DISSERTATION

EFFECTS OF IMMUNOLOGICAL TARGETING OF TWO MOSQUITO ANTIGENS AND ORAL INGESTION OF ANTHELMINTIC DRUGS ON THE YELLOW FEVER MOSQUITO, *AEDES AEGYPTI* (DIPTERA: CULICIDAE)

Submitted by

Kelsey Marie Deus

Department of Microbiology, Immunology and Pathology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2011

Doctoral Committee:

Advisor: Brian D. Foy

Anne Avery
William C. Black, IV
Richard A. Bowen

ABSTRACT

EFFECTS OF IMMUNOLOGICAL TARGETING OF TWO MOSQUITO ANTIGENS AND ORAL INGESTION OF ANTHELMINTIC DRUGS ON THE YELLOW FEVER MOSQUITO, *AEDES AEGYPTI* (DIPTERA: CULICIDAE)

Aedes aegypti is one of the most important mosquito vectors of human arboviruses, including dengue viruses, chikungunya virus, and yellow fever virus. Human infection with these viruses constitutes an enormous global disease burden. Current control methods rely heavily on the use of insecticides, which are rapidly losing their utility due to the spread of insecticide resistance. Anti-vector vaccines and anthelmintic drugs with insecticidal properties have been proposed as novel means to decrease pathogen transmission by reducing the daily probability of mosquito survival. The aims of this dissertation research were to: evaluate the *Ae. aegypti* mosquito lysosomal aspartic protease and the glutamate-gated chloride anion channel as potential mosquitocidal antigens, evaluate drugs frequently used in mass drug administration campaigns for their ability to induce a mosquitocidal effect when imbibed in a blood meal, to assess the variation in susceptibility of *Ae. aegypti* strains to orally imbibed ivermectin, and finally to determine if resistance to ivermectin could be selected for in a genetically diverse laboratory strain of *Ae. aegypti*. Despite the utilization of several immunization regimens, a specific mosquitocidal immune response against the *Ae. aegypti* mosquito lysosomal aspartic protease could not be verified. *In vitro* experiments in which high titer glutamate-gated chloride anion channel serum was fed to mosquitoes

failed to elicit a mosquitocidal response, suggesting that it is an unlikely mosquitocidal antigen. *In vitro* blood feeding experiments with several anthelmintic drugs revealed that high concentrations of macrocyclic lactones (including ivermectin, selamectin and moxidectin) were effective in reducing adult mosquito survival and that sublethal concentrations resulted in reduced fecundity and egg hatch rate. When imbibed in a blood meal, diethylcarbamazine, albendazole-sulfoxide and pyrantel pamoate, which are all currently used in human mass drug administration campaigns for controlling parasitic pathogens in humans, had no effect on adult mosquito survival. Significant differences in susceptibility to ivermectin, according to mosquito strain, were observed, with three permethrin-resistant strains of *Ae. aegypti* being the most refractory to ivermectin, suggesting a possible permethrin-induced cross resistance mechanism to ivermectin. After subjecting a genetically diverse laboratory strain of *Ae. aegypti* to three successive rounds of selection with orally imbibed ivermectin, no resistance to the drug was apparent. Although mass drug administration is unlikely to have any impact on the transmission of *Ae. aegypti* vectored pathogens, *Ae. aegypti* may prove to be a useful model for studying the effects of ivermectin in the mosquito, including studying potential resistance and cross-resistance mechanisms to anthelmintic drugs.

ACKNOWLEDGEMENTS

I'd like to thank my adviser, Dr. Brian Foy. Brian- Thank you for your optimism, encouragement and for giving me that extra push when I needed it! I've always and continue to be amazed by and admire your unquenchable optimism. Thanks for letting me pursue crazy ideas of my own, and for coaching me through some really tough experiments. I've especially appreciated your help and encouragement throughout a daunting task of writing a dissertation whilst working full time. I'm also thankful for the advice and help from rest of my graduate committee: Drs. William C. Black, IV, Richard Bowen, and Anne Avery. Bill- Thank you for all of the many hours you spent patiently helping with data analysis, statistics, and writing. Thank you also for being such an incredible mentor in the classroom. I often find myself drawing on the experiences I had as a GTA in MIP 462 to help me in my own classroom! Dick- Thank you for giving a college freshman from your introductory biology course (yikes…I think that was 10 years ago now) a chance to work in the lab! Thank you also for all of the support and encouragement you've provided through graduate school. You have and continue to be a great mentor! Anne- Thank you for your patience with a bug dork dabbling in the field of immunology. I've truly appreciated your candid advice, patience and tolerance of many silly questions pertaining to immunology.

I'd also like to acknowledge a number of funding sources that helped pay for my graduate schooling, including: the CDC Fellowship Training Program, the Emerging

Viral Disease Unit research grant and the Graduate Teaching Assistant positions provided through the Department of Microbiology, Immunology and Pathology at Colorado State University.

I'm thankful to many members of the Foy and Black labs for their assistance with rearing mosquitoes, and I'd especially like to thank Dr. Karla Saavedra-Rodriguez for providing so many mosquito lines, and for her help with the selection experiments!

I'm also grateful for the support of friends and family. Robyn Raban…you are a crazy woman, and I love you dearly. Thank you for helping me laugh all the way through graduate school. I'm also thankful for two friends in Casper, Evert and Tammy Brown, whose kindness, support and generosity I will be forever grateful for. You have taught me to look for the "pony" in every situation, and, yes Evert, I know…I'm going to get a bill! Most of all, I'm thankful for my family, who has never quit believing me and has provided me with an immeasurable amount of support. I'm especially thankful to my Mom and Grandad, who at a very early age stressed the value and importance of education. Mom- You were and continue to be a great example of what it takes to be both a mother and a student. I appreciate all you have done for our family, and your example has taught me more than a textbook ever could have. Mom, Dad, Steve and Abigail…thank you for your tireless encouragement, love, support and patience. I know that you have all sacrificed a lot to see me through my education, and there's no way I would have made it without you. You have never stopped believing in me, and I love you all more than words can say.

DEDICATION

To:

Miss Abigail Larae Deus and Dr. Dallas Eugene Shafer

TABLE OF CONTENTS

TABLE OF FIGURES

CHAPTER 1: LITERATURE REVIEW

Aedes aegypti

Aedes aegypti is a container-breeding mosquito in the subgenus *Stegomyia* which is globally distributed throughout the tropics and subtropics. There are at least two subspecies of *Ae. aegypti,* which include *Ae. aegypti aegypti* and *Ae. aegypti formosus.* The subspecies *Ae. aegypti formosus* occurs in sub-Saharan Africa, and preferentially oviposits in treeholes, and is rarely associated with urban arbovirus epidemics. The subspecies *Ae. aegypti aegypti* is a synanthropic vector and preferentially oviposits in artificial containers such as tires or trash that have collected rain water. Several characteristics, including extreme anthropophily (a preference to feed on human blood), a long life span and frequent blood feeding behavior make *Ae. aegypti aegypti* an exceptional vector for arboviruses of public health concern (Scott et al. 2000a, Harrington et al. 2001).

The global burden of arboviruses transmitted by *Ae. aegypti.*

Female mosquitoes of most mosquito species, including *Ae. aegypti,* must imbibe a blood meal for egg maturation. *Aedes aegypti* often takes multiple blood meals within a single gonadotropic cycle, thus making it a potent and deadly vector of arthropod-borne viruses (arboviruses). *Aedes aegypti* is one of the most important vectors of human arboviruses, including dengue viruses (DENV), chikungunya virus (CHIKV) and yellow fever virus (YFV) (Gubler 2002, Staples et al. 2009).

Dengue. In the past few decades, the incidence of infection with various arboviruses has increased, but have not surpassed dengue viruses with respect to their economic and public health importance. An estimated 2.5 billion people living in urban

and tropical areas of Southeast Asia, the Pacific, the Americas, the Eastern Mediterranean and in Africa are at risk of infection (WHO 2007). Globally, it is estimated that dengue sickens 50-100 million people, killing an estimated 19,000 annually (Mackenzie et al. 2004). Of significant concern is the rise in the number of dengue fever (DF) and dengue hemorrhagic fever (DHF) cases reported to the World Health Organization, and the fact that the geographical areas in which dengue transmission occurs have expanded (Rigau-Perez et al. 1998, Gubler and Meltzer 1999, Gubler 2004, Wilder-Smith and Schwartz 2005, Guzman et al. 2010). The number of cases reported between 2000-2004 nearly doubled from the number of cases recorded from 1990-1999 (WHO 2007). Dengue epidemics are costly and impart a significant economic burden on areas affected. The costs associated with a 1981 dengue epidemic were estimated at US$103 million (Kouri et al. 1989), and in endemic countries in the Americas and Asia, the burden of dengue is approximately 1,300 disability-adjusted life years (DALYs) per million population; a number on the same magnitude as malaria and meningitis (Gubler and Meltzer 1999, Clark et al. 2005).

Dengue fever (DF), sometimes referred to as "breakbone fever," is caused by infection with a dengue virus (DENV). There are four dengue virus serotypes, referred to as DENV-1, DENV-2, DENV-3 and DENV-4. In most cases, DF is a self-limiting, febrile illness characterized by a high fever, headache, retroorbital pain, bone pain, and a rash (Monath and Heinz 1996). Dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) are more severe forms of disease resulting from DENV infection. In the case of DHF/DSS, spontaneous hemorrhages can occur internally and externally, and other complications such as multiple organ failure and respiratory failure can occur,

leading to severe hypotension and shock (Monath and Heinz 1996). Currently, there is neither a vaccine or an anti-viral therapy that is effective in preventing or treating infection with DENV, and treatment of DF, DHF and DSS rely on supportive therapies such as oral or intravenous fluids and acetaminophen (Guzman et al. 2010).

Dengue viruses circulate through three transmission cycles: the sylvatic, rural/epidemic and urban/epidemic transmission cycles (Gubler 1998). In the sylvatic cycle, DENV is passed between susceptible monkey species and canopy-dwelling *Aedes* mosquitoes. In the rural/epidemic transmission cycle, peridomestic mosquito vectors such as *Aedes polynesiensis, Aedes albopictus,* and possibly *Aedes mediovittatus* bridge the transmission of DENV from the enzootic transmission cycle to the human population. In the urban/epidemic cycle, DENV viruses are transmitted laterally between humans and the mosquito vector alone, with *Ae. aegypti* being the principle vector for epidemic dengue transmission.

Chikungunya. Chikungunya fever is an acute febrile illness resulting from infection with CHIKV. The word, Chikungunya, is derived from the Makonde word meaning, "that which bends up," which references the characteristic stooped posture observed in individuals experiencing the sometimes debilitating arthritic symptoms resulting from infection with CHIKV (Robinson 1955). While death due to infection with CHIKV is rarely observed, the arthralgia resulting from infection can be debilitating and can last from several weeks to months. Like DEN viruses, CHIKV has been found to circulate in a sylvatic cycle between forest-dwelling *Aedes* mosquitoes and nonhuman primates (Jupp and McIntosh 1988). Also similar to DEN viruses, the virus can be transmitted in urban epidemic cycles, circulating between humans and mosquitoes, with

the two principle vectors being *Ae. aegypti* and *Ae. albopictus.* In 2004, a major outbreak of CHIKV occurred. The outbreak originated on the Kenyan coast, and spread rapidly throughout several islands in the Indian Ocean, Southeast Asia and India (Staples et al. 2009), and was imported into at least 18 countries throughout Asia, Europe, and North America where cases of chikungunya fever had previously been unreported (Rezza et al. 2007, Kariuki Njenga et al. 2008, Sergon et al. 2008).

Yellow fever. To date, only one arbovirus, the yellow fever virus, has a safe and effective vaccine for prevention of infection. Despite the availability of the vaccine, yellow fever epidemics continue to occur in parts of Africa and South America, with mortality rates as high as 81 percent (Gould et al. 2008, Ekenna et al. 2010). The epidemiology of yellow fever is quite similar to that of dengue, with the sylvatic cycle and urban epidemic cycles being bridged. Yellow fever is caused by infection with the yellow fever virus, with *Ae. aegypti* being a frequent vector. Indeed, *Ae. aegypti* is so often associated with yellow fever transmission that it is often referred to as the "yellow fever mosquito."

Historical and current vector control strategies

In the 1950's-1960s, under the organization of the Pan American Health Organization, many countries in South and Central America eradicated *Ae. aegypti* through the use of environmental management and insecticides (Gubler 1998). Regrettably, many of these programs were eliminated in the early 1970's and shortly after their discontinuation *Ae. aegypti* re-infested the areas and dengue epidemics resurfaced. Increased urbanization, travel, and a lack of mosquito control measures have lead to the expansion of *Ae. aegypti* habitat (Gubler 2004). The expanse of *Ae. aegypti* habitat,

combined with the emergence of new virus strains and the importation of novel serotypes has lead to a sharp increase in the number of DF and DHF cases.

In recent years, there have been concerted efforts to encourage community participation in the control of *Ae. aegypti,* with emphasis being placed on educating community members of the importance of source (container) reductions (Winch et al. 1991, Leontsini et al. 1993, Lloyd et al. 1994, Rosenbaum et al. 1995). While such programs and campaigns have and continue to be successful, they are difficult to maintain because of the fact that a sustained and consistent effort is required. It is clear that integrated vector elimination efforts are the most effective at preventing epidemic outbreaks of arboviral disease in the human population; however, focus has shifted towards chemical control measures (Lloyd 2003).

Ultra-low volume (ULV) aerosols are recommended for use primarily during an epidemic outbreak of an arboviral disease such as dengue. However, the routine use of ULV application of insecticides is widespread (Gubler 2002). The utility of aerosol insecticides is threatened by the spread of pyrethroid resistance, which has been reported in several locations across the globe (Flores et al. 2006, Jirakanjanakit et al. 2007b, Jirakanjanakit et al. 2007a).

Vectorial capacity

It is clear from the eradication efforts from the 1950's-1960's that vector eradication efforts will, indeed, curb rural epidemic transmission cycles of arboviruses. However, it is also quite clear that such efforts may not be sustainable in the long term. With an increase in global travel and shipping, the risk of introducing *Ae. aegypti* into an

area is ever present. Likewise, as seen in the 2004 CHIKV epidemic, an arbovirus can enter a human population through importation and result in local autochthonous (or local) transmission (Powers and Logue 2007). In the face of the rapid spread of insecticide resistance, it is clear that population suppression is neither a sustainable nor effective practice for reducing arboviral transmission.

The Vectorial capacity (V) is the average number of potentially infective bites delivered by all of the vectors feeding on a single host in one day (Fine 1981, Black and Moore 2005). The equation for V is

$$V = \frac{ma^2 p^n b}{-\ln p}$$

Vectorial capacity takes into account many different aspects of pathogen transmission by an arthropod vector, including the vector density in relation to the host (m), the probability that a vector feeds on a host in one day (a), the proportion of vectors ingesting an infective meal that become infective (b), the probability that the vector will survive one day (p), the duration of the extrinsic incubation period (EIP), given in days, (n), and the duration of the vector's life, in days, after surviving the extrinsic incubation period (1/-ln p). Three variables, a, n and p are the variables that are the most influential on the magnitude of V. However, small changes in p are the most influential on the magnitude of V. The vectorial capacity model predicts that control strategies that may cause even slight reductions in the daily probability of mosquito survival can have a profound impact on pathogen transmission by a mosquito vector. Ultimately, such a strategy would target the ability of a mosquito to survive the EIP. If the vector fails to survive the EIP, it is unable to transmit the pathogen. The body of work presented here investigates two

approaches, mosquitocidal vaccines and oral ingestion of anthelmintic drugs in a blood meal, for their ability to shorten the life span of *Ae. aegypti*. Importantly, the same characteristics that make *Ae. aegypti* such an efficient vector (extreme anthropophily, long life span and frequent blood-feeding behavior) are specifically targeted through either a mosquitocidal vaccine or through the ingestion of an anthelmintic drug circulating in the bloodstream of a human host. Because urban epidemics of dengue, chikungunya, and Yellow Fever are transmitted laterally between humans and mosquitoes, the vaccine or anthelmintic drugs need only be administered to humans. *Aedes aegypti* is also a frequent feeder, and it is estimated that the mosquito will feed on five to ten humans over the course of the EIP (Scott et al. 2000a, Harrington et al. 2001). Only one blood meal needs to be mosquitocidal to interrupt disease transmission. Further, because the mosquito feeds frequently, coverage of the human population would not need to be complete. Mathematical models have predicted that limited vaccine efficacy and coverage that causes only small reductions in the daily probability of mosquito survival can translate into drastic reductions in the transmission of dengue viruses (Billingsley et al. 2008). Wilson (1993) also highlights the potential ability of orally imbibed endectocides to decrease vectorial capacity by interrupting the EIP, rather than through population suppression.

