study does demonstrate that *An. farauti* is susceptible to ivermectin concentrations found in human blood after ingestion of ivermectin at 1.67 times higher dose given during routine ivermectin MDA.

Fritz et al. (2009) documented, for the first time, the effects of ivermectin on colonized *Anopheles gambiae* s.s. and *Anopheles arabiensis* which are two of the most important vectors of *Plasmodium* in Africa (Gillies and de Meillon 1968). *Anopheles gambiae* s.s. Kisumu strain and *An. arabiensis* Dongola strain were stated to have the same LC_{50} value of 19.8 ng/ml. *Anopheles gambiae* s.s. were fed on Zebu bulls that were injected with 600 µg/kg ivermectin, which is three times the recommended concentration used for cattle. The median survivorship of *Anopheles gambiae* s.s. was reduced for mosquitoes that fed on ivermectin-injected bulls compared to saline-injected bulls. Based on this analysis, the authors conclude that mass treatment of cattle in regions dominated by *An. arabiensis* would reduce the transmission of malaria parasites by suppressing the total population of malaria vectors early in the malaria transmission season (see Chapter 1 IV F).

Chaccour et al. (2010) fed *An. gambiae* s.s. G3 strain on people that ingested 200 µg/kg ivermectin one and fourteen days post-ingestion. There was a significant reduction in *An. gambiae* s.s. survivorship between the control and treatment groups when fed on people one day post treatment but not fourteen days post treatment. On days 2, 3, and 4

post blood feed, cumulative mortality of *An. gambiae* s.s. in the ivermectin group was 73, 84, and 89% and in the control group 32, 38, and 44%. This demonstrates that oral treatment of humans with ivermectin is lethal to *An. gambiae* s.s. and that most of the mosquito mortality induced from ivermectin treatment will occur in the first three days post blood meal ingestion. Unfortunately, mosquitoes were not fed on ivermectin treated humans between one and fourteen days post drug ingestion so the point that ivermectin-induced mortality ceased could not be determined. Also, 200 µg/kg ivermectin is 1.33 times higher than the dose given during routine ivermectin MDA for onchocerciasis and LF control.

C) Fecundity

Only a few studies have focused on the effects of ivermectin on mosquito fecundity and egg hatch rates after ingestion of an ivermectin containing blood meal (Tesh and Guzman 1990, Focks et al. 1991, Mahmood et al. 1991, Gardner et al.1993, Fritz et al. 2009).

Tesh and Guzman (1990) demonstrated that female fecundity was reduced for *Ae. aegypti* but not for *Ae. albopictus* after ingestion of 100 ng/ml ivermectin in human blood. Hatch rates of eggs from females of both *Ae. aegypti* and *Ae. albopictus* were severely reduced at 10 ng/ml in human blood. Concentrations of ivermectin that reduced egg hatch by 50% (EC_{50}) were 3.4 and 4.3 ng/ml for *Ae. aegypti* and *Ae. albopictus*, respectively. A second blood meal containing no ivermectin was offered to *Ae. aegypti* previously given a control and ivermectin (100 ng/ml) blood meal and this determined

that fecundity and the hatch rate returned to normal after the second blood meal that did not contain ivermectin.

Focks et al. (1991) demonstrated that *Ae. aegypti* fed on ivermectin-injected rabbits had significant reduction in fecundity and egg hatch rate when 10 mg/kg were injected but not when 2 mg/kg were injected. Again, the concentration of ivermectin in the rabbits was not determined so it difficult to translate the relevance of these results.

Mahmood et al. (1991) performed the most extensive work concerning the reduction of mosquito fecundity post ivermectin ingestion. *Ae. aegypti* that ingested 100 ng/ml of ivermectin in human blood had reduced fecundity and egg hatch rates. Spermathecae were dissected from the females and in all cases sperm appeared to be viable. Both the follicle and yolk length were significantly reduced for ivermectin fed mosquitoes compared to controls at 12, 24, 36, 48, 60, 72, and 96 hours post blood meal. Furthermore, numerous eggs that were successfully laid had distorted shape from that of control mosquito eggs and those with distorted shape were not viable. This work clearly demonstrates that ivermectin has effects on the mosquito reproductive system that diminishes the normal production of eggs.

Gardner et al. (1993) found that *An. quadrimaculatus* had significantly reduced fecundity and egg hatch rates when fed on orally ivermectin-treated dogs, while *Ae. albopictus* only had significantly reduced egg hatch rates. At 0, 6, 12, and 24 µg/kg ivermectin-treated dogs, blood concentrations correlated to 0, 6 ± 1, 11 ± 2, 16 ± 7.5 ng/ml. For *An. quadrimaculatus* fecundity and egg hatch rates decreased with increasing concentrations of ivermectin and at injection of 24 µg/kg no eggs hatched. *Aedes*

albopictus had hatch rates reduced by one half that of controls at ivermectin-injected doses of 15, 30, 60, and 120 µg/kg.

Fritz et al. (2009) found that *An. gambiae* s.s. and *An. arabiensis* did not produce any eggs at ivermectin concentrations of 10 ng/ml or higher. There were no statistically significant differences for *An. gambiae* s.s. or *An. arabiensis* for fecundity or egg hatch rates for concentrations of 0, 0.01, 0.1 or 1 ng/ml of ivermectin. Furthermore, *An. gambiae* s.s. that fed on ivermectin-injected Zebu bulls (600 µg/kg) produced no eggs 1, 4, 7, or 10 days post injection and egg production was reduced compared to controls that fed on bulls days 13 and 17 post-injection.

D) Larval survivorship

The effect of avermectins on mosquito larvae have been assessed when directly applied to water with L1 larvae (Pampiglione et al. 1985, Ali and Nayar 1985) or after the mother had ingested ivermectin (Focks et al. 1991, Mahmood et al. 1991).

Pampiglione et al. (1985) found the 4th instar larvae LC_{50}s for ivermectin to be 2.42, 2.90, and 10.4ng/ml and for abamectin to be 3.94, 5.85, and 23.4 ng/ml for *Cx. pipiens*, *An. stephensi* and *Ae. aegypti* respectively. Ali and Nayar (1985) determined that LC_{50}s of abamectin for fourth instar larvae of *Ae. aegypti* (4.80 ng/ml), *Aedes taeniorhynchus* (0.7 ng/ml), *Anopheles albimanus* (2.21 ng/ml), *An. quadrimaculatus* (6.6 ng/ml), *Culex nigripalpus* (7.84 ng/ml), *Cx. quinquefasciatus* (7.72 ng/ml), *Cx. salinarius* (7.59 ng/ml), and *Wyeomyia mitchelli* (2.25 ng/ml). Although it is apparent that avermectins can reduce the survivorship of larval mosquitoes, it is at concentrations that

would most likely affect non-target species and therefore not an appropriate agent to use for larval control.

Focks et al. (1991) followed the development of *Ae. aegypti* larvae, whose mothers had ingested ivermectin containing blood meals and determined that there was a reduction in the percent survival to the 4th instar. Mahmood et al. (1991) found a significant (12.3 times higher) difference in the number of eggs with fully developed, live larvae that failed to hatch. Furthermore, the larvae of ivermectin-treated females appeared to have no morphological differences from control larvae. The authors suspected that residual ivermectin from the mother may have been introduced to the egg and that this somehow inhibited egg hatch. These studies demonstrate that the offspring of mothers that ingested ivermectin will be further impacted negatively which would apply an even greater reduction or selection pressure of mosquito populations in the field.

E) Effect of ivermectin on pathogen development

The effect of ivermectin on the reduction of development of pathogens in a mosquito is an interesting yet understudied area of research that would further impact vectorial capacity. *Aedes aegypti* were allowed to feed on jirds (*Meriones unguiculatus*) infected with either *B. malayi* or *B. pahangi* and injected with ivermectin (200 µg/kg) (Rao et al. 1990). Mosquitoes that fed on jirds 15 days post ivermectin-treatment had no *B. malayi* or *B. pahangi* develop to the L3 stage in the vector. Mosquitoes fed on *B. malayi*-infected ivermectin-treated jirds had significantly reduced number of L3 recovered. There were no significant differences in the numbers of *B. malayi* mf ingested by *Ae. aegypti* at one or seven days post ivermectin-treatment but there was no recovery

of L2 larvae from mosquitoes fed on ivermectin-treated jirds. Furthermore, there were no sausage stage (the form found after ex-sheathment of the mf) *B. malayi* recovered from thoraxes of mosquitoes that fed on ivermectin-treated jirds. Sugar moieties on the mf from ivermectin-treated jirds were different from untreated jirds in that D-Mannose, N-Acetyl galactosamine, and L-Fucose were present on mf from ivermectin-treated jirds but not untreated jirds. The authors suggest that ivermectin treatment of the jirds altered the surface membrane of the mf and may have inhibited development in the mosquito vector.

Cartel et al. (1990) fed *Ae. polynesiensis* on *W. bancrofti* microfilaremic humans six months post-treatment with ivermectin. They found a reduction in the number of mf that developed to the L3 infective stage which was significant for the group of mosquitoes that fed on ivermectin-treated and untreated humans with > 1000 mf/ml but not 1 – 99 mf/ml. Although not statistically significant, there was an observable delay in the peak average number of L3 stage *W. bancrofti* by three days for mosquitoes that fed on ivermectin-treated humans compared untreated humans. This implies a developmental delay of *W. bancrofti* when mosquitoes fed on an ivermectin-treated human (Cartel et al. 1990).

No studies of the effects of ivermectin on the development of *Plasmodium* in the *Anopheles* vector have been performed to date. Merck researchers found no effect of ivermectin on bacteria or fungi in culture or *Eimeria muris* (a Protozoan) in mice (Burg and Stapley 1989). No effect on *P. falciparum* was observed when *P. falciparum* infected humans were orally treated with 200 µg/kg ivermectin (Lariviere et al. 1989). Regardless, there could be important interactions of ivermectin on *Plasmodium* development in the mosquito vector that should be explored. Mahmood et al. (1991)

found a delay in the formation of the peritrophic membrane and digestion of *Ae. aegypti* which demonstrates that ivermectin affects the mosquito midgut and could impact the interaction of the *Plasmodium* ookinete or oocyst with the midgut.

F) Summary

When these data are taken together there are obvious differences among the *Anopheles*, *Aedes*, and *Culex* genera in their susceptibility to ivermectin. Adult *Anopheles* spp. appear to be far more susceptible to the lethal effects of ivermectin and potentially at concentrations that may be relevant to human pharmacokinetics after a standard oral dose of ivermectin (see Chapter 2). Fecundity after ingestion of ivermectin for both *Anopheles* and *Aedes* spp. is severely reduced at concentrations relevant to human pharmacokinetics, but future experiments are necessary to determine if *Culex* fecundity is reduced. It does not appear that egg hatch rates of *Anopheles* spp. (Fritz et al. 2009) are reduced after ingestion of ivermectin although this should be explored further. The differences between the susceptibility of adult *Anopheles* compared to *Aedes* and *Culex* are intriguing and the molecular mechanisms that cause this should be explored further.

When reading the literature of ivermectin and mosquito interactions, one premise becomes very apparent: most of the authors view ivermectin as a means to reduce mosquito population abundance to delimit vector-borne disease transmission. While there will be an obvious impact on mosquito populations in the field if either ivermectin MDA of humans or zooprophylaxis of domesticated animals (i.e., cattle, goats, etc.) occurs, this would not be the greatest impact on mosquito-borne disease transmission.

Wilson's 1993 review highlights the concept of vectorial capacity (Fig. 1) (Garrett-Jones 1964), which demonstrates that an impact on the daily probability of survivorship (*p*) (i.e., vector longevity) with ivermectin is far more critical for the reduction of vector-borne pathogens than a reduction in overall abundance of the vector population (*m*) (Wilson 1993).

Fig. 1 $$V = \frac{ma^2p^nb}{-\ln p}$$

The vectorial capacity model demonstrates that an intervention that reduces the longevity of a mosquito vector even slightly will drastically reduce transmission of a pathogen. If a vector does not live long enough to fully undergo the extrinsic incubation period (the amount of time required from ingestion of a pathogen to becoming capable of transmitting that pathogen) then there is no chance for that vector to transmit the parasite. Once a vector has lived past the extrinsic incubation period, then any reduction in daily survivorship after this point will limit the number of infectious blood meals it will take and thereby limit transmission of the pathogen. If ivermectin MDA is ever used for vector-borne pathogen control, then it should be viewed primarily as a tool to reduce vector longevity (*p*) and secondarily for reduction of vector abundance (*m*).

Another variable in the vectorial capacity model is the competence of the pathogen to develop in the vector (*n*). If there is a delay in development of the pathogen in the vector by ivermectin as suggested by Cartel et al. (1991b) then this would reduce vectorial capacity.

Ivermectin may have an effect on the feeding frequency of the mosquito vector. The daily probability of a mosquito feeding on a human is (a) which is a factor of the

mosquito host preference index and feeding frequency. If the feeding frequency of a mosquito is delayed by ingestion of ivermectin, then this would reduce vectorial capacity.

The literature highlights a clear need to assess the affects of ivermectin MDA on the transmission of vector-borne diseases with well designed field studies and adequate replication for statistical analyses where humans are given relevant concentrations of ivermectin normally administered during routine ivermectin MDAs. Mosquito survivorship and infection status should be clearly monitored at logical time points post-MDA in both control and treated areas. Further laboratory studies can support this work by assessing variables from the vectorial capacity model that cannot be readily determined in the field.

Chapter 2 – The effect of anthelmintics used for mass drug administration on *Anopheles gambiae* s.s. survivorship

I) Introduction

Mass drug administrations (MDAs) of human populations are performed worldwide with several different anthelmintics to control numerous parasitic diseases. The APOC and the OEPA both rely on once or twice yearly MDA of ivermectin for the control of onchocerciasis in Africa and Latin America, respectively (Hotez 2007). The GPELF distributes various combinations and amounts of albendazole in combination with ivermectin or diethylcarbamazine (DEC), via MDA for the control of LF worldwide (Ottesen et al. 2008). Pyrantel has been distributed by MDA for the control of hookworms, roundworm, and whipworm (Reddy et al. 2007).

Many of the *Anopheles* vectors of human *Plasmodium* spp. occur in regions of the world (Hay et al. 2010b) where MDAs for various nematode control programs are performed (Brooker et al. 2006a, Amazigo 2008, Ottesen et al. 2008). Due to this spatial overlap *Anopheles* vectors will ingest varying concentrations of ivermectin, pyrantel, albendazole or DEC when they blood feed on MDA-treated humans. While these drugs vary in their molecular targets, all show specific activity against invertebrate helminths (Jones and Sattelle 2008), and often effect orthologous targets in blood feeding vectors (Bloomquist 2003, Mounsey et al. 2007). Previous studies have determined that ivermectin can reduce the survivorship of various *Anopheles* species (Pampiglione et al.

1985, Iakubovich et al. 1989, Cartel et al. 1991, Jones et al. 1992, Gardner et al. 1993, Bockarie et al. 1999, Foley et al. 2000, Fritz et al. 2009, Chaccour et al. 2010, see Chapter 1 IV).

The daily probability of mosquito survival (*p*) is the most influential variable in the mosquito vectorial capacity (*V*) (Garrett-Jones 1964, Black and Moore 2005), (see Chapter 1 IV-F). If ivermectin, pyrantel, albendazole, or DEC reduce mosquito longevity and thereby reduce the daily probability of mosquito survival, then MDA of humans may reduce the vectorial capacity of *Anopheles* populations for *Plasmodium*.

An. gambiae s.s. feeds frequently and almost exclusively on humans (Beier 1996, Scott et al. 2006), (see Chapter 4), is a primary vector of *Plasmodium* in Africa (Gillies and de Meillon 1968), and occurs in regions of Africa where ivermectin, pyrantel, and albendazole are administered by MDA (Ottesen et al. 2008, Reddy et al. 2007). DEC is not administered in Africa, due to complications with *O. volvulus* (Awadzi and Gilles 1980). *An. gambiae* s.s. was used as a laboratory model to represent the potential effects of DEC on other *Anopheles* spp. Ivermectin, DEC, pyrantel, and the primary metabolite of albendazole, albendazole sulfoxide, (Mathew and Kalyanasundaram 2007) were examined in this study because they are already approved for human use and are administered throughout the world by MDA (Ottesen et al. 2008, Reddy et al. 2007).

The drugs were serially diluted and mixed with human blood that was imbibed from artificial membrane feeders by laboratory-reared *An. gambiae* s.s. The concentrations at which these four drugs could affect mosquito survivorship were determined, with particular respect to the drug concentrations found in human blood following MDAs (Elkassaby 1991, Fasanmade et al. 1994, Shenoy et al. 2002, Awadzi et

al. 2003). LC_{50} values of each drug were calculated as this is the standard method used to compare the relative susceptibility between insect species (Robertson et al. 2007).

II) Materials and Methods

A) Preliminary experiments

1) Mosquitoes

Anopheles gambiae s.s. G3 strain (origin The Gambia) were raised at 28º-31ºC, 80% relative humidity, and a 14:10 light: dark cycle. Larvae were raised on a diet of ground Tetramin® fish food. Adults were provided water and 10% sucrose solution or raisins *ad libitum*.

2) Drugs

Powdered formulations of ivermectin, DEC, and pyrantel (pyrantel pamoate) were obtained from Sigma Aldrich (St. Louis, USA). Powdered albendazole sulfoxide was obtained from WITEGA (Berlin, Germany). Powdered formulations of each drug were diluted in dimethylsulfoxide (DMSO) to concentrations of 10 mg/ml and aliquots were frozen at -20ºC.

3) Preliminary *In vitro* blood feed experiments

Initial LC_{50} calculations with ivermectin and *An. ga*mbiae s.s. were performed by J. J. Meckel and was done with sheep blood. Originally, ivermectin was diluted into 100 µl of phosphate buffered saline (PBS) and added to 900 µl of sheep blood. Survivorship results were erratic and difficult to interpret. Therefore preliminary experiments were

performed to determine the correct amount of PBS and DMSO to dilute the drug in for blood meals that maximized *An. gambiae* s.s. survivorship. Ivermectin has been shown to avidly bind to human plasma (Klotz et al. 1990) so it was more appropriate to use human blood than sheep blood. Human blood was drawn into vacuette collection tubes by a phlebotomist at the Colorado State University Hartshorn health clinic. Initially, human blood was drawn into Sodium Heparin vacuettes and fed to *An. gambiae* s.s. to determine if varied amounts of PBS or DMSO altered mosquito survivorship. Five mixtures of PBS, DMSO, and human blood were offered to *An. gambiae* s.s.: 1000 µl human blood (control), 900 µl blood with 100 µl PBS, 990 µl blood with 10 µl PBS, 900 µl blood with 100 µl DMSO, and 990 µl blood with 10 µl DMSO, two replicates were performed . This experiment demonstrated that Sodium Heparin anticoagulant may be detrimental to *An. gambiae* s.s. survival, so another experiment was performed to determine the anticoagulant that caused the least mortality. Human blood was drawn into 3.5ml vacuette collection tubes with 7.2 mg K_2EDTA, spray coated Sodium Heparin, or 3.2% Sodium citrate anticoagulant (BD, Franklin Lakes, NJ, USA). One-thousand µl of human blood or 990 µl human blood with 10 µl PBS of each anticoagulant was offered to mosquitoes, and compared to a control where mosquitoes fed on a human hand directly; only one replicate was performed.

Except for the human hand, all blood meals were pipetted into glass bell feeders (Lillie Glass Feeders; Smyrna, GA, USA) sealed with hog sausage casing and warmed to 37 ºC with a circulating-heating water pump. All blood feeds used varied numbers of *An. gambiae* s.s. 2-8 days post-emergence. After blood feeding, mosquitoes were chilled in a refrigerator, sorted on ice-chilled Petri dishes, and only fully engorged mosquitoes were

retained for survivorship analysis. Mosquitoes were held for five days post blood feed in four liter cages with access to water and raisins as a sugar source. *A priori* experiments demonstrated that ingested drugs affected mosquito survivorship within four days post ingestion. Survivorship was monitored every twenty-four hours and dead mosquitoes were removed from the cage at each time point.

B) LC_{50} determination

Human blood, not more than two weeks post-drawn, was mixed with various concentrations of drugs. Drugs frozen in DMSO were thawed and serially diluted in PBS prior to addition to blood meals. Ten µl of varied concentrations of drug in PBS were added to 990 µl of blood to reach the final concentrations that were offered to mosquitoes. Control mosquitoes were fed 990 µl of blood with 10 µl of PBS alone. Multiple concentrations of drugs were fed to mosquitoes to determine the lethal concentration that killed 50% of the mosquitoes (LC_{50}). The concentration of ivermectin and albendazole sulfoxide fed to mosquitoes was estimated from (Awadzi et al. 2003) to match standard plasma concentrations found in humans upon co-ingestion of both drugs. Wing lengths of a subset of mosquitoes were measured to determine if adult mosquito size (Lounibos et al. 1995) affected the susceptibility to the drugs.

A non-linear mixed model with probit analysis was used to calculate LC_{50}s due to ~20% mortality rate in control *An. gambiae* s.s. This non-linear mixed model not only corrected for background control mortality but it also assessed replicate effects. The model is as follows:

Let n_{ij} be the total number of mosquitoes tested at dose level i in rep j, and Y_{ij} be the number of dead mosquitoes at the end of day 5. Let P_{ij} be the true rate of mortality for dose level i and rep j. Assume Y_{ij} follows a binomial distribution with parameters P_{ij} and n_{ij}. The usual estimate of P_{ij} is $\hat{P}_{ij} = Y_{ij} / n_{ij}$; however, the data show that there is background mortality among the controls (dose = 0). Therefore, the drug-related mortality estimates have to be corrected using some method, such as Abbott's formula (Abbott 1925). Let C be the background, then the Abbott's formula is as follows:

$$\text{Corrected mortality} = \frac{(1-C)-(1-\hat{P}_{ij})}{1-C} = \frac{\hat{P}_{ij}-C}{1-C}.$$

It is natural to fit a generalized linear model using a probit or logit link function. The generalized linear model with background mortality is (Collett 1991):

$$\hat{P}_{ij} = C \qquad \text{if dose is 0}$$
$$\hat{P}_{ij} = C + (1-C)\Phi(\beta_0 + \beta_1 x_{ij}) \qquad \text{if dose is not 0,}$$

where x_{ij} is the logarithm of the dose value and $\Phi(x)$ is the standard normal cumulative distribution function. This is a usual generalized linear model for grouped binary data and it does not account for the variance due to replications.

To include a random replication effect in toxicity of the drug the model may be augmented as follows:

$$\hat{P}_{ij} = C + (1-C)\Phi(\beta_0 + \beta_1 x_{ij} + u_j) \qquad \text{if dose is not 0,}$$

where u_j is normally distributed with 0 mean and variance σ^2.

In the above two models the background mortality is the same for all replications. It is reasonable to consider models in which the background mortality also contains a random replication effect, v_j.

$$\hat{P}_{ij} = C + v_j \qquad \text{if dose is 0}$$
$$\hat{P}_{ij} = C + v_j + (1 - C - v_j)\Phi(\beta_0 + \beta_1 x_{ij} + u_j) \qquad \text{if dose is not 0}$$

u_j and v_j are independent normally distributed with 0 mean and variance σ_1^2 and σ_2^2. The above three models can be fit using "Proc Probit" and "Proc NLMIXED" in SAS (SAS Institute Inc.). For the first model LC_{50} (or LC_{95}) can be computed directly, and for the other two models the expectation of LC_{50} is equal to the LC_{50} given the random effects to be zero. The 95% confidence intervals for the LC_{50} can be computed using "Fieller's method" (Fieller 1940) for all three models. Fieller's method allows the calculation of a confidence interval for the ratio of two parameter estimates using their variances and covariance estimates; therefore, the more precise the variance and covariance estimates are, the better the confidence interval is. Wider confidence intervals will be obtained using model 2 and model 3 than using model 1 because the variance due to replications is included in model 2 and model 3. The Akaike's Information Criterion (AIC) was used to compare the three models above. AIC is defined as (Burnham and Anderson 2002):

$$AIC = 2k - 2\ln(L)$$

where k is the number of parameters in the model, and L is the maximized value of the likelihood function for the estimated model.

SAS ProcProbit analysis was used to determine if mosquito adult size affected susceptibility to ivermectin. The two variables analyzed were concentration of ivermectin fed and wing length with death as the dependent variable.

III) Results

A) Preliminary experiments

Mosquitoes fed a mixture of 990 µl human blood with 10 µl PBS appeared to have better survivorship than those that consumed 900 µl human blood with 100 µl PBS (Fig. 1). Mosquitoes fed either mixtures of 900 µl human blood with 100 µl DMSO or PBS and 990 µl human blood with 10 µl DMSO or PBS appeared to have reduced survivorship beyond that of the control (1000 µl human blood) (Fig. 1) when blood was drawn into Sodium Heparin vacuettes. Of the three anticoagulants tested, mosquitoes that fed on the 3.2% sodium citrate appeared to have identical survivorship to that of the mosquitoes that fed directly on a hand (Fig. 2).

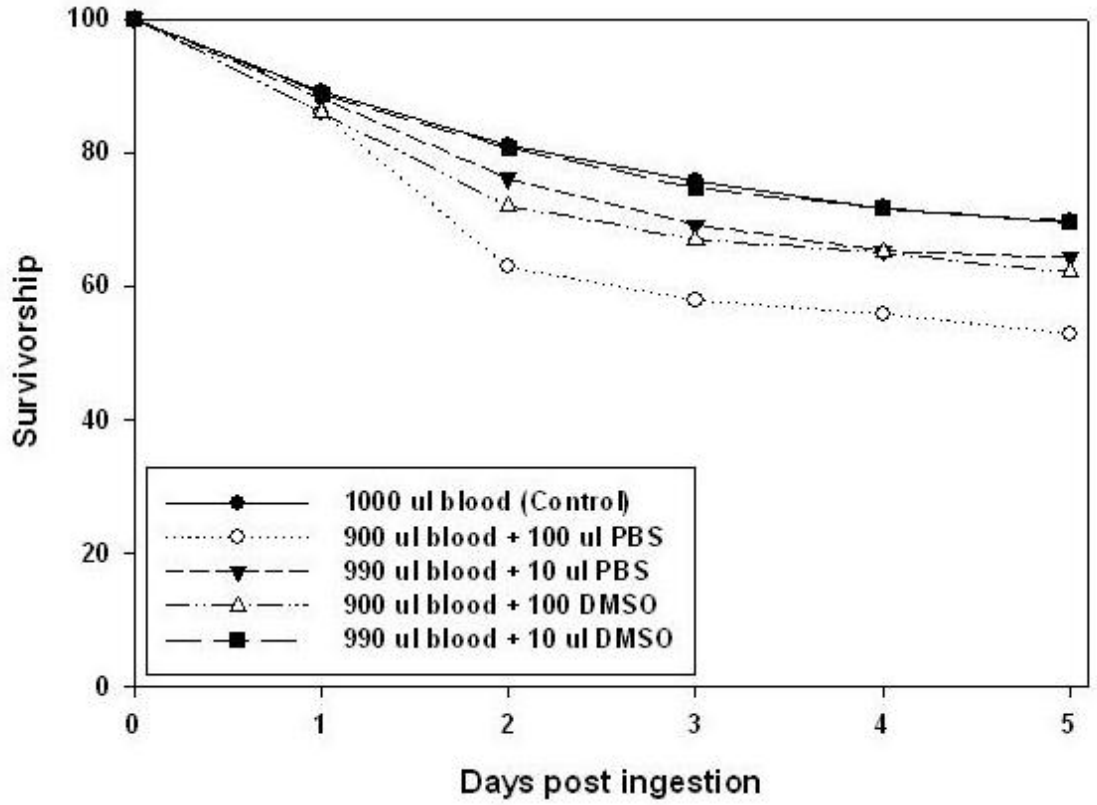

Figure 1. Percent survivorship of An. gambiae s.s. G3 that imbibed mixtures of human blood with either PBS or DMSO. *Anopheles gambiae* s.s. G3 were fed varied concentrations of human blood, PBS and DMSO to determine if either affected mosquito survivorship.

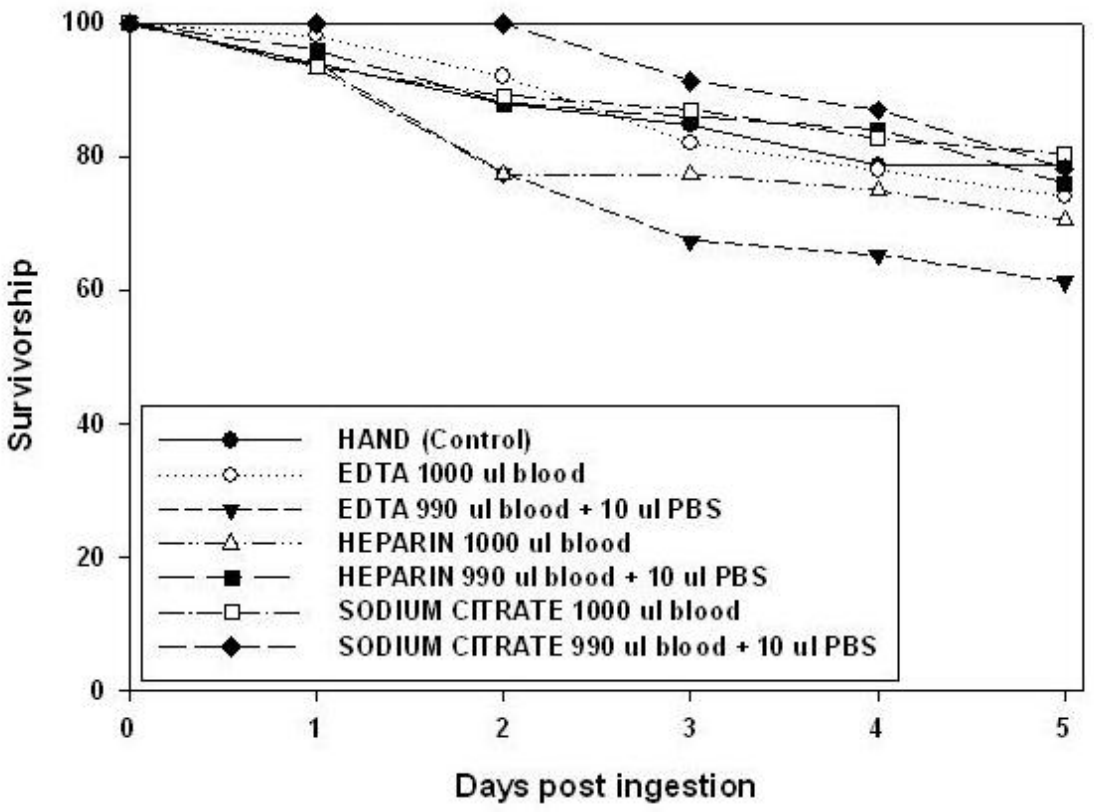

Figure 2. Percent survivorship of *An. gambiae* s.s. G3 that imbibed mixtures of human blood in different anticoagulants with and without PBS. *An. gambiae* s.s. G3 were fed human blood mixed with different anticoagulants and PBS to determine if the anticoagulants affected mosquito survivorship.

B) LC_{50} determination

Ivermectin reduced the survivorship of *An. gambiae* s.s. (LC_{50} 23.00 ng/ml, CI [18.22, 28.61], $P < 0.0001$, n = 2013, 8 replicates, model 3) (Fig. 3). However, pyrantel ($P = 0.1425$, n = 834, 3 replicates, model 1) (Fig. 4), albendazole sulfoxide ($P = 0.9407$, n = 1257, 3 replicates, model 3) (Fig. 5), and DEC ($P = 0.8385$, n = 777, 3 replicates) (Fig. 6, model 1), did not reduce the survivorship of *An. gambiae* s.s. Due to the lack of effect on mosquito survivorship for these drugs, LC_{50} values could not be calculated. The combination of ivermectin and albendazole sulfoxide did not reduce survivorship of *An. gambiae* s.s. when compared to ivermectin alone (Fig. 7). Concentration of ivermectin

($P < 0.0001$, n = 307, 1 replicate) but not adult size of *An. gambiae* s.s. ($P = 0.5935$, n = 307, 1 replicate) altered mosquito susceptibility to ivermectin (Fig. 8).

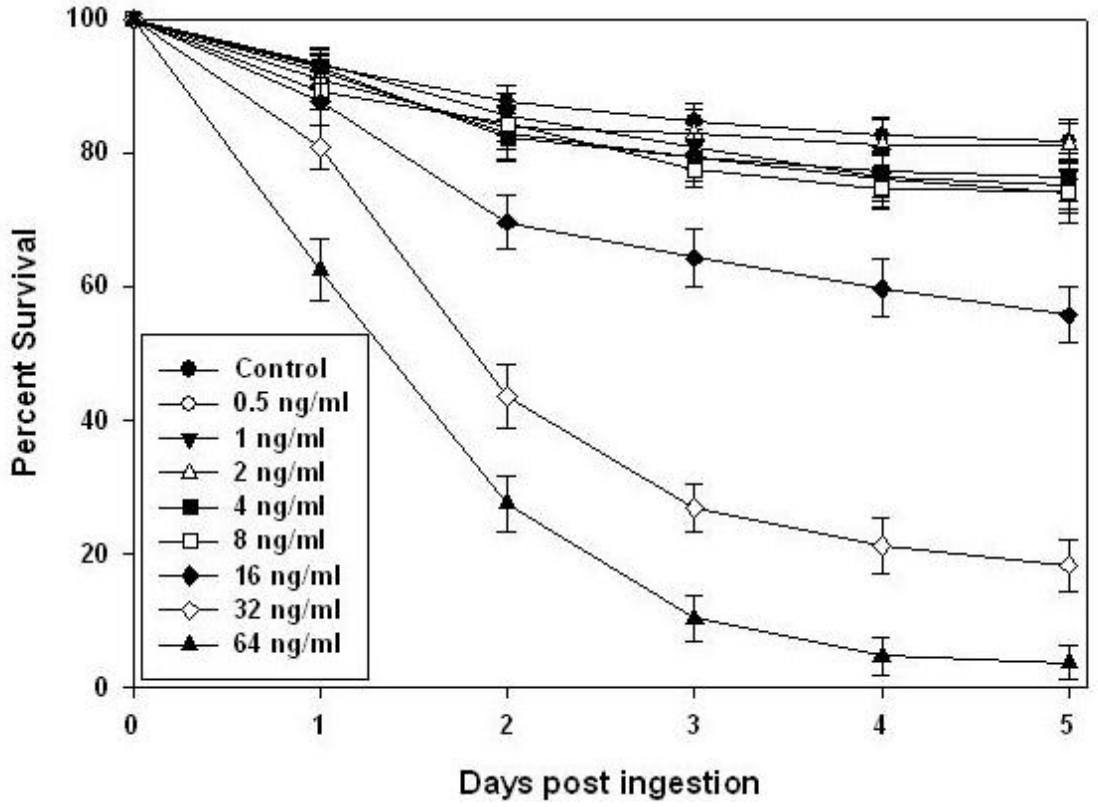

Figure 3. Percent survivorship of *An. gambiae* s.s. G3 that imbibed ivermectin. *An. gambiae* s.s. G3 were fed varied concentrations of ivermectin to determine if ivermectin reduced mosquito survivorship. Ivermectin reduced the survivorship of *An. gambiae* s.s. (LC_{50} 23.00 ng/ml, CI [18.22, 28.61], $P < 0.0001$, n = 2013, 8 replicates).