Anti-vector vaccines

A historical account of anti-vector vaccines

Anti-vector immunity was first demonstrated by William Trager in 1939. Trager immunized guinea pigs and rabbits with extracts from the tick *Dermacentor variabilis*, and observed that ticks which had fed on the immunized animals failed to engorge or

exhibited reduced survival after blood feeding (Trager 1939b, a). In the seven decades since Trager's reports of anti-tick immunity, anti-vector research has been conducted with many arthropod vectors, but limited success has been achieved in identifying specific anti-vector antigens. In the 72 years since Trager's discovery, a handful of anti-vector antigens have been described, and most of these are from ticks (Willadsen 2004).

The development of anti-vector vaccines relies on priming a vertebrate immune system against vector antigens belonging to one of two groups: antigens to which the host is exposed during vector feeding, and "concealed" antigens which reside inside the vector to which the host immune system would be exposed during the host-vector interaction (Willadsen et al. 1993, Willadsen 2004). Ultimately, the vector would imbibe immune components from the host which would prevent the vector from feeding or drastically reduce survival following a blood meal. While anti-vector research has been conducted with many major arthropod vectors, anti-tick research has been the most successful, culminating in the development and deployment of two commercial anti-tick vaccines, both of which incorporate a recombinant glycoprotein (*Bm*86) from the cattle tick, *Boophilus microplus,* as the immunization antigen (Willadsen et al. 1995a, Garcia et al. 2009). The *Bm*86 antigen was identified by first immunizing cattle with crude extracts prepared from homogenates of *B. microplus*, and then subjecting immunized cattle to a tick bioassay (Johnston et al. 1986, Kemp et al. 1986). Isolating the *Bm*86 antigen was laborious, requiring multiple rounds of biochemical fractionation and purification followed by immunizations and tick challenges, with the final purification of the *Bm*86 protein requiring nearly one kilogram of engorged ticks (Willadsen et al. 1989). Remarkably, however, development and licensing of the *Bm*86 vaccines took just slightly

over 12 years to accomplish (Willadsen 2004), a remarkably short time frame when compared to the time required to develop and license pesticides or pharmaceuticals. The two vaccine preparations, TickGARD Plus and GAVAC Plus have been used in Australia and South America, respectively, and are effective in reducing the tick burden of cattle (de la Fuente et al. 1998, de la Fuente et al. 1999, Jonsson et al. 2000, Valle et al. 2004). The success of developing and licensing TickGARD Plus and GAVAC Plus demonstrate that development and deployment of anti-vector vaccines is not only feasible, but may also require less time for development and deployment than traditional pesticides or pharmaceuticals.

While the development and licensing of TickGARD Plus and GAVAC Plus are encouraging, progress in identifying other anti-vector antigens has been slow, and the majority of the described anti-vector antigens are from ticks. Other vectors for which anti-vector antigens have been described include: the sheep blow fly, *Lucilia cuprina,* and the mosquitoes *Anopheles gambiae* and *Aedes albopictus.* Immunologic targeting of two peritrophic matrix proteins of *L. cuprina* is effective in reducing survival of the larvae *in vivo* (Wijffels et al. 1999). To date, only two specific mosquitocidal antigens, the *Anopheles gambiae mucin I* (AgMucI) and *Aedes albopictus* subolesin, have been described (Foy et al. 2003, Canales et al. 2009).

Anti-mosquito immunity was first demonstrated by Alger and Cabrea (1972), who immunized rabbits with homogenate prepared from the midguts of *Anopheles stephensi* mosquitoes. Since Alger and Cabrea's report, a number of other studies demonstrating anti-mosquito immunity have been published (Alger and Cabrera 1972, Sutherland and Ewen 1974, Hatfield 1988, Ramasamy et al. 1988, Ramasamy et al. 1992, Noden et al.

1995, Ramasamy et al. 1996, Almeida and Billingsley 1998, Lal et al. 2001), but results from the reports are difficult to compare because of the disparate methodologies and immunization preparations used in the experiments. Immunized animals included mice, guinea pigs and rabbits, and the methods used to prepare immunizations were not consistent across experiments. Further, some studies fed mosquitoes directly on immunized animals whereas others fed mosquitoes serum harvested from immunized animals through glass membrane feeders.

Cathepsin D aspartic proteases: Biochemistry and their use as immunization antigens against other parasites.

Cathepsin D aspartic proteases (CDAP) are endopeptidases whose catalytic activity requires two aspartic acid residues as active centers, Asp33 and Asp231 (Tang and Wong 1987). The CDAPs are active in an acidic range of pH 3.0-4.5, and are responsible for the majority of proteolytic activity in the cellular lysosomal compartment (Yamamoto 1999, Minarowska et al. 2009). A key characteristic of CDAP is that enzymatic catalytic activity is inhibited by pepstatin A (Barrett 1998). The role of aspartic proteases in the degradation of hemoglobin by blood feeding parasites is well documented, and *in vitro* and *in vivo* experiments targeting the CDAPs of blood feeding nematode parasites, immunologically and through the use of protease inhibitors, have shown deleterious effects on parasite physiology (Bogitsh et al. 1992, Brindley et al. 2001, Williamson et al. 2002, Williamson et al. 2003a).

In nematode parasites, hemoglobin degradation occurs through a cascade of proteolytic cleavages, and there is overwhelming evidence that CDAP are the apical enzyme in the proteolytic cascade involved in hemoglobin degradation (Bogitsh et al.

1992, Brown et al. 1995, Williamson et al. 2002, Williamson et al. 2003b, Williamson et al. 2003a). *In vitro* inhibition of the CDAP of *Schistosoma japonicum* and *Necator americanus* through the use of pepstatin A has been shown to prevent hemoglobin degradation and inhibit nematode growth (Bogitsh et al. 1992, Brown et al. 1995). Also, RNA-interference (RNAi) mediated knockdown of CDAP transcript in *Schistosoma mansonii* has been shown to block hemoglobin digestion and inhibit parasite growth *in vitro* (Morales et al. 2008). Given the critical role of CDAP in hemoglobin digestion and survival of nematode parasites, it is not surprising that CDAP have been investigated as potential antigens for the development of vaccines to protect against infection with these parasites. To date, two vaccines targeted against the CDAP of nematode parasites have been tested *in vivo* (Verity et al. 2001, Hotez et al. 2002).

In a study by Hotez *et al.*(2002), dogs were immunized using a recombinant CDAP from *Ancylostoma caninum*. Dogs challenged with *A. caninum* L_3 larvae exhibited an 18% reduction in worm burden in the small intestine when compared to control immunized dogs. Curiously, worm burden of the colon was elevated in immunized dogs and the cumulative worm burden did not differ between dogs immunized with the recombinant CDAP and control immunized dogs. Greater success in reducing adult worm burdens has been documented in a *S. japonicum* mouse model. In this study, mice were immunized with recombinant CDAP from *S. japonicum,* and then later challenged with *S. japonicum* cercaria. Mean total worm burdens were reduced by 22-40% over control immunized mice, and a reduction in the female worm burden (21-38%) was also observed (Verity et al. 2001).

The mosquito lysosomal aspartic protease

In 1991, the *Ae. aegypti* lysosomal aspartic protease (AaMLAP) was isolated from pre-vitellogenic mosquitoes. The enzyme was classified as a CDAP due to its ability to use hemoglobin as a substrate, and the fact that it was inhibited by pepstatin A (Cho et al. 1991). The protein was found in the head, thorax, midgut and fat body, with the highest concentrations occurring in the fat body. The complementary DNA (cDNA) for AaMLAP was later cloned and sequenced. The AaMLAP mRNA and protein are highly upregulated following a blood meal, and reach peak levels at 24 and 42 hours, respectively (Cho and Raikhel 1992).

In mosquitoes, a multitude of proteolytic enzymes are secreted after the ingestion of a blood meal, with midgut trypsins being the predominant enzyme required for blood meal digestion (Pennington and Wells 2005). However, there is no information on what, if any, role CDAP may play in blood meal digestion or hemoglobin degredation in mosquitoes. Given its strong upregulation following a blood meal, AaMLAP is thought to play a role in the regulation of vitellogenesis (Cho and Raikhel 1992), though a precise mechanism is yet unknown.

Anthelmintics and Mass Drug Administration

History and discovery of the avermectin and milbimycins

Since its discovery, ivermectin has greatly improved life for billions of people across the globe. The broad-spectrum activity, safety, and relatively low cost of the drug have made it a potent tool in combating the devastating effects of onchocerciasis and lymphatic filariasis.

Ivermectin. Ivermectin belongs to the class of anthelmintic drugs known as the avermectins. The avermectins were discovered in 1973 through a collaborative effort between the pharmaceutical company Merck, Sharp and Dohme (USA) and the Kitasato Institute (Tokyo, Japan). The partnership was formed to test bacterial samples for anti-parasitic compounds, and ultimately culminated in the discovery of avermectin, which was extracted from the fermentation products of *Streptomyces avermitilis* (Burg et al. 1979). The ability of avermectin and milbemycin compounds to kill both endo and ectoparasites lead to the name 'endectocide,' and the word avermectin reflects the utility of the drug class (*a*- without, *verm*- worm, *ect*- ectoparasite, *in*- pharmaceutical product) (Egerton et al. 1979).

The avermectins are 16-membered macrocyclic lactones. *Streptomyces avermitilis* produces avermectins as a mixture of eight different components in fermentation, and these compounds are: A_{1a}, A_{1b}, A_{2a}, A_{2b}, B_{1a}, B_{1b}, B_{2a}, and B_{2b} (Shoop et al. 1995). The most potent anthelmintic B components have a methoxy group at the 5-position, whereas the A components have a hydroxy group (Egerton et al. 1980). The major component (1) is distinguished from the (2) component by the presence of a double bond between the 22- and 23- carbon position. During the fermentation process, the A-component is produced in greater proportions than the B-component, although separation of the A and B components is rarely ever completed in commercial production because the components have nearly identical potency.

Ivermectin (Fig 1.1) was the first commercialized drug in the avermectin drug class, and is synthesized by hydrogenating the *cis* 22,23-double bond of avermectin B_1 (Chabala et al. 1980, Egerton et al. 1980). Ivermectin was released for use in

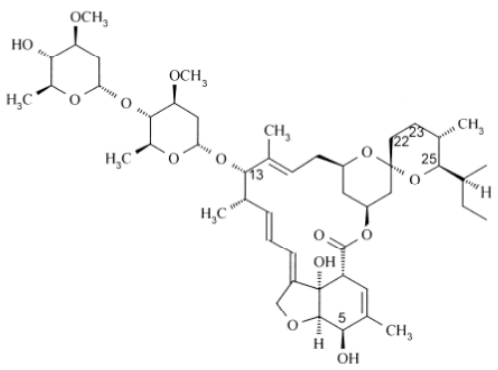
veterinary medicine in 1981, and is now routinely used in human and veterinary medicine as a broad-spectrum endectocide.

Selamectin. Selamectin (Fig 1.1) is a semi-synthetic modification of doramectin, and belongs to the avermectin drug class. Idiosyncratic toxicity of ivermectin in Collies was described shortly after the drug was marketed for use in companion animals (Paul et al. 1987), and selamectin was synthesized to serve as a broad-spectrum alternative**.** In companion animals, selamectin is applied topically, and has a high efficacy against many endo- and ectoparasites including fleas, sarcoptic and otodectic mites, roundworms, hookworm and heartworm (Krautmann et al. 2000, Novotny et al. 2000). Selamectin has not yet been evaluated for safety or tolerability in humans; however, the fact that it can be applied topically and exhibit broad-spectrum activity against a range of parasites could make it an attractive drug for future use in human mass drug administration (MDA) programs.

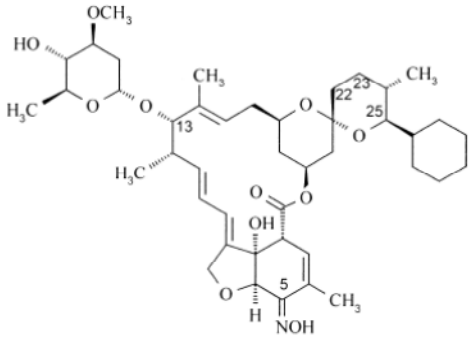
Moxidectin: Moxidectin (Fig 1.1) belongs to the class of drugs known as the milbemycins, which are structurally very similar to the avermectins. Milbemycins were discovered in 1973, and were initially used for crop protection against insects (Nakagawa et al. 1995) , but their utility for animal health applications has rapidly expanded. Naturally occurring milbemycins are fermentation products of *Streptomyces hygroscopicus* and *Streptomyces cyaneogriseus* (Takiguchi et al. 1980, Carter et al. 1988). Like the avermectins, milbemycin compounds are also subdivided into the A- and B-components. Unlike the avermectins, milbimycins have a single bond between the 22- and 23- carbon positions, making them more structurally similar to the avermectin 2-components. The primary difference between the avermectins and milbemycins is that

milbemycins have a disaccharide substituent at the 13-position of the macrolide ring (Fig 1.1), which is lacking in the avermectins.

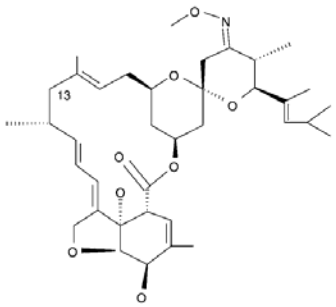
The milbimycin, moxidectin, is used in numerous veterinary applications, including prevention of canine heartworm, and for treatment of endo- and ectoparasites of livestock. Recently, moxidectin was shown to be well-tolerated in humans (Cotreau et al. 2003), and is currently being evaluated for the treatment and prevention of onchocerciasis (Siva 2009).



A. Ivermectin



B. Selamectin



C. Moxidectin

Figure 1.1: Molecular structures of Ivermectin, Selamectin and Moxidectin

Mechanism of action and tolerability of anthelmintic drugs.

The avermectins and milbemycins. Ivermectin was initially thought to exhibit anthelmintic activity by interfering with gamma-aminobutyric acid (GABA) chloride anion channels. This early hypothesis was proposed based on the knowledge that GABA receptors play a critical role in nematode movement (McIntire et al. 1993). Early evidence seemed to support the hypothesis in that ivermectin caused an increase in the chloride ion conductance of GABA channels (Kass et al. 1980, Supavilai and Karobath 1981, Graham et al. 1982). Further, such an effect could be inhibited through treatment with picrotoxin, a known GABA-gated chloride channel blocker (Olsen and Tobin 1990). Electrophysiology studies in nematodes, however, revealed that the concentration of ivermectin required to open GABA channels in nematodes, *in vitro,* was far beyond concentrations that demonstrated clear antiparasitic effects *in vivo* (Holden-Dye and Walker 1990) . Through injecting fractionations of total mRNA prepared from *Caenorhabditis elegans* into *Xenopus* oocytes, two glutamate-chloride anion channel (GluCl) subunits were identified. When the subunits were co-expressed, a GluCl channel that was sensitive to ivermectin was identified (Cully et al. 1994). Further evidence for the GluCl as the target for ivermectin was provided by Dent et. al (2000), demonstrating that a loss of function of the avr-14, avr-15 and glc-1 genes of the GluCl channels of *C. elegans* resulted in a high level of ivermectin resistance (Cully et al. 1996b). Ivermectin agonizes the GluCl channels located at the neuromuscular junctions, resulting in an influx of chloride ions and hyperpolarization of the nerve-cell membrane culminating in flaccid paralysis and death of the nematode or insect (Cully et al. 1996b, Kane et al. 2000). Recently, the X-ray crystal structure of ivermectin bound to the GluCl channel of *C.*

elegans was elucidated, showing that ivermectin integrates between the transmembrane domains of the GluCl channel subunit proteins found in the neuromuscular junctions. Precise mechanisms of action of the milbemycins, including moxidectin, have not yet been proposed; however, one study in *Haemonchus contortus* suggests that ivermectin and moxidectin bind to the same site on the *H. contortus* GluCl ion channel (Forrester et al. 2002).

Ivermectin is well-tolerated in humans and, even at exceptionally high doses, serious adverse effects are rarely observed (Edwards et al. 1988). Most adverse effects following treatment with ivermectin are associated with treatment in individuals with high microfilarial loads, and are thought to result from immunological clearing of dead microfilaria (Mackenzie et al. 2003). Symptoms are generally mild and generally include itching, papular rashes, dermal swelling, headache, nausea and lethargy (De Sole et al. 1989a, De Sole et al. 1989b, Pacque et al. 1989, Zea-Flores et al. 1992, Shoop et al. 1995, Kipp et al. 2003, Ramzy et al. 2006).

Serious adverse effects in humans following treatment with ivermectin are rare, with the exception being when ivermectin is used to treat those co-infected with *Onchocerca volvulus* and *Loa loa* (Chippaux et al. 1996, Boussinesq et al. 1998, Kamgno et al. 2000). *Loa loa,* commonly referred to as "eye worm" is geographically distributed in West and Central Africa. Many infections are asymptomatic, and the infection is usually diagnosed when adult worms are detected migrating under the skin or the sclera of the eye (Boussinesq 2006). Severe adverse effects observed in people that are co-infected with *O. volvulus* and *L. loa* include confusion, lethargy, paralysis, loss of conscientious, and death (Gardon et al. 1997, Boussinesq et al. 1998).

In 2003, the tolerability of Moxidectin was assessed in healthy human volunteers. Moxidectin was shown to be well-tolerated with few side effects at oral doses up to 36 mg. Side effects were mild with no serious adverse effects observed (Cotreau et al. 2003). Field trials are now underway to evaluate the efficacy of moxidectin in onchocerciasis trials (Siva 2009).

Currently, selamectin is only licensed for use in veterinary applications, and no information is available on the tolerability or safety of selamectin in humans.

Diethylcarbamazine. Diethylcarbamazine (DEC) has been used for the control of lymphatic filariasis since 1948 (Gyapong et al. 2005). Although this drug has and remains a mainstay in treatment of filariasis, treatment with DEC fails to clear all microfilaria, and has a limited effect on adult worms (Helmy et al. 2006). Systemic adverse reactions to treatment, including a myriad of neurological symptoms, moderate to severe fever, and haematuria (blood in the urine) have been reported (Dreyer et al. 1994). These adverse effects have been blamed for sometimes poor patient compliance (Babu and Satyanarayana 2003, Babu and Kar 2004). The precise mode of action for DEC remains unclear, although DEC does require host components to exhibit filaricidal activity. The potential of DEC to act as an antifilarial agent was first demonstrated in a wood rat model (Hawking et al. 1948). When wood rats infected with the filarial parasite *Litomosoides carinii* were given an intravenous injection of DEC, the microfilaria were cleared within minutes of treatment. *In vitro* treatment of microfilaria with DEC, even at exceptionally high concentrations of 100mg/ml, failed to kill the microfilaria. Although a precise mechanism of action for DEC is unknown, experiments with gene-knockout mice and pharmacological inhibitors indicate involvement of the

arachidonic acid and nitric oxide pathways in the microfilaricidal activity of DEC *in vivo* (McGarry et al. 2005). When DEC was administered to nitric oxide knockout mice (iNOS-/-) which had been infected with *Brugia malayi*, the microfiliaria levels in the iNOS-/- mice were significantly greater than in the corresponding controls. The involvement of the arachidonic acid pathway in the *in vivo* activity of DEC is supported by the fact that mice infected with *B. malayi,* and then treated with dexamethasone and DEC fail to significantly clear any of the microfilaria when compared to mice treated with only DEC (McGarry et al. 2005). Dexamethasone exhibits anti-inflammatory activity by inhibiting the first stage of the arachidonic acid pathway (Yao et al. 1999).

Albendazole. Albendazole is a broad-spectrum, orally-administered anthelmintic. Albendazole was first licenced for veterinary use, but was licensed for human use in 1982. Upon absorption, albendazole is rapidly metabolized to the active metabolite, albendazole-sulfoxide, which has a high systemic availablility making it a broad-spectrum drug effective in treating difficult parasitic diseases such as echinococcosis (Dollery 1999). When administered alone, and over the course of several months, albendazole is effective at decreasing *Wuchereria bancrofti* microfilaraemia (Gyapong et al. 2005). When given in combination with DEC, the combination therapy is more effective at reducing *W. bancrofti* microfilaraemia than either of the drugs alone (Wamae et al. 2010). Albendazole inhibits the polymerization of β-tubulin, which in turn inhibits the formation of microtubules, organelles required for many important cellular functions including the movement of chromosomes during cellular division, providing cellular structure, and trafficking vesicles and glucose throughout the cell (Martin 1997).

A number of studies have clearly demonstrated that albendazole may be a potent and highly efficacious drug in the treatment of bancroftian filariasis (Addiss et al. 1997, Dunyo et al. 2000b, a, Wamae et al. 2010), however tolerability and safety studies are somewhat conflicting. While 1000 mg oral doses of albendazole were well-tolerated in cancer patients (Pourgholami et al. 1998), there is some evidence that a three-day course of albendazole could impair growth in children (Forrester et al. 1998). Given albendazole's potent and broad-spectrum activity against a number of helminth parasites, it is an attractive drug to consider for MDA, however given the conflicting data with respect to safety, further research is necessary to determine if albendazole is a drug that could be safely and routinely used in MDA regimens.

Pyrantel. Pyrantel is one of four medications used for the treatment and control of soil-transmitted helminth infections, and is frequently administered via MDA (Hotez et al. 2007). Pyrantel agonizes the synaptic nicotinic acetylcholine receptors found in nematode muscle cells. Binding of pyrantel to the nicotinic acetylcholine receptor results in depolarization of the membrane by opening non-selective cation ion channels, and the influx of sodium and potassium ions results in continuous contraction of the muscle leading to paralysis and ultimate death of the parasite (Martin 1997).

Reports of the tolerability and pharmacokinetics for pyrantel in humans is sparse, and the information that is available is conflicting. In a study in Nigeria, nearly half of all patients treated with pyrantel experienced abdominal pain, nausea and dizziness (Kale 1977), whereas another study did not report any (Sinniah et al. 1990).

Mass drug administration for eradication of onchocerciasis and lymphatic filariasis.

Onchocerciasis.

Onchocerciasis, also known as river blindness, is caused by infection with the filarial nematode, *Onchocerca volvulus* (Bush et al. 2001). Onchocericasis is the second leading infectious cause of blindness, and occurs primarily in Africa with additional foci of transmission occurring in Latin America and the Middle East (Boatin and Richards 2006). The *O. volvulus* parasite is transmitted to humans through the bite of a number of species of black flies belonging to the genera *Simulium* (Robles 1917). Larval parasites (microfilaria) move throughout the body, migrating to the dermal tissue and eye. Symptoms of onchocerciasis include severe itching, lymphadenopathy, skin atrophy, and depigmentation. Interestingly, onchocerciasis symptoms are not directly caused by the worm, but rather by the host immune response that is mounted against a *Wolbachia* endosymbiotic bacteria that is released upon death of the microfilaria. If microfilarial death occurs in the eye, the inflammatory response can result in ocular lesions and atrophy leading to blindness (Keiser et al. 2002).

Prior to the use of ivermectin, treatment of onchocerciasis was complicated for a number of reasons. First, the lifespan of adult female worms can be as long as 12-15 years. Second, chemotherapeutic treatments are often ineffective at killing the adult worm (Habbema et al. 1992). Further, the chemotherapeutic treatments with suramin or DEC frequently resulted in severe adverse reactions. Suramin is a highly toxic compound, which is administered by the intravenous route. Patients receiving suramin must be closely monitored due to the high frequency of severe adverse reactions. Diethylcarbamazine causes a rapid clearance of microfilaria, triggering a potent host

inflammatory response which frequently results in blindness (Anderson and Fuglsang 1978).

Shortly after its discovery, ivermectin's safety and efficacy in treating onchocerciasis was assessed in patients infected with *O. volvulus* (Aziz et al. 1982a, Aziz et al. 1982b, Coulaud et al. 1983). Large-scale, field based trials commenced shortly thereafter, demonstrating that ivermectin was well tolerated in the community at large (Pacque et al. 1990b), as well as in patients with underlying health conditions, including heart arrhythmia (Dukuly et al. 1990). Although ivermectin is not administered to pregnant women, inadvertent dosing of pregnant women during community-based treatment for onchocerciasis did not result in a significant number of birth defects in children born to treated mothers (Pacque et al. 1990a). Further, community-based treatment was effective in suppressing the transmission of *O. volvululs* by *Simulium* spp (Cupp et al. 1989, Taylor et al. 1990, Trpis et al. 1990). Ivermectin was later shown to have temporary embryostatic effects on adult female worms (Klager et al. 1996). Although the embryostatic effects are temporary, ivermectin appears to permanently reduce fecundity of female worms by approximately 30% (Plaisier et al. 1995), and one mathematical model has predicted that if a single 150 μg/kg dose of ivermectin is given orally, microfilaremia could potentially be reduced by up to 98% (Basanez et al. 2008). A caveat to this model is that it assumes ivermectin would be ingested at a time when adult females are harboring few microfilaria, and it also fails to take into account differences in absorption and bioavailability of the drug by different patients.

Ivermectin was licensed under the name Mectizan for human use in 1987. Mectizan is generously donated by Merck, Sharpe and Dohme, Inc. for control of and

eradication efforts of onchocerciasis (Collins 2004). The African Programme for Onchocerciasis Control (APOC) was formed in 1995 (Amazigo 2008). The APOC was designed to replace the former Onchocerciasis Control Program (OCP). The OCP relied nearly exclusively on aerial application of insecticides for control of black fly larvae; a practice, although initially effective at interrupting transmission of *O. volvulus,* ultimately proved to be prohibitively expensive and began failing due to the spread of insecticide resistance (Boatin 2008). Through partnerships with the World Health Organization, local governments, non-governmental organizations, the private sector and the World Bank, the APOC is operational in delivering Mectizan to 30 African countries. The Onchocerciasis Elimination Program for the Americas (OPEA) was formed in 1992. Prior to the start of the APOC, human onchocerciasis was reported in 13 foci located in six Latin American countries. In 2008, treatment in a number of locations was stopped because new cases of onchocerciasis were not reported, and transmission had been halted in the region (Sauerbrey 2008). Both the APOC and OPEA utilize mass drug administrations (MDA) of Mectizan once or twice yearly for the control of onchocerciasis, and use an oral dose of 150 μg/kg (Taylor and Greene 1989).

Lymphatic filariasis

Lymphatic filariasis, also known as elephantiasis, is a disease caused by infection with *Wuchereia bancrofti, Brugia malayi* or *Brugia timori.* When left untreated, adult parasites cause blockages of the lymphatic system leading to the condition known as elephantiasis; an extreme swelling of the affected area. Bancroftian filariasis, caused by infection with *W. bancrofti,* generally causes elephantiasis of the genitals. Bancroftian filariasis occurs worldwide in the tropics and sub-tropics. Brugian filariasis, caused by

infection with *B. malayi* and *B. timori,* occurs predominantly in Southeast Asia, and causes elephantiasis of the lower extremities. All of these filariid nematodes are transmitted to humans as microfilaria through the bite of an infected mosquito. Several genera of mosquitoes can serve as vectors for parasite transmission (Marquardt et al. 2000).

In 1998, ivermectin was approved for the treatment of Lymphatic Filariasis, which was the same year that the Global Programme for the Elimination of Lymphatic Filariasis (GPELF) was formed (Hooper et al. 2009). Traditionally, treatment of lymphatic filariasis relied on DEC. However in 1997, albendazole and ivermectin were both shown to be effective for both treatment and control (Ottesen et al. 1997). Shortly after discovering ivermectin and albendazole were also effective treatments, research demonstrated that two drug combinations (albendazole-DEC and albendazole-ivermectin) had enhanced microfilaricidal effects (Ottesen et al. 1999). In 1998, Merck broadened their donation program to include donation of Mectizan for the treatment and control of lymphatic filariasis in areas of Africa where onchocerciasis and lymphatic filariasis both exist (WHO 1998a). Also in 1998, GlaxoSmithKline pledged the donation of albendazole to all countries in need of the drug until eradication is complete (WHO 1998b). Through the use of MDA and vector control programs, the transmission of lymphatic filariasis has been interrupted in China and South Korea, and several other countries have reported considerable declines in transmission after routine MDA treatment of the human populations (Bockarie et al. 2009, WHO 2009). While the goal for elimination of lymphatic filariasis is 2020, it is likely that eradication efforts will need

to extend beyond this period due to the longevity of the adult worm, and the need to implement vector control programs (Bockarie and Deb 2010).

The effect of ivermectin on mosquito survival, fecundity and egg viability

Several reports have shown that ivermectin is effective in reducing the survival and fecundity of numerous vectors of human and veterinary pathogens and arthropod parasites. Ivermectin has been shown to be highly toxic to the larvae of myasis-causing dipterans (Chamberlain 1982, Spradbery et al. 1985) and parasitic botfly larvae (Ostlind et al. 1979, Klei and Torbert 1980, Roncalli 1984). In addition, ivermectin has been demonstrated to reduce the survival of numerous hematophagous insects including: stable and horn flies (Miller et al. 1986), ceratopogonids (Standfast et al. 1984), Tse-Tse flies (Langley and Roe 1984), sucking lice of cattle (Benz et al. 1984), and mosquitoes (Pampiglioni et al. 1985, Iakubovich et al. 1989b, 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). In addition to decreasing adult insect survival, ivermectin has also been shown to decrease fecundity following ingestion of the drug in a bloodmeal. This effect has been observed in mosquitoes (Tesh and Guzman 1990, Focks 1991, Mahmood et al. 1991, Gardner et al. 1993) and in triatome bugs (de Azambuja et al. 1985).

The first report demonstrating that ivermectin could elicit a mosquitocidal response was conducted by Pampiglione et al. (1985). Pampiglione and colleagues investigated the effect of ivermectin to induce a lethal effect in *Culex quinquefasciatus, Aedes aegypti and Anopheles stephensi* larvae and adult mosquitoes. The effect on ivermectin on larvae was assessed by adding ivermectin in known concentrations to water

containing larvae, and then calculating the lethal concentration required to kill 50 percent (LC_{50}) of the larvae. The *Cx. quinquefasciatus* larvae were the most susceptible to ivermectin, followed by *An. stephensi,* and finally *Ae. aegypti.* The effect of ivermectin on adult mosquito survival was assessed by allowing mosquitoes to feed on either cotton soaked with ivermectin or on mice which had been injected with an exceptionally high dose of ivermectin (82 mg/kg). Survival of all mosquitoes was decreased after feeding on the ivermectin-soaked cotton balls and after taking a blood meal from ivermectin treated mice. Curiously, despite the fact that the authors calculated the LC_{50} of ivermectin in larvae, they do not report a LC_{50} for either route of ingestion. Qualitatively, *An. stephensi* was the most susceptible, followed by *Ae. aegypti,* and finally *Cx. quinquefasciatus* so the susceptibility of the mosquito strains to ivermectin cannot be directly compared.

In 1989, Iakubovich and colleagues allowed *An. stephensi, Ae. aegypti,* and *Anopheles sacharovi* to blood feed on rabbits injected with ivermectin and on blood containing ivermectin administered through an artificial membrane feeder. *Anopholes stephensi* was the most susceptible, followed by *Ae. aegypti,* and finally *An. sacharovi*. Again, LC_{50} for ivermectin was not calculated.

The first study to empirically determine the LC_{50} for ivermectin imbibed through a blood meal in mosquitoes was completed by Tesh and Guzman (1990), who employed an artificial membrane feeding assay, allowing for precision in controlling the concentration of ivermectin fed to mosquitoes. The LC_{50} values for the *Ae. aegypti* Rock strain was 126 ng/ml, the *Ae. albopictus* Houston strain was 208 ng/ml, and the *Culex quinquefasciatus* Gainesville strain was 698 ng/ml. Another unique aspect of the study is

that it provides the first report of sub-lethal concentrations of ivermectin inducing a reduction in the number and viability of eggs laid by mosquitoes following a maternal blood meal containing a sub-lethal concentration of ivermectin. The concentration of ivermectin required to prevent 50% of eggs from hatching was estimated for *Ae. aegypti* to be 3.4 ng/ml and was 4.3 ng/ml for *Ae. albopictus.* While the effects of ivermectin on larvae had previously been reported (Ali and Nayar 1985, Pampiglioni et al. 1985), the report by Tesh and Guzman was the first to report that when ingested in a blood meal, ivermectin could significantly decrease the fecundity and egg viabilitiy in mosquitoes. Unlike in the triatome *Rhodnius prolixus* (de Azambuja et al. 1985), the infertility observed in *Ae. aegypti* was temporary, and diminished after feeding on a second blood meal that did not contain ivermectin.