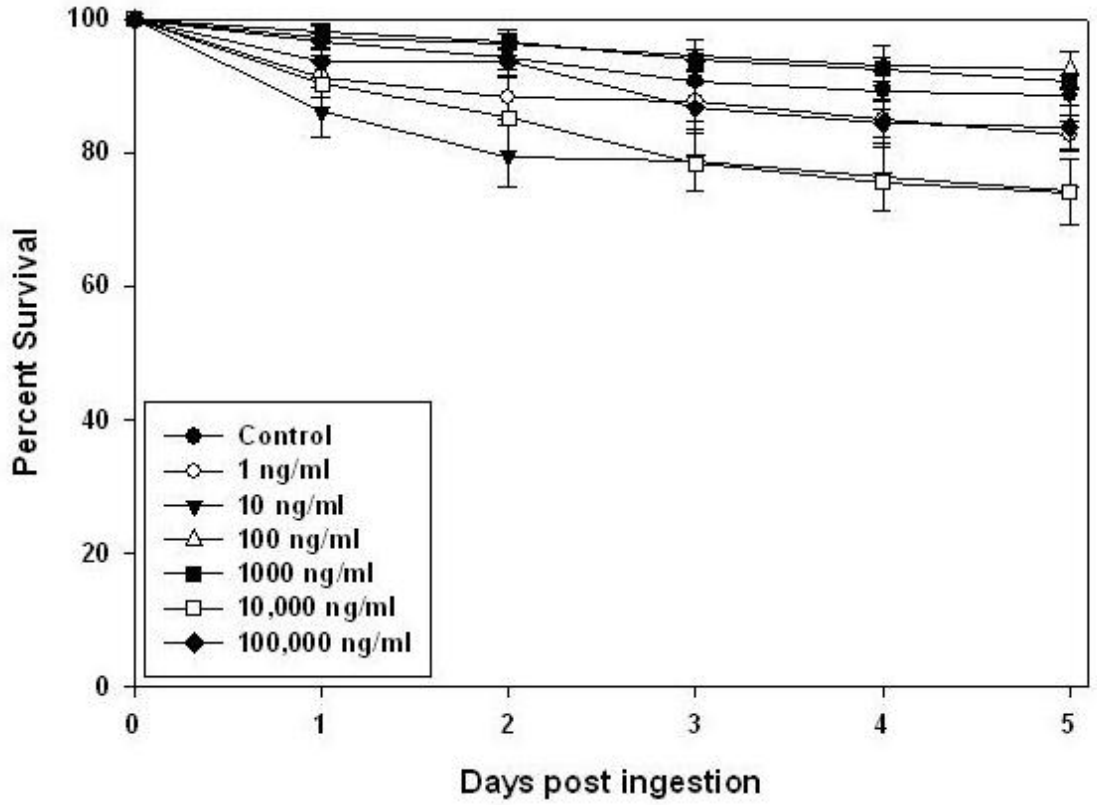

Figure 4. Percent survivorship of *An. gambiae* s.s. G3 that imbibed pyrantel. *An. gambiae* s.s. fed varied concentrations of pyrantel to determine if pyrantel reduced mosquito survivorship, but the drug had no effect on *An. gambiae* s.s.

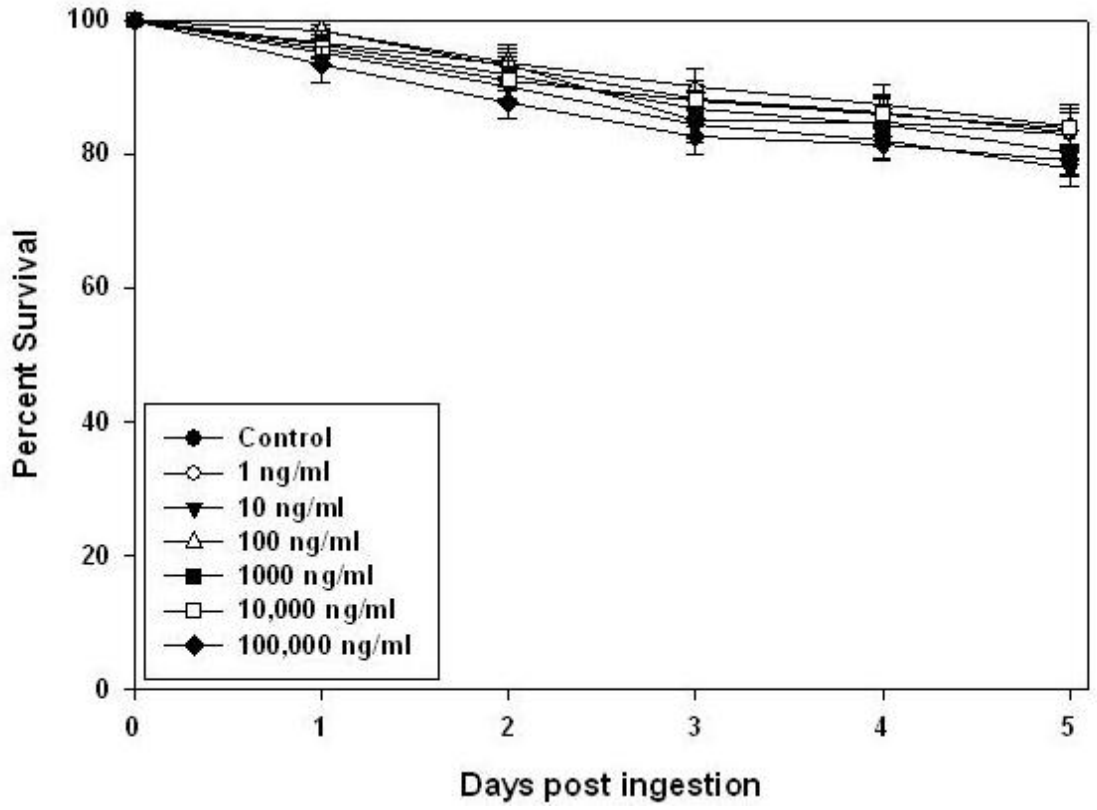

Figure 5. Percent survivorship of *An. gambiae* s.s. G3 that imbibed albendazole sulfoxide. *An. gambiae* s.s. were fed varied concentrations of albendazole sulfoxide to determine if albendazole reduced mosquito survivorship, but the drug had no effect.

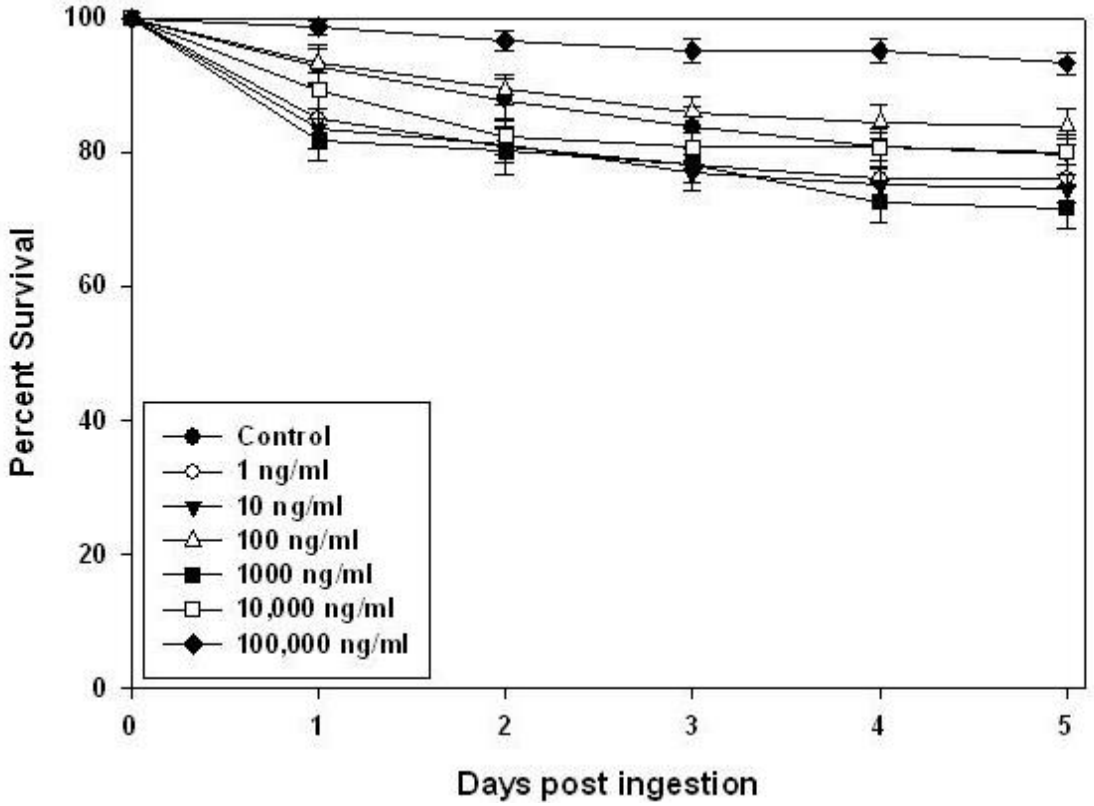

Figure 6. Percent survivorship of *An. gambiae* s.s. G3 that imbibed diethylcarbamazine. *An. gambiae* s.s. were fed varied concentrations of DEC to determine if DEC reduced mosquito survivorship, but the drug had no effect.

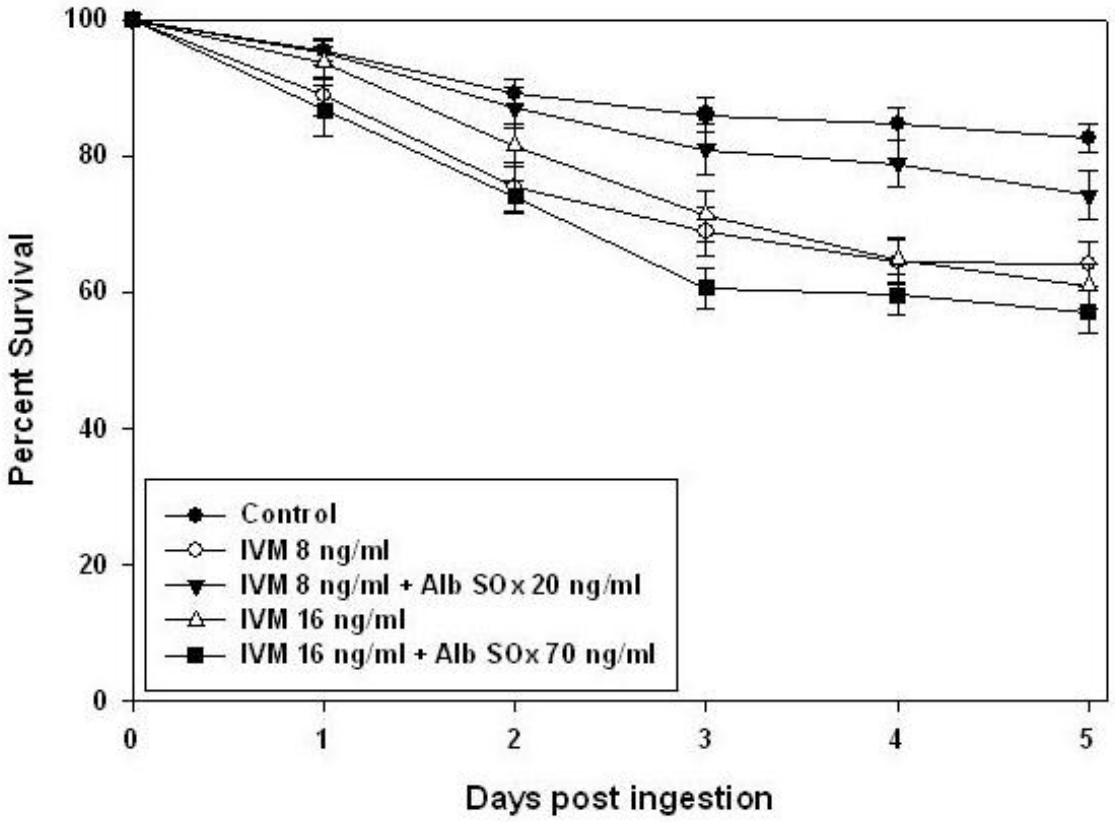

Figure 7. Percent survivorship of *An. gambiae* s.s. G3 that imbibed ivermectin and albendazole sulfoxide. *An. gambiae* s.s. were fed varied concentrations of ivermectin and albendazole sulfoxide to determine if the addition of albendazole to ivermectin reduced mosquito survivorship, but the drug combination did not affect mosquito survival over ivermectin alone (8 ng/ml IVM v 8 ng/ml IVM with 20 ng/ml ALB SOx, P = 0.1918, n = 383, 3 replicates; 16 ng/ml IVM v 16 ng/ml IVM with 70 ng/ml ALB SOx, P = 0.5372, n = 362, 3 replicates, model 2).

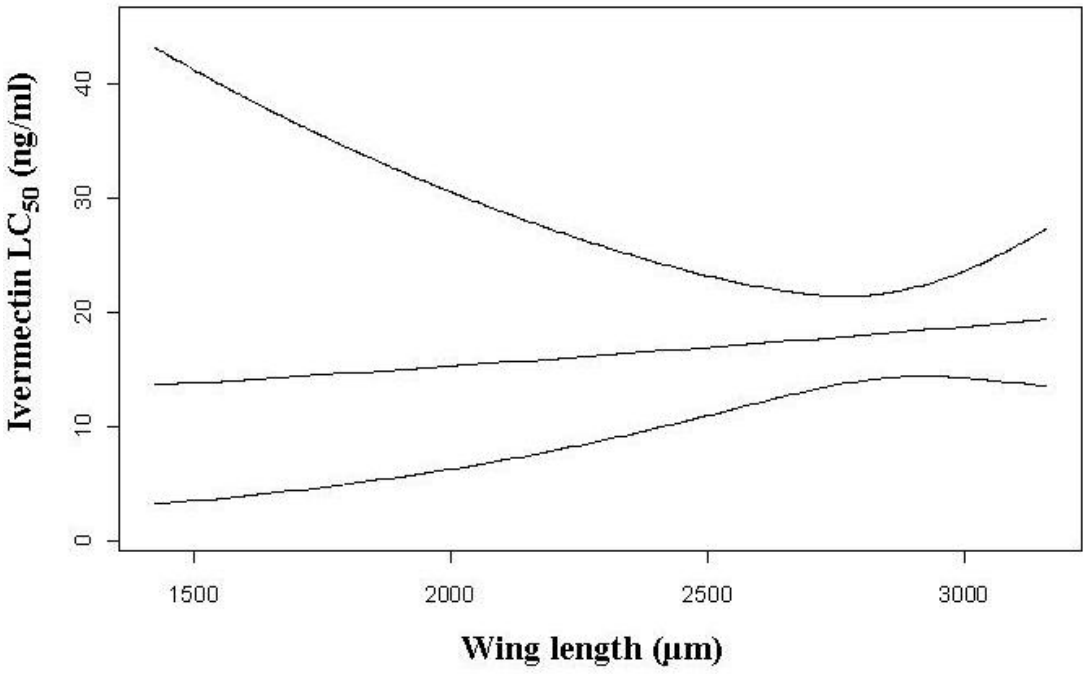

Figure 8. The relationship between adult body size and estimated LC_{50} for ivermectin. *An. gambiae* s.s. were fed varied concentrations of ivermectin and upon death their wing lengths were measured to determine if adult body size altered mosquito survivorship. Concentration of ivermectin ingested ($P < 0.0001$, n = 307, 1 replicate) but not adult size of *An. gambiae* s.s. ($P = 0.5935$, n = 307, 1 replicate) altered mosquito susceptibility to ivermectin. The straight line represents the estimated LC_{50} while the curved lines represent the 95% confidence intervals.

IV) Discussion

Preliminary experiments determined that the best anticoagulant to mix with human blood was 3.2% sodium citrate (Fig. 2), and that all future experiments using drugs diluted into PBS that are imbibed by mosquitoes should use a mixture of 990 µl of blood and 10 µl of PBS (Fig. 1). It was important to determine and standardize the coagulant and ratio of PBS to blood in order minimize erratic survivorship of *An. gambiae* s.s. in these and all future experiments. DMSO at very high concentrations (1:10 or 1:100) in human blood did not appear to alter the survivorship of *An. gambiae* s.s. (Fig. 1). This meant that *An. gambiae* s.s. used for LC_{50} experiments that imbibed

concentrations of DMSO that ranged from 1:10,000,000 to 1:100 did not have survivorship reduced due to DMSO.

Of the four drugs tested, only ivermectin reduced *Anopheles gambiae* s.s. survivorship (Fig. 3). An LC_{50} of 23 ng/ml for ivermectin is relevant in relation to human pharmacokinetics after ingestion of a standard oral dose (150 µg/kg) (Elkassaby 1991), (see Chapter 3). This ivermectin LC_{50} value is similar to that found by Fritz et al. (2009) for *An. gambiae* s.s. Kisumu strain and *An. arabiensis* Dongola strain. If wild *An. gambiae* s.s. are as susceptible as these laboratory strains, then this suggests that a standard ivermectin MDA would affect *An. gambiae* s.s. survivorship in the field (see Chapter 5). Adult size did not appear to alter *An. gambiae* s.s. susceptibility to ivermectin (Fig. 8) which is important as adult *An. gambiae* s.s. size can vary in the field (Lehmann and Diabate 2008).

Pyrantel, albendazole, and DEC had no effect on *An. gambiae* s.s. survivorship. Albendazole sulfoxide disrupts microtubule assembly in worm cells (Martin 1997). DEC, likely, activates the innate immune response of the host, causing rapid sequestration of microfilaria by granulocytes (Maizels and Denham 1992, Martin 1997, McGarry et al. 2005). The efficacy of DEC against various filarioid nematodes is restricted to *in vivo* experiments (Vickery et al. 1985, McGarry et al. 2005), and so the lack of reduced survivorship of *An. gambiae* s.s. fed DEC *in vitro* study does not necessarily mean that DEC will not have an effect on *Anopheles* spp. that blood feed directly on DEC-treated humans. Cartel et al. (1991) demonstrated that DEC reduced the survivorship of *Ae. polynesiensis* that fed on DEC-treated *W. bancrofti* microfilaridermic humans. Future studies should determine if important *Plasmodium* vectors that occur

outside of Africa have reduced survivorship when fed on humans treated with DEC. It is somewhat surprising that pyrantel did not reduce *An. gambiae* s.s. survivorship as this drug affects nicotinic acetylcholine-gated receptors (nAChRs) at nematode neuromuscular junctions (Martin et al. 1997), and these nAChRs are also used by arthropods for fast cholinergic synaptic transmission (Bloomquist 2003). However, multiple subunit genes in this family are common in invertebrate genomes. Ten nAChR paralogues have been identified in *An. gambiae* s.s. (Jones et al. 2005), and this redundancy may prevent pyrantel mortality effects even if it cross-targeted one or more mosquito nAChRs. Other drugs that target nematode nAChRs, such as levamisole, oxantel, and morantel are approved for human use, but these drugs were not examined for activity against mosquitoes in this study because they are not commonly used in current human MDA regimens (Reddy et al. 2007). Neonicotinoids such as nitenpyram and imidacloprid are specifically designed to target arthropod nAChRs, and may be more effective against mosquito vectors, but are currently only used in veterinary practice (Meinke 2001) so they are not as relevant in the context of anthropophilic mosquitoes.

Ivermectin and albendazole are co-administered by MDA for LF (Gyapong et al. 2005) and the combination is effective against soil-transmitted helminths (STHs) (Beach et al. 1999). Awadzi et al. (2003) determined that co-ingestion of albendazole and ivermectin did not reduce plasma levels of ivermectin in humans. Although albendazole sulfoxide does not enhance the mosquito lethal effect of ivermectin (Fig. 7), it does not inhibit it either. This is important to know as the addition of albendazole to ivermectin MDA in Africa would greatly enhance STH control (see Chapter 8).

Chapter 3 – Sublethal effects of ivermectin on *Anopheles gambiae* s.s.

I) Introduction

Ivermectin is the only drug distributed by MDA that reduced the survivorship of *An. gambiae* s.s. (see Chapter 2). The second most influential variable in the vectorial capacity equation is the daily probability of a mosquito feeding on a human (*a*) which is a factor of the mosquito host preference index and feeding frequency. If ingestion of ivermectin, pyrantel, albendazole, or DEC in a blood meal leads to a delay in re-feeding frequency, then the delay would further reduce the vectorial capacity of *Anopheles* for *Plasmodium* transmission. Mahmood et al. (1991) demonstrated that there was a delay in digestion and formation of the peritrophic membrane when *Ae. aegypti* imbibed 100 µg/ml of ivermectin in human blood. Delayed digestion of previous blood meals or delayed egg laying may lead to a delay in re-feeding.

After an ivermectin MDA there will be a decreasing amount of ivermectin in human blood plasma over time (Elkassaby 1991) (Fig. 1). The amount of ivermectin in human blood plasma will rapidly decrease to levels insufficient to kill *An. gambiae* s.s. in the laboratory (see Chapter 2). However, if imbibing several blood meals at sublethal concentrations can compound mosquito mortality, then ivermectin MDA may have an extended killing effect on *An. gambiae* s.s.

Mosquito age has been shown to diminish the susceptibility of *Anopheles* spp. to DDT, malathion, and pyrethroids (Rowland et al. 1987, Hodjati and Curtis 1999, Lines

and Nassor 1991, Rajatileka et al. 2011). If mosquito age increases the susceptibility of *Anopheles* to ivermectin, then this would further increase the impact of ivermectin MDA on malaria parasite transmission by directly reducing the proportion of older, potentially *Plasmodium*-infectious vectors.

A reduction in *Anopheles* fecundity and egg hatch rates has been observed post ivermectin ingestion for *An. quadrimaculatus* (Gardner et al. 1993) and *An. gambiae* s.s. (Fritz et al. 2009) (see Chapter 1 IV C). Neither of these previous studies quantified the effect of ivermectin at relevant human pharmacokinetics on *Anopheles* fecundity and egg hatch rates. An effect on *An. gambiae* s.s. fecundity or egg hatch rates by ivermectin MDA in the field will influence mosquito population structures. This would have an affect on the abundance of the vector population (m) and may therefore alter vectorial capacity.

II) Materials and Methods

A) Ivermectin concentration in human blood post ingestion (150 µg/kg) in relation to *An. gambiae* s.s. feeding times

Only one study to date has measured blood plasma levels of ivermectin beyond 48 hours. Elkassaby (1991) repeatedly measured plasma levels of ivermectin in ten Sudanese adult males infected with *Onchocerca volvulus* after ingestion of a single oral dose (150 µg/kg) for up to seven days and again at day 30. Mean plasma concentrations of ivermectin from Elkassaby (1991) were plotted and these data were used to estimate the concentration of ivermectin found in human blood post ivermectin MDA (Fig. 1). The decay portion of the pharmacokinetic curve was fit by non-linear regression to the

two-phase exponential decay equation ($Y = Span1*e^{-K1*X} + Span2*e^{-K2*X} + Plateau$) (Goodness of Fit, $R^2 = 0.9999$). Ivermectin concentrations from these data were selected based on a typical noontime MDA in the field (personal observation from APOC-directed MDA to villages in southeastern Senegal). To estimate the effects of ivermectin against mosquitoes in the field, mosquitoes were offered blood meals with concentrations of ivermectin that would be predicted during normal mosquito blood feeding times following MDA. *Anopheles gambiae s.s.*, peak biting frequency is at approximately 24:00 hrs (Mathenge et al. 2001), which corresponds to 12 hours post-MDA (Fig. 1).

B) Effects of drugs on mosquito re-feeding frequency and defecation

Age-matched adult mosquitoes were taken from 55 liter rearing bins and moved into four liter cages and held with access to raisins and water. *In vitro* blood meals containing diluted drug in human blood (as described in Chapter 2 B) were offered to these mosquitoes at two days post-emergence. After blood feeding, ten fully engorged female mosquitoes from the control and experimental groups were aspirated and placed individually into 50 ml tubes. The 50 ml tubes were covered with organdy. Mosquitoes were held without access to sugar for the remainder of the experiment and a wet cotton ball was placed at the bottom of each 50 ml tube to maintain humidity and provide access to water. Individually held mosquitoes were offered a human arm laid on top of the tubes for five minutes, at 24 hour intervals following their initial blood feed. The same person who donated blood for the *in vitro* feed, fed the mosquitoes in subsequent re-feedings. Following the daily chance to re-blood feed, mosquitoes were immediately scored for survival, defecation and re-feeding. The experiment ended for each mosquito

when it had either re-blood fed or died. Three independent replicates were performed for each drug concentration fed to the mosquitoes. Time to first defecation was also recorded. Concentrations of DEC, pyrantel, and albendazole sulfoxide were offered to mosquitoes at the maximum concentrations found in human plasma after ingestion of standard clinical dose regimens: DEC = 6mg/kg dose, maximum plasma level = 2100 ng/ml; pyrantel = 750 mg, maximum plasma level = 50 ng/ml; albendazole = 400 mg, maximum plasma level of albendazole sulfoxide = 700 ng/ml (Fasanmade et al. 1994, Shenoy et al. 2002). Maximal concentrations of DEC, pyrantel, and albendazole sulfoxide failed to change the re-feeding frequency of *An. gambiae* s.s., so lower concentrations of the drugs were not investigated. Re-feeding experiments were conducted using ivermectin concentrations determined above. Due to a lack of effect of DEC, pyrantel, and albendazole sulfoxide on *An. gambiae* s.s. survivorship or re-feeding frequency, none of these drugs effects on mosquitoes were investigated further.

C) Effects of cumulative ivermectin-containing blood meals on *An. gambiae* s.s. survivorship

To measure the effects of cumulative ivermectin exposures in a blood meal, two day post emergence adult *An. gambiae* s.s. were offered two consecutive blood meals, three days apart. The blood contained ivermectin concentrations that were predicted based on average mosquito feeding times matched to the pharmacokinetic curve of ivermectin in humans following a single oral dose of 150 µg/kg of ivermectin administered at noon. Immediately following their first blood meal, mosquitoes were sorted, and only fully engorged females were retained in a four liter cage, without sugar,

but with access to water. The second blood meal was offered to these mosquitoes three days later, control mosquitoes for each group were given only blood containing 10 µl of PBS. Following their second blood meal, individual fully engorged mosquitoes were placed in 50 ml tubes and maintained with access to water, but not a sugar source, until they died. Mortality was checked daily. Experiments with groups receiving primary blood meals containing 11.26 ng/ml and 6.58 ng/ml ivermectin were replicated twice and the data were pooled. Due to the high death rate and inhibited re-blood feeding frequency of mosquitoes that ingested a primary blood meal that contained 26.21 ng/ml of ivermectin, this experiment was performed only once.

D) The effect of ivermectin on *An. gambiae* s.s. knockdown

For each assay, 20 newly-emerged cohort female *An. gambiae* s.s. G3 strain mosquitoes per replicate were placed in 8 L volume cages (one, 4 L cages fastened on top of another 4 L cage) and held with access to water and 10% sucrose until they were 2 days post emergence. At this age, they were offered 990 µl freshly-drawn human blood spiked with 10 µl of ivermectin corresponding to the LC_{25} and LC_5 concentration, or 10 µl of vehicle-only control solution (a PBS-DMSO mixture of volumes equivalent to the concentration found in each matched drug concentration). Immediately prior to the blood feed, sticky fly paper (Olson Products, Inc, Medina, OH, USA) was lined on the bottom of the cage. Mosquitoes that blood fed and then landed on the bottom of the cage (as opposed to resting on the sides) were trapped by the fly paper and defined as 'knocked-down'. Knockdown rates of blood fed mosquitoes were calculated at 1, 3, and 24 hours post blood feed. Four replicate experiments were performed for each drug concentration tested, and drug groups were compared to matched controls by Logrank analysis.

E) The effect of ivermectin on *An. gambiae* s.s. recovery

For each assay, newly-emerged cohort female *An. gambiae* s.s. G3 strain mosquitoes were placed in 4 L volume cages and held with access to water and 10% sucrose until they were 2 days post emergence. At this age, they were offered 990 µl freshly-drawn human blood spiked with 10 µl of ivermectin corresponding to the LC_{25} and LC_5 concentration, or 10 µl of vehicle-only control solution (a PBS-DMSO mixture of volumes equivalent to the concentration found in each matched drug concentration). Immediately following the blood feed, 20 fully-engorged blood fed females were placed in the bottom chamber of a Mosquito Breeder, consisting of an emergence cone leading into a top chamber (BioQuip Products, Inc., Rancho Dominguez, USA), and a wet cotton ball and raisins were placed in the sides of the top chamber. Mosquitoes able to recover from the toxic effects of a sub-lethal blood meal were able to sense the food and water source and use coordinated flight to gain access to the top chamber; the proportion 'recovered' was defined by the proportion of mosquitoes in the top chamber and calculated at 1, 3, and 24 hours post-blood feed. Four replicates were performed for each drug concentration tested, and LC_{25} and LC_5 groups were compared to DMSO-concentration matched controls Logrank analysis.

F) Age-induced susceptibility of *An. gambiae* s.s. to ivermectin – survivorship, fecundity and hatch rates

In order to determine if *An. gambiae* s.s. were more or less susceptible to ivermectin at different ages, age-matched adult *An. gambiae* s.s. were fed varied concentrations of ivermectin two days post-emergence and every four days thereafter. Eight blood meals were fed to each group of mosquitoes. The concentrations of ivermectin fed to mosquitoes matched the human pharmacokinetic curve discussed above. Feeding schedules and concentrations are outlined in table 1. All feeds had the same amount of DMSO added to the blood meal that would occur in the highest

concentration of ivermectin fed to mosquitoes. Mortality was observed daily and all dead mosquitoes were recorded and removed. A Petri dish lined with cotton batting, a filter paper, and moistened with 25ml of room temperature tap water was placed in each cage as an oviposition substrate. The egg paper was replaced daily. Any eggs laid were counted, recorded and placed into 50 ml of room temp water to hatch. After the first replicate, only ≤ 180 eggs were placed in water to hatch. Two days later larvae were counted and recorded.

Table 1. Feeding schedule and concentration of ivermectin (ng/ml) fed for age-induced susceptibility of *An. gambiae* s.s. to ivermectin study

	BF*	1	2	3	4	5	6	7	8
Group	DPE†	2	6	10	14	18	22	26	30
1	D	11.7‡	2.75	-	-	-	-	-	-
	E	-	11.7	2.75	-	-	-	-	-
	F	-	-	11.7	2.75	-	-	-	-
	C	-	-	-	-	-	-	-	-
2	G	6.8	2.1	-	-	-	-	-	-
	H	-	6.8	2.1	-	-	-	-	-
	I	-	-	6.8	2.1	-	-	-	-
	C	-	-	-	-	-	-	-	-

*** BF- blood feed**
† DPE - days post emergence
‡ concentration of ivermectin (ng/ml)

G) Statistical analysis

Survival analysis was performed on the re-feeding frequency experiments, time to first defecation, the consecutive ivermectin blood feed experiments, knockdown, and recovery assay. In the former two experiments, mosquitoes that died instead of re-blood feeding or depositing feces over the course of the experiment were censored data (up-ticks marked on each graph line). Replicates were pooled and analyzed by the Logrank Test (Mantel-Haenszel method; proportional hazards model) and the hazard ratio with 95% confidence intervals. The age-susceptibility assay data were analyzed by the Logrank Test (Mantel-Haenszel method; proportional hazards model). Fisher's exact tests were used to determine if there was a difference in age-susceptibility after the ingestion of ivermectin. All data were analyzed with Graph Pad Prism (GraphPad Software, Inc.) and SAS (SAS, Cary Institute Inc.).

III) Results

A) Ivermectin concentrations in human plasma post ingestion (150 µg/kg) in relation to *An. gambiae* s.s. feeding times assuming MDA at 12:00 pm

The first ivermectin-containing blood meal that *An. gambiae* s.s. would imbibe correlated to a mean plasma concentration of 26.21 ng/ml. Subsequent concentrations of ivermectin given to *An. gambiae* s.s. were interpolated at 24 hour time points thereafter (36 hours = 11.73 ng/ml, 60 hours = 6.58 ng/ml, 84 hours = 4.03 ng/ml, 108 hours = 2.75 ng/ml, 132 hours = 2.1 ng/ml) (Fig. 1).

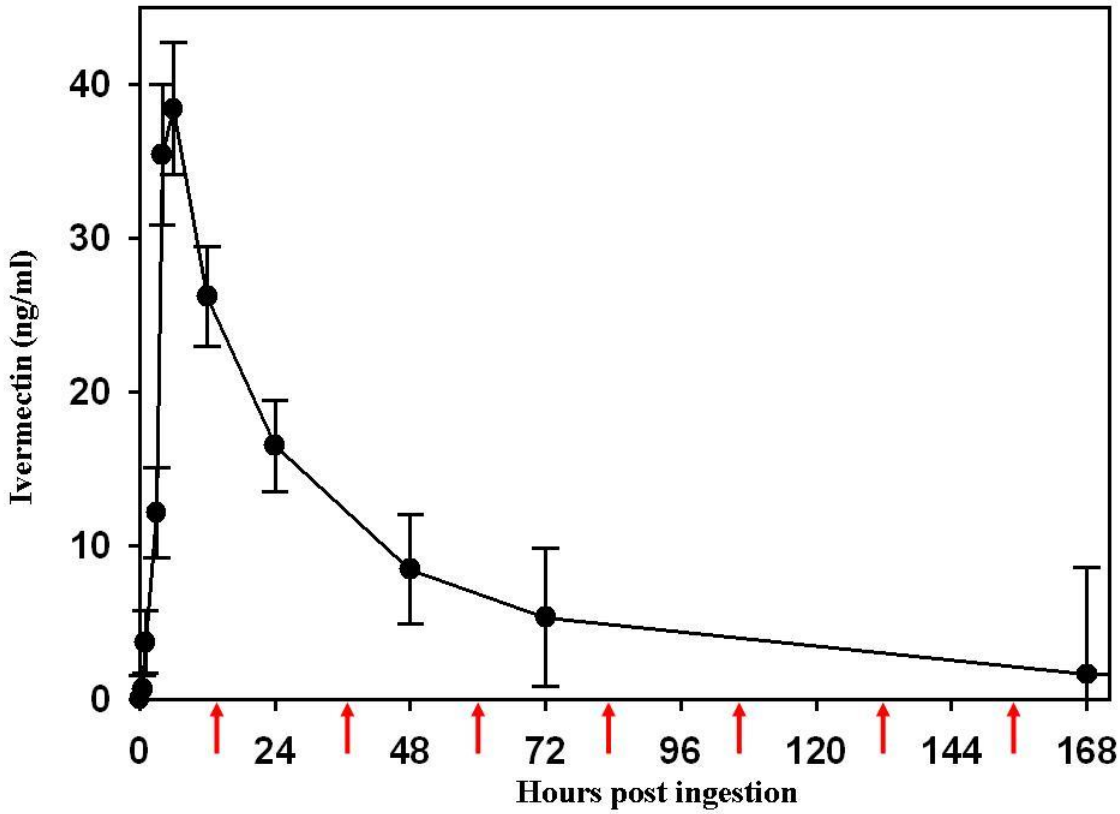

Figure 1. Mean ivermectin plasma concentration found in humans post ingestion (150 µg/kg) matched to *An. gambiae* s.s. feeding times. Mean ivermectin plasma concentration of ivermectin found in humans post ingestion of a standard oral dose (150 µg/kg) matched to approximate feeding times of *An. gambiae* s.s. assuming a 12:00 pm MDA, represented by red arrows.