The effects of ivermectin on the ovarian development and egg viability were further investigated by Focks et al. (1991) and Mahmood et al. (1991). Focks and colleagues injected rabbits with ivermectin at a concentration of ivermectin 10 and 50 times greater than the labeled dose recommended in cattle. Mosquitoes who fed on the rabbits injected with the high dose exhibited reduced survival. Mosquitoes that survived oviposited significantly fewer eggs than control-fed mosquitoes, and eggs were less likely to hatch, and survival of larvae was lower than observed in controls. Mosquitoes that had fed on rabbits injected with the lower dose of ivermectin did not experience a reduction in survival, and no significant effects on fecundity or hatch rate were observed relative to control fed mosquitoes. A drawback to the study design used by Focks et al. (1991) is that the concentration of ivermectin imbibed by blood feeding mosquitoes was

not quantified, making it difficult to relate the results from the study to relate the findings of the study to potential impact of ivermectin on field populations of mosquitoes.

The effect of ivermectin on the ovarian development of *Ae. aegypti* was investigated by Focks et al. (1991). Utilizing the artificial membrane feeding assay described by Tesh and Guzman (1991), *Ae. aegypti* (Rock strain) mosquitoes were fed on blood containing ivermectin at a concentration of 100 ng/ml. Mosquitoes which had ingested blood containing the ivermectin experienced a 23.5% decrease in survival over controls. Mosquitoes which had ingested blood containing ivermectin exhibited a significant reduction in follicle and yolk length when compared to controls. A significant reduction in egg production was also observed by females which had ingested ivermectin. Eggs that were laid were abnormal in size, and exhibited reduced hatching. Interestingly, many of the eggs which failed to hatch contained viable larvae. Based on the lipophilic nature of ivermectin, the authors hypothesize that ivermectin may be incorporated into the egg and prevent hatching. This hypothesis remains untested, and the precise mechanism through which ivermectin induces infertility and prevents egg hatching has not yet been elucidated.

The first study to report a reduction in mosquito survival after feeding on humans that had been treated with an MDA drug was conducted by Cartel et al. (1991). The study was designed to evaluate the safety and efficacy of a single dose of ivermectin for the treatment of bancroftian filariasis, and to evaluate the efficacy of three treatment regimens for their effect on transmission by laboratory raised *Aedes polynesiensis* mosquitoes. Surprisingly, *Ae. polynesiensis* which had fed on ivermectin and DEC treated patients at one, three and six months post-treatment experienced significantly

higher mortality when compared to mosquitoes fed on placebo-treated patients. Mortality was significantly greater in mosquitoes fed on carriers treated with ivermectin than those treated with DEC. The six-month longevity of the mosquitocidal response following treatment with ivermectin has not been replicated in human studies or any other animal system, and the results remain rather perplexing. The authors speculate that the longevity of the mosquitocidal response may have been due to deposition and persistence of ivermectin in human tissues. Since the report by Cartel et al. (1991), a handful of studies have investigated mosquito survival following MDA treatment of human populations (discussed below), and none have seen such a long-lived mosquitocidal effect, suggesting that deposition of ivermectin in tissues is not a likely cause for the results reported.

The first study to quantify circulating concentrations of ivermectin at the time of a mosquito blood feed was conducted by Jones et al. (1992). Dogs were injected with varying concentrations of ivermectin. Twenty-four hours after ivermectin administration, blood from the dogs was collected and offered to *Anopheles quadrimaculatus* mosquitoes through a membrane feeder. A portion of the blood was reserved for quantification of ivermectin in the blood. Mosquitoes which had imbibed blood from dogs containing ivermectin at a concentration of 33-37 ng/ml experienced 65% mortality. The mean maximal concentration of ivermectin in human blood following a standard MDA dose of ivermectin is 46 ng/ml (Elkassaby 1991). The findings of Jones et al. (1992) are significant in that they were the first to demonstrate that a mosquito was susceptible to a concentration of ivermectin which could be expected in vertebrate blood following a treatment with ivermectin. Gardner et al (1993) conducted a similar study in which dogs were given oral doses of ivermectin. Laboratory raised *Ae. albopictus*, and field caught

An. quadrimaculatus and *Culex salinarius* were allowed to blood feed directly on the dogs. After the blood feed, dogs were bled and the concentration of ivermectin circulating at the time of blood feed was quantified. Depending on the oral dose of ivermectin administered to the dog, the concentrations of ivermectin circulating in the blood ranged from 0 ng/ml (controls) to 16 ng/ml (dogs given a 24 µg/kg dose of ivermectin). After feeding on ivermectin-treated dogs, increased mortality was observed in *An. quadrimaculatus* which had fed on dogs that had received either a 12 or 24 µg/kg dose of ivermectin. No reduction in survival was observed in *Ae. albopictus* or in *Cx. salinarius* after feeding on treated dogs, even at an oral dose of 120 µg/kg. The fecundity and hatch rates of *An. quadrimaculatus* were significantly decreased after feeding on dogs treated with increasing concentrations of ivermectin, and egg hatching was completely inhibited from blood meals taken from dogs treated with a 24 µg/kg dose of ivermectin. A reduction in hatch rate, but not overall fecundity, was observed in *Ae. albopictus* after a maternal blood meal from dogs treated with 15, 30, 60, and 120 µg/kg of ivermectin. Fecundity and hatch rate of *Cx. salinarius* was not affected.

Chandre and Hougard (1999) observed a 25% reduction in the survival of *Cx. quinquefasciatus* after feeding on chickens injected with a 2,000 µg/kg dose of ivermectin, and did not see any death in mosquitoes fed on humans treated with a therapeutic dose of 170 µg/kg. Since the report by Chander and Hougard (1999), focus has shifted towards investigating the effects of ivermectin on *Anopheles* spp. mosquitoes.

The first field study to investigate the effects of MDA treatment with anthelmintic drugs on the survival of endemic populations of mosquitoes was conducted shortly after the formation of the GPELF, and investigated the effects of experimental MDA doses

(given to evaluate their efficacy to interrupt transmission of *W. bancrofti*) on the survival of *Anopheles punctulatus* mosquitoes. The two MDA regimens included: mass treatment of a village with a single dose of 400 µg/kg ivermectin + 6 mg/kg DEC, and 400 µg/kg of ivermectin only. For the 400 µg/kg ivermectin + 6 mg/kg DEC MDA treatment, blood fed mosquitoes were collected from homes for three consecutive days immediately prior to and after the MDA. At 28 days post-MDA, blood fed mosquitoes were again collected. Survival of collected mosquitoes was monitored for nine days. The survival of mosquitoes after MDA treatment was significantly reduced (n=36), with 100% mortality reported within nine days. Survival of mosquitoes caught post-treatment over the course of nine days was significantly lower survival of mosquitoes captured pre-treatment or 28 days post-treatment.

To evaluate the effect of a single 400 µg/kg ivermectin dose of ivermectin on mosquito survival, the authors compared the survival of mosquitoes caught in the MDA treated village to survival of mosquitoes caught in a control village. The control village had, however, been treated with DEC two months prior to the experiment, but did not receive ivermectin. Mosquito survival was monitored for 48 hours, and was significantly lower (94%) in the ivermectin treated village when compared to the control village. At 38 days post-treatment, mosquito survival was similar between villages. The study by Bockarie et al. (1999) suffers from some experimental design flaws. The sample sizes of mosquitoes for the 400 µg/kg ivermectin + 6 mg/kg DEC study group were relatively small, and were only collected from nine homes. Secondly, the control village used for the 400 µg/kg ivermectin MDA treatment was not an optimal control. The control village had been treated two months prior with DEC whereas the village treated with ivermectin

only had not. However, it does not appear from the results that DEC had any effect on the survival of mosquitoes in the control village. Despite some experimental design flaws, the findings from this report are significant in that they are the first to show that a MDA dose of ivermectin, albeit higher than the 150 µg/kg dose now used, can be effective in reducing the daily survival of field caught mosquitoes.

The effect of ivermectin on the survival of other *Anopheles* species has also been investigated. Foley et al. (2000) found that survival of a laboratory strain of *Anopheles farauti* was decreased after feeding on a human volunteer for close to a week following ingestion of a single 250 µg/kg dose of ivermectin. An important downfall of the study design is that an untreated volunteer was not included as a control. Mortality data collected at the various time points post-feeding was compared to survival of mosquitoes that fed on the human volunteer prior to administration of the ivermectin. Presumably, each blood feed used a different cohort of mosquitoes. Given that survival of mosquitoes was assessed at different times, one cannot rule out environmental factors that may have contributed to the survivorship of mosquitoes. Regardless, this study appears to provide some evidence that *An. farauti* is susceptible to ivermectin in human blood following oral ingestion, although the 250 µg/kg dose ingested by the human volunteer is 100 µg greater than the dose frequently used in human MDA campaigns.

Anopheles gambiae s.s. and *Anopheles arabiensis* are two of the most important vectors of human *Plasmodium* in Africa, and both were demonstrated to be susceptible to ivermectin at concentrations which could be expected to be found circulating in human blood(Fritz et al. 2009). Using an *in vitro* feeding assay, Fritz and colleagues determined the LC_{50} of *An. gambiae* s.s. (Kisumu strain) and *An. arabiensis* (Dongola strain) to be

19.8 parts per billion (19.8 ng/ml). The authors also observed a significant reduction in survivorship of *An. gambiae* s.s. after feeding on ivermectin treated bulls, relative to *An. gambiae* that had fed on saline injected cattle. From their findings, they conclude that treatment of cattle with ivermectin could be used to reduce zoophilic vector populations that are responsible for some transmission of human malaria. These conclusions may be premature, as the cattle were injected with a 600 µg/kg dose of ivermectin; a dose three times greater than the recommended dose in cattle. Further, their experimental design utilized *An. gambiae* s.s., which is known to be a highly anthropophillic, not a zoophilic vector (Besansky et al. 2004).

Fritz et al (2009) also observed that ingestion of ivermectin at a concentration of greater than 10 ng/ml was associated with complete infertility in both *An. gambiae* and *An. arabiensis.* Egg production in *An. gambiae* mosquitoes which had fed on ivermectin treated bulls appeared to be inhibited if they had fed within 10 days of the treatment, and oviposition by mosquitoes that had taken blood meals at 13 and 17 days post treatment exhibited a significant reduction in overall fecundity when compared to control fed mosquitoes. Mosquitoes which had fed at 20 and 23 days post treatment did not exhibit reduced fecundity. Neither *An. gambiae* or *An. arabiensis* produced eggs after feeding on ivermectin at concentrations of 10, 100 and 1,000 ng/ml. Neither egg production nor hatch rate was affected at concentrations of 0.01, 0.1 or 1.0 ng/ml.

An. gambiae was later demonstrated to be susceptible to ivermectin circulating in the blood of humans following ingestion of a 200 µg/kg dose (Chaccour et al. 2010). Twenty-four hours after treatment, the mean survival of mosquitoes fed on ivermectin-treated humans was 2.3 days, whereas mean survival in the control-fed mosquitoes was

5.5 days. Mosquito survival was not affected after feeding on ivermectin treated individuals 14 days after treatment.

Recently, MDA of ivermectin was shown to significantly reduce the survival of field caught *An. gambiae* s.l. and *An. arabiensis* (Sylla et al. 2010). Significant reductions in *An. gambiae* s.l. survival were observed for up to six days following a single MDA treatment of ivermectin (150 μg/kg), which covered approximately 80% of the villagers in the study sites. From the experimental data, it was estimated that the daily probability of mosquito survival was decreased by at least 10% during the six days after MDA of ivermectin. Given the estimated reduction of daily probability of mosquito survival and an age-structure model for malaria transmission, it was estimated that a single MDA dose of ivermectin could reduce the reproductive rate of malaria, suggesting that repeated MDAs of ivermectin could have a considerable impact on the transmission of malaria. The model proposed by Sylla et al. (2010) was supported by field data in 2011 (Kobylinski et al. 2011). Kobylinski et al. (2011) observed that the sporozoite rate of *An. gambiae* mosquitoes captured in MDA treated villages decreased by nearly 80%, whereas sporozoite rates in a nearby, untreated control village did not.

Ivermectin resistance and cross-resistance.

Ivermectin was licensed for veterinary use in 1981, and shortly thereafter was approved for use in humans. Ivermectin and closely related compounds are widely used in agriculture, veterinary and human medicine. Resistance in nematode parasites and insects has been documented, and is reviewed below.

Ivermectin resistance in nematodes.

Reports of suboptimal treatment responses of *O. volvulus* microfilaria with ivermectin have been reported (Addiss et al. 1991, Awadzi et al. 2004b, Awadzi et al. 2004c, Awadzi et al. 2004a, Osei-Atweneboana et al. 2007, Osei-Atweneboana et al. 2011). In all of these reports, treatment with ivermectin resulted in an immediate reduction of microfilaria observed in skin snips. However, despite multiple treatments with ivermectin, microfilaria counts at later time points were elevated. The initial dose of ivermectin is not effective at killing adult worms and has no effect on their reproduction, and subsequent and repeated treatments with ivermectin are required, and are generally successful, in impairing the ability of adult female worms to produce microfilaria (Chavasse et al. 1992). Thus, the higher than expected microfilaria counts suggest that multiple treatments with ivermectin failed to decrease microfilaria production by adult female worms. In a recent study by Osei-Atweneboana et al. (2011), nodules containing adult *O. volvulus* were surgically removed from patients that had failed to respond to repeated treatments with ivermectin. Adult worms from the nodules were observed, and morphological examination revealed that nearly 90% of the worms were older worms, providing phenotypic evidence that resistance to ivermectin could be developing in *O. volvulus.*

Ivermectin resistance has been documented in numerous other nematode parasites, some of which include: *Haemonchus contortus* (Paiement et al. 1999), *Trichostrongylus* (Traversa et al. 2007), and *Cooperia oncophora* (Njue et al. 2004). Precise mechanisms of resistance to ivermectin have not been elucidated, and may vary between parasites. In *C. elegans,* high-level resistance to ivermectin appears to be

polygenic, requiring the simultaneous mutation of three GluCl α-type subunits (Dent et al. 2000). In *H. contortus,* radio-labeled ivermectin did not differ in its ability to bind to membranes of the worm, suggesting that target site-mutations are not responsible for ivermectin resistance observed in a laboratory-selected strain (Paiement et al. 1999). Yet another potential mechanism for ivermectin resistance is P-glycoprotein mediated drug efflux. P-glycoproteins are known to pump drugs across membranes thereby preventing them from reaching their target sites (Kerboeuf et al. 2003b), which has been associated with macrocyclic lactone resistance in several nematode parasites (Blackhall et al. 1998, Xu et al. 1998, Le Jambre et al. 2000, Kerboeuf et al. 2003a, James and Davey 2009).

Ivermectin resistance in insects and other arthropods.

The most comprehensive selection studies with any avermectin in an insect have been conducted in the Colorado potato beetle (Argentine and Clark 1990). Argentine and colleagues generated, by different procedures, two different abamectin resistant strains. One of the strains was generated by placing a field strain through intensive laboratory selection, and the other was produced by treating a susceptible laboratory strain with a chemical mutagen in conjunction with discriminatory dose selection with abamectin. Crosses of the resistant lines with the susceptible laboratory strain indicated that resistance in both strains was incompletely recessive, based on the finding that the F_1 offspring resulting from a mating between the susceptible and resistant strains was less resistant to abamectin than what would be expected in an intermediate phenotype (Argentine et al. 1992). Log dose versus logit mortality regression lines were calculated for the susceptible laboratory strain, the abamectin-resistant strains, and their F_1 offspring. Overlap of the regression lines indicated that inheritance of abamectin

resistance in both selected strains was polygenic (Georghiou 1969). Although a specific mechanism for abamectin-resistance was never identified in the resistant lines, experiments utilizing metabolic synergists are indicative of metabolic resistance (Argentine et al. 1992). Treatment of the resistant lines with piperonyl butoxide (a mixed function oxidase inhibitor) resulted in increased toxicity of abamectin to both lines, and treatment with *S,S,S*-tributyl phosphorotrithioate (an esterase inhibitor) resulted in moderate levels of increased toxicity in the abamectin-resistant strains. Treatment of the strains with glutathione-*S*-transferase inhibitors did not elicit an increased toxicity effect of abamectin. Collectively, the results are suggestive of a metabolic resistance mechanism involving mixed function oxidases, and perhaps to a lesser degree, esterases. Studies with radiolabeled avermectin were not suggestive of a decreased cuticular penetration mechanism of resistance.