B) Effects of drugs on mosquito re-feeding frequency and defecation

Re-blood feeding was delayed in *An. gambiae* s.s. that ingested a 26.21 ng/ml of ivermectin (Chi square = 23.83, df = 1, $P < 0.0001$, Hazard ratio = 7.656 [3.487, 18.63]) and an 11.73 ng/ml of ivermectin (Chi square = 3.845, df = 1, $P = 0.0499$, Hazard ratio = 1.603 [1, 4.459]), but not 6.58 ng/ml of ivermectin (Chi square = 2.036, df = 1, $P = 0.1536$) (Fig. 2). Maximum plasma concentrations of pyrantel (50 ng/ml), albendazole sulfoxide (700 ng/ml), and DEC (2100 ng/ml) did not delay the re-blood feeding frequency of *An. gambiae* s.s.

Time to defecation following the initial blood meal was delayed for *An. gambiae* s.s. that ingested ivermectin concentrations: 26.21 ng/ml (Chi square = 22.05, df = 1, $P < 0.0001$, Hazard ratio = 2.62 [3.679, 23.78]) and 11.73 ng/ml (Chi square = 8.904, df = 1, $P = 0.0028$, Hazard ratio = 1.836 [1.541, 8.059]), but not 6.58 ng/ml (Chi square = 0.6129, df = 1 , $P = 0.4337$, Hazard ratio = 1.141 [0.5771, 3.6]) (Fig. 3). Maximum plasma concentrations of pyrantel (50 ng/ml), albendazole sulfoxide (700 ng/ml), and DEC (2100 ng/ml) did not delay the time to defecation of *An. gambiae* s.s.

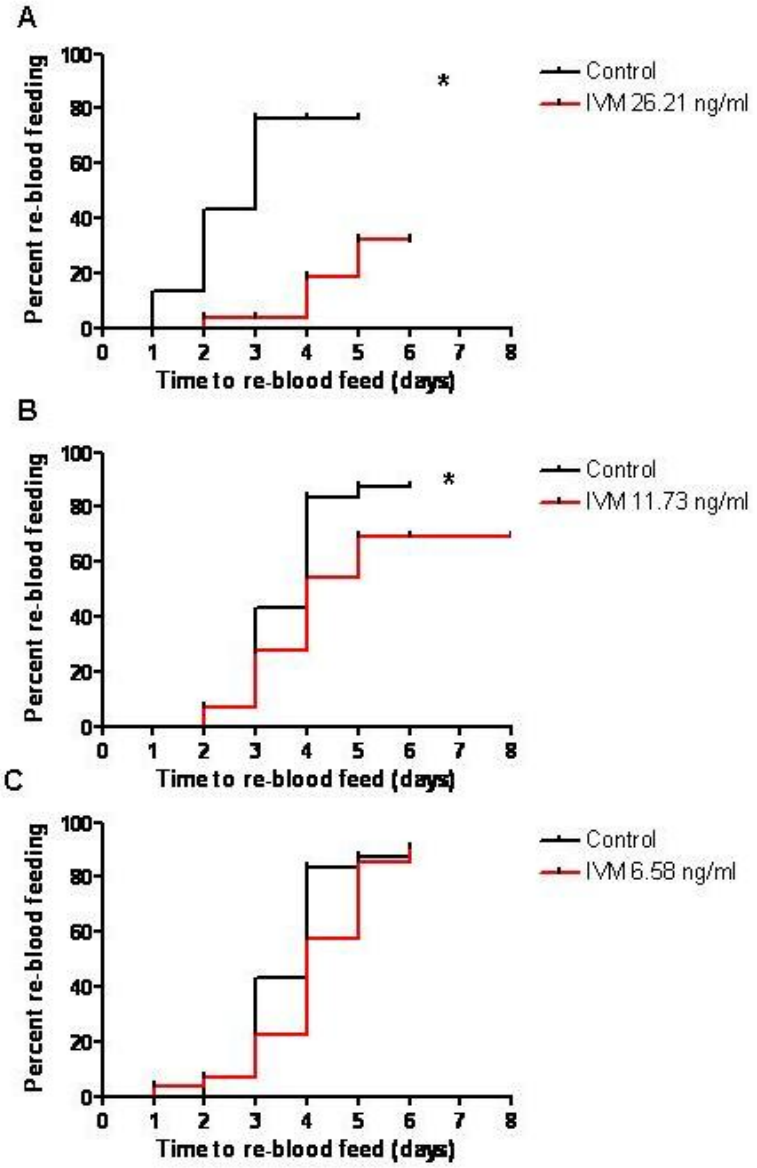

Figure 2. Re-blood feeding frequency of *An. gambiae* s.s. after a primary blood meal that contained ivermectin. Re-blood feeding frequency was assessed for *An. gambiae* s.s. with an initial blood meal that contained ivermectin concentrations: 26.21 ng/ml (A), 11.73 ng/ml (B), and 6.58 (C). Asterisks denote statistical significance.

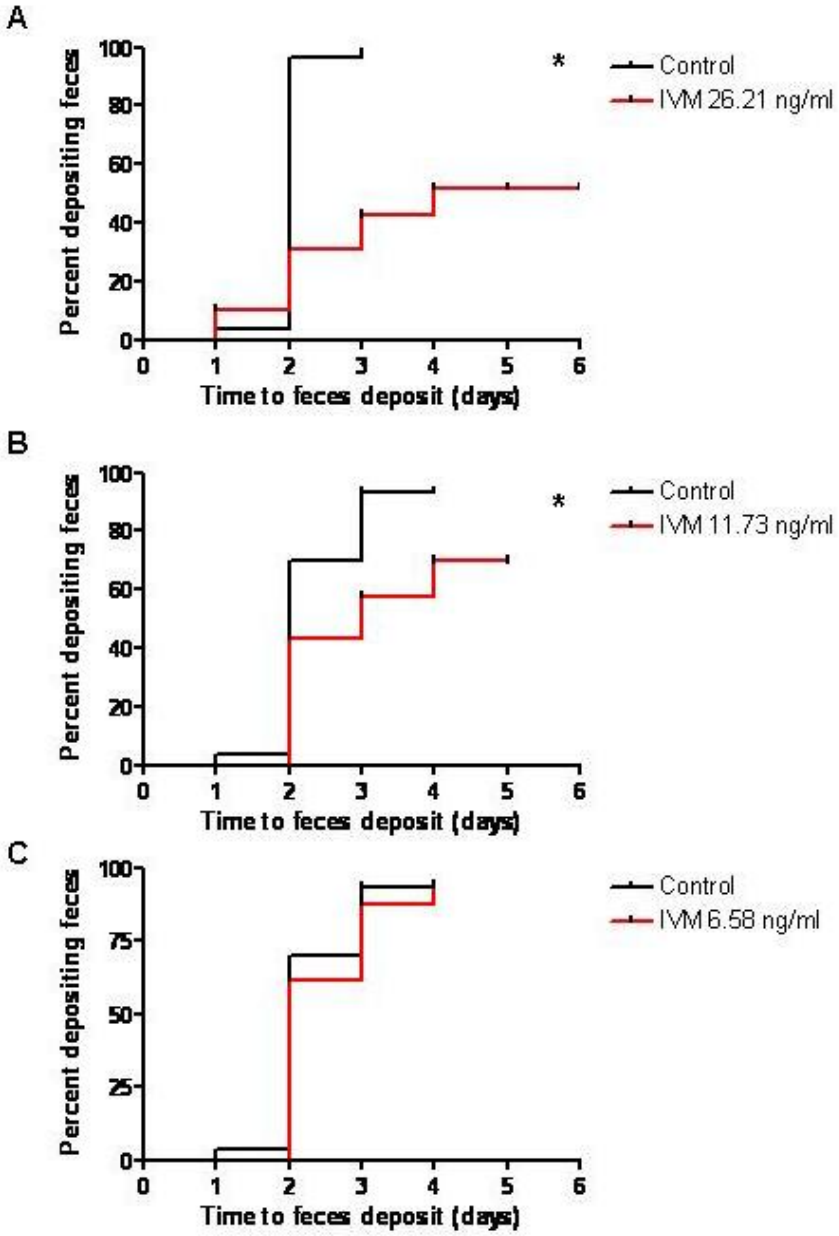

Figure 3. Time to defecation of *An. gambiae* s.s. following a primary blood meal that contained ivermectin. Time to defecation was assessed for *An. gambiae s.s.* with a blood meal that contained ivermectin concentrations: 26.21 ng/ml (A), 11.73 ng/ml (B), and 6.58 (C). Asterisks denote statistical significance.

C) Effects of cumulative ivermectin-containing blood meals on *An. gambiae* s.s. survivorship

Anopheles gambiae s.s. that survived their primary blood meal containing ivermectin concentrations predicted on the first night (26.21 ng/ml) and second night (11.73 ng/ml) post-MDA, had significantly reduced survivorship when re-blood fed on the predicted ivermectin concentration three days later compared to controls ('IVM 26.21-PBS' (n = 16) versus 'IVM 26.21-4.03' (n = 16), Chi square = 9.008, df = 1, P = 0.0027, Hazard ratio = 0.449 [0.086, 0.598]); ('IVM 11.73-PBS' (n = 38) versus 'IVM 11.73-2.75' (n = 26), Chi square = 7.512, df = 1, $P < 0.0061$, Hazard ratio = 0.571 [0.2, 0.766]). *Anopheles gambiae* s.s. that fed on an initial ivermectin concentration predicted at three days (6.58 ng/ml) post-MDA, and then re-blood fed on a concentration of ivermectin predicted three days later (2.1 ng/ml), had significantly elevated survivorship compared to their PBS controls ('IVM 6.58-PBS' (n = 39) versus 'IVM 6.58-2.1' (n = 27), Chi square = 4.027, df = 1, P = 0.045, Hazard ratio = 1.429 [1.016, 3.922] (Fig 5). This is not biologically relevant because both groups of mosquitoes that initially fed on 6.58 ng/ml ivermectin that re-fed on ivermectin or PBS were all dead by the same time point whereas the mosquitoes that fed on the other two initial (26.21 and 11.73 ng/ml) concentrations were all dead at different time points.

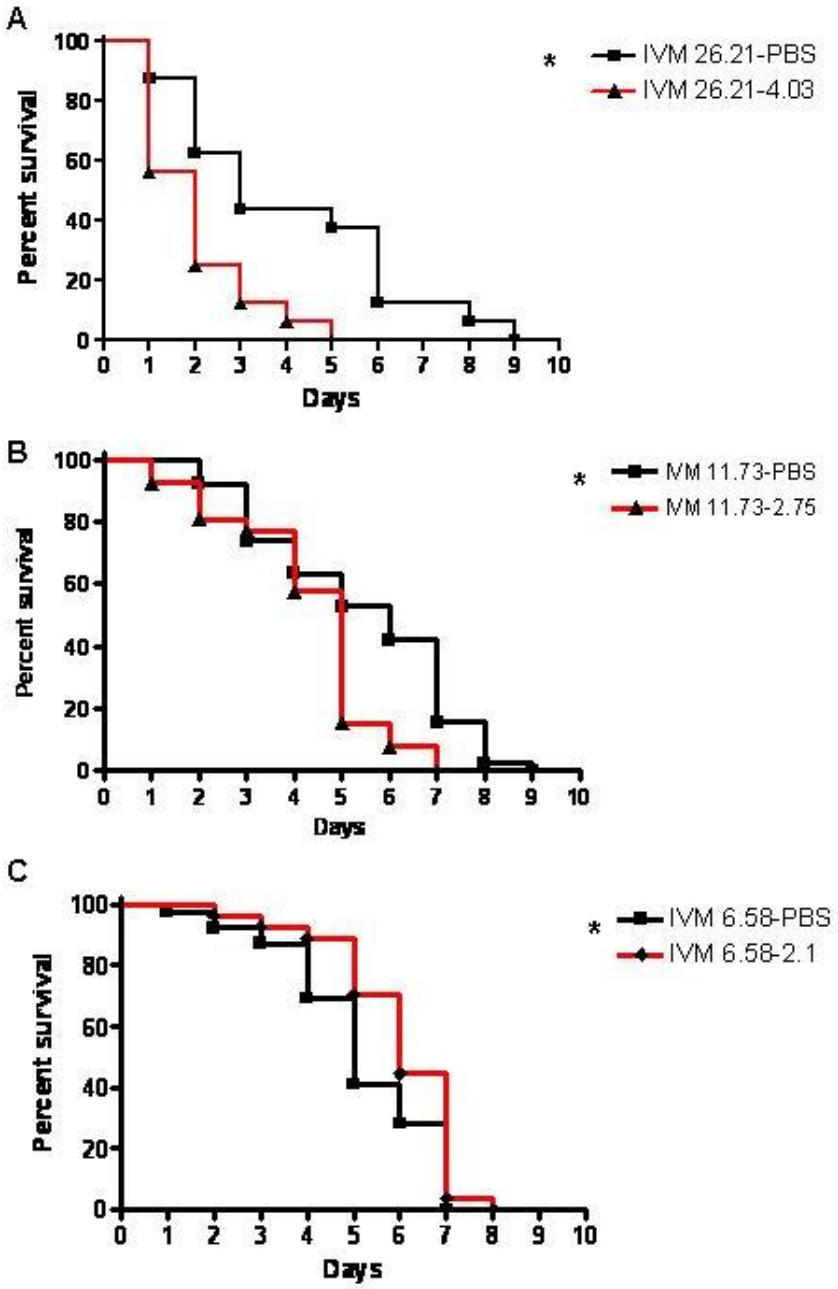

Figure 4. Percent survivorship of *An. gambiae* s.s. after imbibing two consecutive blood meals that contained ivermectin. *An. gambiae* s.s. were given two consecutive blood meals (3 days apart) that contained ivermectin concentrations corresponding to those interpolated from the ivermectin pharmacokinetic curve: (A) 1º blood meal = 26.21 ng/ml, 2º blood meal = 4.03 ng/ml; (B) 1º blood meal = 11.73 ng/ml, 2º blood meal = 2.75 ng/ml; (C) 1º blood meal = 6.58 ng/ml, 2º blood meal = 2.1 ng/ml. Asterisks denote statistical significance.

D) The effect of ivermectin on *An. gambiae* s.s. knockdown

Anopheles gambiae s.s. that ingested ivermectin at the LC_{25} (12.94 ng/ml) (Chi square = 5.918, $P = 0.015$, Hazard ratio = 1.821 [1.192, 5.129], n = 66) and LC_5 (7.66 ng/ml) (Chi square = 4.592, $P = 0.0321$, Hazard ratio = 2.355 [1.092, 7.221], n = 57) concentrations displayed a knockdown effect compared to controls (Fig. 5).

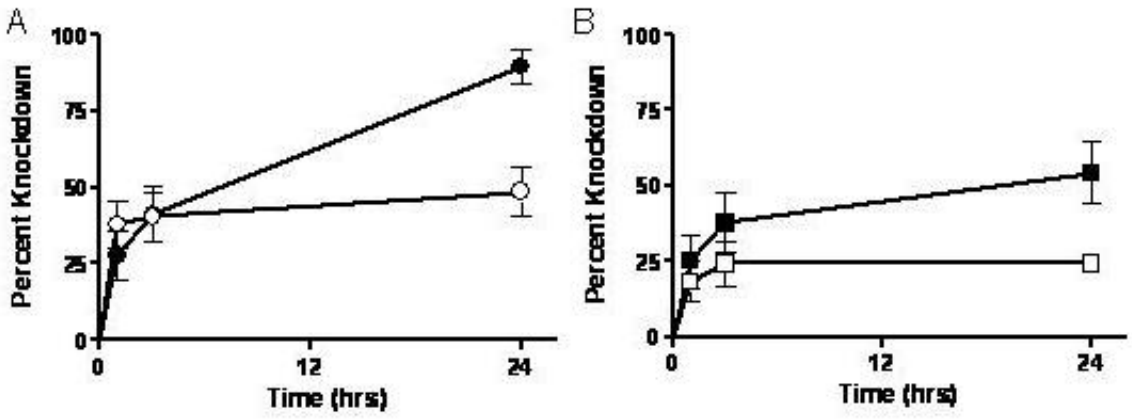

Figure 5. The percent knockdown of *An. gambiae* s.s. that ingested ivermectin. The percent knockdown of *An. gambiae* s.s. at 1, 3, and 24 hours post ingestion of ivermectin at the LC_{25} (12.94 ng/ml) (Fig. 5A) and the LC_5 (7.66 ng/ml) (Fig. 5B) concentrations. There was a significant increase in the percent knockdown at the LC_{25} and LC_5 concentrations (closed symbols) compared to controls (open symbols).

E) The effect of ivermectin on *An. gambiae* s.s. recovery

Anopheles gambiae s.s. that ingested ivermectin at the LC_{25} (12.94 ng/ml) (Chi square = 49.21, $P < 0.0001$, Hazard ratio = 0.411 [0.377, 0.158], n = 120) and LC_5 (7.66 ng/ml) (Chi square = 28.38, $P < 0.0001$, Hazard ratio = 0.573 [0.05, 0.272], n = 120) concentrations had reduced recovery rates compared to controls.

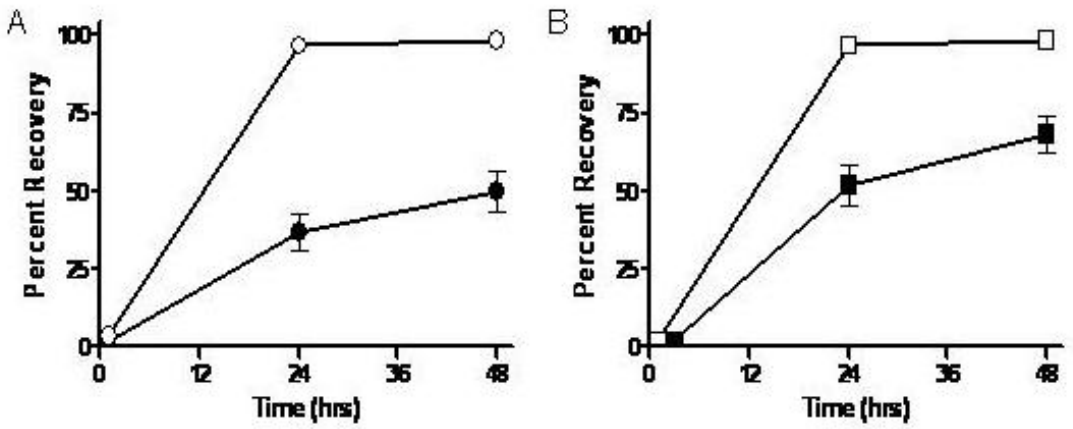

Figure 6. The percent recovery of *An. gambiae* s.s. that ingested ivermectin. The percent recovery of *An. gambiae* s.s. at 1, 3, and 24 hours post ingestion of ivermectin at the LC_{25} (12.94 ng/ml) (Fig. 6A) and the LC_5 (7.66 ng/ml) (Fig. 6B) concentrations. There was a significant decrease in the percent recovery at the LC_{25} and LC_5 concentrations (closed symbols) compared to controls (open symbols).

F) Age-induced susceptibility of *An. gambiae* s.s. to ivermectin

1) Susceptibility

For group 1 (11.7 ng/ml) all *An. gambiae* s.s. blood feed regimens (i.e., Table 1 – DEFC) survivorship curves differed from the control: DEFC (Chi-square = 172.7, $P < 0.0001$), D v C (Chi-square = 105.7, $P < 0.0001$), E v C (Chi-square = 30.32, $P < 0.0001$), and F v C (Chi-square = 34.85, $P < 0.0001$) (Fig. 7A). In order to determine if survivorship of *An. gambiae* s.s. later in life was affected by previous ingestion of sublethal concentrations of ivermectin, each survivorship curve was compared to the control from the point that each blood feeding regimen received its first non-ivermectin containing blood meal after ivermectin ingestion. There were no differences in survivorship compared to control after each blood feed regimen was fed 0 ng/ml of ivermectin: D v C (Chi-square = 0.09878, $P = 0.7533$), E v C (Chi-square = 0.1739, P = 0.6767), and F v C (Chi-square = 0.2624, $P = 0.6085$). In order to determine if *An.*

gambiae s.s. susceptibility to ivermectin increased later in life, the slopes of survivorship curves for each blood feed regimen were compared for the following four days after an ivermectin-containing blood meal. There were greater reductions in survivorship curve slopes when mosquitoes imbibed their first ivermectin-containing blood meal compared to their second (D1 v E2 Chi-square = 15.543, $P < 0.0001$) and third (D1 v F3 Chi-square = 11.858, $P = 0.0007$) ivermectin-containing blood meals (Table 2A). Fecundity (number of eggs/female on each day) and egg hatch rates (percent larvae hatched/eggs from each blood meal) results are presented in table 3A and 4A, respectively. Only one replicate has been performed, therefore no statistical analyses were performed on this data.

For group 2 (6.8. ng/ml), none of the *An. gambiae* s.s. blood feed regimens (i.e., Table 1 – GHIC) survivorship curves differed from the control: GHIC (Chi-square = 4.801, $P = 0.1869$) (Fig. 7B). There were no differences in survivorship compared to control after each blood feed regimen was fed 0 ng/ml of ivermectin: G,H,or I v C (Chi-square = 0, $P = 1$). There was greater reduction in the survivorship curve slope when mosquitoes imbibed their third ivermectin-containing blood meal compared to their second (I3 v H2 Chi-square = 7.2094, $P = 0.0124$) (see table 2B). Fecundity and egg hatch rates results are presented in table 3B and 4B, respectively. Only one replicate has been performed, therefore no statistical analyses were performed on this data.

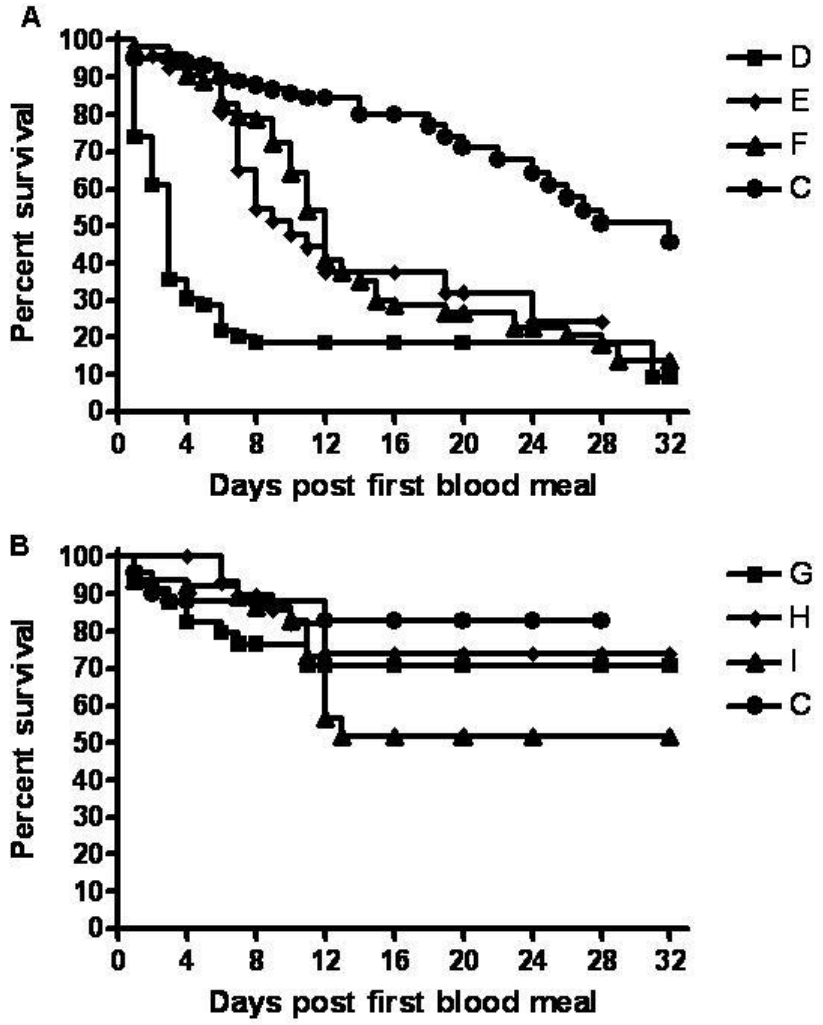

Figure 7. *An. gambiae* s.s. survivorship curves after ingestion of ivermectin at different blood feed regimens. *An. gambiae* s.s. survivorship curves after ingestion of ivermectin at different blood feed regimens and different concentrations: group 1 (11.7 ng/ml) (A) and group 2 (6.8 ng/ml) (B).

Table 2. Statistical comparisons of *An. gambiae* s.s. survival curve slopes four days after ingestion of ivermectin-spiked blood meals

A) Group 1 (11.7 ng/ml)

E2	E3	F3	F4	
15.543[§Φ]	-	**11.858**	-	D1
<0.0001	-	**0.0007**	-	
	0.3862	-	0.7876	D2
	0.7409	-	0.5075	
		0.2735	-	E2
		0.6344	-	
			0.0837	E3
			0.7814	

B) Group 2 (6.8 ng/ml)

H2	H3	I3	I4	
1.2439	-	2.9481	-	G1
0.2936	-	0.0988	-	
	0.5772	-	0.8282	G2
	0.6428	-	0.5101	
		7.2094	-	H2
		0.0124	-	
			1.65	H3
			0.5343	

[§] Chi-square values on top of each cell, P-values on bottom of each cell
[Φ] Values in bold are statistically significant

Table 2. Chi-square and P-values for all *An. gambiae* s.s. survival curve slope comparisons four days after the ingestion of ivermectin. For Group 1, there was a greater reduction in survivorship after ivermectin ingestion (11.7 ng/ml) at the first blood meal (D) compared to the second (E) and third (F) blood meals (see Fig. 7A). For group 2, there was a greater reduction in survivorship after ivermectin ingestion (6.8 ng/ml) at the third (I) blood meal compared to the second (H) blood meal.

2) Fecundity and egg hatch rates

In all instances it appears that the ingestion of ivermectin at 11.7 ng/ml or 6.8 ng/ml reduced the number of eggs per female (Table 3), and the effect appeared more to be greater when mosquitoes ingested 11.7 ng/ml compared to 6.8 ng/ml. Only one

replicate has been performed so statistical analysis was not possible. The percent egg hatch rate data (Table 4) are difficult to interpret without further replication and statistical analyses. In some instances, so few eggs were produced that if any eggs hatched, it inflated the percent hatch rate to appear higher than when blood without ivermectin was ingested.

Table 3. Number of eggs produced per female on each day

A) Group 1 (11.7 ng/ml) ivermectin

BF	1				2				3				4			
DP1BF	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
D	0*	0	0	0	0	0	0	0	5.0	0	0	0.2	0	0	0	0
E	0	0	0	1.8	9.9*	0	0.2	0.8	1.7	0	0	6.2	5.9	0	0	25.2
F	0	0	0	6.9	10.6	0.3	5.4	22.6	4.4*	0	0	0	0	0	0	0.0
C	0	0	0	0.0	1.3	0.3	3.2	7.6	3.2	0	0.6	7.5	3.2	1.4	0	12.2

A) Cont'd

BF	5				6				7				8				END
DP1BF	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
D	13.5	0	0	0	2.3	0	0	0	0	0	0	0	0	0	0	0	0
E	0	0	0	17.2	10.0	0	0	23.3	29.3	0	0	2.0	0	-	-	-	-
F	1.5	0	0	9.0	6.6	0	2.6	13.5	0	0	0	3.9	0	0	0	0	0
C	4.2	0	0	17.0	0.1	0	15.6	0	0	0	0	6.1	3.8	0	0	0	0

B) Group 2 (6.8 ng/ml) ivermectin

BF	1				2				3				4			
DP1BF	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
G	0*	0	0	0.6	3.0	0	0	0	11.0	0	0	19.1	0	0	0	15.4
H	0	0	0	14.9	8.4*	0	0	11.3	15.0	0	0.2	26.4	1.0	0	0	11.6
I	0	0	0	0	2.7	0	1.3	10.1	29.2*	0	0	0	3.9	0	0	0
C	0	0	0	6.6	1.3	0	0	8.7	42.3	0	0	29.2	1.3	0	0	25.8

B) Cont'd

BF	5				6				7				8				END
DP1BF	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
D	4.8	0	0	22.8	0	0	0	4.3	0	0	0	6.5	2.3	0	0	0	0
E	8.2	0	0	30.1	14.8	0	0	22.0	17.5	0	0	0.3	0	0	0	16.7	0
F	0	0	0	0	0.8	0	0	0	0	0	0	0	0	0	0	0	0
C	0.4	0	0	21.2	13.4	0	0	0	17.7	0	0	0	0	-	-	-	-

* indicates dates where the highest concentrations of ivermectin were fed to mosquitoes
† DP1BF – days post first blood feed

Table 3. The number of eggs produced per female on each day. In all cases it appears that the ingestion of ivermectin at the highest concentrations reduces the number of eggs laid per female. On average it takes two days for eggs to develop and be laid. Days when eggs were deposited before the two-day developmental period represent eggs that were produced from the previous blood meal and held longer than normal.

Table 4. Percent egg hatch rate

A) Group 1 (11.7 ng/ml) ivermectin

BF	1	2	3	4	5	6	7	8
DP1BF	0	4	8	12	16	20	24	28
D	-*	60.0	50.0	11.1	71.4	-	-	-
E	69.6	25.2*	55.8	75.3	82.2	38.1	50.0	-
F	74.5	70.7	-*	94.7	66.2	32.4	11.2	-
C	80.4	72.7	81.6	51.6	57.5	73.8	69.6	-

B) Group 2 (6.8 ng/ml) ivermectin

BF	1	2	3	4	5	6	7	8
DP1BF	0	4	8	12	16	20	24	28
G	62.6*	73.3	78.2	76.9	58.4	41.2	71.4	-
H	73.4	73.1*	81.5	70.0	80.9	65.8	50.0	0.0
I	72.8	77.4	70.1*	-	33.3	-	-	-
C	66.7	71.6	62.4	79.9	71.1	75.5	-	-

* blood meals that had the highest concentration of ivermectin fed to mosquitoes

Table 4. The egg hatch rates grouped by all eggs laid due to each blood feed. As described above, eggs produced on dates prior to days post the subsequent blood feed were included in the prior feed (see Table 3).

IV) Discussion

While the effects of ivermectin against numerous *Anopheles* species have been shown in the laboratory (Pampiglione et al. 1985, Iukabovich et al. 1989, Jones et al. 1992, Gardner et al. 1993, Fritz et al. 2009), this study differs because it specifically addresses ivermectin pharmacokinetics in humans following ingestion of a standard ivermectin MDA dose (150 µg/kg) (Fig. 1) coupled to a focus on *An. gambiae* s.s. daily feeding habits (Beier 1996, Scott et al. 2006). Since a typical ivermectin MDA results in more than 70% of the people being treated (Boatin and Richards 2006), most adult *An. gambiae* s.s. in the MDA-treated area are likely to imbibe a concentration of ivermectin that could affect survivorship, re-feeding rates, blood meal digestion, knockdown and

recovery rates, fecundity, or egg hatch rates. These experiments were performed in a controlled laboratory environment so these effects could be tested at specific human-relevant ivermectin concentrations.

Ivermectin is lipophilic and found at higher concentrations in fat and dermal tissue than in venous plasma (Baraka et al. 1996). A caveat of the experiments described in this chapter is that the ivermectin blood pharmacokinetics in humans was only measured from venous-drawn plasma samples (Elkassaby 1991). This means that a mosquito that fed on an ivermectin-treated human may potentially ingest a different amount of ivermectin when they imbibe blood from sub-dermal capillaries. Therefore, all results from these experiments are most likely underestimates of what may occur in the field in relation to the time post-ingestion of ivermectin. Specific ivermectin concentrations in any one person following MDA in the field can vary by more than 3-fold (Elkassaby 1991) and ivermectin concentrations in humans or in blood fed mosquitoes cannot be determined unless costly high-performance liquid chromatography or mass spectrometry is used.

Of the four drugs tested, only ivermectin delayed *An. gambiae* s.s. re-blood feeding frequency. This ivermectin induced re-blood feeding delay (Fig. 2) was observed at ivermectin concentrations present in human plasma two days post ingestion of ivermectin (Fig. 1). Therefore, *An. gambiae* s.s. that survived ivermectin-containing blood meals taken within two nights post MDA would still have a reduced vectorial capacity due to a delay in re-feeding frequency.

The delay in the re-feeding frequency of *An. gambiae* s.s. could potentially be explained by a delay in blood meal digestion. Mahmood et al. (1991) demonstrated that

ingestion of ivermectin led to a delay in the development of and thinner peritrophic membranes for *Ae. aegypti*. Ivermectin-treated *Ae. aegypti* had white feces in their posterior midguts, ileum, and malpighian tubules while time matched blood fed controls had no such white feces detected. The inhibited defecation of *An. gambiae* s.s. (Fig. 3) probably demonstrates delayed blood meal digestion, which may have led to the reduction in re-blood feeding frequency (Fig. 2) at ivermectin concentrations up to two nights post MDA (Fig. 1).

Even if blood feeding frequency is delayed in the field, it is likely that *An. gambiae* s.s. will imbibe several blood meals that may contain ivermectin after MDA. Figure 4 demonstrates that consecutive blood meals that contain ivermectin will further compound mosquito mortality and thus further reduce the daily probability of survivorship.

Numerous studies have noted mosquito paralysis, uncoordinated locomotion, and lethargy post ingestion of ivermectin (Tesh and Guzman 1990, Mahmood et al. 1991, Fritz et al. 2009, Chaccour et al. 2010) but none have attempted to quantify these effects. Ivermectin has an immediate knockdown effect on *An. gambiae* s.s. at concentrations found in humans up to three days post MDA (Fig. 5). Furthermore, ivermectin reduces the recovery rate of *An. gambiae* s.s. up to 48 hours post ingestion (Fig. 6). An ivermectin attributed increase in the knockdown rates and decrease in the recovery rates means that, in the field, *An. gambiae* s.s. that ingest ivermectin may succumb to predation or desiccation. Therefore, ivermectin may have greater lethal effects in the field beyond those estimated by direct mortality effects.

Comparison of *An. gambiae* s.s. survival curves after ingestion of the first non-ivermectin-containing blood meal post prior ivermectin ingestion indicates that there are not delayed effects from ingestion of sublethal ivermectin concentrations which manifests themselves later in life. One limitation of this study was that not all mosquitoes will re-feed at each blood meal, which means that many mosquitoes are lost to re-feeding as opposed to death. Due to this fact, it can not clearly be determined if *An. gambiae* s.s. survivorship later in life is impacted by prior ivermectin ingestion.