Low-level ivermectin resistance was achieved in sheep blow fly larvae after 60 successive rounds of selection, achieving an eight-fold increase in tolerance to ivermectin when relative to the parental strain (Rugg et al. 1998). Increased monooxygenase activity, measured through a microsomal bioassay, were slightly elevated in the resistant strain, but were not significantly greater than observed in the parental line.

Ivermectin has been used successfully for the treatment of crusted scabies, a skin condition caused by infestation with the mite, *Sarcoptes scabiei* (Lawrence et al. 2005), and has been used successfully for treatment of severe infestations. Recently, however, recrudescent infestations after treatment with ivermectin have been reported, requiring multiple doses of ivermectin or high concentrations of topical permethrin to cure patients of the infestation (Huffam and Currie 1998, Walton et al. 1999, van den Hoek et al.

2008). The precise cause for the recrudescent infestations has not been determined, and the failure of ivermectin to cure the infestations is not enough to conclusively state whether or not resistance is developing in *S. scabiei.* Other factors such as patient compliance and living conditions may play a role in the cure rate. In one report, Currie and colleagues (2004) claim the first documentation of *in vivo* and *in vitro* ivermectin resistance in *S. scabiei* mites that were obtained from two patients, both of whom failed to respond to over 30 oral doses of ivermectin. Mites were collected from patients at various points over the course of five years. At each collection, mites were immediately subjected to an *in vitro* assay where their time to death was measured after exposure to a predetermined concentration of ivermectin. In one of the patients, the time to death for mites collected in the year 2000 was significantly greater than the time to death for mites collected in 1999 or 2001. The patient is reported to have received 30 doses of ivermectin between 1995 and 2000, but no discussion is provided as to how patient compliance with the regimen was assessed. Further, the *in vitro* assays on mites collected at different times were not conducted concurrently, rather they were separated by years. For a number of reasons, it is questionable as to whether the data from the two experiments should be directly compared. Nonetheless, the occurrence of ivermectin failure in the treatment of scabies is of notable concern and should continue to be monitored.

Cross-resistance between avermectins and other insecticides.

Cross-resistance between the avermectins and other classes of insecticides is poorly understood, and reports of its occurrence and lack thereof are conflicting. The most recent report of cross-resistance to ivermectin demonstrated that *Drosophila*

melanogaster which had been laboratory selected for resistance to nodulisporic acid were 3-fold cross-resistant to ivermectin. Both nodulisporic acid and ivermectin target the GluCl channel. Sequencing of the GluCl alpha subunit revealed a proline to serine mutation. Recombinant mutant GluCl channels expressed in *Xenopus* oocytes were highly insensitive to activation by nodulisporic acid and ivermectin, indicating that a target site mutation was responsible for both resistance to nodulisporic acid as well as nodulisporic acid-induced cross-resistance to ivermectin (Kane et al. 2000).

Other than the study by Kane et al. (2000), most cross-resistance studies have used abamectin (avermectin B_{1a}). The first report of cross-resistance between abamectin and permethrin was observed in a laboratory-selected permethrin resistant strain and a field-collected strain of *Musca domestica* (Scott 1989). The laboratory-selected permethrin resistant strain and field collected strain had a 25-fold and 5.9-fold cross-resistance (relative to a laboratory susceptible strain) to abamectin. A topical bioassay was used to determine cross-resistance ratios. The field strain used in the study was collected from a dairy reporting failure of permethrin insecticides, and was shown to have a high level of permethrin resistance relative to the standard susceptible strain. To determine whether the increased tolerance to abamectin in the laboratory-selected permethrin resistant strain was truly due to a cross-resistance mechanism rather than a difference in tolerance between strains, the researchers employed an F_1 backcross where susceptible females with known genetic markers were crossed with F_1 males. Offspring from the mating were treated with a diagnostic dose of abamectin, and the effect of each chromosome was estimated using an arcsine of the percentage of survival, allowing for statistical analysis of a specific gene's contribution to resistance. From the genetic

analysis, three possible mechanisms for the observed resistance were identified: increased metabolism via mixed-function oxidase (MFO), *knock-down resistance (kdr)* insensitivity, and decreased cuticular penetration. Treatment of flies with piperonyl butoxide (an inhibitor of MFO) suppressed resistance to abamectin in the permethrin-resistant line and increased the toxicity of the drug in the susceptible strain. Given that permethrin and abamectin have different molecular targets, the researchers concluded that the cross-resistance was likely due to increased metabolic resistance and decreased cuticular penetration.

While cross-resistance between other insecticides and abamectin have been reported in other insects, including the German cockroach (Scott 1991) , the diamond back moth (Abro et al. 1988) , and the sheep blow fly larvae (Hughes and Levot 1990) , the studies by Kane et al. (2000) and Scott (1989) are the only ones to propose possible mechanisms for cross-resistance to avermectin compounds. Abro et al. (1988) used a topical bioassay to examine the level of cross-resistance to abamectin in a "field strain [of the diamond-back moth, *Plutella xylostella*] showing multiple resistance to insecticides." The authors propose that a "low-level of cross-resistance to avermectin could be present in the field strain," however they also point out that the field strain was equally susceptible to abamectin as a baseline colony, making it difficult to interpret whether or not a cross-resistance mechanism was really observed. Scott (1991) observed significantly higher LD_{95} values for abamectin in two multiresistant German cockroach lines, relative to a laboratory susceptible strain. The resistance to other insecticides in the cockroach lines was poorly defined, and it was impossible to determine which insecticide or insecticides were associated with observed cross-resistance to abamectin.

Low level cross-resistance to ivermectin and abamectin was observed in an organophosphate and carbamate laboratory selected line of the sheep blowfly (Hughes and Levot 1990); however, after selecting the same strain for pyrethroid and carbamate resistance, the strain was later shown to be susceptible to ivermectin (Rugg et al. 1995).

While there is a handful of reports that suggest cross-resistance between avermectins and other insecticides may occur, there is an equal number of reports that have found no evidence of this phenomenon; further, reports are conflicting. One study found no evidence of cross-resistance to avermectins in house flies known to be resistant to diazinon, dieldren, DDT and permethrin (Roush and Wright 1986a). The literature is also conflicting in the case of the German cockroaches. While Scott (1991) observed a possible cross-resistance phenomenon, another study in the German cockroach found that field-selected pyrethroid-resistant cockroaches were fully susceptible to abamectin (Cochran 1990). Pyrethroid-induced cross-resistance was not observed in a strain of tobacco budworm which displayed both metabolic and target site resistance to pyrethroids (Campanhola and Plapp 1989). Similarly, cross-resistance studies in a multiple insecticide resistant strain of Colorado potato beetle showed no evidence of cross-resistance to abamectin (Argentine and Clark 1990). To date, the only studies of cross-resistance to ivermectin in hematophagous insects have been in head lice, and to date studies indicate that permethrin-resistant headlice are susceptible to ivermectin both *in vitro* (Strycharz et al. 2008), and *in vivo*(Chosidow et al. 2010). Ultimately, the occurrence of cross-resistance between the avermectins and other insecticide classes in insects is both poorly understood and ill-defined. With the exception of head lice, most research has focused on domestic and agricultural insect pests. Further, all studies

except for those in the German cockroach and head louse have employed topical bioassays to evaluate the occurrence of cross-resistance.

The effect of oral ingestion of an avermectin compound in hematophagous insects is largely unexplored. The uses of ivermectin are rapidly expanding beyond endo and ectoparasite control. Some have pointed out that ivermectin could someday be used to control mosquito-borne diseases such as malaria (Foy et al. 2011). Given the wide-spread reports of ivermectin resistance in many organisms, and in light of some evidence of cross-resistance (coupled with the fact that insecticide resistance is wide-spread and expanding), investigating the likelihood for the development of ivermectin-resistance or cross-resistance in hematophagous vector species, such as the mosquito is warranted.

Summary

Arboviral pathogens transmitted by *Ae. aegypti* impart a tremendous global burden of disease. Traditional control methods for many of these pathogens are failing due to the rapid spread of insecticide resistance. Global travel and shipping routes present a constant risk of introducing both *Ae. aegypti* and pathogens transmitted by the vector into new geographic locations. Novel methods for interrupting transmission of mosquito-borne pathogens are needed, and it has been suggested that mosquitocidal vaccines and MDA of anthelmintic drugs could be effective tools (Billingsley et al. 2008, Foy et al. 2011). The work presented here was designed to evaluate antigens of *Ae. aegypti* for their potential for inducing mosquitocidal activity (Chapter 2), to evaluate the effect of anthelmintic drugs on *Ae. aegypti* (Chapter 3), and to evaluate differences in susceptibility of different strains of *Ae. aegypti* to ivermectin and to investigate whether ivermectin resistance could be selected for in *Ae. aegypti* (Chapter 4).

CHAPTER 2: THE MOSQUITO LYSOSOMAL ASPARTIC PROTEASE AND THE GLUTAMATE GATED CHLORIDE ANION CHANNEL AS ANTIGENS FOR GENERATING MOSQUITOCIDAL IMMUNITY AGAINST *AEDES AEGYPTI*

Research Objective and Hypotheses.

The objective of the research outlined in this chapter was to evaluate the *Aedes aegypti* mosquito lysosomal aspartic protease (AaMLAP) and glutamate gated chloride anion channel (GluCl) as antigens for inducing mosquitocidal immunity in mice. The hypotheses for this chapter are the following:

1. If the AaMLAP is a mosquitocidal antigen, then mosquitoes that imbibe blood meals from mice immunized against AaMLAP will experience reduced survival because immune components ingested in the blood meal will attack the native AaMLAP protein in the mosquito.
2. If the glutamate gated chloride anion channel is a mosquitocidal antigen, then mosquitoes that imbibe GluCl antiserum will experience reduced survival because binding of the antibody to the GluCl channel will disrupt channel function.

Introduction.

Ae. aegypti is one of the most important mosquito vectors of human arboviruses. To date, the most successful attempts at controlling transmission of arboviruses vectored by *Ae. aegypti* in human populations have involved the use of insecticides (World Health Organization 2006), but their utility is rapidly diminishing due to the rapid emergence and spread of insecticide resistance (Castle et al. 1999, Jirakanjanakit et al. 2007b, Jirakanjanakit et al. 2007a, Pethuan et al. 2007). Consequently, novel methods to control the mosquito vector are urgently needed, and some have argued that the use of

mosquitocidal vaccines could be a novel method to help contol the transmission of mosquito-vectored pathogens (Foy et al. 2002, Billingsley et al. 2008).

Hematophagous insects, such as *Ae. aegypti,* imbibe blood for nutrition and egg production and maturation. When a mosquito feeds on a host, it ingests immune components circulating in the host's bloodstream. A mosquitocidal vaccine (reviewed in Chapter 1) would immunize a host against antigens from the mosquito tissue. If immune components mounted against mosquito antigen were imbibed by a blood feeding mosquito, then they could bind the antigen within the mosquito, ultimately leading to mosquito death (Foy et al. 2002).

Anti-vector immunity was first demonstrated in 1939, when William Trager immunized guinea pigs and rabbits with crude tick extracts from the tick *Dermacentor variabilis.* Ticks which had fed on immunized animals experienced significant mortality, or failed to engorge (Trager 1939b, a). Nearly sixty years after Trager's discovery, two recombinant protein anti-tick vaccines, were developed and licensed for use in cattle for protection against *Boophilus microplus,* and both vaccines incorporate the recombinant protein, *Bm*86 (Willadsen et al. 1995b, de la Fuente et al. 1999). These vaccines have been shown to only reduce tick-burden on treated animals (Rodriguez et al. 1995a, Rodriguez et al. 1995b, Canales et al. 1997), and may also reduce the transmission of tick-borne pathogens (Pipano et al. 2003, Labuda et al. 2006).

Progress in identifying mosquitocidal antigens has been slow. Historically, mosquitocidal immunity has been demonstrated through immunizing animals against crude mosquito homogenates (Alger and Cabrera 1972, Hatfield 1988, Almeida and

Billingsley 1998, 1999, Lal et al. 2001, Almeida and Billingsley 2002). To date, only two mosquitocidal antigens have been described; the *Anopheles gambiae* Mucin-1(*AgMucI)* protein (Foy et al. 2003) and the *Aedes albopictus* subolesin protein (Canales et al. 2009). No reports of specific mosquitocidal antigens for *Ae. aegypti* have been published. In this report, we evaluated the *Ae. aegypti* mosquito lysosomal aspartic protease (AaMLAP) and the glutamate-gated chloride anion channel (GluCl) as potential mosquitocidal antigens.

In 1991, the AaMLAP was isolated from previtellogenic mosquitoes. Due to its ability to use hemoglobin as a substrate and inhibition of activity by pepstain A, the AaMLAP was identified as belonging to the cathepsin D family of proteases (Cho et al. 1991). The protein was found in the head, thorax, mid-gut, and fat body, with the highest concentrations occurring in the fat body, and a putative secretion signal sequence was identified. The cDNA coding for AaMLAP was later cloned and sequenced, and AaMLAP mRNA and protein were shown to be highly upregulated after blood feeding. The AaMLAP mRNA abundance increased between six and twelve hours following a bloodfeed and reached peak levels at 24 hours post bloodmeal. AaMLAP protein levels reached peak concentrations between 36 and 42 hours following a bloodmeal (Cho and Raikhel 1992).

Orthologs of the AaMLAP protein have been described in several blood feeding nematodes, including in *Schistosoma japonicum, Schistosoma mansonii,* and *Ancylostoma caninum. In vitro* and *in vivo* immunologic targeting of these parasites has been shown to induce pathology in the parasite and decrease survival. *In vitro* incubation of *S. japonicum* with bovine antiserum against *S. japonicum* cathepsin-D, resulted in

decreased hemoglobin degradation and disruption of the tegument and gastrodermus of the worm (Bogitsh et al. 1992). Dogs immunized with a recombinant *A. caninum* cathepsin-D aspartic protease protein exhibited an 18% reduction of worm burden of the small intestine (Hotez et al. 2002), and mice immunized with a recombinant *S. japonicum* cathepsin-D aspartic protease protein exhibited 22-40% reductions in overall worm burden (Verity et al. 2001).

In mosquitoes, a multitude of proteolytic enzymes are secreted after the ingestion of a bloodmeal with midgut trypsins being the predominant enzyme required for bloodmeal digestion (Pennington and Wells 2005). While it has been proposed that AaMLAP is important in the regulation of vitellogenesis (Cho and Raikhel 1992), whether or not the enzyme plays a role in mosquito blood meal digestion is unknown A sequence alignment revealed an amino acid sequence identity of 57.3% between AaMLAP and *S. japonicum* CDAP. When conserved replacements are considered, the similaratiy is 85%. Given that AaMLAP mRNA and protein are upregulated following a blood meal, and immunizations against orthologous cathepsin-D aspartic proteases are effective in decreasing worm burdens *in vivo,* we hypothesized AaMLAP could be used as an antigen to elicit mosquitocidal immunity in Balb/c mice. To test this hypothesis, we generated four different immunization constructs, and tested them for their ability to induce mosquitocidal immunity in mice by using different immunization regimens. The immunization constructs included: two DNA immunization plasmids, a recombinant Sindbis virus vector containing the AaMLAP gene, and a recombinant AaMLAP protein produced in *Escherichia coli* cells. The four different immunization regimens included: immunization with the DNA immunization plasmids co-administered with mouse IL-12

(mIL-12) adjuvant plasmid (administered either by intramuscular (i.m.) injection or delivered intradermally (i.d.) using a gene gun), i.m. DNA immunization followed by a boost with the live AaMLAP-Sindbis virus expression vector, and finally subcutaneous (s.c.) immunization with a polyacrylamide gel slurry containing the recombinant protein expressed from *E. coli* cells.

In this chapter, we also investigated the *Ae. aegypti* GluCl anion channel as a potential mosquitocidal target. GluCl ion channels belong to the cys-loop superfamily of neurotransmitters, and are responsible for modulating synaptic inhibition in neurons and muscle fibers of many invertebrate animals, including insects (Cleland 1996). Avermectins, such as ivermectin, are GluCl receptor agonists which bind selectively to GluCl receptors in the nerve and muscle cells of invertebrates (Arena et al. 1992, Cully et al. 1996a, Cully et al. 1996b). Ivermectin has been shown to be highly effective in killing mosquitoes when it is imbibed with a bloodmeal (Pampiglioni et al. 1985, Tesh and Guzman 1990, Cartel et al. 1991, Jones et al. 1992, White 1997, Foley et al. 2000, Fritz et al. 2009, Kobylinski et al. 2010, Sylla et al. 2010). Given the success of ivermectin in inducing mosquitocidal activity when ingested with a bloodmeal, we hypothesized that *Ae. aegypti* fed on heterologous GluCl antisera would exhibit reduced survival when compared to mosquitoes fed on control serum. We tested this hypothesis by feeding *Ae. aegypti* on rabbit polyclonal antisera which had been raised against the extracellular domain of recombinant *An. gambiae* GluCl protein using an *in vitro* feeding system. The amino acid sequence identity between the extracellular domains of the GluCl anion channel of *Ae. aegypti* and *An. gambiae* is 75%. When conserved amino acid replacements are considered, the sequence similarity is 90%.

Materials and Methods.

Mosquitoes.

The *Ae. aegypti* Rexville D- Higgs White Eye (HWE) strain was used for all of the experiments in this chapter. Mosquitoes were reared at 28°C ± 2°C, 80% relative humidity, under a photoperiod of 14:10 (L:D). Larvae were reared in 28 liter (L) containers filled with approximately 15 L of tap water. Mosquito larvae were fed a diet of ground Tetramin® fish food mixed with ground mouse food. Adult mosquitoes were offered water and raisins as a sugar source *ad libitum.*

Production and administration of AaMLAP-DNA immunization construct(1).

Two different immunization regimens were used to evaluate the efficacy of the AaMLAP-DNA immunization construct(1). The AaMLAP-DNA immunization construct(1) was administered to mice by injection (i.m.), or by delivering the vaccine (i.d.) via gene gun particle bombardment. A summary of all immunization constructs and immunization regimens is presented in Table 2.1

Production of the immunization construct.

All work outlined in this section is unpublished and was completed by Dr. Brian Foy. To prepare the AaMLAP-DNA immunization construct(1), the AaMLAP gene was inserted into the pCDNA3.1(+) mammalian plasmid expression vector (Invitrogen, Carlsbad, CA, USA). Briefly, complementary DNA (cDNA) was cloned via reverse transcriptase polymerase chain reaction (RT-PCR) from total RNA extracted from a pool of five mosquito midguts. Cloning primers were constructed as follows: a 5' primer was created containing the *Hind*III restriction site, Kozak sequence, ATG start codon and 18

nucleotides of gene specific sequence; a 3' primer was created containing the *Xba*I site, termination codon and 18 nucleotides of gene specific sequence. The AaMLAP cDNA was cloned into the pCDNA3.1(+) vector, and the resulting AaMLAP-pCDNA3.1(+) plasmid was amplified in XL1 cells (Stratagene, La Jolla, CA). A high concentration stock was produced using the Qiagen Endotoxin-Free Giga-Prep kit (Qiagen, Valencia, CA) (Foy 2005- personal communication).

The resulting AaMLAP-pCDNA3.1(+) plasmid was administered concurrently with a mouse interleukin-12 (mIL-12) adjuvant plasmid (Aldevron, Fargo, ND, USA), and the plasmid mixture will be referred to as the AaMLAP-DNA immunization construct(1). The i.m. preparation of the AaMLAP-DNA immunization construct(1) was prepared as follows: Each dose contained 100 µg of the AaMLAP-pCDNA3.1(+) plasmid plus 100 µg of mIL-12 adjuvant plasmid contained in 100 µl of sterile PBS. Control mice were immunized with 100 µl of the empty pCDNA3.1(+) plasmid plus 100 µl of the mIL-12 adjuvant plasmid contained in 100 µl of sterile PBS.

Immunizations.

Intra-muscular administration of the AaMLAP-DNA immunization construct(1).

Drs. Foy, Douglas Brackney and Tereza Magalhaes performed the i.m. immunizations and mosquitocidal bioassays using the AaMLAP-DNA immunization construct(1). The data are unpublished. Two replicates of the experiment were conducted, and female Balb/c mice, 6-8 weeks old were used in both replicates. In the first replicate, each experimental and control group consisted of four mice. In the second replicate, each experimental and control group consisted of two mice. Prior to immunization, each mouse was bled by nicking the tip of the tail with a razor blade and collecting blood into

a microtainer tube (BD Biosciences, San Jose, CA, USA) for serum separation. Pre-immune serum was pooled for each experimental group and stored at -20°C.

Mice were immunized a total of three times, with two weeks between each immunization. Each immunization was administered by injecting 50 µl of the immunization construct into both the right and left hind quadriceps muscle. Two weeks following the final immunization, mice were restrained between wire mesh secured to a board by push pins, then placed into a cage of mosquitoes. Immediately following the mosquito feed, mice were euthanized via carbon dioxide inhalation and terminally bled via cardiac puncture. Blood was collected in serum separation microtainer tubes (BD Biosciences). Serum was collected and stored at -20°C (Foy, B.D., personal communication, 2005).

Administration of the AaMLAP-DNA immunization construct(1) via particle bombardment. In this experiment, female Balb/c mice were immunized against AaMLAP via particle bombardment using the Helios Gene Gun (Biorad). The plasmids used for immunization were the same as described in the previous section, and were kindly provided by Dr. Foy. The experimental and control groups each consisted of 3 total mice, however four weeks following initial immunization, one mouse from the control group died.

Immunization doses were prepared as follows. Immunization plasmids (either AaMLAP-pCDNA3.1(+) or the empty pCDNA3.1(+) plasmids were co-precipitated with the mIL-12 plasmid onto 1 µm gold beads (BioRad, Hercules, CA, USA) according to the manufacturer's instructions. Briefly, 50 mg of gold beads and 100 µl of 0.05M

spermidine were combined in a microfuge tube, vortexed and then sonicated in an ultrasonic cleaner for 5 seconds. One hundred μg of mIL-12 and 100 μg of AaMLAP-DNA immunization construct-1 were added to the gold beads and vortexed. One hundred μl of 1M $CaCl_2$ was added to the tube, drop wise, while periodically vortexing the tube. The mixture was incubated at room temperature for 10 minutes and then centrifuged at 5000 x g for 30 seconds. The supernatant was removed and discarded, and the pellet was washed with 1 ml of absolute ethanol. The coated gold beads were then loaded onto gold coat tubing using the Tubing Prep Station (BioRad) according to the manufacturer's instructions. Unevenly coated tubing was discarded and the remaining tubing was cut into 1.3 cm cartridges. Control vaccine cartridges were prepared with the empty plasmid vector and mIL-12 plasmids according to the procedure outlined above. The amount of DNA per cartridge was estimated by combining a cartridge and 500 μl of TE buffer into a microfuge tube. Gold beads were dislodged from the tubing by placing the tubes into an ultrasonic cleaner water bath. Gold beads were pelleted by a brief centrifugation, and DNA was quantified using a Nanodrop UV spectrophotometer. The estimate for total DNA per AaMLAP DNA vaccine construct-1 + mIL-12 was 0.5 μg of DNA per cartridge. The control vaccine cartridges were estimated to contain 1.0 μg of DNA per cartridge.

Prior to immunization, mice were bled from the tail vein, and blood was collected into serum separation microtainer tubes (BD Biosciences). Blood was allowed to clot at 4°C overnight. Serum was separated by centrifugation at 7,500 x g in a microcentrifuge and then stored at -20°C. Mice received a total of two immunizations via intradermal particle bombardment, with two weeks in between the immunizations. Each

immunization delivered a total of 1 μg of total plasmid DNA. Prior to vaccine administration, the ventral side of the mouse was shaved. Cartridges containing gold beads coated with the immunization plasmids were loaded into the Helios gene gun, which was positioned over the shaved immunization site. Gold beads were dislodged from cartridges by a helium burst delivered at 200 pounds per square inch. Six weeks after the second immunization, mice were restrained (described above), and were then placed into a cage of mosquitoes. Immediately following the mosquito feed, mice were anesthetized using a Ketamine/Xylazine cocktail, bled via cardiac puncture and euthanized via cervical dislocation. Blood was placed into serum separation tubes and serum was collected as described above.

Production of a recombinant AaMLAP-SINV virus, and its use in an AaMLAP DNA: prime-alphavirus-boost immunization regimen.

Production of the recombinant AaMLAP-SINV virus.

Construction of the AaMLAP-SINV infectious clone. The Sindbis virus (SINV) vector used to generate the recombinant AaMLAP-SINV vaccine was the 5' double-subgenomic SINV, 5'dsMRE16, which contains a double subgenomic promoter and multiple cloning site (Myles et al. 2003, Foy et al. 2004). Gene specific primers for AaMLAP were designed to facilitate cloning of the gene into the 5'dsMRE16 plasmid vector by incorporating the *Not*I restriction recognition sequence on both the forward primer (AaM-SINV-Fwd) and reverse primer (AaM-SINV-Rev). Additionally, a start codon was incorporated into the AaM-SINV-Fwd primer, and a stop codon was incorporated into the AaM-SINV-REV codon. Primer sequences are reported in Table 2.2.

A full-length AaMLAP amplicon, flanked by the *Not*I restriction recognition sequence at the 5' and 3' end was generated via PCR using the AaM-SINV-Fwd primer, the AaM-SINV-Rev primer amplicon and 500 ng of the purified AaMLAP-DNA immunization construct-1 plasmid as template DNA. The PCR reaction consisted of: 50 µl of 2X MasterMix containing *Taq* Polymerase (Promega, Madison, WI), 1 µl of forward primer at a 10 µM concentration, 1 µl of reverse pimer at a 10 µM concentration, and ultra pure water added to reach the final reaction volume of 100 µl. The product was amplified using the following program on an MJ Research thermal cycler: 1 cycle of 95°C for 5 minutes, 35 cycles of 95°C for 30 seconds, 55°C for 1 minute, 72°C for 60 seconds, followed by a 10 minute extension step at 72°C. After verifying the presence of a single 1.1 kilobase pair (kb) PCR product by agarose gel electrophoresis, the amplification product was purified using the QIAGEN PCR purification kit according to the manufacturer's instructions (Qiagen). The amplification product was inserted into the 5'dsMRE16 infectious clone, and proper orientation and DNA sequence were confirmed by DNA sequencing.

Production of recombinant AaMLAP-5'dsMRE16 virus. Recombinant AaMLAP-5'dsMRE16 virus particles were produced using an *in vitro* transcription system (Olson et al. 2000). Briefly, 5 µg of the AaMLAP-5'dsMRE15 infectious clone was linearized using the restriction endonuclease *Asc*I (New England Biolabs, Ipswich, MA, USA), and then digested with Proteinase K (Ambion, Austin, TX, USA) overnight at 37°C. The reaction was extracted with cold saturated phenol, extracted once more using 24:1 chloroform:isoamyl alcohol, and then precipitated using 3M sodium acetate (pH 5.2) and ethanol. Linearized DNA was pelleted by centrifugation at 15,000 x g at 4°C in a

microcentrifuge, resolubilized in nuclease free water and quantified using a Beckman spectrophotometer (Beckman-Coulter, Brea, CA, USA). The following *in vitro* transcription reaction was used to generate positive sense single stranded RNA: 1 µg of linear plasmid, 10 µl of a 75 mM mix of ribonucleotid triphosphates (rNTPs), 1.5 µl of 1mM m^7-G(5')ppp(5')G cap analog, 5 µl of 5X transcription buffer, 40 units of SP6 polymerase (Ambion) and nuclease free water to a final volume of 50 µl was incubated at 39°C for one hour. After completion of the transcription reaction 25 µl of the resulting product was electroporated into 4 x 10^6 C6/36 cells by pulsing the cells at: 250 V, 25 Ω and 550 µF. Cells were grown for one week, and then cells and supernatant were collected. The virus was passaged once more in C6/36 cells, titrated and stored in 1 ml aliquots at -80°C.

Immunizations.

The DNA prime-virus-boost vaccination utilized the AaMLAP DNA-immunization construct(1) and the recombinant AaMLAP-SINV described above. A 5'dsMRE16 virus containing enhanced green fluorescent protein (SINV-GFP) was used as a control virus. The control and experimental groups consisted of five female Balb/c mice, aged 5-8 weeks at the time of the first immunization. Prior to immunization, mice were bled by nicking the tail vein with a razor blade, and pre-immune serum was collected as described above. The immunization regimen consisted of three i.m. DNA immunizations as described above, followed by one subcutaneous (s.c) injection using the recombinant 5' dsMRE16 viruses. Mice were injected with 10^3 plaque forming units of the virus contained in 100 µl of sterile PBS. Two weeks following inoculation with virus, mice were restrained as described above, and placed into a cage of mosquitoes.

After the feed mice were anesthetized using a Ketamine/Xylazine cocktail and processed as described above.

Production and administration of the AaMLAP-DNA immunization construct(2).

Additional experiments utilizing the AaMLAP-DNA immunization construct-1 were planned, so the plasmid was re-propagated by electroporating the plasmid into XL-1 cells. A high concentration stock was generated using an endotoxin-free gigaprep (Qiagen). The newly purified plasmid and the original plasmid were submitted for DNA sequencing, which revealed an incomplete start codon in both plasmids. The original sequencing data for the AaMLAP DNA immunization construct-1 was not available; therefore cDNA of AaMLAP was re-cloned and ligated into the pCDNA3.1(+)-TOPO DNA immunization vector (Invitrogen). The pCDNA3.1(+)-TOPO plasmid is identical in sequence to the pCDNA3.1(+) vector used to generate AaMLAP DNA immunization construct-1, but does not require the use of restriction endonucleases and was chosen to simplify the cloning process.

Production of the immunization construct.

Aedes aegypti HWE mosquitoes were blood fed on defimbrinated sheep blood (Colorado Serum Company, Denver, CO, USA) through an artificial membrane feeder (Lillie Glassblowers, Smyrna, GA, USA). A pool of fully engorged mosquitoes was retained in a 500 ml cardboard container covered with organdy fabric, with access to water and raisins. Twenty-four hours following the blood meal, midguts were dissected from five mosquitoes and pooled. Total RNA was harvested using Trizol (Invitrogen) according to the manufacturer's instructions. The AaMLAP cDNA was cloned via the RT-PCR procedure described above using the gene specific primers AaM-pCDNA Fwd

and AaM-pCDNA Rev. Sequences for the primers are reported in Table 2.2. The forward primer contained the Kozak sequence (ACC) followed by the start codon (ATG). Following verification of a single 1.1 kb PCR product, the PCR product was cloned into the pCDNA3.1(+)-TOPO plasmid according to the manufacturer's instructions. Chemically competent, TOP10, *E. coli* cells (Invitrogen) were transformed with the ligation reaction and then plated onto LB-Amp agar. Individual colonies were grown in LB-Amp broth. Plasmids were purified using the Qiagen Miniprep kit (Qiagen), and then sequenced. After confirming proper gene insertion orientation and open reading frame, a high concentration stock was produced using the Qiagen Endotoxin-Free Giga-Prep Kit (Qiagen), according to the manufacturer's instructions. The plasmid was reconstituted in sterile PBS and stored at -20˚C.

Immunizations.

The AaMLAP-DNA immunization construct(2) was administered, i.m., using the same procedure described for the AaMLAP-DNA immunization construct(1). Female Balb/c mice, aged 6-8 weeks at the time of immunization were used for the experiment, and the experimental and control groups each consisted of five mice. Prior to immunization, mice were bled by nicking the tail vein with a razor blade, and serum was collected as described above.

Production and administration of a recombinant AaMLAP protein immunization construct.

Production of the immunization construct.

The pBAD/TOPO ThioFusion expression vector , which will be abbreviated as pBAD, (Invitrogen) was selected because it contains a C-terminal polyhistidine tag for purification, a V5 epitope for identification, and an inducible promoter (araBAD), allowing for inducible expression of the protein in the presence of L-arabinose (L-ara).