Previous studies demonstrated that older *Anopheles* are more susceptible to organophosphates, organochlorines, carbamates, and pyrethroids (Rowland and Hemingway 1987, Lines and Nassor 1991, Hodjati and Curtis 1999, Rajatileka et al. 2011). The results obtained here with ivermectin are difficult to interpret. There was a statistically significant decrease in survivorship curve slopes for *An. gambiae* s.s. that ingested 11.7 ng/ml at the first blood meal compared to the second and third blood meals (Table 2, Fig. 7). While the mosquitoes with reduced survivorship were younger, they had also not yet imbibed a blood meal, which confounds these results. To the contrary, *An. gambiae* s.s. that imbibed ivermectin at 6.8 ng/ml had a significant decrease in survivorship curve slopes at the third blood meal when compared to the second, which may indicate that indeed older mosquitoes are more susceptible to ivermectin. A different experimental design will be necessary to determine the effects of mosquito age and susceptibility to ivermectin. It has also been demonstrated that blood meal ingestion within two days post insecticide exposure increases mosquito tolerance to insecticides (Barlow and Hadaway 1956, Davidson 1958, Halliday and Feyereisen 1987, Hunt et al. 2005). The unique mode of delivery of ivermectin bypasses any confounding of blood

meal ingestion on mosquito survivorship, as the mosquito only contacts ivermectin at the point of imbibement.

An. gambiae s.s. fecundity was reduced post-ingestion of ivermectin at 11.7 and 6.8 ng/ml. A reduction in fecundity in the field may lead to diminished *Anopheles* population sizes. A reduction in fecundity may also affect potential resistance development in wild *An. gambiae* s.s. populations. Factors such as larval density dependence, reduced larval survivorship of offspring whose mothers imbibed ivermectin, heritability of resistance genes, and the fact that the ivermectin resistance mechanism has not been determined yet in mosquitoes confound the effects fecundity may have on resistance development.

These studies clearly demonstrate that there are further sublethal effects of ivermectin on *An. gambiae* s.s. that may occur in the field post ivermectin-MDA that would impact vectorial capacity beyond outright reduction of the probability of survival after one ivermectin blood meal exposure.

Chapter 4 – Bionomics of *Plasmodium* vectors in southeastern Senegal

I) Introduction

The burden of clinical malaria in Senegal is associated with *P. falciparum*, *P. ovale*, and *P. malariae*, listed in descending order of importance (Trape et al. 1994, Lemasson et al. 1997, Dia et al. 2003). In all regions, *Plasmodium* transmission is seasonal and typically peaks at the end of the rainy season and beginning of the dry season (Carrara et al. 1990, Trape et al. 1994, Dia et al. 2003, Brasseur et al. 2007, Dia et al. 2008). Roughly 12.5 million people live in Senegal (PMI 2010), a vast majority of whom live in the Dakar and Thies regions at the central-western most portion of the country along the Atlantic Ocean north of the Gambia. This area is characterized by dense human populations in urban centers with very low levels of clinical malaria cases (Trape et al. 1992, Kleinschmidt et al. 2001). The northwestern portion of Senegal has seasonal *Plasmodium* transmission along the Senegal River and delta regions (Carrara et al. 1990, Dia et al. 2008). The north-central portion of Senegal is sparsely populated but does have documented malaria transmission (Lemasson et al. 1997). The Gambia has seasonal *Plasmodium* transmission which is linked primarily to the flooding of the low-lying areas that surround the Gambia River (Majambere et al. 2010). Just below the Gambia is the region known as the Casamance, which has had political instability since the 1980s (Lambert 1998). Due to this, less public health aid and assistance is provided to the people in this region and very few malaria transmission studies are performed

there. Seasonal transmission of *P. falciparum* and *P. ovale* does occur in the Casamance (Sokhna et al. 2001, Brasseur et al. 2007). The most intense malaria transmission documented in Senegal occurs in south-eastern Senegal, which lies in the Sudano-Guinean phytogeographic zone, characterized by high annual rainfall (average of 1250 mm/year) (Dia et al. 2003).

Senegal receives aid for malaria control and treatment through the President's Malaria Initiative (PMI), which is heavily funded by US tax payers and the BMGF (PMI 2010). Unfortunately, many of the LLINs, Rapid Diagnostic Tests (RDTs), and Artemisinin-based Combination Therapies (ACTs) that are sent to Senegal never leave the West-central and north-west regions (PMI 2010). IRS is only performed in the urban centers located along the coastline north of Dakar (PMI 2010). Free distribution of LLINs in southeastern Senegal is performed by Net Life, the Against Malaria Foundation, United Nation's Children Funds, and Peace Corps volunteers (PMI 2010, personal observation). Primary health care services (i.e., Poste de la Sante) sell subsidized LLINs for 1000 CFA (~$2.2) (PMI 2010, personal observation). Commercial suppliers of LLINs reach 13 of 14 regions in Senegal, but not the Kedougou region. In south-eastern Senegal APOC-directed ivermectin MDAs are performed for onchocerciasis control (Diawara et al. 2009).

Senegal is broken into four distinct phytogeographic areas. In a north to south longitudinal gradient are the Sahelian, Sahelo-Sudanian, Sudanian, and Sudano-Guinean phytogeographic areas (Fontenille et al. 1997). Twenty *Anopheles* spp. have been documented in Senegal (Diagne et al. 1994). Of these, *An. gambiae* s.s., *An. arabiensis*, *An. funestus*, and *An. nili* are the primary *Plasmodium* vectors in various regions. Several

secondary or potential *Plasmodium* vectors occur in Senegal, including *An. pharoensis*, *An. melas*, *An. coustani*, *An. paludis*, *An. squamosus*, and *An. ziemanni*. In northern and central Senegal, in the Sahelian and Sahelo-Sudanian phytogeographic areas, most *Plasmodium* transmission is dominated by *An. arabiensis* and *An. gambiae* s.s. (Lemasson et al. 1997, Robert et al. 1998), with some *An. funestus* involvement closer to the coast (Dia et al. 2008). In north-western Senegal, along the Senegal River basin, especially at the delta, transmission is dominated by *An. pharoensis*, and secondarily by *An. arabiensis* and *An. gambiae* s.s (Carrara et al. 1990, Dia et al. 2008). The Sudanian and Sudano-Guinean phytogeographic zones see a decrease in the proportion of *An. arabiensis* as the habitat becomes wetter with a concurrent increase in *An. gambiae* s.s. and *An. funestus* becomes more critical for *Plasmodium* transmission (Fontenille et al. 1997). At the mouth of the Gambia River transmission is dominated by *An. melas* but upriver there is a shift in *An. gambiae* s.l. composition to more *An. gambiae* s.s. and *An. arabiensis* dominated *Plasmodium* transmission (Diop et al. 2002, Bogh et al. 2003). Scant entomological data are present for the Casamance region, but *Plasmodium* transmission is likely dominated by *An. gambiae* s.s. and *An. funestus* due to location in the Sudano-Guinean phytogeographic zone and most likely *An. melas* closer to the coast. In southeastern Senegal, also located in the Sudano-Guinean phytogeographic zone but farther inland, *Plasmodium* transmission is dominated by *An. gambiae* s.s., *An. funestus*, *An. arabiensis*, and in certain localities, *An. nili* (Dia et al. 2003). Southeastern Senegal has twelve or more *Anopheles* spp. routinely captured during human landing catches or pyrethrum spray catches (Dia et al. 2003, personal observation), whereas most other

regions only document three to seven *Anopheles* spp. (Lemasson et al. 1997, Fontenille et al. 1997, Carrara et al. 1990, Dia et al. 2008).

In all cases, in Senegal, *Anopheles* spp. *Plasmodium*-vector composition shifts throughout the transmission seasons (Fontenille et al. 1997, Dia et al. 2003, Lemasson et al. 1997) and between closely spaced villages (Fontenille et al. 1997, Dia et al. 2008). Many studies only test *An. gambiae* s.s., *An. arabiensis*, and *An. funestus* for *Plasmodium* sporozoites, but the diversity of *Anopheles* vectors and transmission dynamics in Senegal exemplify the need to test all other vectors that have been potentially incriminated in *Plasmodium* transmission from other regions of Africa. *Anopheles nili* is the main *Plasmodium* vector in southern Cameroon (Carnevale et al. 1992), and an important primary *Plasmodium* vector in Kinshasa, Zaire (Coene 1993) and south-eastern Senegal (Dia et al. 2003). *Anopheles pharoensis* is the dominant *Plasmodium*-vector in north-western Senegal (Carrara et al. 1990, Dia et al. 2008), Egypt (Barber and Rice 1973, El Said et al. 1983, El Said et al. 1986), an important *Plasmodium* vector in the Mwea Rice Irrigation Scheme in Kenya (Mukiama and Mwangi 1989), and a secondary *Plasmodium* vector in Chad (Kerah-Hinzoumbe et al. 2009) and Cameroon (Antonio-Nkondjio et al. 2006). Of the *An. funestus* group, two species besides *An. funestus* that have had some involvement in *Plasmodium* transmission in Tanzania are *An. rivulorum* (Wilkes et al. 1996, Temu et al. 2007) and *An. leesoni* (Temu et al. 2007). The following vectors have been identified with sporozoites from various countries in sub-Saharan Africa: *An. ziemanni* - Cameroon (Antonio-Nkondjio et al. 2006), Chad (Adam 1956, Kerah-Hinzoumbe et al. 2009), *An. coustani* - Cameroon (Vincke 1946, Gillies 1964, Antonio-Nkondjio et al. 2006), *An. paludis* - Cameroon - (Wolfs 1945, Lips 1962, Antonio-

Nkondjio et al. 2006), *An. hancocki* – Cameroon (Antonio-Nkondjio et al. 2006), Uganda (Gibbins 1932)**,** Sierra Leone (Lewis 1956), Nigeria (Hamon et al. 1956), *An. squamosus* - Tanzania (Gillies 1964), Rhodesia (Gillies and de Meillon 1968), and *An. brunnipes* - Zaire (Wanson and Berteaux 1944, Coene 1993).

This chapter will elucidate, the primary *Plasmodium*-vectors found in each village. Blood meal identification will also determine the host preference of the most abundant blood-fed vectors collected indoors by aspiration. This information can be used to formulate future field site selections and potential bionomic seasonality studies.

II) Materials and Methods

The study was conducted in the villages of Boundoucondi, Damboucoye, Nathia, Ibel and Ndebou, in rural south-eastern Senegal (Fig. 1). The five villages are located along a 15 km stretch of road heading west out of Kedougou. The study area is located in the hilly Sudano-Guinean phytogeographic zone. The rainy season lasts from June to October-November with an average yearly rainfall of 1250 mm (Dia et al. 2003). Most of the people in this area are subsistence farmers. They live in extended family compounds with 2-10 sleeping huts, and cultivate maize, sorghum and groundnuts between these compounds and in separate fields outside the village. Cattle, sheep, goats, dogs, and chickens are the primary domesticated animals in the villages. The five villages were chosen based on their proximity to the main road (Fig. 1), small size (250 – 600 people), high abundance and diversity of *Anopheles* spp. (Dia et al. 2003), active malaria cases (personal communication with Mactar Mansaly), and ivermectin MDAs performed for onchocerciasis control (Diawara et al. 2009). In 2008, two villages were

sampled, Ibel and Ndebou, and in 2009, four villages were sampled, Ndebou, Boundoucondi, Damboucoye, and Nathia. Permission to work in these villages was granted first by the Senegalese MoH and then by the residents of each village. The study was also reviewed by the Colorado State University Institutional Review Board prior to being conducted.

In 2009, oral surveys were conducted to determine the number of people that lived in each hut, whether or not they were treated by ivermectin MDA, and number of people that use an ITN. A GPS MAP 60 CSx unit (Garmin, Olathe, KS, USA) was used to record the location of each structure in the village. Each compound was designated by a number and each hut within the compound a letter.

Anopheles mosquitoes were primarily captured in order to determine the effects of ivermectin MDA on *Anopheles* survivorship (Chapter 5) and the sporozoite rate (Chapter 6). Therefore, the bionomic data presented in this chapter lack the routine collection time points necessary to determine the effects of seasonality on *Anopheles* population dynamics. Furthermore, this study was conducted with a minimal number of personnel and vehicles; future field studies requiring more in depth analyses will require more personnel and resources or altered objectives.

Indoor resting, wild, blood fed *Anopheles* mosquitoes were collected in the morning from huts people had slept in the previous night using backpack aspirators (John W. Hock, Gainesville, FL, USA). In 2008, only one team of four people performed aspirations at a time so only one village could be aspirated each day. In 2009, two teams of four people performed aspirations which allowed for two villages to be sampled on the same day. After capture, *Anopheles* were transferred by mouth aspirators from backpack

aspirator cups to 473 ml cardboard containers screened with organdy. The containers were labeled and designated by village, date collected, and the specific hut from which mosquitoes were collected (see Chapter 5 for survivorship analysis).

Centers for Disease Control (CDC) light traps (John W. Hock, Gainesville, FL, USA) were hung next to people that slept under ITNs in the late-afternoon in two villages and collected the following morning. CDC light traps are an effective and ethically safer replacement of the standard human landing catch (HLC) to estimate *An. gambiae* s.l. abundance (Mbogo et al. 1993, Magbity et al. 2002). Eight CDC traps per sampling night were hung indoors in 2008 and 2009, and in 2009, two CDC traps were hung outdoors next to a person who slept under an ITN. Indoor CDC traps were randomly chosen each night (Magbity and Lines 2002), while outdoor CDC traps were hung in the same two locations every time.

In 2009, standard window traps were made from metal, with collapsible frames, with mosquito netting mesh to form a funnel (Silver 2008). Window traps were placed on the outside of windows of eight huts in Boundoucondi and Ndebou in 2009. Unfortunately, only 4 *Anopheles* spp. were captured in window traps so their use was discontinued (data not shown).

Mosquitoes were identified morphologically to species (Diagne et al. 1994, Hervy et al. 1998) in the field insectary immediately following their death. The abdomens were separated from the thoraxes of all *Anopheles* spp. and placed into two separate 1.5 ml microfuge tubes with silica gel desiccant T.H.E. (EMD Chemicals, NJ, USA). The tubes were labeled with the village, collection date, and hut location, and all relevant information was recorded on matching log sheets. Processed mosquitoes were shipped

back to Colorado State University for further molecular analysis. DNA was extracted with the Qiagen DNeasy kit (Qiagen Sciences, Maryland, USA) and a Qiacube robot (Qiagen Sciences, Maryland, USA) in 2009, and by phenol chloroform extraction in 2008. Multiplex polymerase chain reaction was used to molecularly identify members of the *An. gambiae* s.l. complex (Scott et al. 1993), *An. funestus* group (Koekemoer et al. 2002), and *An. nili* group (Kengne et al. 2003). A subset of twelve or fewer *An. gambiae* s.l. or *An. funestus* group from each collection day, that died within one day of capture and contained undigested blood at the time of processing, had their blood meals analyzed by the multiplex polymerase chain reaction to determine the source of blood (Kent and Norris 2005). The annealing temperature of the Kent and Norris (2005) protocol was adjusted from 58°C to 50°C.

Thoraxes were tested for *P. falciparum* or *P. ovale*, *P. vivax*, and *P. malariae* by Taqman-PCR (Bass et al. 2008). Laboratory-confirmed *P. falciparum* sporozoite-infected *An. gambiae* s.s. (provided by Dr. Alvaro Molina-Cruz) and plasmid-*P. vivax*, *P. ovale*, and *P. malariae* were used as positive controls for calibration. Aspirated *Anopheles* were held for up to five days for survivorship analysis (see Chapter 5). This means that the extrinsic incubation period (EIP) was artificially extended for *Anopheles* that were held in the insectary after their collection. Therefore, a weighting system was developed to equalize any differences in *Plasmodium* development that may have occurred by extending the life of the mosquito beyond the point of collection. A conservative estimate for the EIP of 10 days for *Plasmodium* development in *An. gambiae* s.s. was assumed. For each day post collection that a *Plasmodium*-positive

Anopheles was held, a 0.1 multiplier was applied (eg. if a positive mosquito survived two days post capture it was multiplied by 0.8, four days post capture 0.6, etc.).

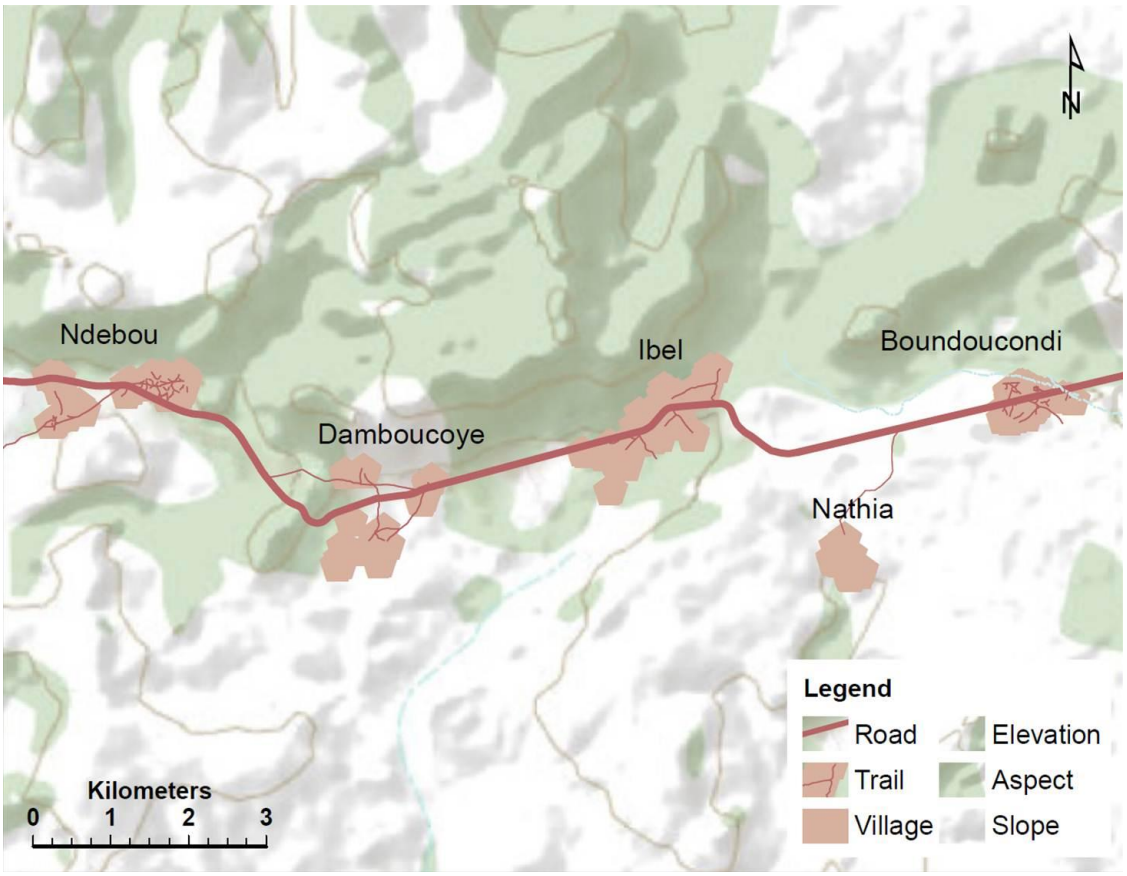

Figure 1. Field site map

III) Results

Surveyed people reported anywhere from 53.5 to 94% of people utilized ITNs across the four villages in 2009: Ndebou 53.5% (228/426), Boundoucondi 92.2% (273/296), Nathia 94% (188/200), and Damboucoye 91.8% (214/233). Although Ibel and Ndebou were not surveyed for ITN use in 2008, these villages most likely had similar ITN usage, based on presence of ITNs in the huts in 2008 and that the Net Life distribution in that area had occurred two years prior (personal communication, Peace

Corp volunteers). The data regarding the percent of people that ingested ivermectin will be presented in Chapter 5.

Eighteen of the twenty morphologically distinguishable *Anopheles* species previously reported in Senegal (Diagne et al. 1994) were captured by either aspiration or CDC light traps. There were distinct temporal and spatial differences in *Anopheles* collections from the various villages. A total of 7089 *Anopheles* mosquitoes were collected over the course of this study: in 2008, 315 by CDC trap and 330 by aspiration, and in 2009, 3875 by CDC trap and 2569 by aspiration. A total of 490 CDC light trap nights were performed; 112 in 2008, and 376 in 2009. Of the 178 *Anopheles* that were *Plasmodium*-positive, 174 (97.8%) were *P. falciparum* positive, 3 (1.7%) were either *P. ovale, P. malariae,* or *P. vivax,* and one (0.6%) *An. gambiae* s.s. was positive for *P. falciparum* and either *P. ovale*, *P. malariae*, or *P. vivax*. *An. gambiae* s.s., *An. arabiensis*, *An. funestus*, *An. leesoni*, and *An. coustani* were *Plasmodium*-positive (Table 2). Of the *An. gambiae* s.l. and *An. funestus* group mosquitoes identified as blood fed in the field, 63.9% (493/772) produced successful blood meal identification by PCR. The following figures depict the percent and numbers of each mosquito species caught per night by CDC light trap (Figs. 2, 3 and 4), indoors v outdoors (Figs. 5 and 6), molecular species composition of the *An. funestus* group (Fig. 7) and *An. gambiae* s.l. (Fig. 8), aspiration composition (Figs. 9, 10 and 11), blood meal composition of aspirated (Figs. 12 and 13) and CDC light trap caught mosquitoes (Fig. 14) from 2008 and 2009 from all five villages.

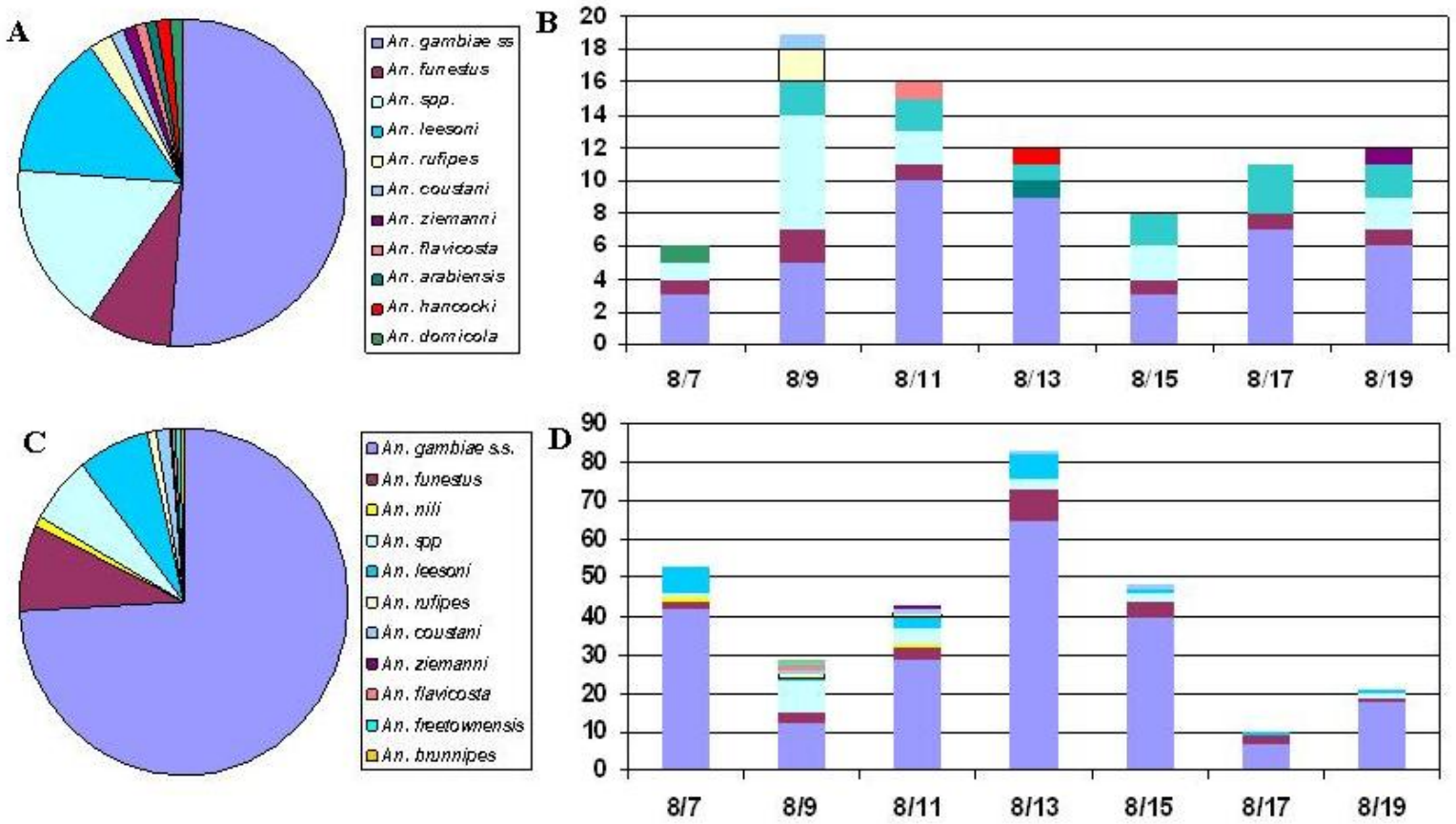

Figure 2. *Anopheles* composition from CDC light traps from Ibel (A,B) and Ndebou (C,D) in 2008. *Anopheles* spp. composition from Ibel (A,B) n = 86, and Ndebou (C,D) n = 229 captured by CDC light trap in 2008. Pie charts (A) and (C) represent the total percent composition each *Anopheles* species comprises, while stacked bar graphs (C) and (D) represents the number of *Anopheles* caught (y-axis) each aspiration by date (x-axis).

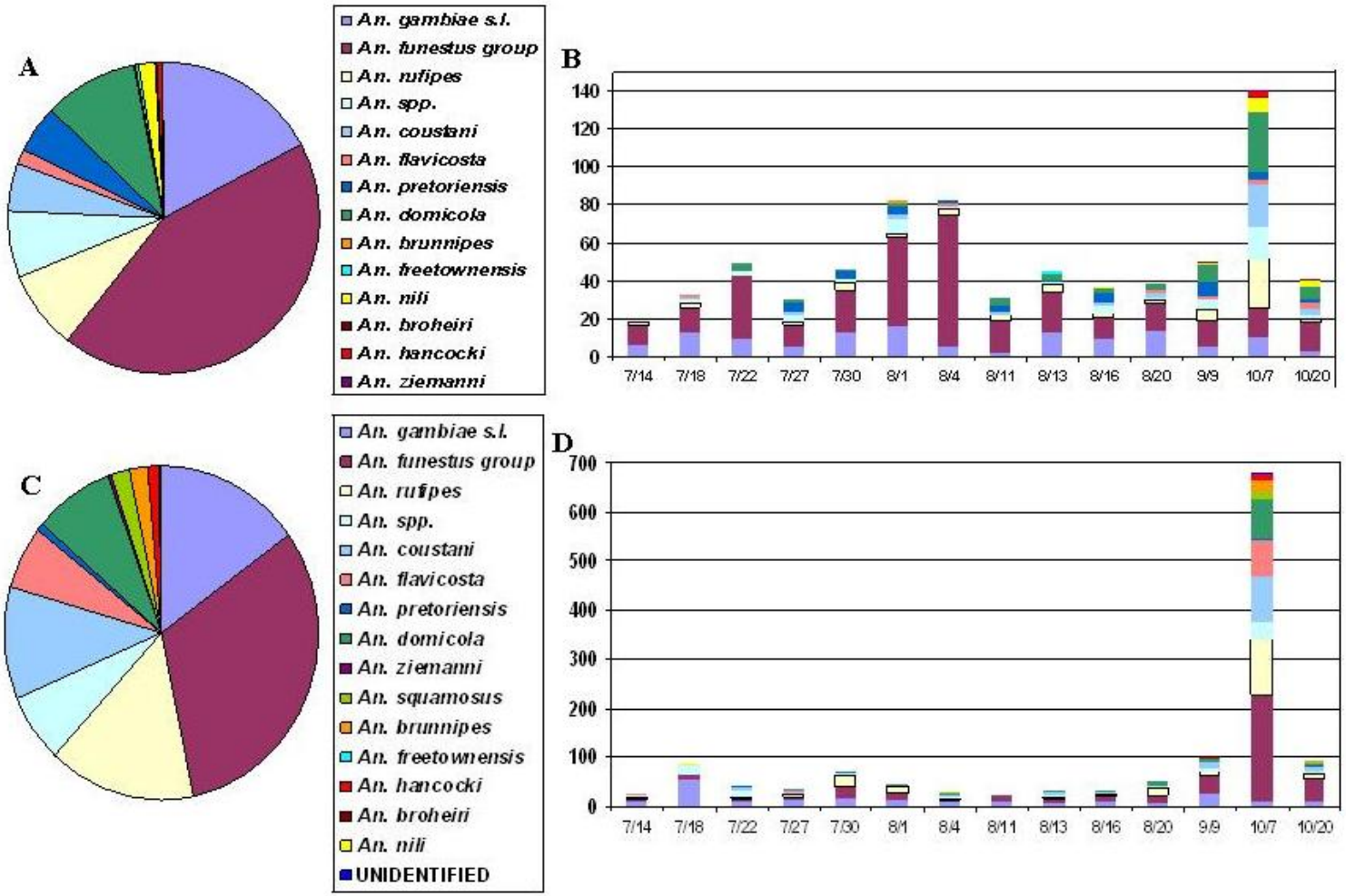

Figure 3. *Anopheles* composition from CDC light traps from Ndebou (A,B) and Boundoucondi (C,D) in 2009.

Anopheles spp. composition from Ndebou (A,B) n = 725, and Boundoucondi (C,D) n = 1347 captured by CDC light trap in 2009. Pie charts (A) and (C) represent the total percent composition each *Anopheles* species comprises, while stacked bar graphs (C) and (D) represents the number of *Anopheles* caught (y-axis) each aspiration by date (x-axis).

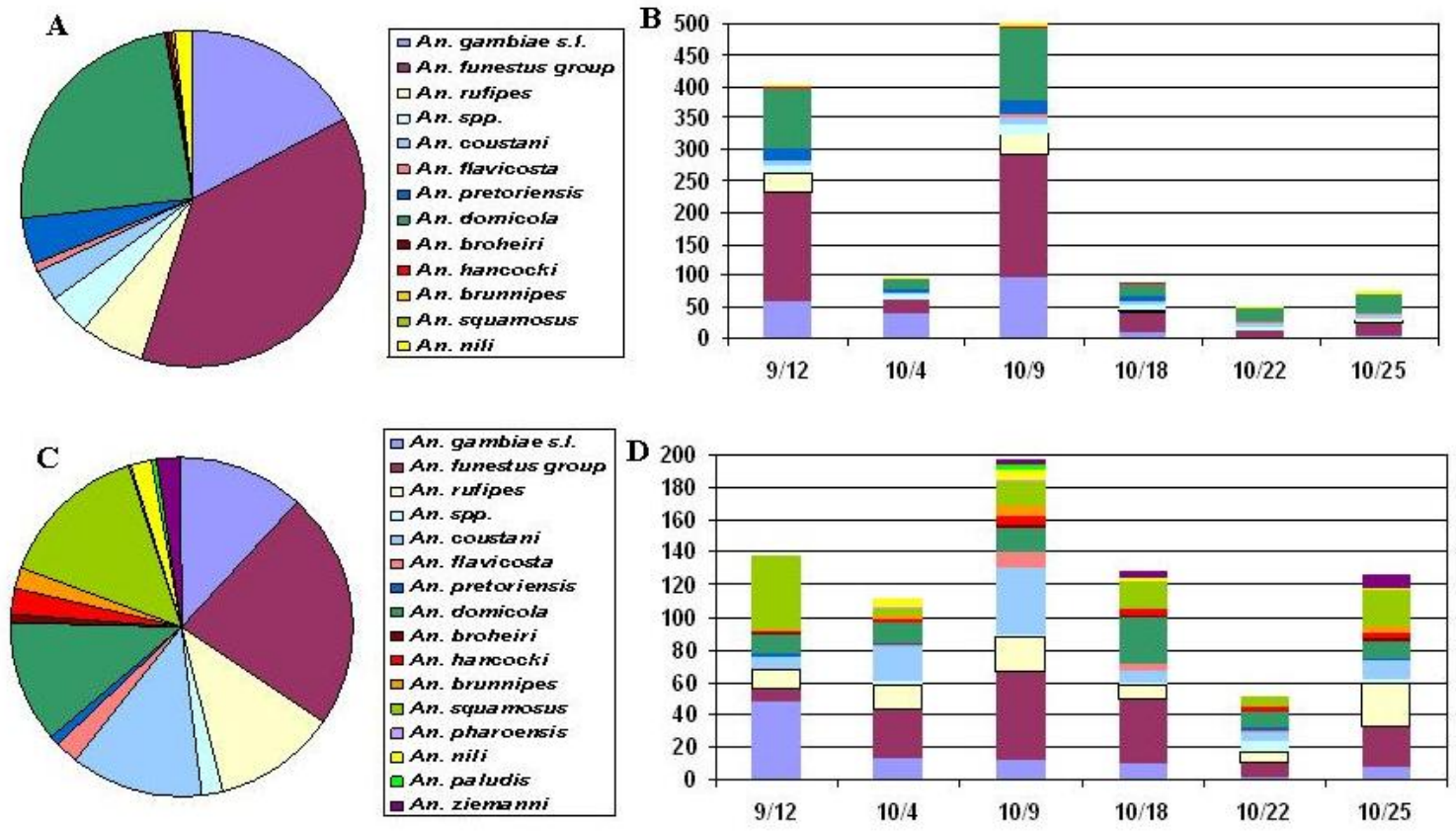

Figure 4. *Anopheles* composition from CDC light traps from Damboucoye (A,B) and Nathia (C,D) in 2009. *Anopheles* spp. composition from Damboucoye (A,B) n = 1195, and Nathia (C,D) n = 608 captured by CDC light trap in 2009. Pie charts (A) and (C) represent the total percent composition each *Anopheles* species comprises, while stacked bar graphs (C) and (D) represents the number of *Anopheles* caught (y-axis) each aspiration by date (x-axis).

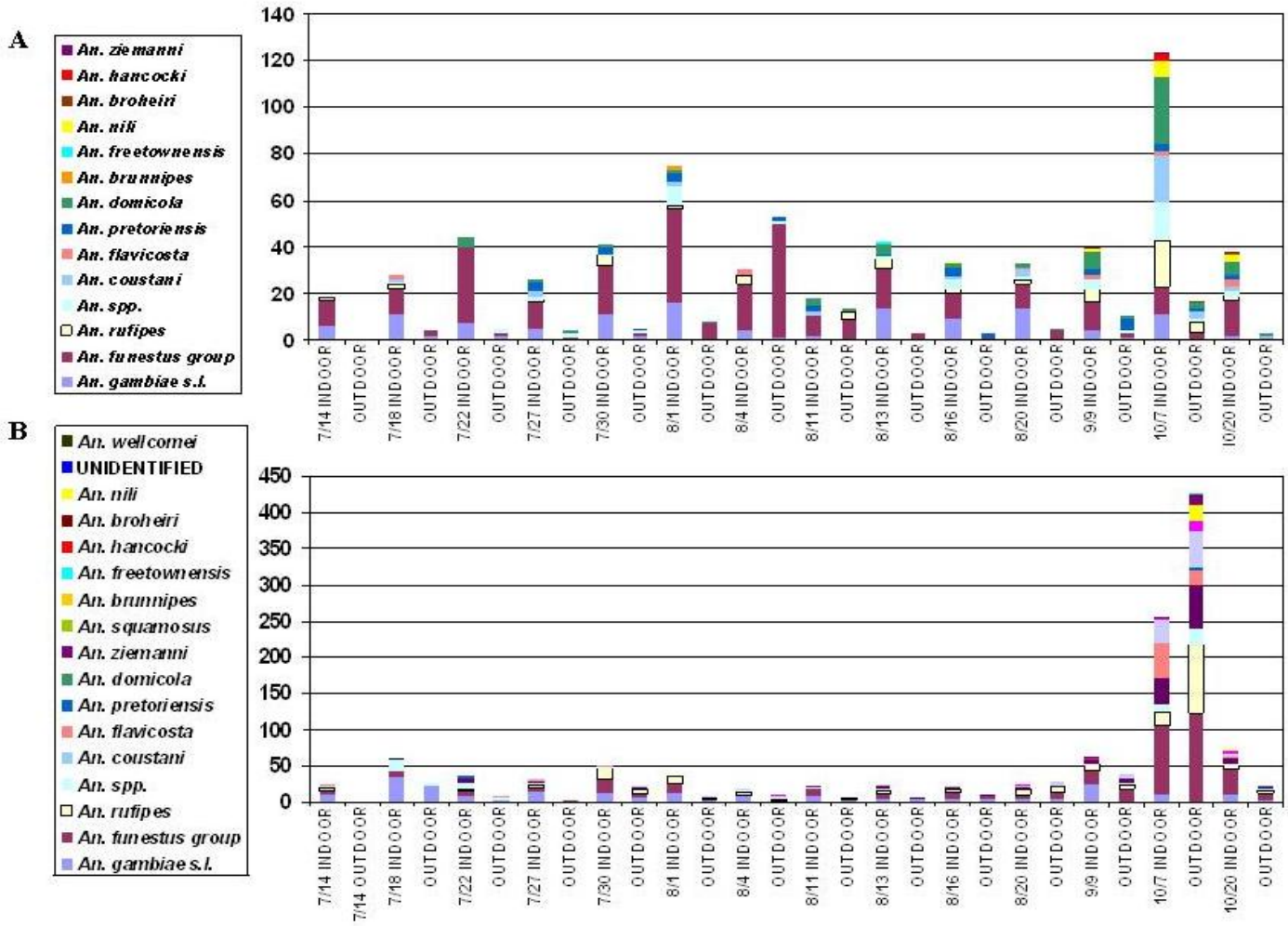

Figure 5. The number of *Anopheles* caught by CDC light trap indoors and outdoors from Ndebou (A) and Boundoucondi (B). The total number of *Anopheles* caught by CDC light trap by date and location (indoors versus outdoors) from Ndebou (A) and

Boundoucondi (B) in 2009. Stacked bar graphs represent the number of *Anopheles* caught (y-axis) each aspiration by date (x-axis) and whether they were collected indoors or outdoors.

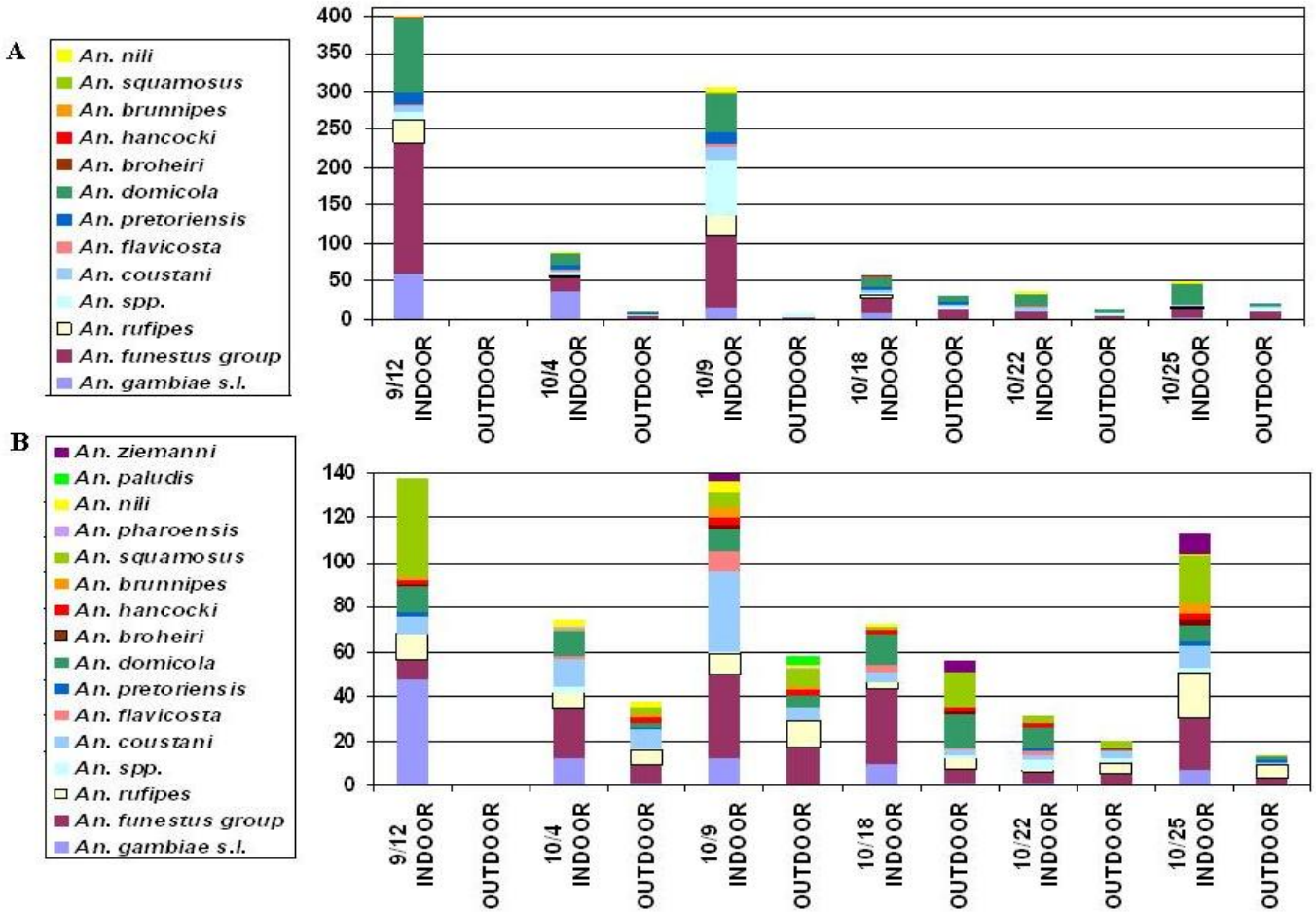

Figure 6. The number of *Anopheles* caught by CDC light trap indoors and outdoors from Damboucoye (A) and Nathia (B). The total number of *Anopheles* caught by CDC light trap by date and location (indoors versus outdoors) from Damboucoye (A) and Nathia (B) in 2009. Stacked bar graphs represent the number of *Anopheles* caught (y-axis) each aspiration by date (x-axis) and whether they were collected indoors or outdoors.

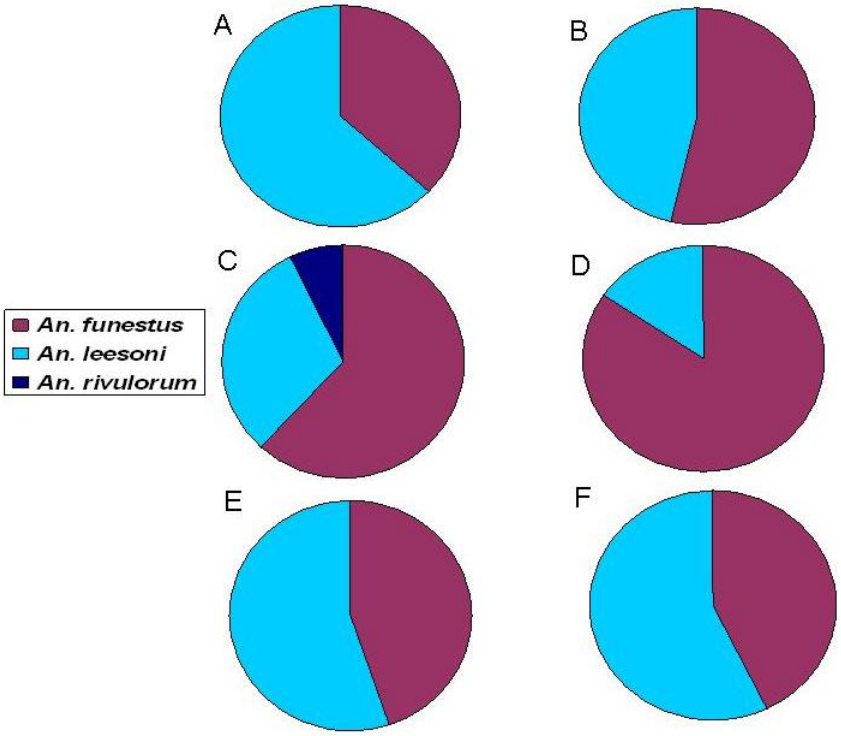

Figure 7. Percent composition of *Anopheles funestus* group collected in CDC light traps. Percent composition of *An. funestus* group collected by CDC light traps: A – Ibel 2008 n = 20, B – Ndebou 2008 n = 43, C – Ndebou 2009 n = 42, D – Boundoucondi 2009 n = 45, E – Damboucoye 2009 n = 29, F – Nathia 2009 n = 63.

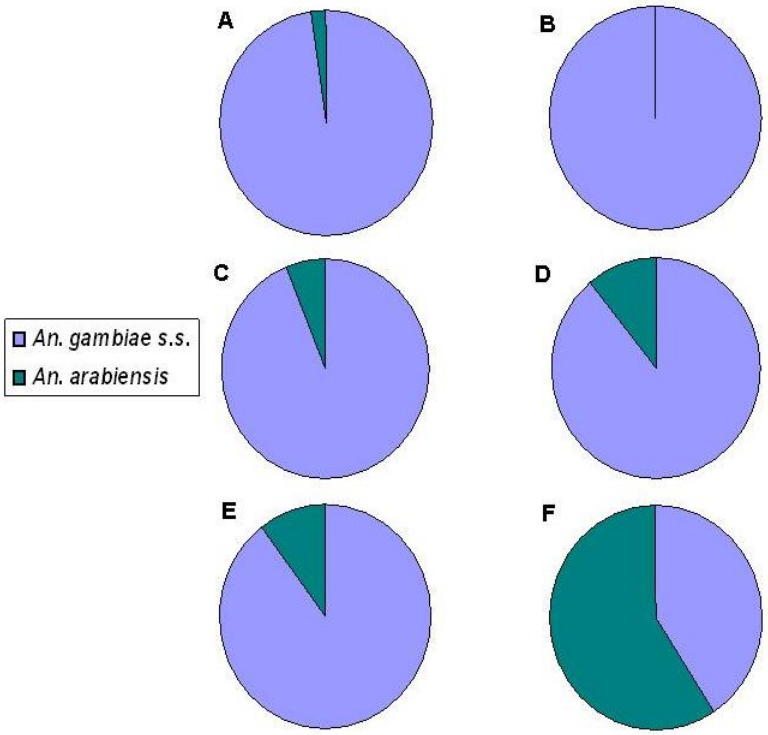

Figure 8. Percent composition of *Anopheles gambiae* s.l. collected in CDC light traps. Percent composition of *An. gambiae* s.l. collected by CDC light traps: A – Ibel 2008 n = 44, B – Ndebou 2008 n = 213, C – Ndebou 2009 n = 114, D – Boundoucondi 2009 n = 192, E – Damboucoye 2009 n = 126, F – Nathia 2009 n = 54.

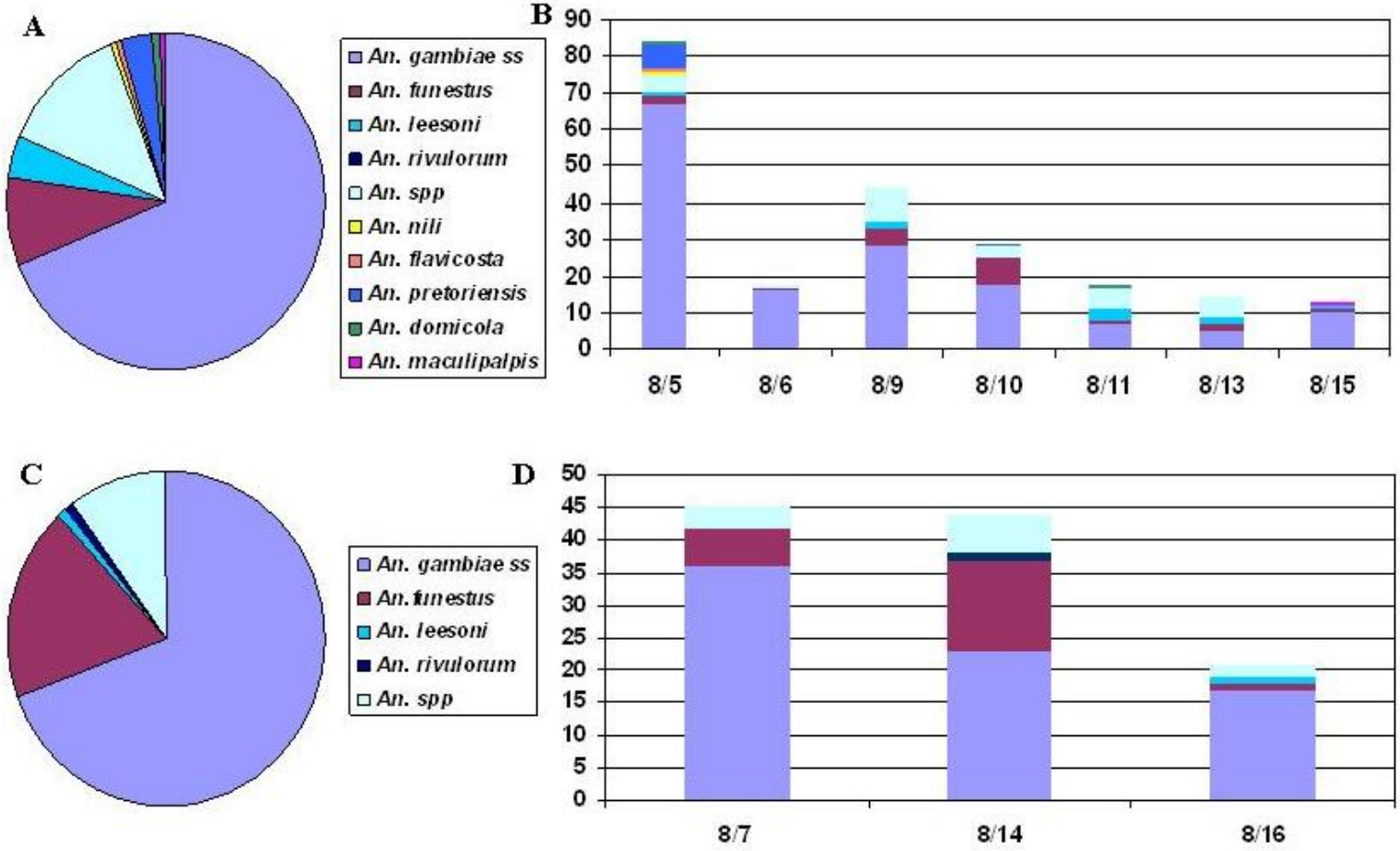

Figure 9. *Anopheles* composition from aspiration collections from Ibel (A,B) and Ndebou (C,D) in 2008. *Anopheles* spp. composition from Ibel (A,B) n = 220, and Ndebou (C,D) n = 110 captured by aspiration in 2008. Pie charts (A) and (C) represent the

total percent composition each *Anopheles* species comprises, while stacked bar graphs (C) and (D) represents the number of *Anopheles* caught (y-axis) each aspiration by date (x-axis).

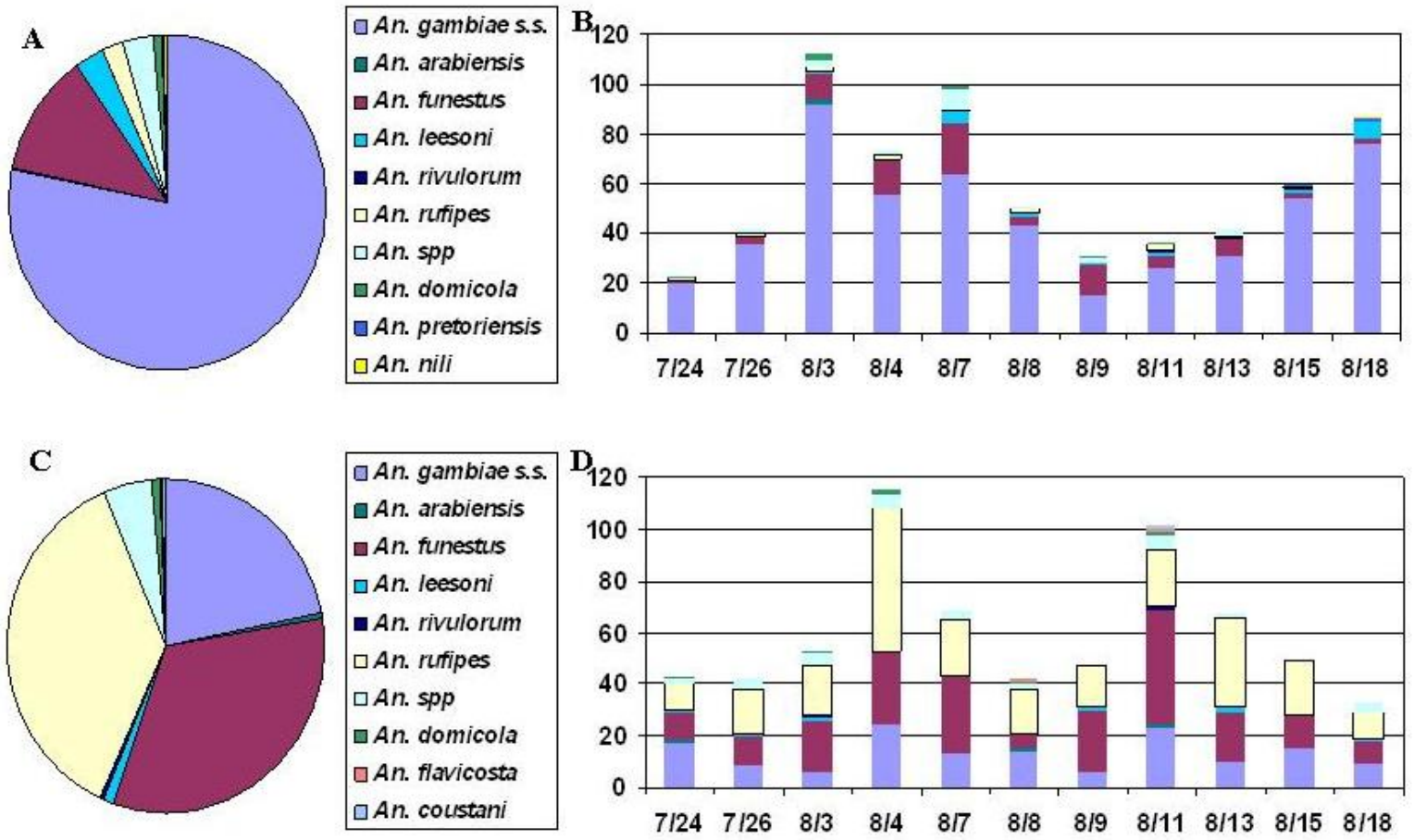

Figure 10. *Anopheles* composition from aspiration collections from Ndebou (A,B) and Boundoucondi (C,D) in 2009.
Anopheles spp. composition from Ndebou (A,B) n = 657, and Boundoucondi (C,D) n = 661 captured by aspiration in 2009. Pie charts (A) and (C) represent the total percent composition each *Anopheles* species comprises, while stacked bar graphs (C) and (D) represents the number of *Anopheles* caught (y-axis) each aspiration by date (x-axis).

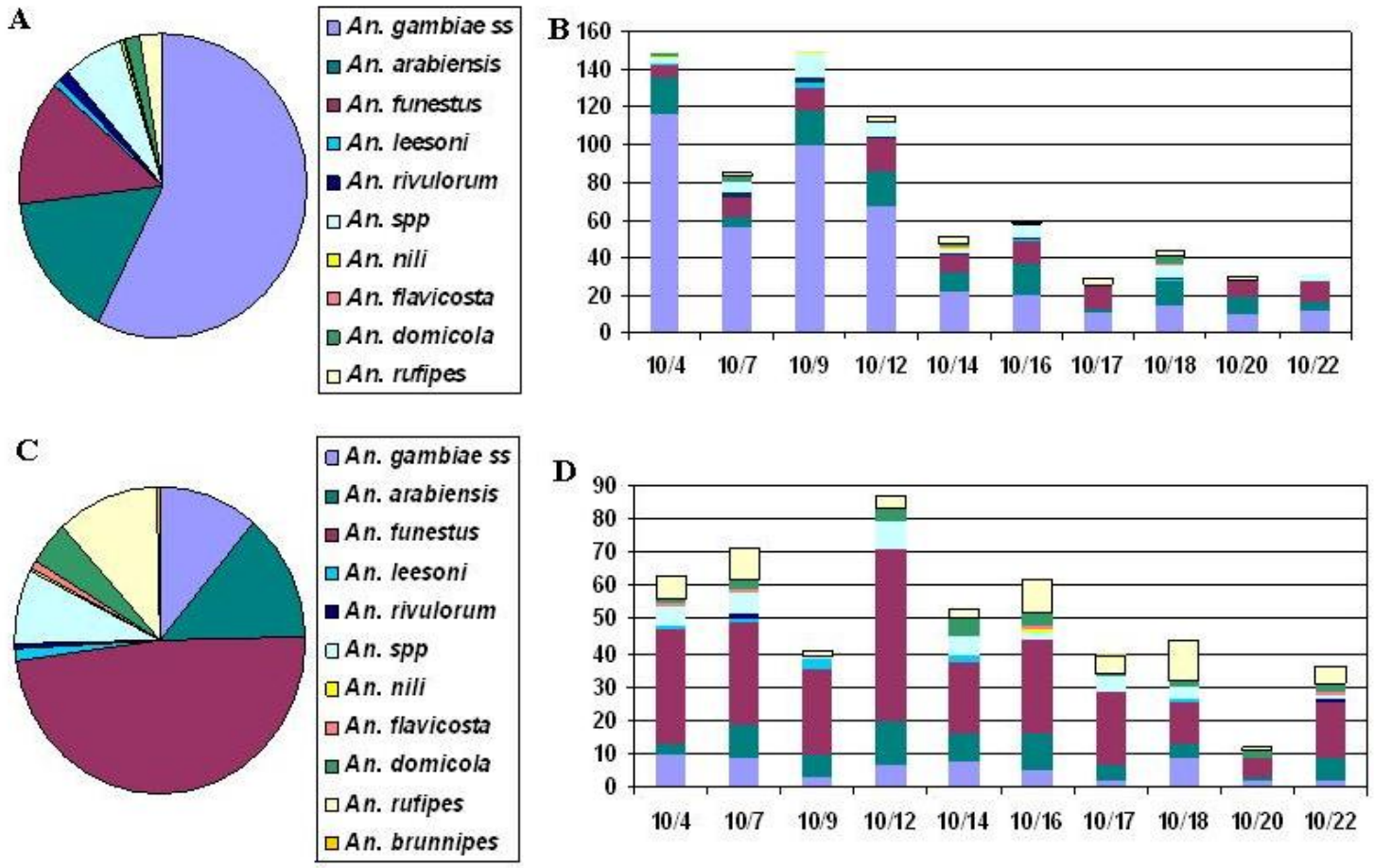

Figure 11. *Anopheles* composition from aspiration collections from Damboucoye (A,B) and Nathia (C,D) in 2009.
Anopheles spp. composition from Damboucoye (A,B) n = 742, and Nathia (C,D) n = 509 captured by aspiration in 2009. Pie charts (A) and (C) represent the total percent composition each *Anopheles* species comprises, while stacked bar graphs (C) and (D) represents the number of *Anopheles* caught (y-axis) each aspiration by date (x-axis).

Table 1. Percent of *Anopheles* spp. *Plasmodium* sporozoite-positive by year, village, and collected by aspiration

		An. gambiae s.s.	*An. arabiensis*	*An. funestus*	*An. leesoni*	*An. rivulorum*	*An.* spp.
2008	**Ibel**	3.3 (151)	-	0 (18)	7.1 (7)	-	3.9 (13)
	Ndebou	3.3 (76)	-	9.5 (19)	50 (1)	0 (1)	0 (8)
	Total	3.1 (227)	-	4.9 (37)	12.5 (8)	0 (1)	2.4 (21)
2009	**Ndebou**	3.1 (507)	0 (2)	0.7 (72)	0 (18)	0 (1)	0 (5)
	Boundoucondi	0.8 (145)	0 (4)	0 (197)	0 (7)	0 (4)	-
	Damboucoye	13.4 (423)	2.5 (104)	0 (89)	0 (3)	0 (7)	0 (2)
	Nathia	7.3 (55)	0.8 (66)	0.3 (181)	0 (6)	0 (2)	0 (2)
	Total	6.8 (1130)	1.9 (176)	0.2 (539)	0 (34)	0 (14)	0 (9)

Table 1. The percent *Plasmodium* sporozoite-positive and (number tested) of each species caught by aspiration, tabulated by year and village.

Table 2. Percent of *Anopheles* spp. *Plasmodium* sporozoite-positive by year, village, and collected by CDC light trap

	Anopheles	***gambiae*** s.s.	***arabiensis***	***funestus***	***leesoni***	spp.	***nili***	***coustani***	***ziemanni***	***hancocki***	***squamosus***	***brunnipes***
2008	**Ibel**	2.2 (45)	0 (1)	0 (7)	0 (12)	0 (14)	-	0 (1)	0 (1)	0 (1)	-	-
	Ndebou	3.4 (204)	-	15.8 (19)	0 (20)	0 (10)	0 (1)	0 (4)	-	-	-	0 (1)
	Total	3.2 (249)	0 (1)	11.5 (26)	0 (32)	0 (24)	0 (1)	0 (5)	0 (1)	0 (1)	-	0 (1)
2009	**Ndebou**	0.9 (111)	0 (7)	-	-	0 (13)	0 (9)	0 (37)	0 (1)	0 (6)	-	0 (2)
	Boundoucondi	1.2 (170)	0 (21)	-	-	0 (8)	0 (1)	2.9 (35)	0 (1)	0 (5)	-	-
	Damboucoye	6.2 (113)	0 (13)	-	-	0 (8)	0 (13)	0 (43)	-	0 (5)	0 (3)	0 (1)
	Nathia	0 (22)	0 (29)	-	-	0(4)	0 (14)	0 (52)	0 (11)	0 (20)	0 (108)	0 (14)
	Total	2.4 (416)	0 (70)	-	-	0 (33)	0 (37)	0.6 (167)	0 (13)	0 (36)	0 (111)	0 (17)

Table 2. The percent *Plasmodium* sporozoite-positive and (number tested) of each species caught by CDC light trap, tabulated by year and village.

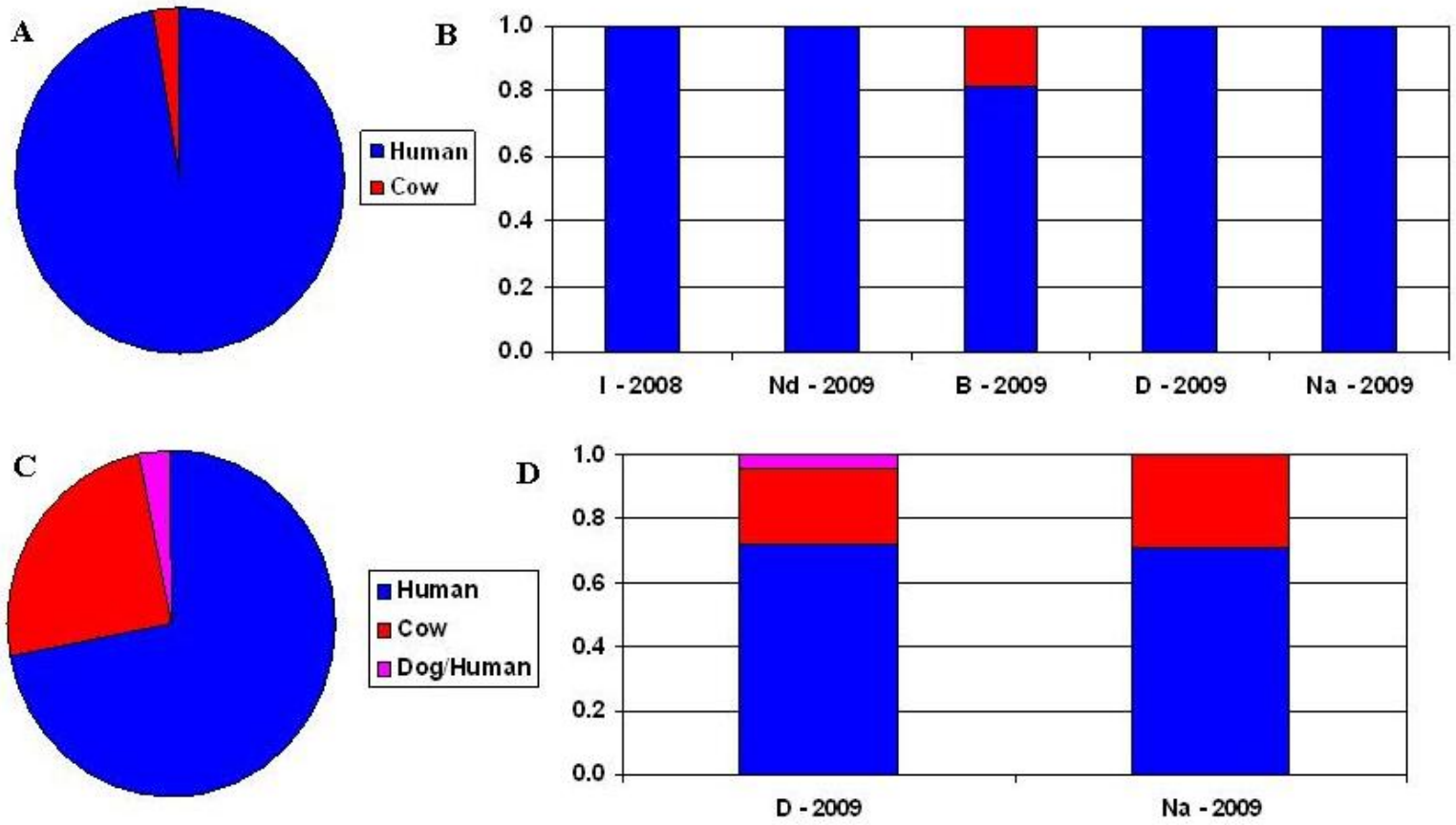

Figure 12. Blood meal composition for *An. gambiae* s.s. (A,B) and *An. arabiensis* (C,D) collected by aspiration.
Pie charts (A,C) depict the total percent blood meal composition for *An. gambiae* s.s. (A) n = 125 and *An. arabiensis* (B) n = 32. Stacked bar graphs (B,D) depicts the percent blood meal composition by village for *An. gambiae* s.s. (B) Ibel – 2008 n = 23, Ndebou – 2009 n = 43, Boundoucondi – 2009 n = 16, Damboucoye – 2009 n = 37, and Nathia – 2009 n = 6, and *An. arabiensis* (D) Damboucoye – 2009 n = 25 and Nathia – 2009 n = 7.

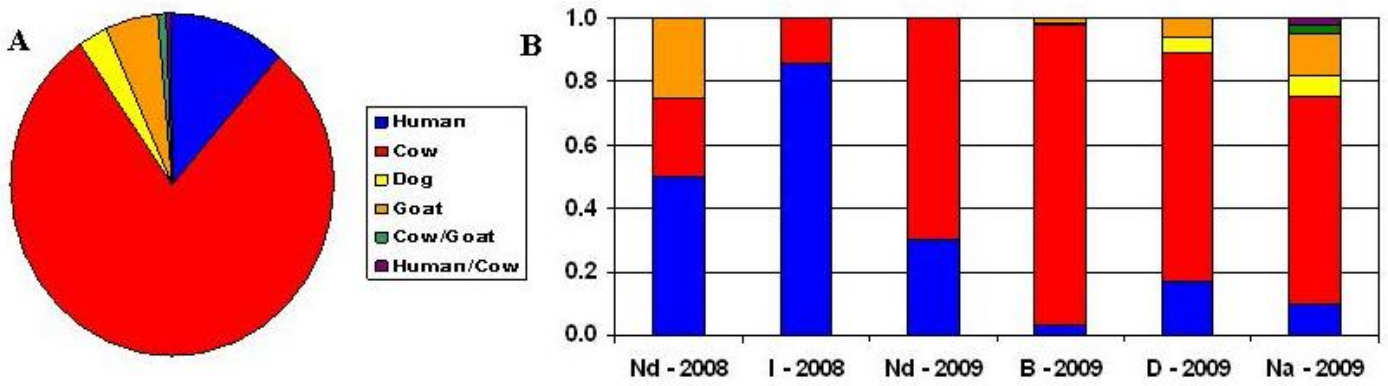

Figure 13. Blood meal composition of *Anopheles funestus* collected by aspiration. The percent of all aspirated *An. funestus* blood meal IDs is represented in the pie chart (A) n = 225. The stacked bar graph (B) depicts the percent blood meal composition by village for *An. funestus* Ndebou – 2008 n = 4, Ibel – 2008 n = 7, Ndebou – 2009 n = 10, Boundoucondi – 2009 n = 107, Damboucoye – 2009 n = 36, and Nathia – 2009 n = 61.