Cloning of AaMLAP into the pBAD plasmid. The AaMLAP sequence was amplified by RT-PCR using total mosquito RNA from 24-hour post blood fed female *Ae. aegypti* mosquitoes as a template, and the gene specific primers AaMpBAD Fwd and AaMpBAD Rev. Sequences for the primers are reported in Table 2.2. The forward primer contained the start codon. The stop codon was omitted from the reverse primer to allow translation of the polyhistidine tag and V5 epitope. Total RNA was extracted from mosquitoes using Trizol (Invitrogen) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized as follows: 500 ng of total RNA was incubated with 1 µl of 10 mM dNTPs, 1 µl of reverse primer at a 10 mM concentration and 9 µl of nuclease free water for 5 minutes at 65°C. The reaction was subsequently placed onto ice, and the following reagents were added: 4 µl of first strand buffer, 2 µl of 0.1M DTT, 1 µl of RNaseOUT, and 1 µl of Superscript II reverse transcriptase (Invitrogen). The reaction was heated to 50°C for 1 hour, and then heated at 70°C for 15 minutes.

The resulting cDNA, AaMpBAD fwd primer and AaMpBAD rev primer were then used to generate an AaMLAP amplicon via PCR as described above. Following verification of a single 1.1 kb PCR product, the PCR product was cloned into the pBAD plasmid according to the manufacturer's instructions. Chemically competent TOP10 *E. coli* cells (Invitrogen) were transformed with the ligation reaction and then plated onto

LB- agar containing 100 µg/ml of ampicillin (LB-Amp). Individual colonies were grown in LB-Amp broth. Plasmids were purified using the Qiagen miniprep kit (Qiagen), and then sequenced to verify proper orientation of the insert, presence of the start codon and the proper open reading frame to allow expression of the AaMLAP protein, polyhistidine tag and V5 epitope.

Expression of recombinant AaMLAP. After verifying that the AaMLAP gene was properly inserted into the pBAD vector, the plasmid was grown in TOP10 *E. coli* cells (Invitrogen), which were grown in a shaking incubator at 37°C to mid-log phase, as determined by an optical density of 0.5 read at a wavelength of 600 nm (OD_{600}). Once the cultures reached an OD_{600} of 0.5, L-ara was added at a final concentration of 0.02%. The 0.02% L-ara concentration was determined to be the optimal concentration for inducing protein production by testing multiple concentrations of the L-ara. Bacterial cells were harvested by centrifugation in a table top centrifuge at 6,000 x g for 5 minutes at 25°C, supernatant was removed and the pellets were frozen overnight at -20°C.

Detection of recombinant AaMLAP. After being held overnight at -20°C, the bacterial cell pellet was resuspended in 100 µl of 2X sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, heated to 90°C for 5 minutes, then centrifuged at 15,000 x g for 15 minutes at room temperature. To confirm recombinant protein was expressed from the AaMLAP-pBAD plasmid, 10 µl of the resulting bacterial lysate was loaded onto a 10% SDS-PAGE gel, which was run in duplicate. Gels were run at 200V using a BioRad Mini-Protean II cell (BioRad) under reducing conditions. One of the SDS-PAGE gels was submerged in coomassie brilliant blue stain [0.25% coomassie brilliant blue (w/v), 50% methanol (v/v) and 10% glacial

acetic acid (v/v)] for one hour. The gel was de-stained using a solution of 5% methanol and 7.5% glacial acetic acid. The remaining gel was subjected to western blotting analysis. Proteins contained in the SDS-PAGE gel were transferred to a polyvinylidene fluoride (PVDF) membrane, which was blocked for one hour at room temperature using 5% non-fat dry milk diluted in phosphate buffered saline containing 0.1% tween-20 (PBST). The membrane was washed three times with PBST for 5 minutes, and then incubated with the an anti-V5-horseraddish peroxidase (HRP) antibody (Invitrogen), diluted 1:5000 in blocking buffer, for one hour at room temperature. The membrane was then washed three times with PBST, developed with the ECL Plus Western Blotting Detection Kit (GE Health Sciences, Arlington Heights, IL, USA), exposed to X-ray film and developed using an automatic film developer (Kodak, Rochester, NY, USA).

Efforts to purify the recombinant protein expressed from the pBAD-AaMLAP plasmid failed to yield sufficient quantities of recombinant protein for immunizations, so SDS-PAGE gel strips containing the protein were used for immunizations. Five 10 ml cultures containing *E. coli* transformed with the pBAD-AaMLAP plasmid were grown as described above and induced with L-ara at a concentration of 0.02%. Bacterial cells were pelleted as described above, resuspended in 100 µl of 2X SDS-PAGE loading buffer, heated to 90°C for five minutes, and then centrifuged at 15,000 x g for 15 minutes in a microcentrifuge at room temperature. For each dose of the recombinant AaMLAP protein vaccine, 20 µl of the lysate was loaded into the lane of a 10% SDS-PAGE gel and run under denaturing and reducing conditions. Three bovine serum albumin (BSA) standards, 1.0, 2.5, and 5 µg per well were run on each SDS-PAGE gel. The SDS-PAGE gel was stained with coomassie brilliant blue and de-stained as described previously, then

washed in sterile deionized water. The region of the gel containing the AaMLAP recombinant protein was excised using a razor blade, and the quantity of recombinant AaMLAP protein in each lane was estimated to be 2.5 μg by visually comparing the AaMLAP band to the BSA standards. To prepare the AaMLAP polyacrylamide gel slurry used for immunizations, five gel strips, each containing the excised AaMLAP protein, were combined into a ceramic mortar and ground under liquid nitrogen to a fine powder. The powder was resolubilized in 500 μl of sterile PBS, which was subsequently passed through an 18 gauge needle. Each immunization dose consisted of 100 μl of the PBS-gel strip slurry, and was estimated to contain 2.5 μg of recombinant AaMLAP protein. Control vaccine doses consisted of 2.5 μg of BSA, which was run on an SDS-PAGE gel and excised according to the methods described above.

Immunizations.

Balb/c mice, aged 5-8 weeks at the time of first immunization, were used in this study. Prior to immunization, mice were bled by nicking the tail vein with a razor blade, and pre-immune serum was processed as described above. Mice were given either three (n= 2 mice) or four (n=3 mice) immunizations with the AaMLAP polyacrylamide gel slurry, with two week intervals between each immunization. For each immunization, mice were injected (s.c.) with 100 μl of the AaMLAP-polyacrylamide gel slurry. Two weeks after the final immunization, mice were restrained as described above, and placed into a cage of mosquitoes. After the blood feed mice were anesthetized using a Ketamine/Xylazine cocktail and processed as described above.

Mosquitocidal bioassay.

Immunization efficacy was assessed using the following bioassay. One day prior to blood feeding on immunized mice, two to three day old adult *Ae. aegypti* HWE mosquitos were transferred to 4 L cages. Prior to the blood feed, mosquitoes were deprived of sugar and water for 24 and 12 hours, respectively. Each live mouse was restrained as described above. Once immobilized, the mouse was placed into a cage of mosquitoes. A separate cage of mosquitoes was used for each mouse. After one hour, mice were removed, bled and euthanized (described above). Mosquitoes were cold-anesthethized in a refrigerator, placed onto a glass Petri dish maintained on ice, and sorted for the presence of a blood meal. Only fully engorged mosquitoes were retained for survival analysis. Mosquitoes were retained for seven days following the blood feed, and mortality was recorded every day.

Statistical analysis.

For each experimental group, mosquito mortality tabulated seven days after feeding on each mouse was compared individually with all other mice using a Fisher's exact test, with the significance level set at $P < 0.05$. If no significant within-group differences in mosquito survivorship were observed, data within groups were pooled and significant differences in mosquito survival between the immunized and control group were analyzed using a Fisher's exact test.

Antibody detection via western blotting.

To test for antibodies against AaMLAP, pre and post-immunization serum from all immunization regimens, and *Schistosoma japonicum* cathepsin-D antiserum (kindly

provided by Dr. Paul Brindley) were probed against mosquito homogenate and the bacterial lysate containing the recombinant AaMLAP protein (described above).

Protein preparation, separation and transfer.

The mosquito homogenate was prepared by first blood feeding *Ae. aegypti* HWE mosquitoes on defimbrinated sheep blood. Given that peak AaMLAP protein production occurs 42 hours following a blood feed (Cho and Raikhel 1992), whole mosquito homogenates were prepared by grinding a pool of ten 42 hour post-blood fed mosquitoes into 100 µl of SDS-PAGE sample buffer, boiling the mixture for five minutes, then pelleting insoluble material by centrifugation at 700 x g for three minutes. One mosquito equivalent was estimated to be 10 µl of the prepared homogenate. One mosquito equivalent was loaded into each well of a 10% SDS-PAGE gel.

The recombinant AaMLAP protein was produced in *E. coli* cells according to the methods described above, and 10 µl of the bacterial lysate was loaded into each well of a 10% SDS-PAGE gel.

Proteins were separated using SDS-PAGE electrophoresis under denaturing and reducing conditions, transferred to a PVDF membrane which was blocked in 5% non-fat dry milk in PBST for one hour at room temperature. The membrane was cut into strips, with each strip corresponding to a well from the SDS-PAGE gel.

Antibody detection.

To absorb antibodies developed against *E.coli* proteins that were included with SDS-PAGE gel strip immunization, prior to western blotting, post-immune mouse sera

from the recombinant protein immunizations were incubated with beads coated with *E. coli* lysate (Pierce Protein Research Products, Rockford, IL, USA).

For each immunization regimen, a pool of post immune serum was created by combining 2 µl of serum from each mouse into an microfuge tube, and then mixing the serum by pipetting. A pre-immune serum pool was produced by combining 2 µl from all mice into a microfuge tube and mixing the serum by pipetting. Serum was then diluted to a working concentration of 1:250 in blocking buffer, and then incubated on a blocked PVDF membrane strip gel strip containing either the mosquito homogenate or recombinant AaMLAP protein. The PVDF membrane strips were then washed three times with PBST for 15 minutes, and incubated for one hour at room temperature with a horseradish peroxidase (HRP) conjugated polyclonal rabbit anti-mouse secondary antibody (Abnova, Walnut, CA, USA) diluted 1:5000 in blocking buffer. The PVDF membrane strips were washed three times with PBST for 15 minutes, and finally developed using the Vector VIP Substrate (Vector laboratories).

Evaluation of rabbit AgGluCl antiserum following oral ingestion in an *in vitro* blood meal.

An *in vitro* feeding assay was used to evaluate the effect of rabbit AgGluCl anti-serum on the survival of *Ae. aegypti*. One ml of human blood preserved in 3.2% sodium citrate was centrifuged at 7,500 x g in a microcentrifuge. Serum and the buffy coat were removed, and the remaining erythrocytes were washed twice in 1 ml of sterile PBS. A volume of rabbit AgGluCl antiserum (kindly provided by Dr. Brian Foy) equivalent to the volume of discarded human serum was mixed with the washed erythrocytes. The resulting mixture of rabbit serum and human erythrocytes was placed into a water-

jacketed glass bell feeder (Lillie Glass Blowers) which had been sealed with hog gut sausage casing. The membrane feeder was warmed to and maintained at 37°C using a circulating water pump for the duration of the feed. Washed erythrocytes re-constituted with pre-immune rabbit serum and heat inactivated normal rabbit serum (Sigma-Aldrich, St. Louis, MO, USA) were used as controls. Survival analysis was carried out as described above.

Table 2.1: Summary of Immunizations against the AaMLAP

Description of Immunization	Immunization Regimen; Route of administration (frequency)
1. DNA immunization	AaMLAP- DNA immunization construct(1); i.m. (3X) OR i.d. (2X) AaMLAP- DNA immunization construct(2); i.m. (3X)
2. DNA immunization prime-alphavirus-boost	AaMLAP- DNA immunization construct(1); i.m. (2X) followed by AaMLAP-SINV; s.c. (1X)
3. Protein immunization	SDS-PAGE strip slurry; s.c. (3X) or (4X)

Table 2.2: Primers used to clone AaMLAP into different plasmid expression vectors

Primer name	Primer Sequence[a]
AaM-SINV Fwd	5' -ATT GTT **GCG GCC GC***A CC*A TGC TAA TTA AAT CAA- 3’
AaM- SINV Rev	5' -ATT GTT **GCG GCC GC**A CCT TAG ACA GCA GTG GCA AAT CC- 3'.
AaM- pBAD Fwd	5' - ATG CTA ATT AAA TCA ATT ATT GCC CTG GTT TGC- 3'
AaM- pBAD Rev	5' -GAC AGC AGT GGC AAA TCC AAC GCT- 3'
AaM- pCDNA Fwd	5’- *ACC* ATG CTA ATT AAA TCA ATT ATT G- 3’
AaM-pCDNA Rev	5’ – TTA GAC AGC AGT GGC AAA TCC 3’

[a] The *Not*I restriction recognition sequence is denoted in **bold** font. Start codons sequences are denoted by text underlined once. Stop codons are denoted by text underlined twice, and the Kozak recognition sequence is denoted in *italicized* font.

Results.

Mosquitocidal bioassay of the AaMLAP-DNA immunization construct(1) immunization regimen.

Intramuscular administration. Two experimental replicates were conducted, and in both experimental replicates, mosquito survival was reduced after feeding on mice immunized (i.m.) with the AaMLAP-DNA immunization construct(1).

In the first experimental replicate, analysis of mosquito survival, by mouse, within treatment groups did not reveal significant differences in either the control group (Fig 2.1A) or the group that had received three injections (i.m.) with AaMLAP-DNA immunization construct(1) (Figure 2.1B). Mosquito survival data within treatment groups were pooled and analyzed. After seven days, the percentage of mosquitoes surviving after feeding on mice injected three times with the AaMLAP-DNA immunization construct(1) was significantly reduced, 1.23 fold, relative to control-immunized mice ($P<0.0001$) (Figure 2.1C).

In the second experimental replicate, analysis of mosquito survival, by mouse, within treatment groups did not reveal significant differences in either the control group (Figure 2.2A) or in the group that had received three injections (i.m.) with the AaMLAP-DNA immunization construct(1) (Figure 2.2B). Mosquito survival data within treatment groups were pooled and analyzed. After seven days, the percentage of mosquitoes surviving after feeding on mice injected three times with the AaMLAP-DNA immunization construct(1) was marginally reduced relative to control immunized mice ($P=0.048$) (Figure 2.1C).

Intra-dermal administration. Following i.d. immunization with the AaMLAP-DNA immunization construct(1), mosquito survival was reduced after feeding on two of the mice (mice 1 & 2), but was not reduced after blood feeding on mouse 3 (Figure 2.3A). Mosquito survival did not differ significantly between mouse 1 and mouse 2, however mouse 3 differed significantly from both mouse 1 and mouse 2 ($P = 0.011$ and $P = 0.036$, respectively). One of the control immunized mice died prior to the mosquito feed; however, of the remaining two mice, there were not significant within group differences in mosquito survival after blood feeding (Figure 2.3B). A cumulative, between group, comparison of mosquito survival was made by omitting the data from mouse-3 in the AaMLAP immunization group, and then pooling the data within groups. The resulting comparison revealed that mosquito survival was significantly reduced after blood feeding on the mice immunized (i.d.) with the AaMLAP-DNA immunization construct-1 when compared to control immunized mice ($P=0.017$) (Figure 2.3 C).