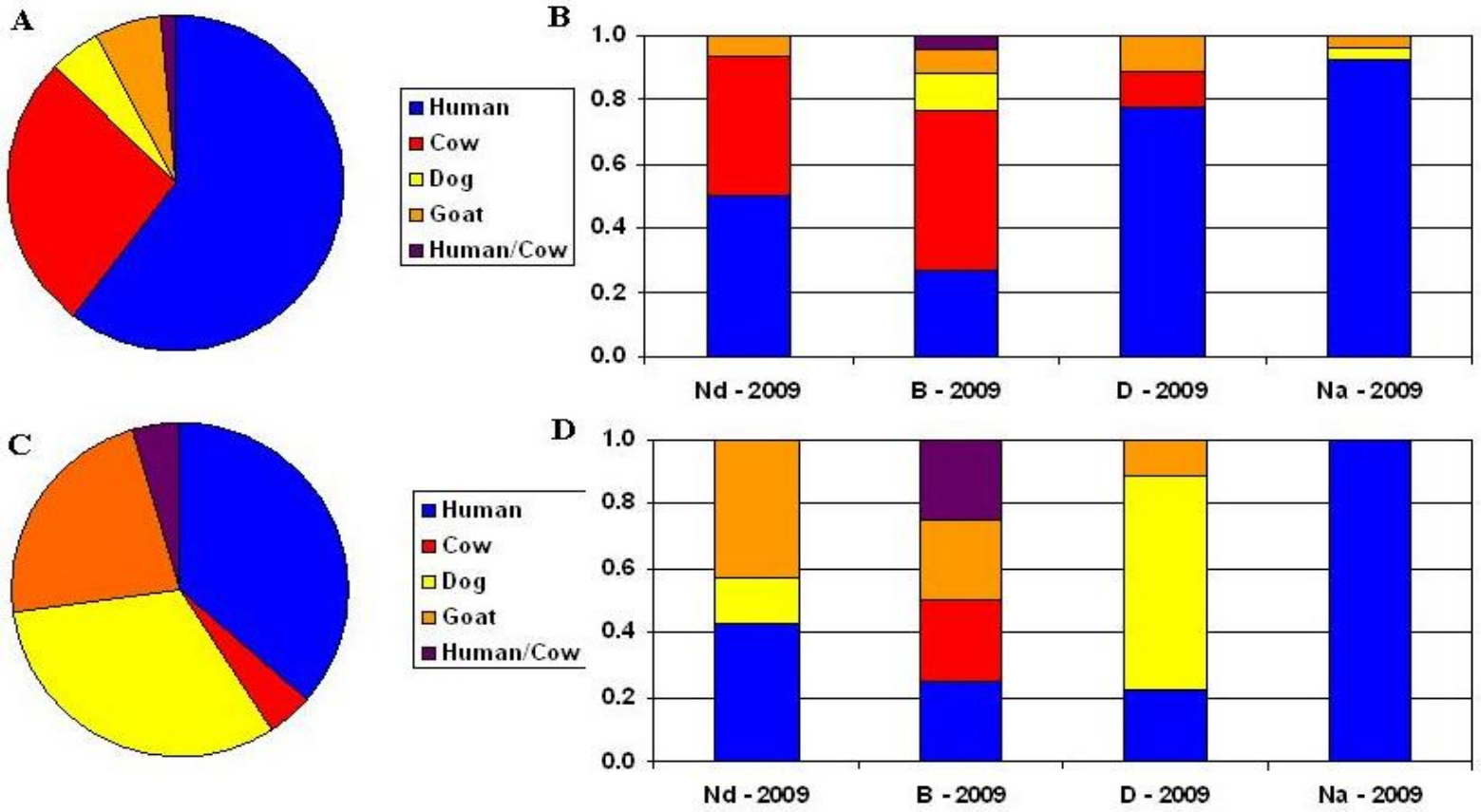

Figure 14. Blood meal composition for *An. funestus* (A,B) and *An. leesoni* (C,D) collected by CDC light trap. Pie charts (A,C) depict the total percent blood meal composition for *An. funestus* (A) n = 78 and *An. leesoni* (B) n = 22. Stacked bar graphs (B,D) depicts the percent blood meal composition by village for *An. funestus* (B) Boundoucondi – 2009 n = 26, Ndebou – 2009 n = 16, Damboucoye – 2009 n = 9, and Nathia – 2009 n = 27, and *An. leesoni* (D) Boundoucondi – 2009 n = 4, Ndebou – 2009 n = 7, Damboucoye – 2009 n = 9, and Nathia – 2009 n = 2.

IV) Discussion

Based on the high level of human blood meals (Fig. 13), sporozoite rate (Tables 1 and 2), and frequency of capture (Figs. 2, 3, 4, 5, 6, 8, 9, 10, and 11) *An. gambiae* s.s. appears to be the most important *Plasmodium* vector in these villages, followed by *An. funestus* and *An. arabiensis*. Dia et al. (2003) demonstrated peak *An. arabiensis* abundance at the end of the rainy season and beginning of the dry season (October-November). *An. arabiensis* was only caught by aspiration or CDC light trap in large numbers from Damboucoye and Nathia in October (Figs. 8, 11). The low abundance of *An. arabiensis* limits its involvement in *Plasmodium* transmission in this area.

The *An. funestus* results are difficult to interpret. In 2008, the *An. funestus* sporozoite rate by aspiration was 4.9% of 37 tested but in 2009 it was only 0.2% of 539 tested across all of the villages sampled (Table 1). Ndebou aspiration collections had the highest sporozoite rates for *An. funestus* in both, 2008, 9.5% of 19 and 2009, 0.7% of 72 tested. The precipitous drop in sporozoite rates from 2008 to 2009 aspiration collections indicated that processing the *An. funestus* caught by CDC light trap in 2009 would not be worthwhile due to cost and time efforts required to do so. Ndebou was treated by ivermectin MDA in 2009 and it is possible this could have affected *An. funestus* survivorship (see Chapter 5) and therefore the resultant sporozoite rate (see Chapter 6). Dia et al. (2003), in nearby Ngari, found total sporozoite rates across one field season (July – November 2001) to be similar to *An. gambiae* s.s. but the entomological inoculation rate (EIR) for *An. gambiae* s.s. was three times greater because the abundance of *An. gambiae* s.s. feeding on humans as determined by the HLC was greater. Unfortunately, in the current study, the EIR cannot be determined as the number of

humans sleeping indoors on the night of CDC trapping was not recorded. Dia et al. (2003) caught almost twice as many *An. funestus* group outdoors as indoors, however, molecular speciation was not performed. The results from the current outdoor versus indoor collections are difficult to interpret because sampling efforts were not standardized (i.e., 2 permanent outdoor collection sites versus 8 indoor collections sites that were randomly selected each trap night). Only blood fed *An. funestus* group specimens from CDC light trap collections were processed and identified molecularly to species which further complicates interpretation of results.

Based on 30 separate publications across sub-Saharan Africa, it was determined that *An. funestus* had a human blood meal index (HBI) of 0.980, which was the highest HBI for any of the 33 *Plasmodium* vectors assessed worldwide (Kiszewski et al. 2004). Results obtained in the current study found *An. funestus* to feed more frequently on domesticated animals, especially cattle, than humans (Figs. 13, 14). Cattle are kept on the edges of all of the villages and they are commonly sold during the rainy season. Furthermore, cattle frequently migrate through the study area as they are herded from surrounding villages to Kedougou for transport to Dakar (personal observation). In the Gambia, it was demonstrated that various *Anopheles* species enter houses to either blood feed or to utilize the structure as a resting site (Snow 1987). This was observed with *An. rufipes* that fed outdoors on cattle and then entered homes to rest. In the current study, it appears that *An. funestus* frequently rest indoors after blood feeding outdoors. This is evidenced by the high proportions of cattle blood meals and the fact that cattle are not kept indoors with people. Goats, dogs, and chickens are sometimes allowed to enter homes at night in the study area, but the infrequency of goat and dog blood meals

compared to cattle indicate that *An. funestus* are not entering homes to feed on these vertebrates.

In Burkina Faso, there is chromosomal evidence of incipient speciation of *An. funestus* 'Folonzo' and 'Kiribina' forms (Guelbeogo et al. 2005). The two forms differ spatially in their larval habitat distribution, with Kiribina occurring in irrigated agricultural areas and Folonzo in classic savannah (Dabire et al. 2007), and the distribution of the two forms were shown to differ temporally within and across years (Guelbeogo et al. 2009). *An. funestus* from the Kedougou area were the Folonzo form (Dia et al. 2000). In the Kedougou area, 81.3% of 471 *An. funestus* blood meals were human (Lochouarn et al. 1998). In nearby Wassadou, dominated by the Folonzo form as well, *An. funestus* were more zoophilic, only 33.3% of 30 blood meals were human. In Dielmo and Kouvar/Sankagne, Senegal, *An. funestus* was identified as the Folonzo form with 91.0% of 1149 and 74.3% of 179 blood meals identified as human, respectively. As evidenced by Guelbeogo et al. (2009) it is possible that cytogenetic forms may differ from year to year in the same area, however there have not been any direct observations of *An. funestus* forms differing in their blood feeding propensities on humans. Evidence from these studies does not suggest that the *An. funestus* cytogenetic form specifically determines anthropophilic or zoophilic blood feeding behavior. As there are no molecular assays to determine *An. funestus* cytogenetic forms; Folonzo and Kiribina forms cannot be determined in this study.

One of eight *An. leesoni* were found *P. falciparum*-positive in Ndebou in 2008. The positive mosquito was held for five days in the insectary, which may have artificially enhanced development of *Plasmodium*. No other *An. leesoni* examined in 2009 were

Plasmodium-positive. In this study, *An. leesoni* appears to be more zoophilic than *An. funestus* (Fig. 14). Temu et al. (2007), in Tanzania, found higher than expected proportions of *An. leesoni* thoraxes *P. falciparum*-positive and this is the only study to date to incriminate *An. leesoni* in *Plasmodium* transmission. The lack of evidence for *An. leesoni* as a *Plasmodium* vector may be because a molecular assay to determine this species from the morphologically indistinguishable adult members of the *An. funestus* group was only recently developed (Koekemoer et al. 2002). Future field studies should always molecularly distinguish the *An. funestus* group.

Surprisingly, *An. nili* was not frequently found in this study. In nearby Ngari, Senegal, Dia et al. 2003 calculated as many as 60 bites/human/night for *An. nili* in October by HLCs. Service (1964) found that very few *An. nili* rested indoors as evidenced by pyrethrum spray catches but were frequently collected in window exit traps, which indicates that this species is endophagic but exophilic. This may explain why *An. nili* was infrequently collected by aspiration in the current study. Ngari is 13 km north of Kedougou, but in the current study, the villages are all west of Kedougou by ≥15 km. The distance between the villages may imply different habitat types which may alter the abundance of *An. nili*. Indeed, the Fangoli River, adjacent to Ngari flows during the rainy season (Dia et al. 2003), and is most likely the larval habitat for *An. nili* (Gillies and de Meillon 1968). It is possible that seasonal environmental changes may cause fluctuations of *An. nili* abundance. Another explanation of infrequent *An. nili* capture is that *An. nili* may not be attracted to CDC light traps, but studies that directly compared HLCs to CDC light trap catches would be necessary to determine this.

One of 35 *An. coustani* from Boundoucondi in 2009 was positive for either *P. ovale, P. malariae or P. vivax*. As no other *An. coustani* from the other villages were *Plasmodium*-positive, this vector most likely only plays a minor role in *Plasmodium* transmission in this region.

These villages have diverse and abundant *Anopheles* population dynamics. *An. gambiae* s.s., *An. arabiensis,* and *An. funestus* appear to be the dominant *Plasmodium*-vectors while *An. leesoni* and *An. coustani* may be minor, secondary *Plasmodium*-vectors in these villages. The zoophagic blood feeding habits of *An. funestus* are unique compared to other published literature and warrant further field studies. It may be worthwhile to determine the cytogenetic 'Folonzo' and 'Kiribina' forms to determine if these play a factor in blood meal choice. Human landing catches would be necessary for future field studies to determine the EIR for the various *Anopheles* species to assess seasonal bionomics.

Chapter 5 - Survivorship of field-caught *Anopheles* from ivermectin MDA-treated villages in southeastern Senegal

I) Introduction

The development of novel methods to reduce *Plasmodium* transmission that can integrate with and enhance current malaria control measures, as well as other health priorities, is critical. Chapter 2 clearly demonstrated that ivermectin at relevant human pharmacokinetics directly reduces the survivorship of *An. gambiae* s.s. The goal of this study was to determine if ivermectin MDA of humans in Senegal for onchocerciasis control could simultaneously reduce the survivorship of wild African malaria vectors. In 2008 and 2009, blood-fed *Anopheles* spp. were captured from inside peoples' huts before and after ivermectin MDA in three replicate pairs of villages in south-eastern Senegal. Survivorship of the mosquitoes was assessed by holding them in a field insectary for five days.

II) Materials and Methods

A) Study site

The study site was previously described in Chapter 4. Ibel was treated by MDA with 150 µg/kg of ivermectin (Mectizan™, Merck & Co., Inc) on August 8, 2008, while Ndebou was not treated and served as the paired control village. In 2009, two pairs of villages were sampled. Ndebou and Boundoucondi were the first pair of villages sampled

and MDA occurred on August 6, 2009 in Ndebou, with Boundoucondi serving as the control. Damboucoye and Nathia were the second pair of villages sampled and MDA occurred on October 11, 2009 in Damboucoye, with Nathia serving as the control. MDA was coordinated by APOC in Senegal and the Senegalese Ministry of Health, and performed through community-directed treatment by the local nurses. Permission to conduct mosquito sampling surrounding these MDAs was granted first by the Senegalese MoH and then by the residents of each village. The study was also reviewed by the Colorado State University Institutional Review Board prior to being conducted.

B) Mosquito collections

Indoor resting, wild, blood fed *Anopheles* mosquitoes were collected in the morning from huts people had slept in the previous night using backpack aspirators (John W. Hock, Gainesville, FL, USA). After capture, *Anopheles* mosquitoes were transferred by mouth aspirators from backpack aspirator cups to 473 ml cardboard containers screened with organdy. The containers were labeled and designated by village, date collected, and the specific hut from which mosquitoes were collected. Containers were placed into a large basket and two moist towels were placed over the top of the basket to keep the mosquitoes humid and cool. Immediately following morning aspirations, the mosquitoes were transported back to the insectary in Bandafassi (2008) or Kedougou (2009) and maintained on shelves. Insectaries were designated rooms of houses and had screened and slatted windows so that indoor temperatures and humidity naturally fluctuated with the outside ambient temperature and humidity. Temperature and humidity within the insectary ranged from (27 - 30ºC) and (66 - 86%). Any dead, non-

blood fed, or non-*Anopheles* mosquitoes were removed from the containers upon placement in the insectary. The containers had a moistened sponge and raisins placed on top to serve as water and sugar sources for the mosquitoes. Survivorship was checked daily at 12:00pm and dead mosquitoes were removed from the containers. The containers were then randomly placed back onto the shelves. All mosquitoes that survived for five days were frozen and counted as alive on day five post-capture. In 2009, both the treated and the control village were sampled on the same day by two separate field teams. In 2008, only one village was sampled on each day. Mosquitoes were processed in the laboratory as described in Chapter 4.

C) Survivorship model and statistics

A generalized linear mixed model (ProcGlimmix in SAS) was used to assess the effects of ivermectin MDA on mosquito survivorship. Mosquito survivorship results for each village at each sampling date were classified by treatment (whether or not they originated from a treatment or control village), replicate (the three pairs of villages sampled over the two field seasons), and phase. The three phases are groups of sampling dates from each village before MDA (phase 1), one to six days after MDA (phase 2), and seven days or more after MDA (phase 3). The one to six day grouping immediately after MDA was chosen based on the differential survivorship curves generated from the three replicates (Figure 1). Treatment and phase were treated as fixed effects. Replicates were treated as random effects with sample date nested within replicate and phase. The model was fit by the SAS ProcGlimmix procedure using maximum likelihood estimation with three Gaussian quadrature points (SAS Institute, Cary, NC, USA). The percent

survivorship of mosquitoes was tested for interaction of treatment by phase, and if significant, then post-tests were performed to determine which treatment by phase groups significantly differed from the others. The efficacy of ivermectin to reduce mosquito survivorship would be reflected by a significant drop in the treated village survivorship of the phase 2 group compared to control and pre-treatment groups.

III) Results

A) Species composition of aspirated mosquitoes

The majority, 78.1% (1997/2556) of the indoor resting blood-fed *Anopheles* collected by hut aspiration were *An. gambiae* s.s., *An. funestus*, and *An. arabiensis* (see Chapter 4) which are three of the most important *Plasmodium* vectors found in this region. Adequate numbers of *An. gambiae* s.s. were collected from all three replicates to allow pooled replicate statistical comparisons of the effects of ivermectin on mosquito survivorship. *Anopheles funestus* was collected in sufficient numbers for statistical analysis from the August and October 2009 replicates, while *An. arabiensis* was only collected in sufficient numbers from villages in October 2009.

B) Mosquito survivorship analysis

Figure 1 depicts the percent survivorship of *An. gambiae* s.s. from all three replicates grouped by treatment and phase. There is an observable reduction in *An. gambiae* s.s. survivorship after ivermectin MDA (phase 2) in the treated villages that lasts for six days. The survivorship of *An. gambiae* s.s. in Ibel during phase 3 is low (Figure 1), but this line only represents ten mosquitoes caught from one collection. A total of

1,265 *An. gambiae* s.s. from three replicates were captured and held for survivorship analysis. The model of estimated mosquito survivorship for *An. gambiae* s.s. identified a treatment by phase interaction, indicating that the difference between treated and control survivorship depends on phase (F-value = 18.27, $P < 0.0001$) (Figure 2). In post-test comparisons, treatment at phase 2 significantly differed from control at phase 2 (t-value = 4.01, $P = 0.0003$), and it also significantly differed from both treatment at phase 1, pre-MDA (t-value = 8.31, $P < 0.0001$) and treatment at phase 3, seven days and after ivermectin MDA (t-value = -4.61, $P < 0.0001$). The conclusion of this analysis is that ivermectin MDA significantly reduced the survivorship of *An. gambiae* s.s. for six days past the date of the MDA.

Adequate numbers for survivorship analysis of *An. arabiensis* were only caught during the third replicate (Damboucoye and Nathia, n = 153). There appears to be a reduction in survivorship of *An. arabiensis* following ivermectin MDA (Figure 3), but treatment by phase comparisons were not significantly different from each other (F-value = 0.66, $P = 0.5332$). However, treatment alone was significant (F-value = 7.01, $P = 0.0191$), therefore the overall survivorship of *An. arabiensis* was lower in the treated village compared to the control village (Figure 4).

A total of 542 *An. funestus* from two replicates were captured and held for survivorship analysis. The model of estimated mosquito survivorship for *An. funestus* identified a treatment by phase interaction, indicating that the difference between treated and control survivorship depends on phase (F-value = 4.22, $P = 0.0230$) (Figure 6). In post-test comparisons differences occurred as follows: treatment at phase 1 (pre-MDA) versus treatment at phase 2 (1-6 days post MDA) (t-value = 2.04, $P = 0.0488$), treatment

at phase 1, (pre-MDA) versus treatment at phase 3 (≥7 days post MDA) (t-value = 2.39, $P = 0.0255$), control at phase 3 versus treatment at phase 3 (t-value = 2.04, $P = 0.0496$).

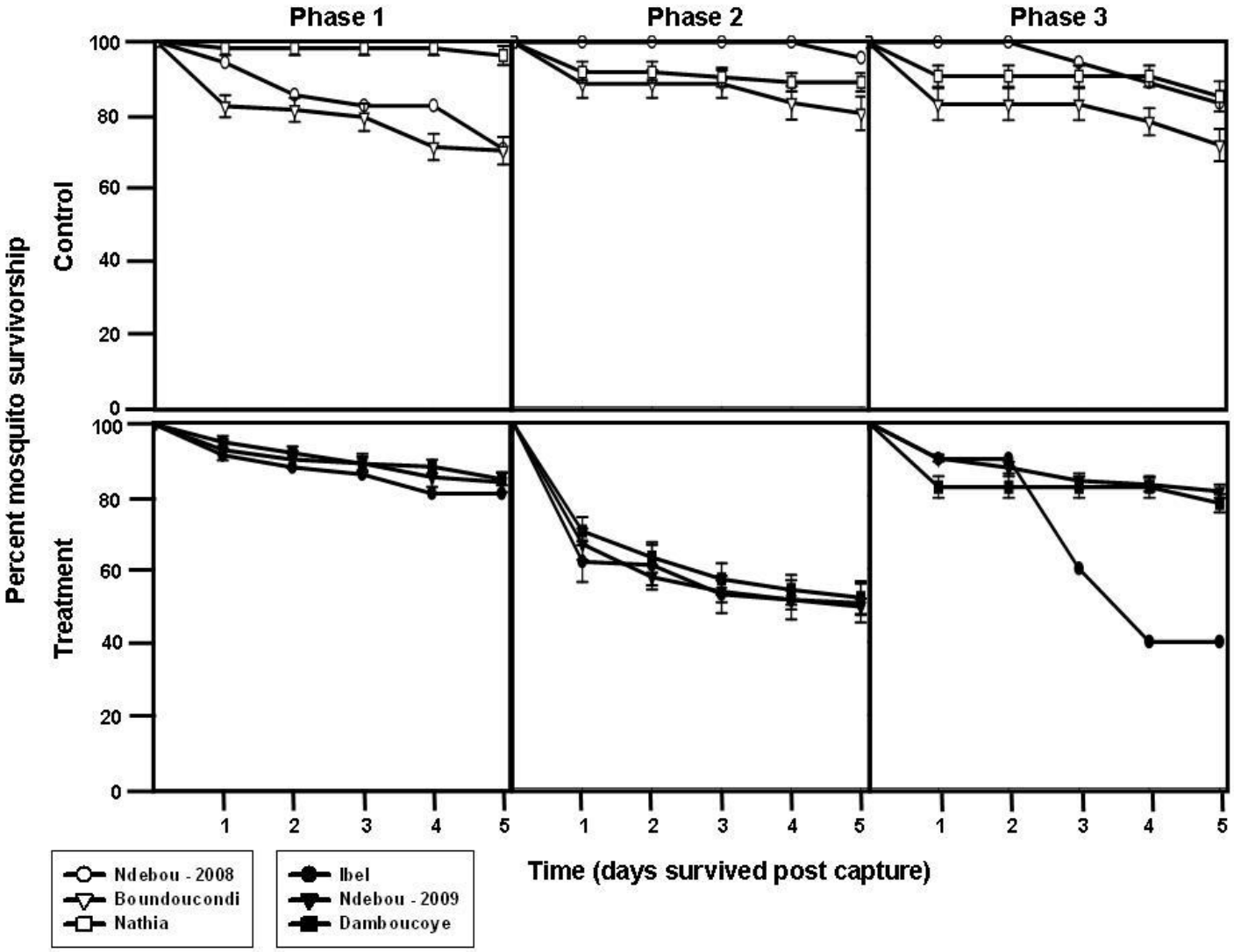
Phase 1
Phase 2
Phase 3
Percent mosquito survivorship
Control
Treatment
100
80
60
40
20
0
1
2
3
4
5
Time (days survived post capture)
Ndebou - 2008
Boundoucondi
Nathia
Ibel
Ndebou - 2009
Damboucoye

Figure 1. Percent survivorship of aspirated *An. gambiae* s.s. grouped by treatment and phase. Percent survivorship of all aspirated *An. gambiae* s.s. (n= 1265) held five days post-capture from all three replicates. Standard error bars represent the percent survivorship variation of all mosquitoes from each village grouped within each treatment and phase.

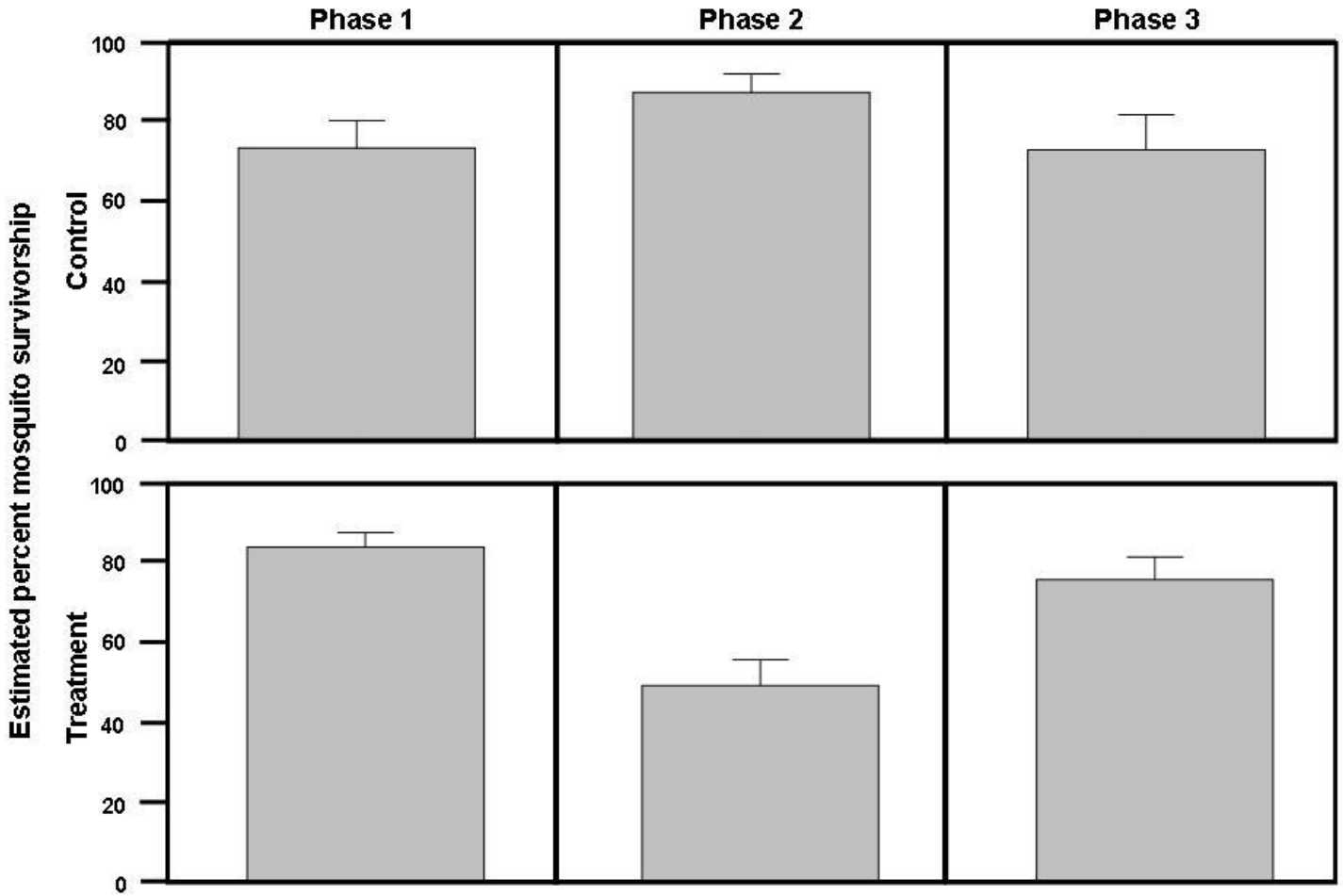

Figure 2. Glimmix model estimated percent survivorship of *An. gambiae* s.s. grouped by treatment and phase. Glimmix model estimated percent survivorship of all *An. gambiae* s.s. (N=1265) held five days post-capture from all three replicates. Treatment by phase was significant (F-value = 18.27, $P < 0.0001$). Treatment at phase 2 significantly differed from control at phase 2 (t-value = 4.01, $P = 0.0003$), treatment at phase 1 (t-value = 8.31, $P < 0.0001$), and treatment at phase 3 (t-value = -4.61, $P < 0.0001$).

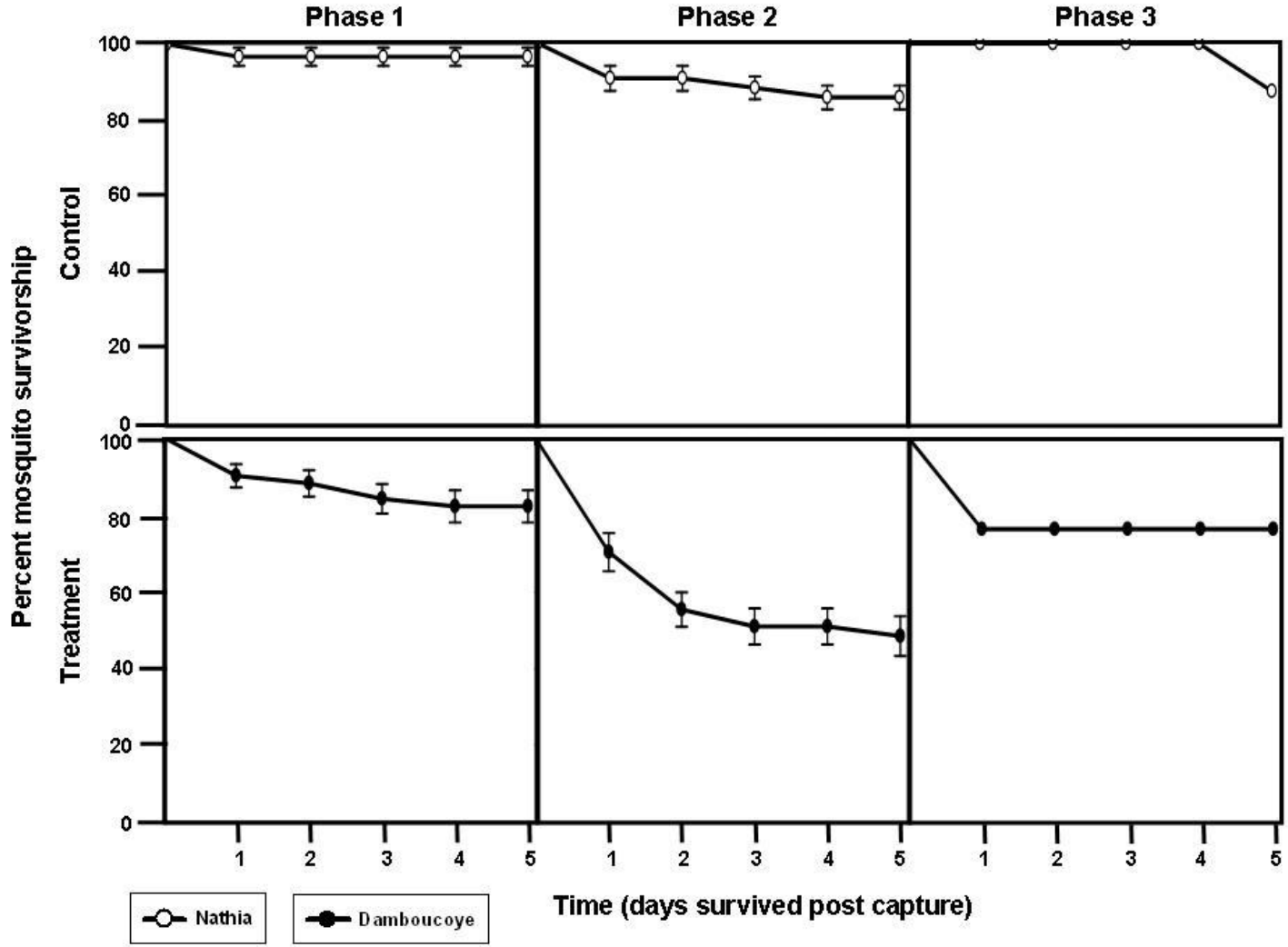

Figure 3. Percent survivorship of aspirated *An. arabiensis* grouped by treatment and phase. Percent survivorship of all aspirated *An. arabiensis* (n = 153) held five days post-capture from Nathia and Damboucoye. Standard error bars

represent the percent survivorship variation of all mosquitoes from each village grouped within each treatment and phase.

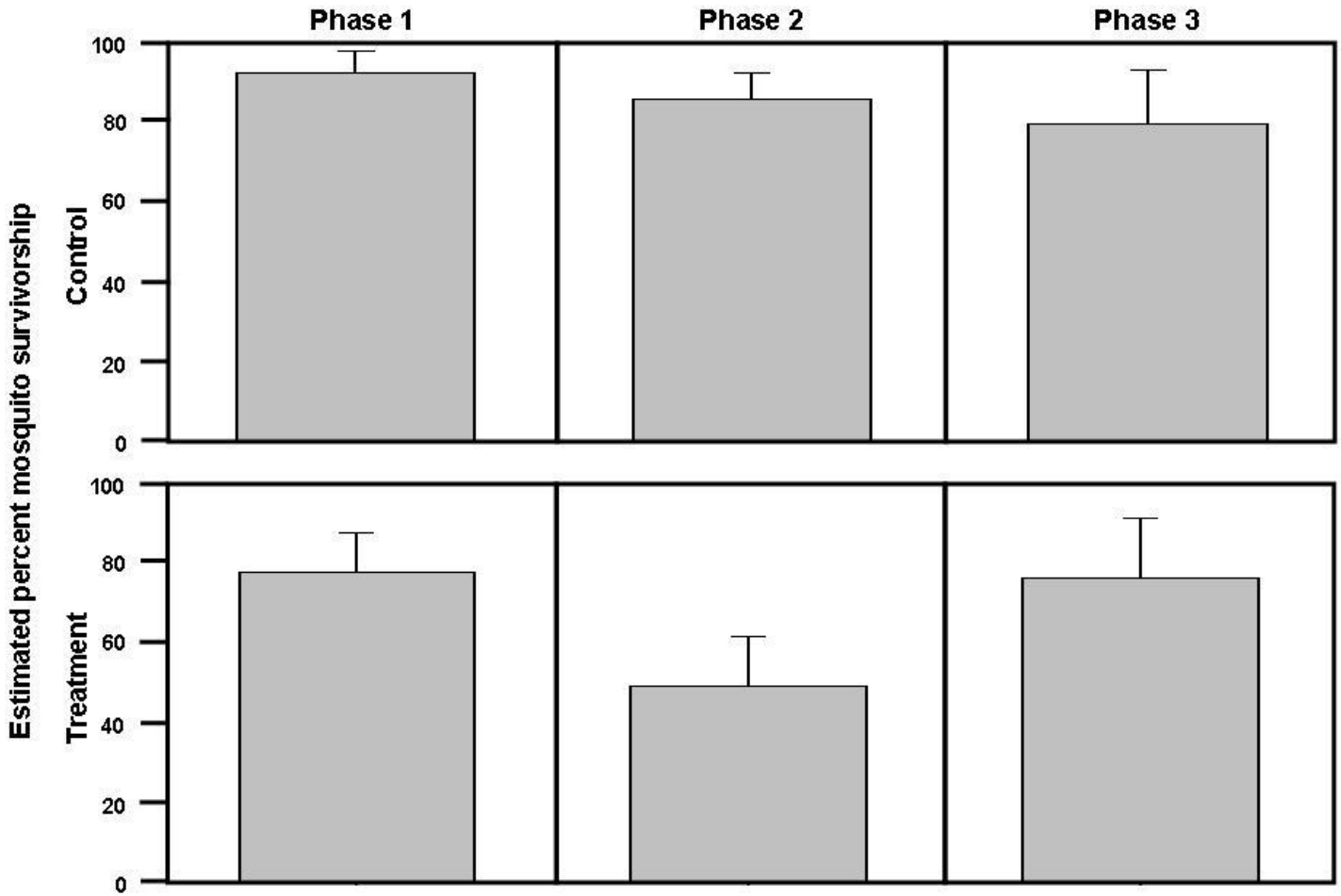

Figure 4. Glimmix model estimated percent survivorship of *An. arabiensis* grouped by treatment and phase. Glimmix model estimated percent survivorship of all *An. arabiensis* (n = 153) held five days post-capture from Nathia and Damboucoye. Treatment compared to control was significant (F-value = 7.01, *P* = 0.0191).

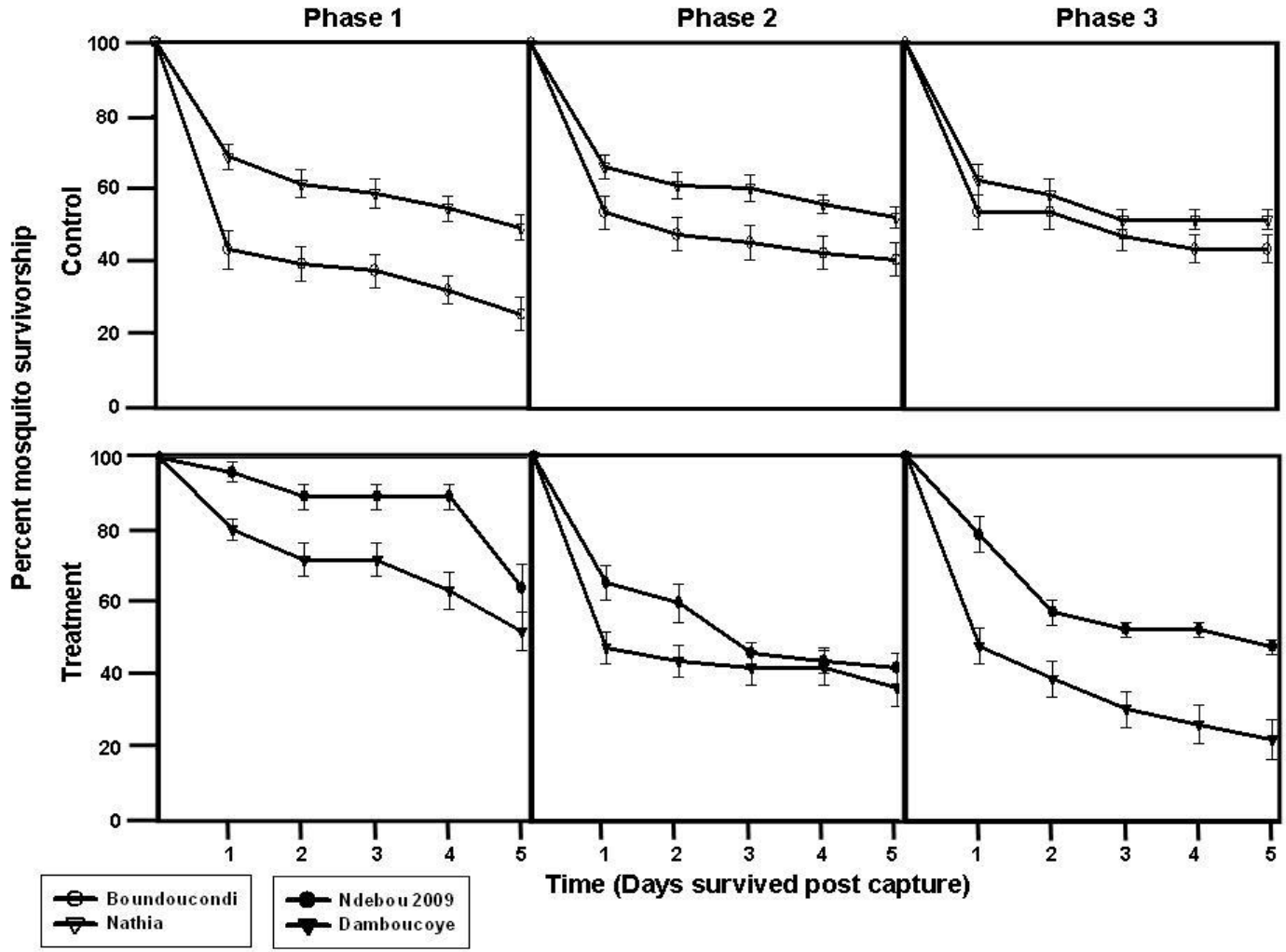

Figure 5. Percent survivorship of aspirated *An. funestus* grouped by treatment and phase. Percent survivorship of all aspirated *An. funestus* (n = 542) held five days post-capture from the two replicates performed in 2009. Standard

error bars represent survivorship variation of all mosquitoes from each village grouped within each treatment and phase.

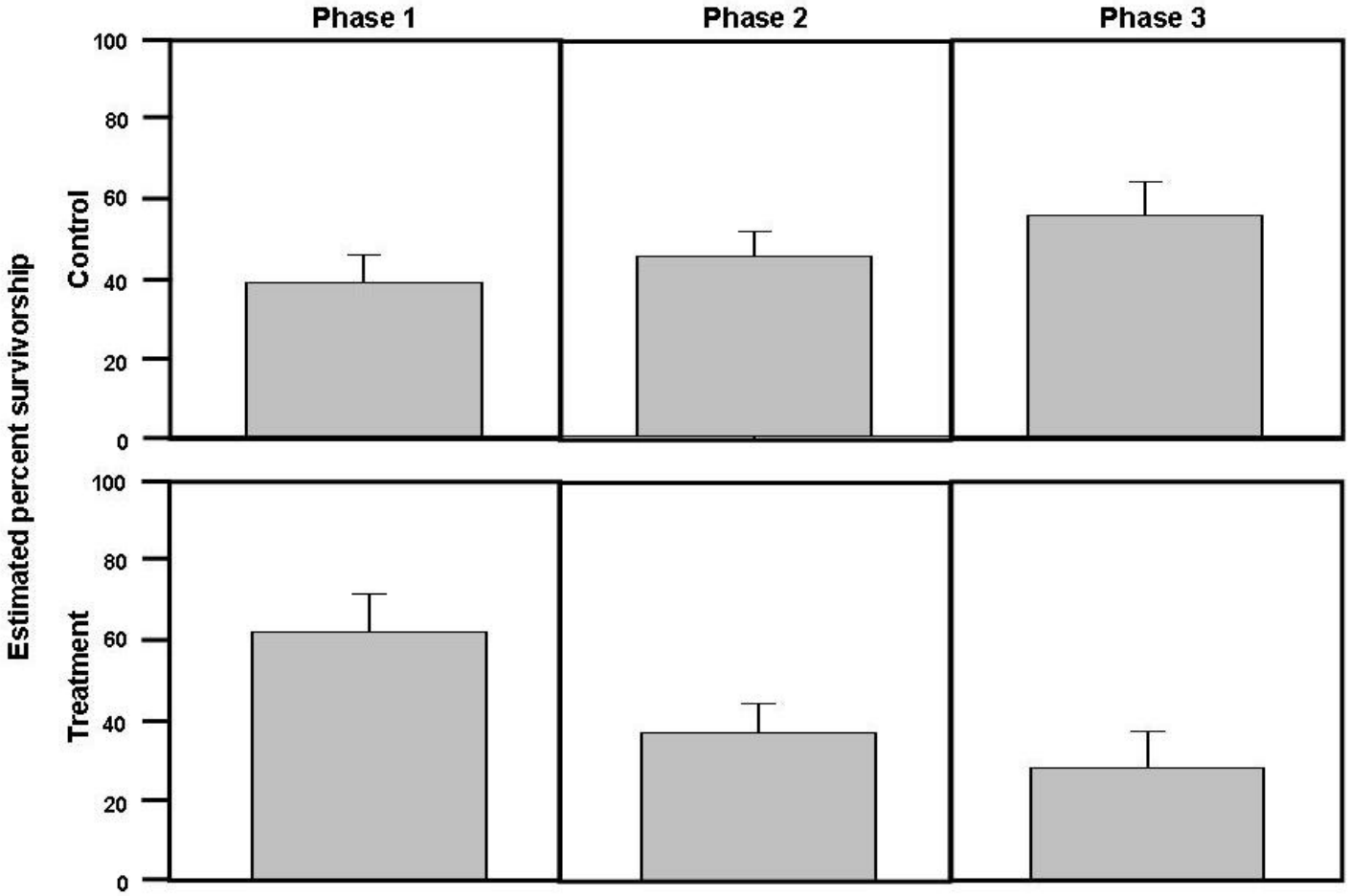

Figure 6. Glimmix model estimated percent survivorship of *An. funestus* grouped by treatment and phase. Glimmix model estimated percent survivorship of all *An. funestus*. (n = 542) held five days post-capture from all three replicates. Treatment by phase was significant (F-value = 4.22, *P* = 0.0230). Treatment at phase 1 significantly differed from treatment at phase 2 (t-value = 2.04, *P* = 0.00488) and at phase 3 (t-value = 2.39, *P* = 0.0255), and control at phase 3 versus treatment at phase 3 (t-value = 2.04, *P* = 0.0496).

IV) Discussion

Laboratory-based evidence showed that colonized *An. gambiae* s.s. is susceptible to ivermectin at concentrations relevant to human pharmacokinetics after a typical MDA (Chapter 2), and that colonized *An. gambiae* s.s. fed on ivermectin-treated humans one day post-treatment had reduced survivorship (Chaccour et al. 2010). The current study demonstrates that routine MDA of ivermectin to people significantly reduces the survivorship of wild *An. gambiae* s.s. for up to six days post MDA. This six day lethal effect is longer than would be predicted based on laboratory evidence (Chapter 2, Fritz et al. 2009), and this effect occurs despite incomplete MDA coverage in treated villages. Three field replicates were performed over space and time in different villages to make this study a rigorous assessment of the effects of ivermectin MDA on three *Anopheles* spp.

There were no significant differences in *An. arabiensis* treatment by phase survivorship (F-value = 0.66, $P = 0.5332$) but this was almost certainly due to sampling, in that adequate numbers (n = 153) for survivorship analysis were only captured in the third replicate MDA. The third replicate MDA was performed in October 2009, toward the end of the rainy season when *An. arabiensis* is more prevalent (Chapter 4, Dia et al. 2003). There was a 38% reduction in mosquito survivorship from phase 2 treatment collections compared to phase 2 control collections (Figure 4). The fact that treatment alone was significant (F-value = 7.01, $P = 0.0191$), means that the overall survivorship of *An. arabiensis* was lower in the treated village compared to the control village (Figure 4). Furthermore, only 75% (24/32) of *An. arabiensis* blood meals were from humans (Chapter 4), which reduces the probability that mosquitoes held for survivorship analysis

may have ingested an ivermectin-containing blood meal. Fritz et al. (2009) reported that colonized *An. gambiae* s.s. and colonized *An. arabiensis* have almost identical susceptibility to ivermectin. When these data are considered together, it is reasonable to assume that upon further replication wild *An. arabiensis* will be shown to be as susceptible to ivermectin MDA as wild *An. gambiae* s.s.

The *An. funestus* survivorship results were difficult to interpret, which was most likely due to the fact that wild *An. funestus* did not adequately survive our methods of back pack aspiration and housing in cardboard containers, as observed from the less than 60% survivorship in the control and treated villages before ivermectin MDA occurred (Figs. 5 and 6). Kiszewski et al. (2004) calculated the median daily survival rate (MDSR) of 33 *Plasmodium* vectors found worldwide and based on records from 30 publications, *An. funestus* had the lowest MDSR of 0.690. This lower MDSR may explain why *An. funestus* (Figs. 5 and 6) had reduced survivorship in control collections compared to that of *An. gambiae* s.s. (Figs. 1 and 2) and *An. arabiensis* (Figs. 3 and 4) which have MDSR values of 0.860 and 0.790, respectively. A majority, 80.0% (180/225) of *An. funestus* collected from all six villages were found to have blood fed on cattle rather than humans (Chapter 4), despite the fact that they were caught resting in houses. Only 19.6% (9/46) of *An. funestus* blood meals came from humans in the treated villages. This questions the biological relevance of statistical differences observed for *An. funestus*. Alternative methods will need to be developed in order to determine if wild *An. funestus* are affected by ivermectin MDA.

Based on clinical records, 84.2% (203/241) of people in Damboucoye and 82.1% (311/379) of people in Ndebou were treated with ivermectin during these two MDAs.

Pregnant women and children under 90 cm did not receive the drug, following the manufacturer guidelines. Mosquitoes that were held for survivorship analysis for five days had completely digested their blood meals, which made it impossible to detect ivermectin from individual mosquitoes. Yet it is impressive that mosquito survival was still significantly reduced despite not knowing whether any one mosquito fed on a treated person. Incomplete coverage may actually be beneficial to the overall concept of repeated ivermectin MDAs for *Plasmodium* transmission control in that it may provide a refugia of untreated human hosts for mosquitoes to feed on which could reduce the likelihood of ivermectin resistance development in the mosquito population.

Of people accounted, 78.2% (903/1,155) utilized ITNs across the four villages surveyed in 2009. Even with high ITN coverage, human blood fed *An. gambiae* s.s., *An. arabiensis*, and *An. funestus* were frequently collected from inside the huts (Chapter 4), demonstrating that ITNs have limitations in preventing *Anopheles* from feeding on people. The human antibody response to *An. gambiae* SG6 salivary protein was measured and it was determined that although human-vector contact is reduced after ITN deployment, there are still *Anopheles gambiae* that successfully bite people that use ITNs (Drame et al. 2010). Antibody levels cannot determine whether the blood feeding occurred while the person was under the ITN or if the blood feeds occurred outside the ITN and home. However, ivermectin would affect *Anopheles* survivorship if the blood meal was obtained while the person was under the ITN or outside the ITN and home. Russell et al. (2011) demonstrated that ITNs dramatically reduced *Plasmodium* transmission indoors, but this shifted *Plasmodium* transmission to occur outdoors. Ivermectin MDA would integrate well with ITN use as ivermectin MDA may be one of

the few methods that can directly target these exophagic, exophilic, and crepuscular-feeding *Plasmodium* vectors.

Ivermectin has a different mode of action (Cully et al. 1994, Kane et al. 2000) from the insecticide classes currently used for ITNs and IRS (i.e., carbamates, pyrethroids, and organochlorines) (Hemingway and Ranson 2005). Once or twice yearly ivermectin MDA has been occurring in this region for over fifteen years (Diawara et al. 2009), so the fact that a reduction in survivorship of *An. gambiae* s.s. was still detectable is a promising sign that resistance by *Anopheles* spp. may be slow to develop against this drug. Furthermore, the novel mode of action of ivermectin compared to the currently used insecticides for malaria control should potentially minimize issues of cross-resistance where ivermectin MDA may be used in combination with IRS and ITNs (see Chapter 8).

Orally ingested ivermectin has been found to have a higher bioavailability in human plasma of adult females compared to males as evidenced by higher total body CL/F values, 0.35 ± 0.167 and 0.196 ± 0.057, respectively (Vanapalli et al. 2003). Higher concentrations of ivermectin in the plasma of females compared to males have also been demonstrated in sheep (Ndong et al. 2007) and cattle (Toutain et al. 1997). As Elkassaby (1991) only measured plasma concentrations of ivermectin in adult male humans, the concentrations derived may have been less representative of concentrations found in all humans treated by ivermectin MDA in a given area. This may explain why the effect of ivermectin on mosquito survivorship in the field study was greater than would have been expected based on laboratory results. Chaccour et al. (2010) did not report differences in survivorship of *An. gambiae* s.s. that fed on ivermectin-treated

humans, but this would most likely have been due to the fact that mosquitoes were only fed on people one and fourteen days post ivermectin ingestion. A direct comparison of the survivorship of colonized, pathogen-free *Anopheles*, after blood feeding at several time points on ivermectin-treated male and female humans from malaria-endemic areas could determine if increased bioavailability of ivermectin in females causes a greater reduction in mosquito survivorship.

Lastly, our *Anopheles* survivorship measures from one to six days post-MDA are likely an underestimate of the true effect of ivermectin MDA, because these experiments only examined direct mortality of field-caught mosquitoes, protected from environmental hazards, and supplied constant water and sugar sources. The effect of ivermectin on mosquitoes to delay of re-feeding, cause knockdown, and delay recovery (see Chapter 3) would likely enhance mortality in the field due to predation, desiccation, and reduced nutrition.

Chapter 6 – Ivermectin mass drug administration to humans disrupts *Plasmodium* transmission in Senegalese villages

I) Introduction

Chapter 5 demonstrated that the survivorship of field-caught *An. gambiae* s.s. and most likely *An. arabiensis* is reduced for up to six days post ivermectin MDA to humans. *Anopheles gambiae* s.s. often requires two blood meals to complete its initial gonotrophic cycle (Gillies 1955, Charlwood et al. 2003) and thereafter will often take multiple blood meals per gonotrophic cycle (Gillies 1953, Briegel and Horler 1993, Beier 1996) and feeds almost exclusively on humans (Chapter 4, Dia et al. 2003). These blood feeding characteristics, coupled to the fact that the extrinsic incubation period for *Plasmodium* spp. is 9-14 days, means that most malaria transmission by *An. gambiae* s.s. will occur only after *Plasmodium* parasite-harboring mosquitoes have taken multiple non-sporozoite transmitting blood meals from humans (Billingsley et al. 2008). If human population clusters were simultaneously treated with ivermectin MDA, then most adult *An. gambiae* s.s. in the MDA area would imbibe a concentration of ivermectin that would reduce their survivorship. The predicted effect has the potential to temporarily shift the *An. gambiae* s.s. population age structure, which would reduce the reservoir of adult sporozoite-transmitting *An. gambiae* s.s. in the MDA area.

Sylla et al. (2010) modified a previously published (Billingsley et al. 2008) mosquito population age-structured model that incorporated mosquito field survivorship

data (see Chapter 5) to demonstrate that ivermectin MDA would shift the population structure and thus reduce the proportion of *Plasmodium*-infectious *Anopheles*. The model predicted that the low sporozoite rate in the resulting mosquito population would temporarily reduce the basic reproductive number (R_0) of *Plasmodium* below the base number for approximately eleven days post ivermectin MDA. The age-structured model predicts that strategically administered, repeated ivermectin MDAs would achieve sustained reductions in *Plasmodium* transmission (Sylla et al. 2010). Such repeated MDAs may only be logistically feasible for areas with seasonal malaria transmission, such as the Sahel, or in areas experiencing malaria epidemics (http://www.mara.org.za/trview_e.htm).

The calculation of the R_o model requires the determination of many entomological and human epidemiological, field-based parameters (Macdonald 1952, 1955, Reisen 1989). Since not all of the variables necessary to calculate R_o were determined and too few sporozoite-positive *An. funestus* and *An. arabiensis* were collected (see Chapter 4), only the sporozoite rate of *An. gambiae* s.s. was assessed in relation to ivermectin MDA.

II) Materials and Methods

The study area was the same as that described in Chapter 4, with the same ivermectin MDA schedule described in Chapter 5. Mosquitoes were collected, held in an insectary (Chapter 5), and processed (Chapter 4) as described previously. As demonstrated in Chapter 4, very few *Anopheles* caught by CDC light traps as compared to aspiration were *Plasmodium*-positive, so only *Anopheles gambiae* s.s. captured by

aspiration were used for statistical analysis. Since statistical analyses required discreet numbers for comparison, the method to estimate the percent of infectious *Anopheles* caught by aspiration, as described in Chapter 4, could not be used. Therefore, only *Anopheles gambiae* s.s. held in the insectary for survivorship analysis that survived for five days were statistically analyzed so that no bias occurred by comparing mosquitoes held in the insectary for variable amounts of times. Mosquitoes that survived to day 5 were chosen as this was the largest proportion of mosquitoes maintained in the insectary.

Using accepted rates of adult blood feeding frequency (Beier 1996, Briegel and Horler 1993), it was conservatively estimated that it would take three days for all potentially *Plasmodium*-infectious *An. gambiae* s.s. present in the area at the time of MDA to imbibe a blood meal from treated people. Therefore, mosquitoes collected from 14 days before ivermectin MDA to three days post-treatment were placed in the 'before' group, while mosquitoes collected from three days post-treatment to twelve days post-treatment were placed in the 'after' group. However, post-hoc analyses revealed that significant differences were retained between the 'before' and 'after' sporozoite rates even if mosquitoes caught one, two and three days post ivermectin MDA were placed in the 'after' group.

For individual replicates, infection rates were analyzed by logistic regression with effects for village (treated, untreated), period (before, after) and village by period interactions. A combined analysis for all three replicates included effects for village, period, replicate, village by period, and village by replicate. In both analyses, the village by period interaction tests whether the change in infection rate over period differs between treated and untreated villages. For estimation of means, the village by replicate

interaction was removed from the model because the second replicate control village had zero infection rates. Computations were performed with SAS Proc GENMOD (SAS 2002).

III) Results

Figure 1 demonstrates a 79% reduction in the mean proportion of *Plasmodium falciparum* sporozoite-infectious *An. gambiae* s.s. collected two weeks following ivermectin MDA in villages from three replicates, while there was a 246% increase in the mean proportion of sporozoite-infectious *An. gambiae* s.s. collected in pair-matched control villages at the same time (treatment by period, df = 1, chi-square = 12.18, P = 0.0005, n = 934).

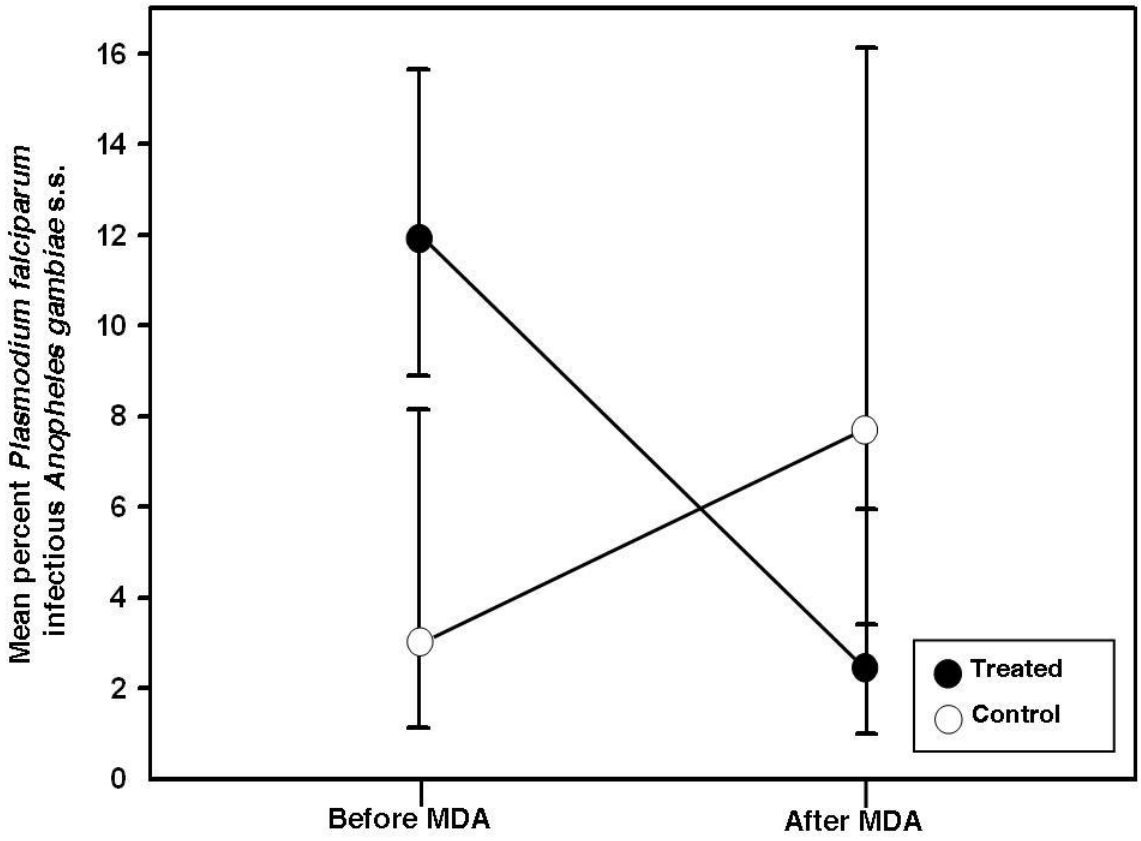

Figure 1. The proportion of *Plasmodium falciparum* infectious *Anopheles gambiae* s.s. before and after ivermectin MDAs. The mean percent of *P. falciparum* sporozoite-infectious *An. gambiae* s.s. were estimated from three replicate collections of mosquitoes in treated and pair-matched untreated, control villages in southeastern Senegal. Closed (●) and open (○) circles represent the means of ivermectin treated and untreated control villages respectively. Error bars represent Exact 95% confidence intervals.

IV) Discussion

Direct measurements presented here show that *Plasmodium* transmission is indeed significantly disrupted after ivermectin MDA and the effect is sustained for at least two weeks. This study was conducted on a small spatial scale. All villages are located along a ~12 km stretch of road (Chapter 4, Figure 1) where humans, and possibly vectors, moved between treated and control villages, yet there was still a demonstrable

effect restricted to treatment villages highlighting that localized *Plasmodium* transmission control can be achieved in a single village by ivermectin MDA.

Current ivermectin MDAs for onchocerciasis control in Africa are performed only once or twice per year, and do not always coincide with local malaria transmission seasons. Annual applications would not be expected to lower malaria transmission long enough to see any noticeable reductions of parasite prevalence, intensity or disease in people. Indeed, malaria is hyperendemic in APOC-control areas of southeastern Senegal despite more than fifteen years of annual ivermectin MDAs.

Initial laboratory experiments by Merck found ivermectin ineffective against *Eimeria muris* (a protozoan) when fed to infected mice. Ivermectin also had minimal to no effect on any bacteria, fungi, or yeast species tested (Burg and Stapley 1989). Glutamate-gated and GABA chloride channels regulate neuromuscular function in invertebrates (Bloomquist 2003), and are the primary target of ivermectin in invertebrates (Duce and Scott 1983, Dent et al. 2000, Kane et al. 2000). These channels are not present in protozoans, bacteria, fungi, or yeast, which may explain the inactivity of ivermectin on these organisms. However, the interaction of ivermectin on physiological functions of mosquitoes may interfere with the development of *Plasmodium* in the mosquito. Formation of the peritrophic membrane and blood meal digestion was delayed in *Ae. aegypti* that had imbibed a sublethal concentration of ivermectin (Mahmood et al. 1991). The delay in blood meal digestion and defecation observed in *An. gambiae* s.s. after ivermectin ingestion (see Chapter 3) was probably associated with a delay in the formation of the peritrophic membrane. Numerous interactions occur between *Plasmodium* and the *Anopheles* midgut that could potentially be influenced by ivermectin

and include: exflaggelation of male gametocytes, zygote and ookinete development, ookinete motility through the peritrophic membrane, oocyst development, release of sporozoites, hemolymph circulation and sporozoite motility. The field data do not exclude the possibility that sub-lethal ivermectin concentrations inhibited the development of *Plasmodium* in mosquitoes. If such an effect can occur, it may have contributed to the reduction in sporozoite rates and should be tested further.

Other factors may have contributed to the reduction of *P. falciparum* prevalence in *An. gambiae* s.s. post ivermectin MDA beyond those of the outright lethal effect of a single ivermectin-containing blood meal. These factors potentially include: the reduced feeding frequency after ingestion of ivermectin, compounding effect of multiple ivermectin-containing blood meals on mosquito survivorship, and reduced survival due to increased predation or desiccation after knockdown and delayed recovery (see Chapter 3). Although these effects have been demonstrated in the laboratory; it is not known if they will occur in the field.

This study demonstrates that a single ivermectin MDA can significantly reduce the proportion of sporozoite-infectious malaria vectors for at least two weeks; further studies are needed to determine the duration of *Plasmodium* transmission reduction. If given more frequently, in spaced intervals defined by the duration of control, ivermectin MDAs may be effective for reducing malaria parasite transmission during epidemics or delineated malaria transmission seasons that occur throughout large regions of Africa (http://www.mara.org.za/trview_e.htm) and other continents.

Chapter 7 - Mermithid nematodes in adult *Anopheles* spp. from southeastern Senegal

I) Introduction

Nematodes from the family Mermithidae (Enoplea: Mermithida) parasitize aquatic and terrestrial invertebrates including Isopoda, Copepoda, Aranea, and most insect orders. Seven genera of Mermithidae are parasites of mosquitoes: *Culicimermis*, *Empidomermis*, *Hydromermis*, *Octomyomermis*, *Perutilimermis*, *Romanomermis*, and *Strelkovimermis* (Poinar 2001). Over 25 species of mermithid nematodes that parasitize mosquitoes have been described (Blackmore 1994). Mermithid nematodes from mosquitoes have been documented from Asia, Europe, North America, South America, and Africa (Petersen 1985, Platzer 2007).

Surprisingly, only two mermithid species, *Empidomermis cozii* and *Reesimermis* (*Octomyomermis*) *muspratti*, have been documented from mosquitoes in Africa (Muspratt 1945, Coz 1966, Poinar 1977). *Octomyomermis muspratti* was isolated from various larvae of *Aedes* and *Culex* spp. found in tree holes in Livingstone, Zambia (Muspratt 1945). Subsequent colonies were established and *O. muspratti* was found to be capable of parasitizing *Ae. aegypti*, *Ae. polynesiensis*, *Cx. pipiens*, *An. stephensi*, and *An. albimanus* (Obiamiwe and MacDonald 1973). Only *Ae. aegypti* and *Cx. pipiens* are present in Africa which demonstrates that mermithid nematodes are capable of parasitizing a wide range of mosquito species that they have not previously encountered.

Empidomermis cozii was first found in adult *An. funestus* from Burkina Faso (Coz 1966). Since then, Poinar (1977) returned to the same area to collect and describe *E. cozii*. Again, *E. cozii* was only found in adult *An. funestus*.

In 2008, we suspected mermithid nematodes may have been observed in some of the mosquitoes that were collected in Senegal. Upon our return, in 2009, mermithid nematodes were identified from several *Anopheles* spp.

II) Materials and Methods

Mosquitoes were collected from huts by aspiration and from CDC light traps as discussed previously (see Chapter IV). During routine morphological examination, mosquitoes were also inspected visually for possible mermithid infection. Adult mermithids range in length from 10 to 100 mm (Platzer 2007) so they can be readily observed visually without dissection of the mosquito (see Figure 2).

Two mermithid-positive *An. gambiae* s.s. were dissected and the mermithids were separated from the mosquitoes so that mermithid DNA could be extracted with the Qiagen DNeasy kit (Qiagen Sciences, Maryland, USA) and a Qiacube robot (Qiagen Sciences, Maryland, USA). Primers were designed to amplify regions of the 18S ribosomal RNA gene by PCR. Primers were designed by comparing sequences from other mermithids obtained from Genbank. Primer sequences were:
MERM 3 forward – 5'-CAAGGACGAAAGTTAGAGGTTC-3' and
MERM 3 reverse – 5'-GGAAACCTTGTTACGACTTTTA-3'.
The 20 µl master mix recipe was: 10 µl Fermentas 2X Master Mix, 2 µl forward and reverse primers at 10 µM, 2 µl DNA, and 4 µl of RNAse free water. The thermocycler

protocol was 5 minutes at 95°C, followed by 40 cycles of 30 seconds denaturing at 95°C, 30 seconds annealing at 50°C, and 45 seconds extension at 72°C with an additional 10 minute extension step, all performed on a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA, USA). This protocol was then used to assess a subset of mosquito abdomens that previously had DNA extracted for blood meal identification (see Chapter 4). The primers amplified an 804 base pair fragment of mermithid DNA and also a 900 base pair fragment of *Anopheles* spp. DNA. Thus, when these primers were used to screen wild *Anopheles* mosquitoes one band would always be present when a mosquito was processed, but when the mosquito was infected with a mermithid parasite, there would be two bands present (see Figure 1).

Extracted DNA from mermithid only isolates were cloned into a chemically competent *E. coli* vector with the TOPO-TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA, USA), selected on Kanomycin agar plates, screened by PCR, miniprepped with QIAprep Spin Miniprep Kit (Qiagen, Gaithersburg, Maryland, USA) and submitted for sequencing by the proteomics and metabolomics facility at CSU. Sequenced mermithid DNA was then used to identify 147 of the most similar sequences, based on E-score, published in GenBank. These sequences were then used to create a bootstrapped Maximum Parsimony tree. The Maximum Parsimony tree was chosen because it is the only method that assesses gaps in DNA sequences, which are common in the 18S ribosomal RNA gene. There were four levels of out grouping with 100 rounds of bootstrapping performed. Nematodes from the Mermithidae family formed a monophyletic clade and therefore only sequences from these were compared for the final tree.

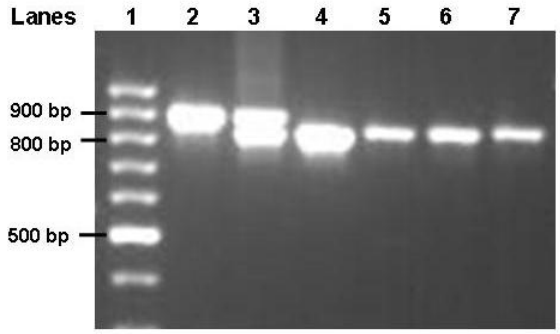

Figure 1. Gel electrophoresis image of samples amplified with mermithid primers. Gel electrophoresis image of multiple samples amplified with mermithid primers: Lane 1 – 100 base pair ladder, Lane 2 – uninfected laboratory-reared *An. gambiae* s.s., Lane 3 – Field-collected *An. gambiae* s.s. infected with mermithid parasite, Lanes 4–7 – Mosquito-free mermithid samples dissected from field-collected mosquitoes.

III) Results

A total of ten (1.8%) out of 551 *Anopheles* spp. were positive for mermithid parasites. Four *Anopheles* spp. were visually observed with mermithid parasites and six others were detected by PCR One *An. gambiae* s.s. had a live mermithid but attempts to colonize the mermithid failed. Mermithid-positive *Anopheles* spp. were found in Ndebou, Boundoucondi, and Damboucoye in 2009. Five of 74 (6.8%) *An. gambiae* s.s. from Ndebou, two of 91 (2.2%) *An. funestus* and one *An. rufipes* (Fig. 2) from Boundoucondi, and two of 51 (3.9%) *An. funestus* from Damboucoye were positive for mermithid parasites.

The Maximum Parsimony tree confirmed that the nematode parasite found in *Anopheles* from Senegal is indeed a mermithid parasite. The Senegalese mermithid is most closely related to *Strelkovimermis spiculatus*, a mermithid parasite of mosquitoes.

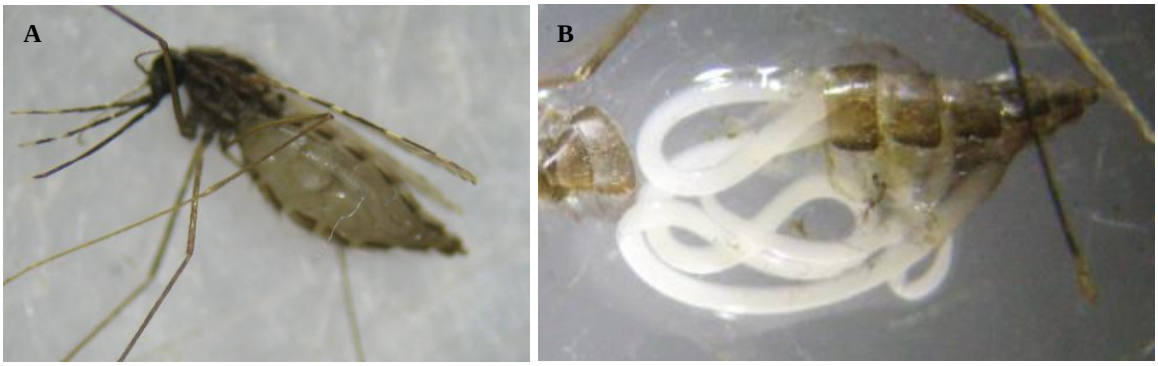

Figure 2. Photograph of a Senegalese mermithid. Photograph of a mermithid parasitized *An. rufipes* (A) and dissected mosquito abdomen with mermithid parasite (B).

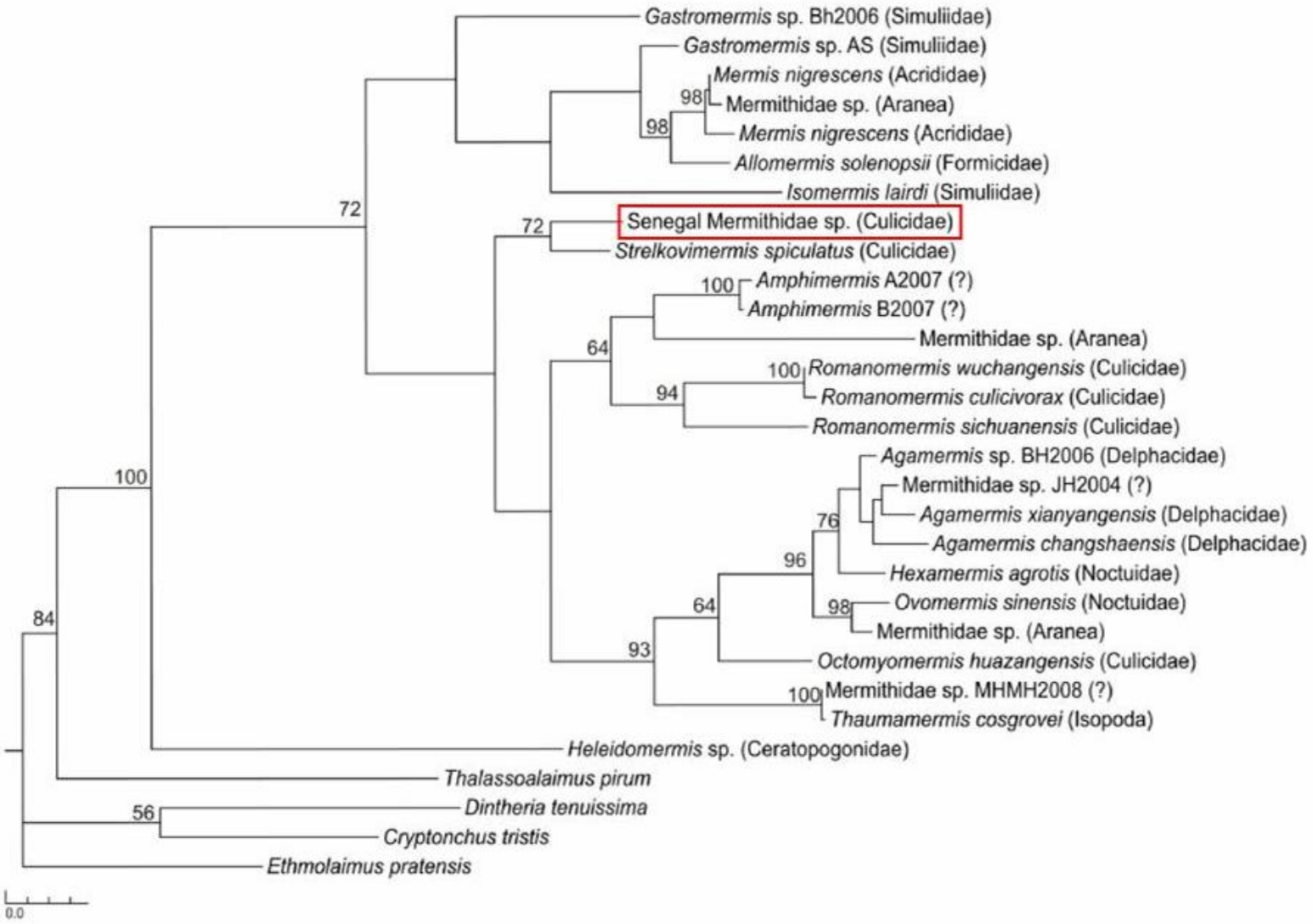
Gastromermis sp. Bh2006 (Simuliidae)
Gastromermis sp. AS (Simuliidae)
Mermis nigrescens (Acrididae)
98
Mermithidae sp. (Aranea)
98
Mermis nigrescens (Acrididae)
Allomermis solenopsii (Formicidae)
Isomermis lairdi (Simuliidae)
72
72
Senegal Mermithidae sp. (Culicidae)
Strelkovimermis spiculatus (Culicidae)
100
Amphimermis A2007 (?)
Amphimermis B2007 (?)
Mermithidae sp. (Aranea)
64
100
Romanomermis wuchangensis (Culicidae)
94
Romanomermis culicivorax (Culicidae)
Romanomermis sichuanensis (Culicidae)
Agamermis sp. BH2006 (Delphacidae)
100
Mermithidae sp. JH2004 (?)
76
Agamermis xianyangensis (Delphacidae)
Agamermis changshaensis (Delphacidae)
96
Hexamermis agrotis (Noctuidae)
64
98
Ovomermis sinensis (Noctuidae)
84
Mermithidae sp. (Aranea)
93
Octomyomermis huazangensis (Culicidae)
100
Mermithidae sp. MHMH2008 (?)
Thaumamermis cosgrovei (Isopoda)
Heleidomermis sp. (Ceratopogonidae)
Thalassoalaimus pirum
56
Dintheria tenuissima
Cryptonchus tristis
Ethmolaimus pratensis
0.0

Figure 3. Maximum Parsimony tree for the family Mermithidae. The Maximum Parsimony tree demonstrates that the nematode found in various *Anopheles* spp. from Senegal is indeed a mermithid parasite. Numbers at nodes represent the degree of bootstrap support. The scale represents the number of steps. Mermithid species names are in italics and the family or order the parasite was isolated from in parentheses.

IV) Discussion

Mermithid parasites have been considered as possible biological control agents of mosquito populations (Petersen 1985, Platzer 2007). Platzer (2007) summarized several review articles and listed the advantages of mermithids for biological control of mosquito populations to include: "the ease of application, environmental safety, host specificity, laboratory manipulation of life history, lethality, mass rearing *in vivo*, and potential for long-term recycling."

The life cycles of mermithid parasites are characterized as follows: egg hatch in water, attachment to and penetration of mosquito larvae by the pre-parasitic stage, development of parasitic larvae in the mosquito, emergence of the parasite while the mosquito is a larvae, pupae, or adult, escape from the mosquito host, development of post-parasitic stages into adults, mating, oviposition, and development of eggs (Platzer 2007).

Unfortunately, no mermithid field release study has proven successful enough to warrant the development of mermithids for mosquito population control (Petersen 1985). All of the field studies summarized in Petersen (1985) used *Romanomermis culicivorax* due to the ease in which it can be reared in large enough numbers sufficient for mass release (Petersen and Willis 1972) and the ability of *R. culicivorax* to infect numerous genera and species of mosquitoes (Vyas-Patel 1988). A major drawback to *R. culicivorax* as a mosquito control agent is that it enters the mosquito larvae, develops, and

emerges from the mosquito larvae (Gaugler et al. 1984). While the emergence is lethal to the mosquito, this would only affect larval population structure which means that a high rate of infection and mosquito death would be necessary to overcome density dependent mortality effects. Furthermore, there is no chance for dispersal of *R. culicivorax* to other mosquito larval habitats by adult mosquitoes. This requires all mosquito larval habitats to be treated with *R. culicivorax* larvae which can be a daunting task for certain species of mosquitoes with diverse and ephemeral larval habitats such as *An. gambiae* s.s.

The Senegalese mermithid was most closely related to *Strelkovimermis spiculatus*, although a bootstrap value of 72 may not indicate that it belongs to the *Strelkovimermis* genera. Only two *Strelkovimermis* species have been identified as parasites of mosquitoes (Platzer 2007), *S. spiculatus* (Poinar and Camino 1986) and *S. peterseni* (Petersen and Chapman 1970). Both *S. spiculatus* (Campos and Sy 2003) and *S. peterseni* (Petersen and Willis 1974) exit the mosquito in its larval stage thus diminishing the capacity of these two species to control adult mosquito populations or mosquito-borne diseases.

Unfortunately, there are few submissions of 18S ribosomal RNA gene sequences in GenBank for mermithid nematodes that parasitize mosquitoes. The two previous mermithid species isolated from Africa, *E. cozii* (Coz 1966, Poinar 1977) and *O. muspratti* (Muspratt 1945, Obiamiwe and MacDonald 1973) do not have 18S ribosomal RNA sequences in GenBank. The distance of the Senegalese mermithid from *Octomyomermis huazongensis* in the Maximum Parsimony tree (Fig. 3) suggests that the unidentified parasite does not belong to the *Octomyomermis* genera. There are no *Empidomermis* spp. DNA entries in GenBank.

Three isolations of *Empidomermis* spp. from mosquitoes have been documented: *E. cozii* from Burkina Faso (Coz 1966, Poinar 1977), *Empidomermis riouxi* from France (Doucet et al. 1979), and *Empidomermis* spp. from New York, USA (Gaugler et al. 1984). In all cases, *Empidomermis* was reported to emerge from the adult mosquitoes, similar to the Senegalese mermithid (personal observation). Both *E. cozii* (Coz 1966) and *E. riouxi* (Doucet et al. 1979) were reported to sterilize adult mosquitoes. Doucet et al. (1979) demonstrated arrested ovarian development in parasitized females and inhibited mating activity of parasitized males. It appears that *E. cozii* cannot fully develop in the adult mosquito unless a blood meal has been ingested by the mosquito (Poinar 1977), but the number of blood meals nor the time necessary for post-parasitic stage emergence was not determined. All mermithids isolated in our study came from *Anopheles* spp. that were blood engorged at the point of capture.

A mermithid parasite that develops in and emerges from the adult mosquito may be more effective for mosquito-borne disease control as this will reduce the daily probability of adult survivorship (see Chapter 1 IV). Furthermore, a mermithid parasite that emerges from the adult mosquito stage may be dispersed by the adult mosquito to cryptic mosquito larval habitats. This enhances the likelihood of mermithid contact with mosquitoes in other larval habitats. However, a mermithid that emerges in the mosquito adult stage may be more difficult to rear in large numbers for mass release. If the mermithid sterilizes adult mosquitoes, then this will have an effect on mosquito population size. A mermithid with a broad mosquito host range is more likely to be recycled in the environment which would reduce the number of releases necessary for mosquito population control. There is the possibility that the mermithid may inhibit

Plasmodium development in the mosquito. The Senegalese mermithid was observed or detected in *An. gambiae* s.s., *An. funestus*, and *An. rufipes*, which means that it is not species specific within the *Anopheles* genera.

Re-isolation of the Senegalese mermithid should be feasible. Aspiration collections should be performed and blood-fed mosquitoes held in cardboard containers. After 3-5 days the cups should be filled with water. When the mosquitoes die, the post-parasitic larvae will emerge into the water. The mermithids should then be transferred to Petri dishes filled with water and a thin layer of sand to serve as an oviposition substrate (Poinar 1977). Specimens should be prepared and submitted to a nematode taxonomist to make a formal description of the species. Laboratory colonies should be established that utilize rearing techniques published for several other mermithids from mosquitoes. The mermithid life cycle and interactions with various mosquito species should also be determined to assess the potential effectiveness of the Senegalese mermithid as a biological control agent.

Chapter 8 - Conclusions and remarks

I) Summary of previous findings

This dissertation has demonstrated that ivermectin at human relevant pharmacokinetics affects *An. gambiae* s.s.: survivorship and that cumulative ivermectin blood meals compounds mortality, blood feeding frequency, knockdown, and recovery. Ivermectin MDA to humans was shown to reduce the survivorship of wild-caught *An. gambiae* s.s. and probably *An. arabiensis* up to six days post-administration. Most importantly, ivermectin MDA to humans in a natural field experiment was shown to reduce the proportion of field-caught *P. falciparum*-infectious *An. gambiae* s.s. for at least 12 days post-treatment. The exact duration of *Plasmodium* transmission control with a single ivermectin MDA remains to be determined. The population age-structured model presented in Sylla et al. (2010) predicts that more frequent ivermectin MDAs can achieve a sustained reduction in malaria parasite transmission control. Clinical field studies need to be performed to determine if multiple ivermectin MDAs in a malaria transmission season does indeed achieve sustained *Plasmodium* transmission control.

II) Caveats and benefits to ivermectin MDA for malaria parasite transmission control

A) Ivermectin MDA - Synergism of targeting malaria with NTDs

There are several advantages for the use of ivermectin MDAs for malaria parasite transmission control. Ivermectin MDA is extremely safe for humans to take (Aziz et al. 1982, Pacque et al. 1990), only minimal training is needed for the drug to be administered without the supervision of medical practitioners (Brown 1998). Furthermore, ivermectin has been shown to reduce the incidence and intensity of not only onchocerciasis and lymphatic filariasis, but also other neglected tropical diseases (NTDs) such as scabeiasis, pediculosis, hookworm, trichuriasis, ascariasis, and strongyloidiasis (Campbell 1991, Dunne et al. 1991, Whitworth et al. 1991, Gann et al. 1994, Marti et al. 1996, Beach et al. 1999, Bockarie et al. 2000, Belizario et al. 2003, Heukelbach and Feldmeier 2004, Heukelbach et al. 2004, Wen et al. 2008, Gutman et al. 2010).

Several NTDs affected by ivermectin MDA are co-endemic with malaria across sub-Saharan Africa, including: onchocerciasis, LF, scabiasis, pediculosis, hookworm, trichuriasis, ascariasis, and strongyloidiasis (Brooker et al. 2006b, Nkuo-Akenji et al. 2006, Hay et al. 2009, Olsen et al. 2009, Clements et al. 2010, Glinz et al. 2010, Manguin et al. 2010). Ivermectin has been shown to be extremely effective at reducing the human burden of onchocerciasis, LF, ascariasis, strongyloidiasis, scabeiasis, and pediculosis with moderate effects against trichuriasis, and less effect against hookworms (Campbell 1991). However, ivermectin is not currently recommended for MDA for the control of soil-transmitted helminths (STHs) (Keiser and Utzinger 2008). The STHs, *Ascaris lumbricoides*, *Trichuris trichiura*, *Strongyloides stercoralis*, and the hookworms

(*Ancylostoma duodenale* and *Necator americanus*) are difficult to control with a single ivermectin MDA due to the high re-infection rates as the eggs or larvae present in soil are not affected by ivermectin treatment of humans (Anderson and Medley 1985, Freedman et al. 1989, Naquira et al. 1989, Whitworth et al. 1991, Marti et al. 1996, Beach et al. 1999, Belizario et al. 2003, Moncayo et al. 2008). *Pediculus capitus* and *Sarcoptes scabei* adults are affected by ivermectin MDA but ivermectin is not ovicidal, therefore re-infestation by unaffected eggs or contact with infested human carriers precludes a lasting treatment effect after a single ivermectin MDA (Heukelbach and Feldmeier 2004). Indeed, Heukelbach et al. (2004) found a dramatic reduction in the number of people infected with STHs, scabies and pediculosis at one month post ivermectin MDA but at nine months ascariasis, trichuriasis and pediculosis prevalence rates had begun to increase.

More frequent ivermectin MDAs, which will be necessary if ivermectin MDA is used to reduce malaria parasite transmission (see Chapters 6, Sylla et al. 2010), would undoubtedly reduce the prevalence and intensities of the NTDs (Ranque et al. 2001, Geary et al. 2010). STHs, especially hookworm, infections contribute to anemia due to intestinal blood loss (Brooker et al. 2006a). *Plasmodium* parasites can also cause severe anemia by haemolysis of red blood cells (RBCs) and adherence of asexual *P. falciparum* infected RBCs to the placenta of pregnant women (Miller et al. 2002). Concomitant infection with these parasites exacerbates anemia (Nacher et al. 2001) which leads to worsened child development (Midzi et al. 2010) and more adverse pregnancy outcomes (Yatich et al. 2009, Yatich et al. 2010) than these diseases would cause on their own. Therefore, the combined effects of ivermectin MDA on multiple parasites could

potentially reduce anemia in the human population which would lead to an overall improvement in human health beyond what would be expected from malaria control alone. As an example, in Nigeria, it was demonstrated that albendazole-treatment administered every four months not only reduced the prevalence and intensity of *A. lumbricoides* infections (Kirwan et al. 2009) but also reduced the likelihood of *Plasmodium* infection in children 1-4 years of age (Kirwan et al. 2010). The disease synergies between malaria and STHs, and the possibility of combined control of nematodes and malaria with ivermectin MDA may be a powerful incentive to integrate more frequent ivermectin MDAs into primary health care programs in co-endemic areas.

Numerous publications have highlighted the importance of combining efforts to control multiple diseases simultaneously (Molyneux and Nantulya 2004, Molyneux et al. 2005, Fenwick 2006, Lammie et al. 2006, Sachs and Hotez 2006, Hotez and Molyneux 2008, Hotez et al. 2011). (Druilhe et al. 2005) suggests that a reduction in STH infection prevalence will reduce the severity of malaria infections in the same population. Albendazole is commonly administered for the control STHs (Utzinger and Keiser 2004). Albendazole is very effective against ascariasis and hookworm, but less effective against trichuriasis (Keiser and Utzinger 2008). Therefore, the addition of albendazole to ivermectin MDA will have a greater reduction of STH prevalence and intensity than ivermectin or albendazole alone (Beach et al. 1999, Ismail and Jayakody 1999, Belizario et al. 2003, Olsen 2007, Ndyomugyenyi et al. 2008, Knopp et al. 2010). Ivermectin and albendazole combination is safe to administer by MDA (Olsen 2007) and MDA precludes the need for expensive clinical diagnosis of STH infected individuals (Brooker et al. 2006a). Although Ottesen et al. (2008) does not measure the impact of ivermectin and

albendazole MDA for LF eradication in Africa, they do demonstrate that millions of people are treated annually with these two drugs and that this will most likely impart some degree of STH control in treated communities. The addition of albendazole to ivermectin MDA should not interfere with the ability of ivermectin to affect *Anopheles* survivorship (see Chapter 2) and therefore not reduce the efficacy of dual drug MDA for malaria parasite transmission control.

More frequent ivermectin MDAs would increase the potential of resistance development in STHs. There has been no documented ivermectin resistance in human STHs, but this is most likely due to the difficulty of technical and ethical issues associated with resistance detection, than a lack of resistance (Prichard 2007). As evidenced by ivermectin resistance in veterinary nematodes (Kaplan 2004, Wolstenholme et al. 2004), it is likely that ivermectin resistance of human STHs will develop. The addition of albendazole to ivermectin MDA could help minimize resistance development by using agents with two different modes of actions.

B) Compliance with ivermectin MDA

Compliance with ivermectin MDA is based on concepts of the Health Belief Model (HBM). In disease terms, the HBM states that if a person perceives a disease risk, there is an increased likelihood that the person will comply with the prevention or treatment. This requires the person to believe that they are susceptible to the disease, the disease is serious enough to take action against, prevention will benefit them, and that barriers to prevention are not insurmountable (Rosenstock 1974). By providing ivermectin free of charge during MDA campaigns the barrier to prevention has been

eliminated, but education will be necessary to reinforce the issues of disease susceptibility, seriousness, and prevention. For onchocerciasis, there was a direct link of compliance with ivermectin MDA and knowledge of the benefits of ivermectin. This was achieved through education of local communities that participate in ivermectin MDA for onchocerciasis control (Okeibunor et al. 2011) and education will definitely be an important part any ivermectin MDA efforts for malaria parasite transmission control. In a survey of 1600 people from four countries, Okeibunor et al. (2011) found that 84.7% of people thought that ivermectin had benefits and that 89.7% felt that ivermectin was beneficial to their community. It is important to not only maintain high levels of awareness in communities of the direct personal benefits of ivermectin (i.e., reduction in intensity of scabiasis, pediculosis, STHs) but that ivermectin MDA is important for the community as a whole, whether it be for onchocerciasis, LF, STH, or malaria parasite transmission control. Maintaining high levels of treatment compliance will become difficult if community interest wanes as the perceived community benefit of treatment are less obvious as disease prevalence diminishes (Amazigo et al. 2002a).

The CDTI platform requires the appointment by the community of people within the community to serve as CDDs. The CDDs plan the periods, dates, and locations of village-level distribution (APOC/WHO 1998) which provides sustainability, empowerment of community participation, and recognition and tolerance of social customs (Amazigo et al. 1998). The use of CDTI increases coverage of villages within a region (WHO 1996, (Akogun et al. 2001) and CDDs increase treatment coverage and overall compliance within villages (Katabarwa et al. 2000). If ivermectin MDA for

malaria parasite transmission control is implemented, then it should rely on CDTI to reach the highest levels of coverage and compliance.

Compliance will definitely become a major factor if the frequency of ivermectin MDA is increased if it were used for *Plasmodium* transmission control. Other benefits of CDTI will be required to increase compliance and acceptance of ivermectin MDA. The CDTI platform has been integrated with primary health care systems to provide access to other public health measures such as the distribution of vitamin A (Remme and C.D.I.StudyGroup 2010), the malaria chemoprophylaxis sulfadoxine-pyrimethamine for intermittent preventive treatment for pregnant women (Ndyomugyenyi et al. 2009), and ITNs (Remme 2010 and C.D.I Study Group 2010). By incorporating multiple drug/intervention deliveries into the same CDTI platform (Molyneux et al. 2005) more benefits would directly be tangible to participating people and there would be a better chance of compliance remaining at levels necessary for control of several diseases.

Treatment refusal is always an issue and occurs for many reasons but is typically associated with the perceived likelihood of experiencing an AE or SAE. Indeed, in Cameroon 29.2% of people not treated by ivermectin MDA were direct refusals, which was three to four times higher than refusal rates in Nigeria, Tanzania, and Uganda. Surveys indicated that many of the refusals in Cameroon were associated with the fear of a SAE in regions where *L. loa* ivermectin-induced SAEs have occurred before (Amazigo et al. 2002b). Onchocerciasis control is still feasible in these areas with the use of doxycycline (Hoerauf et al. 2001, Wanji et al. 2009) but the threat of *L. loa*-induced SAEs would likely reduce compliance with ivermectin MDA for malaria parasite transmission control efforts.

Original specifications by the Mectizan Expert Committee (MEC 2000) stated that alcohol should be abstained from during ivermectin MDA over concerns that alcohol may increase AEs or SAEs. Several anecdotal occurrences of AEs were suspected to be caused by heavy ingestion of alcohol on the day of ivermectin MDA (Takougang et al. 2008). Many ethnic groups in Africa consider the consumption of alcohol to be important and imbibe alcohol on a daily basis. Recommendation that alcohol should be abstained from during ivermectin MDAs could reduce MDA compliance. Indeed, (Shu et al. 2000) found that CDDs of ivermectin encountered hostility when alcohol restrictions were enforced. A case-control study determined that routine ingestion of alcohol did not lead to an increase in SAEs during ivermectin MDAs (Takougang et al. 2008). In a controlled laboratory experiment, Shu et al. (2000) demonstrated that the consumption of alcohol with oral ivermectin (150 µg/kg) significantly increased plasma concentrations of ivermectin by approximately 160%. *Anopheles gambiae* s.s. is more attracted to humans that have consumed alcohol compared to those that consumed water (Lefevre et al. 2010). This means malaria vectors would not only be more attracted to people that imbibed alcohol just after MDA but elevated concentrations of ivermectin in human blood would further reduce *Anopheles* survivorship. These facts taken together indicate that the recommendation to abstain from alcohol during ivermectin MDA for malaria parasite transmission control is unnecessary and would only diminish compliance and reduce control efficacy.

C) Resistance development of ivermectin in mosquitoes

Ivermectin resistance development in *Anopheles* vectors would reduce the efficacy of ivermectin MDA for malaria parasite transmission control. Once or twice yearly ivermectin MDA has been occurring in southeastern Senegal for over fifteen years (Diawara et al. 2009), so the fact that a reduction in survivorship of *An. gambiae* s.s. was still detectable (see Chapter 5) is a promising sign that ivermectin resistance may be slow to develop in *Anopheles* spp. There are several intrinsic factors of ivermectin MDA to humans and mosquito biology that may limit the likelihood of resistance development in *Anopheles*. Unlike insect exposure to insecticides used in agricultural settings; only adult female mosquitoes ingest ivermectin, therefore no selection pressure for resistance occurs in males. Roughly 70 – 80% of people are treated during routine ivermectin MDAs. This provides a refugia of untreated humans that mosquitoes may blood feed upon, therefore not all *Anopheles* will be exposed to ivermectin after MDA. Developing larvae and pupae will not be exposed immediately after ivermectin MDA, which will reduce selection pressure. *Plasmodium* vectors that are partially zoophagic will imbibe blood from untreated livestock and thus not be exposed to ivermectin as frequently. Compared to DDT and other long-lasting insecticides used for IRS, ivermectin is only present in human plasma for a short duration which will diminish selection pressure as ivermectin is eliminated from human plasma. Circulating *Anopheles* spp. present outside primary *Plasmodium* transmission seasons would not be exposed to ivermectin as MDAs for *Plasmodium* transmission control would not be performed. All of the above listed intrinsic factors fulfill some of the properties of 'evolution-proof' malaria control strategies (Read et al. 2009).

The novel mode of action of ivermectin compared the currently used insecticides for malaria control could potentially minimize issues of cross-resistance where ivermectin MDA may be used in combination with IRS and ITNs. Abamectin, an avermectin with similar properties as ivermectin, is frequently used for pest control (Dybas 1989). Pyrethroid-resistant *Blattella germanica* (i.e., German cockroach) had no cross-resistance to ingested abamectin (Cochran 1990). (Roush and Wright 1986) tested six *Musca domestica* (i.e., house fly) insecticide-resistant strains (organochlorines – DDT and dieldrin; organophosphates; and pyrethroid - permethrin) and found none of them were cross resistant to abamectin via tarsal exposure. (Scott 1989) demonstrated that pyrethroid-resistant *M. domestica* strains were cross-resistant to abamectin when it was applied directly to the notum. Field-caught house flies selected for abamectin resistance were not cross-resistant via tarsal exposure to: organophosphates - crotoxyphos, dichlorvos, dimethoate, and tetrachlorvinphos; organochlorines – dieldrin and lindane; or pyrethroids - permethrin (Scott et al. 1991). Scott et al. (1991) admit that their findings that pyrethroid-resistant *M. domestica* were cross-resistant to abamectin but that abamectin-resistant *M. domestica* were not cross-resistant to pyrethroids were contradictory to each other and required further evaluation. These studies suggest that permethrin-resistance may not confer cross-resistance to avermectins in insects. However, as mosquitoes ingest ivermectin during blood feeding and not by tarsal contact; results with mosquitoes may differ. The use of insecticide-resistant *Anopheles* strains will be necessary to determine if insecticide-resistance confers cross-resistance to imbibed ivermectin in *Anopheles* mosquitoes.

In Benin, pyrethroid-based ITNs were ineffective against pyrethroid-resistant *An. gambiae* s.s. due to previous agricultural exposure (N'Guessan et al. 2007). Abamectin is frequently used to manage numerous arthropod crop pests (Dybas 1989), however, abamectin is not an insecticide used in Africa due to cost limitations (personal communication with Dr. Georgina Bingham). This means that *Anopheles* exposure to avermectins in Africa is restricted solely to blood meals from treated vertebrates.

Although ivermectin-resistance in *Anopheles* is a major concern if ivermectin MDAs were used for *Plasmodium* transmission control, no data exists concerning potential ivermectin-resistance or cross-resistance mechanisms in *Anopheles*. This is an area of laboratory and field-based research that will be necessary to expand upon were ivermectin MDA used for *Plasmodium* transmission control.

III) Applications of ivermectin other than human MDA for *Plasmodium* control

A) Ivermectin MDA to animals for *Plasmodium* transmission control

Ivermectin MDA to domesticated animals for malaria parasite transmission control operates under the same principal that ivermectin MDA to humans does; direct reduction in the daily probability of survivorship, feeding frequency, and density of malaria vectors. The concept of ivermectin MDA to domesticated animals for vector-borne disease control was suggested by several authors (Iakubovich et al. 1989, Focks et al. 1991, Wilson 1993, Gardner et al. 1993, Foley et al. 2000, Fritz et al. 2009). This method would be most appropriate where malaria vectors are zoophagic, such as *An. arabiensis* in sub-Saharan Africa (Lemasson et al. 1997), *An. farauti* in the south Pacific (Hii et al. 1995), or *An. stephensi* and *An. culicifacies* in West Asia (Reisen and Boreham

1982). Ivermectin MDA to domesticated animals might be more effective in rural areas where the ratio of livestock to humans is higher, and therefore the likelihood of malaria vectors feeding on livestock as opposed to humans increases, as seen with *Anopheles minimus* in southeast Asia (Trung et al. 2005). In many areas of the world, people keep their domesticated animals in or near their homes when they sleep. Nomadic people without permanent standing structures to treat with IRS would also benefit from ivermectin MDA to domesticated animals.

Rowland et al. (2001) topically treated cattle with Deltamethrin, a pyrethroid, in settled Afghan refugee camps in Pakistan. There was a 56% decrease in *P. falciparum* and 31% *P. vivax* clinical human cases across three years in experimentally treated compared to untreated villages. This exemplifies that malaria incidence in humans can be reduced by mass zooprophylaxis, defined here as treatment of domesticated animals. These results are similar to IRS but at 20% the cost and it was cheaper and more effective than previous ITN distribution. There was a great deal of enthusiasm from the local communities, not due to the substantial reduction in malaria burden, but instead to the benefits of the intervention on their cattle, which were apparent in elevated weight gains and milk yields (Rowland et al. 2001).

Ivermectin is very safe and effective against numerous endo- and ecto-parasites of livestock (Benz et al. 1989), therefore the direct benefits of ivermectin MDA to livestock would be apparent to owners and should ensure high levels of compliance. Several delivery methods have been developed for sustained release of ivermectin through bolus implantation or injections to extend the half life and duration of ivermectin control in livestock (Jackson 1989). Since ivermectin is a systemic insecticide there would not be

problems of inadequate coverage of individual livestock as observed for *An. arabiensis* with cattle and the pour-on formulation of deltamethrin (Habtewold et al. 2004).

Ivermectin MDA to livestock would rely on trained, traveling veterinarians so it may not be as feasible and cost effective as CDTI to humans. There is the possibility that *Plasmodium* vectors may be diverted from ivermectin-treated livestock to untreated humans which may increase *Plasmodium* transmission to humans. *Culicoides imicola* olfactory systems were found to be less responsive to ivermectin-treated sheep compared to untreated sheep (Sollai et al. 2007); but this has not been demonstrated with mosquitoes. Ivermectin treatment of domesticated animals too close to slaughter and milk consumption restrictions with treated dairy cattle are an issue in the United States, but not necessarily in developing countries (Benz et al. 1989).

A severe drawback of ivermectin MDA to domesticated animals would be the ensuing increase in ivermectin resistance in veterinary parasites. Veterinary parasite resistance to ivermectin has already been reported for numerous nematode parasites of sheep, goats, cattle, and horses (Kaplan 2004, Wolstenholme et al. 2004). Increasing the frequency of parasite exposure to ivermectin by ivermectin MDA to domesticated animals will undoubtedly exacerbate veterinary parasite resistance to ivermectin.

Although there are drawbacks to ivermectin MDA to livestock, this control strategy could have potential to reduce *Plasmodium* transmission in limited areas of Africa and abroad where humans are in close contact with their domesticated animals.

B) Treatment with ivermectin to possibly prevent drug-resistant *Plasmodium* transmission

Antimalarial drug-resistant *Plasmodium* spp. strains are a major threat to the current malaria eradication campaign (Plowe 2009). Chaccour et al. (2010) suggest that the combination of ivermectin with antimalarials could help prevent the spread of drug-resistant *Plasmodium*. *Plasmodium* drug resistant strains can be difficult to identify, especially in areas with diminished PHC services. The combination of ivermectin with antimalarial drugs would reduce *Anopheles* survivorship and thereby reduce transmission potential of *Plasmodium* drug-resistant strains. This could be achieved by incorporating ivermectin into blister packaging that is frequently used to contain Artemisinin-based Combination Therapy (ACT) antimalarial drugs (Diap et al. 2010). The cost of ivermectin distribution would be dramatically reduced if ivermectin was delivered to local ACT manufacturers and packaged in blister packs with ACTs. If a *Plasmodium* parasite is drug-resistant, then it will most likely be present in the human well after the concentration of ivermectin necessary to reduce *Anopheles* survivorship has been exceeded. Therefore, frequent ivermectin MDAs to humans regardless of *Plasmodium* infection status may be the better method to reduce the likelihood of transmission of drug-resistant *Plasmodium* parasites.

Historically, the major epicenter of antimalarial drug resistant *Plasmodium* development is along the Thai-Cambodian border. Chloroquine, sulfadoxine-pyrimethamine, and now possibly artemisinin drug-resistant *Plasmodium falciparum* strains have originated from the Thai-Cambodian border (Plowe 2009, Noedl et al. 2010). Mass screening and treatment (MST) of *Plasmodium* carriers in western Cambodia are

currently being employed to contain and eliminate malaria (White 2008). The combination of ivermectin with MST in western Cambodia may also help prevent the spread of drug-resistant *Plasmodium* in this area.

IV) Concluding Remarks -malERA vector control research agenda

Ivermectin MDA to humans meets many of the criteria set forth by the malERA CGoVC. Although not specifically demonstrated, ivermectin MDA to humans should target exophagic and exophilic vectors (see Chapter 5). As demonstrated by numerous studies ivermectin effectively reduces the survivorship of numerous important *Anopheles Plasmodium* vector species. Ivermectin MDA was clearly shown to integrate with ITN use and have a demonstrable reduction in *Plasmodium* transmission in an area with high ITN coverage (Chapter 6). Furthermore, the CDTI platform has been utilized for the successful distribution of ITNs (Remme 2010). Ivermectin has a mode of action (Kane et al. 2000) that differs from currently used insecticides for ITNs and IRS (Hemingway and Ranson 2005) and cross-resistance may be unlikely (Rhoush and Wright 1986, Cochran 1990, Scott et al. 1991). The only behavioral mechanism that could occur in mosquitoes to avoid imbibing ivermectin from treated humans would be to blood feed on untreated animals, which would still reduce vectorial capacity by reducing the frequency of blood feeding on humans (see Chapter 1 IV F). As discussed above, ivermectin MDA has numerous intrinsic factors that interact with natural mosquito biology to reduce the likelihood of physiological resistance development. Ivermectin MDA specifically alters the vector population age-structure (Sylla et al. 2010).

Future field studies are necessary to determine: the duration of *Plasmodium* transmission reduction that occurs after a single MDA, if multiple ivermectin MDAs in a malaria transmission season indeed cause a sustained reduction in *Plasmodium* transmission, how more frequent MDAs impact STH prevalence and intensity, and if ivermectin MDAs truly can lead to health benefits in humans as measured by a reduction in *Plasmodium* incidence and anemia in humans. Future laboratory studies should determine: if ivermectin inhibits sporogony in *Anopheles* vectors, the precise mode of action of ivermectin in *Anopheles*, and ivermectin-resistance and cross-resistance mechanisms in *Anopheles*. The mermithid parasite found in southeastern Senegal should: be re-isolated, formally described, colonized, and life history characterized to determine its relevance as a potential biological control agent.

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