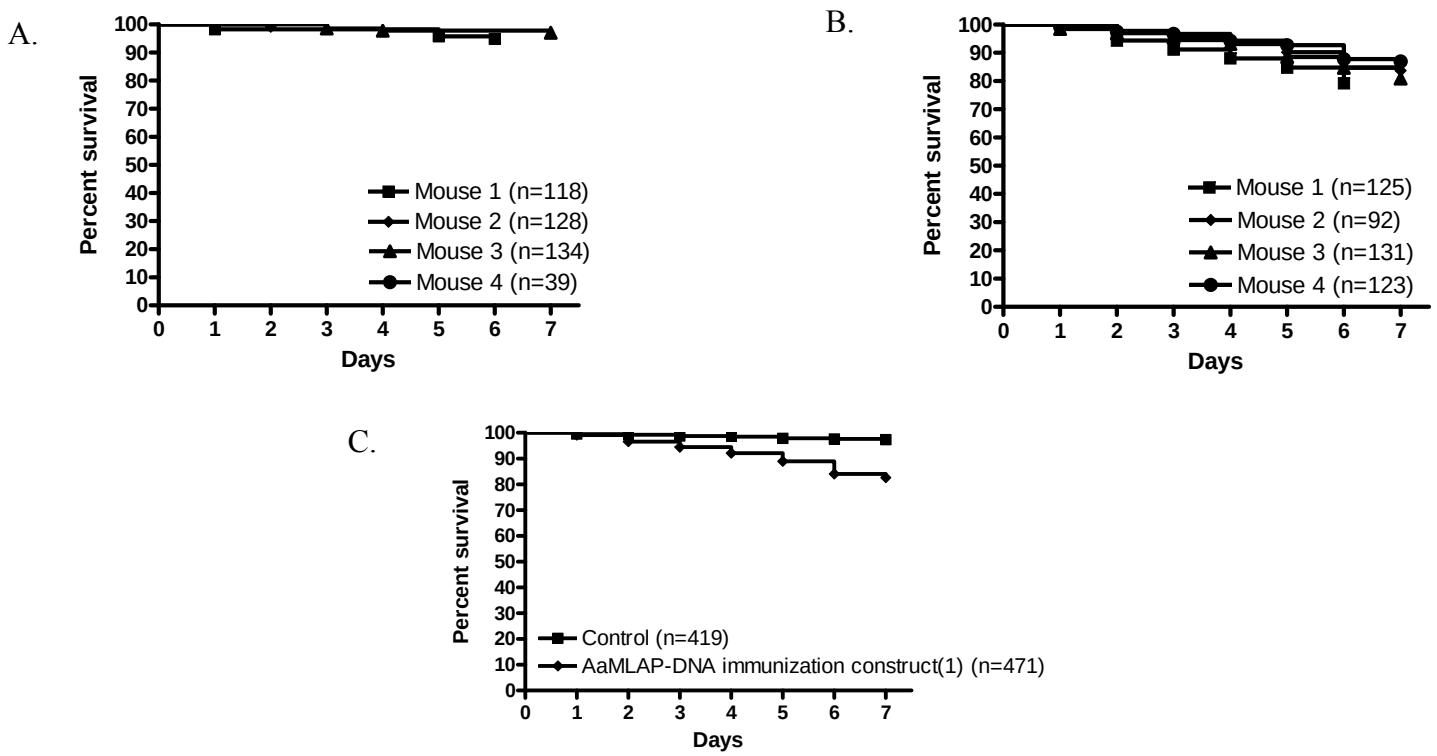


Figure 2.1: Survival of mosquitoes after blood feeding on mice immunized (i.m) with the AaMLAP-DNA-immuzation construct(1) (replicate 1). Two weeks following the final immunization, all mice were fed upon by *Ae. aegypti.* Percent survival of mosquitoes blood fed on each mouse that had been immunized with pCDNA3.1(+) plasmid only + mIL-12 (A) or AaMLAP-DNA immunization construct(1) (B) are shown. Within group comparisons of mosquito mortality by mouse revealed no significant differences, and data were pooled (C). Mortality of mosquitoes that fed upon mice immunized with the AaMLAP-DNA immunization construct(1) was significantly greater ($P<0.001$) than mortality observed in mosquitoes fed on control immunized mice. n= the total number of blood fed mosquitoes.

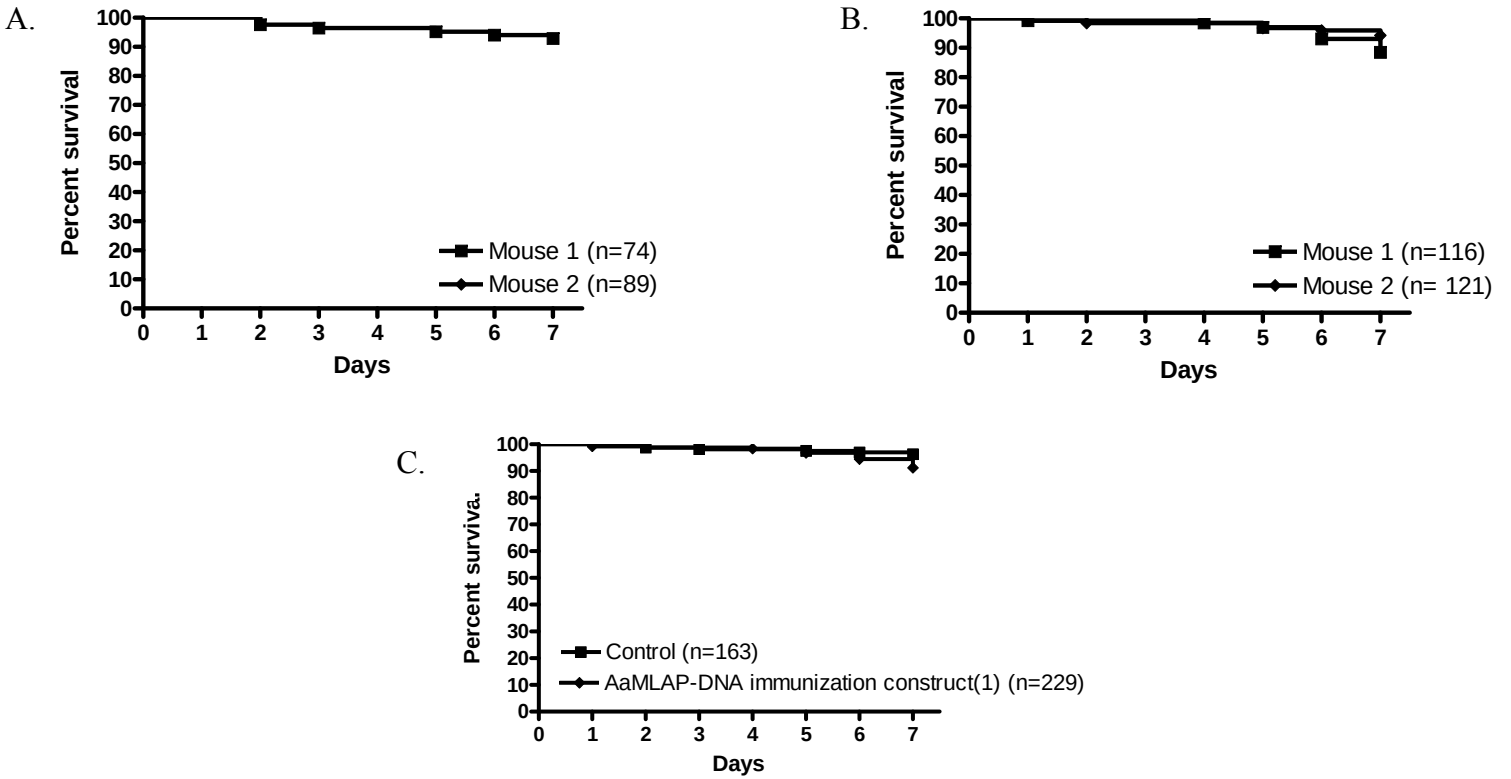


Figure 2.2: Survival of mosquitoes after blood feeding on mice immunized (i.m) with the AaMLAP-DNA-immuzation construct(1) (replicate 2). Two weeks following the final immunization, all mice were fed upon by *Ae. aegypti.* Percent survival of mosquitoes blood fed on each mouse that had been immunized with pCDNA3.1(+) plasmid only + mIL-12 (A) or the AaMLAP-DNA immunization construct(1) (B) are shown. Within group comparisons of mosquito mortality by mouse revealed no significant differences, and data were pooled (C). Mortality of mosquitoes that fed upon mice immunized with the AaMLAP-DNA immunization construct(1) was marginally greater ($P<0.0485$) than mortality observed in mosquitoes fed on control immunized mice. n= the total number of blood fed mosquitoes.

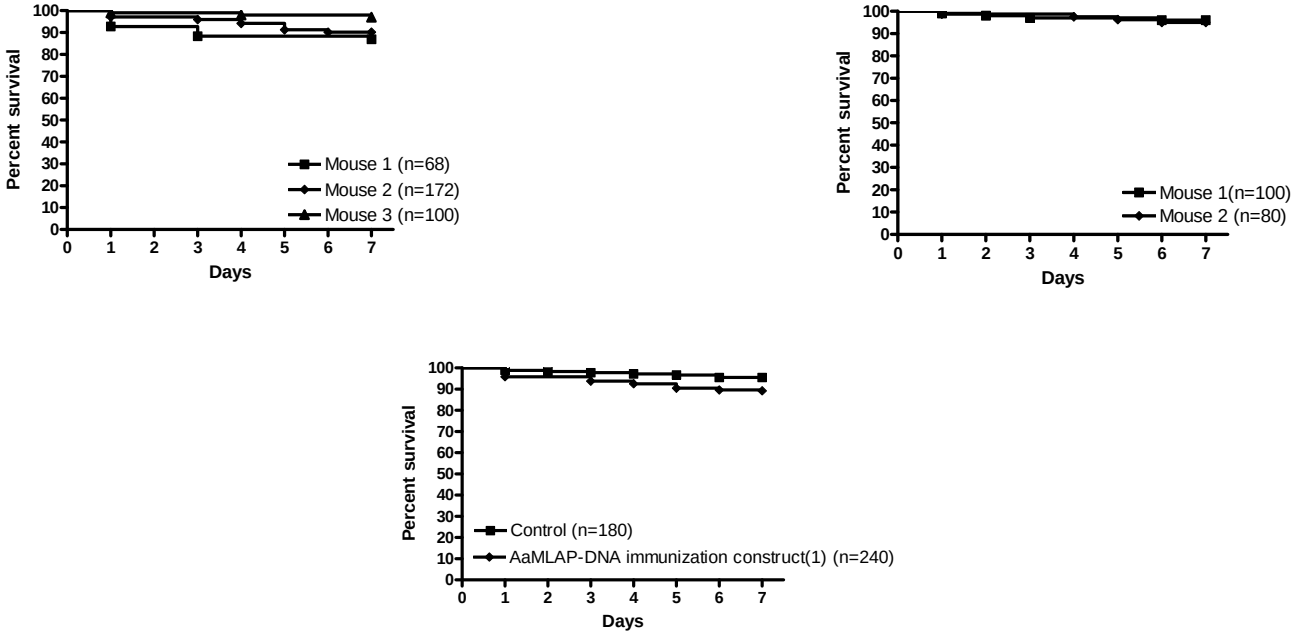


Figure 2.3: Survival of mosquitoes after blood feeding on mice immunized (i.d.) with the AaMLAP-DNA-immuzation construct(1). Two weeks following the final immunization, each mouse from the experimental and control group was fed upon by *Ae. aegypti.* Percent survival of mosquitoes blood fed on each mouse that had been immunized with (A) pCDNA3.1(+) plasmid only + mIL-12 (B) AaMLAP-DNA immunization construct-1 + mIL-12 are shown. Within group comparisons of mosquito mortality by mouse revealed no significant differences between control mice. In the AaMLAP immunization group, mosquito mortality after feeding on mouse 3 was significantly lower than mice 1 and 2 ($P<0.05$). Mosquito mortality after feeding on AaMLAP mouse 3 did not differ significantly from either of the control mice. (C) Mortality of mosquitoes that fed upon AaMLAP mice 1 and 2 mice immunized with the AaMLAP-DNA immunization construct 1 was significantly lower ($P=0.017$) than mortality observed in mosquitoes fed on control immunized mice. n= the total number of blood fed mosquitoes.

Mosquitocidal bioassay of the AaMLAP-DNA prime-alphavirus-boost immunization regimen.

Analysis of mosquito survival, by mouse, within treatment group did not reveal significant differences in either the control group (Figure 2.4A) or in the group treated with the AaMLAP-DNA-prime-alphavirus-boost immunization regiment (Figure 2.5B). Mosquito survival data within treatment groups were pooled and analyzed. No significant ($P>0.05$)differences in mosquito mortality were observed between mosquitoes which had fed on mice immunized via the AaMLAP-DNA prime-alphavirus-boost regimen when compared to mice immunized with the control regimen (Figure 2.4C).

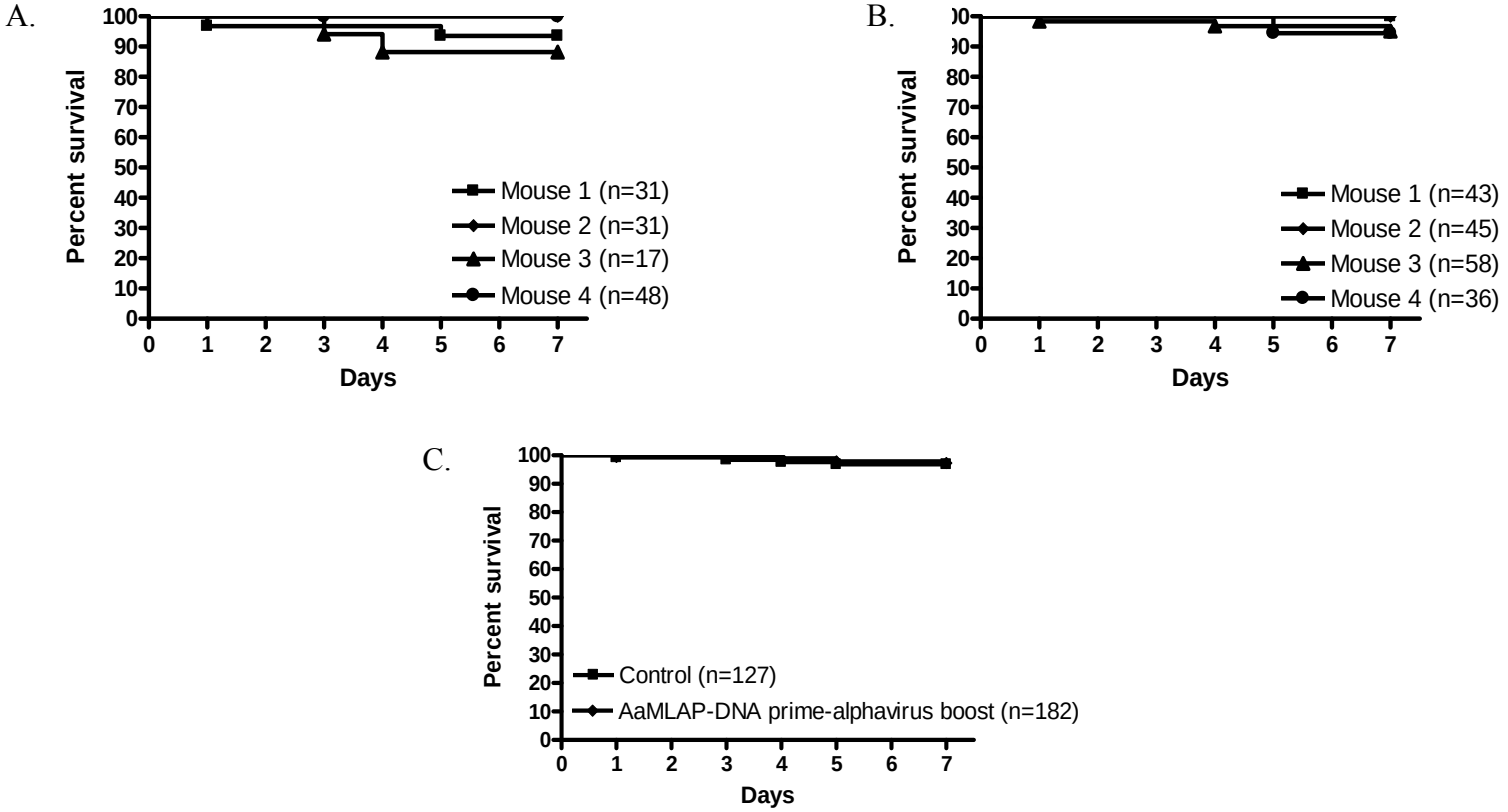


Figure 2.4: Survival of mosquitoes after blood feeding on mice treated with the AaMLAP-DNA: prime-alphavirus-boost immunization regimen. Two weeks after the inoculation with recombinant SINV, all mice were fed upon by *Ae. aegypti*. Percent survival of mosquitoes blood fed on mice treated with the control immunization regimen (A) and mice treated with the AaMLAP-DNA-prime-alphavirus boost immunization regimen (B) are shown. Within group comparisons of mosquito mortality by mouse revealed no significant differences, and data were pooled (C). No significant difference ($P>0.05$) in mosquito mortality was observed between mosquitoes that fed on control immunized mice and mice immunized with the AaMLAP-DNA: prime-alphavirus-boost regimen.

Mosquitocidal bioassay of the AaMLAP-DNA immunization construct(2) immunization regimen.

Analysis of mosquito survival, by mouse, within treatment groups did not reveal significant differences in either the control group (Figure 2.5A) or the group that received three injections (i.m.) of the the AaMLAP-DNA immunization construct(2) (Figure 2.5B). Mosquito survival data within treatment groups were pooled and analyzed. No significant difference ($P>0.05$) in mosquito mortality was observed between mosquitoes which had fed on mice immunized with the AaMLAP-DNA immunization construct(2) when compared to the corresponding control immunized mice (Figure 2.5C).

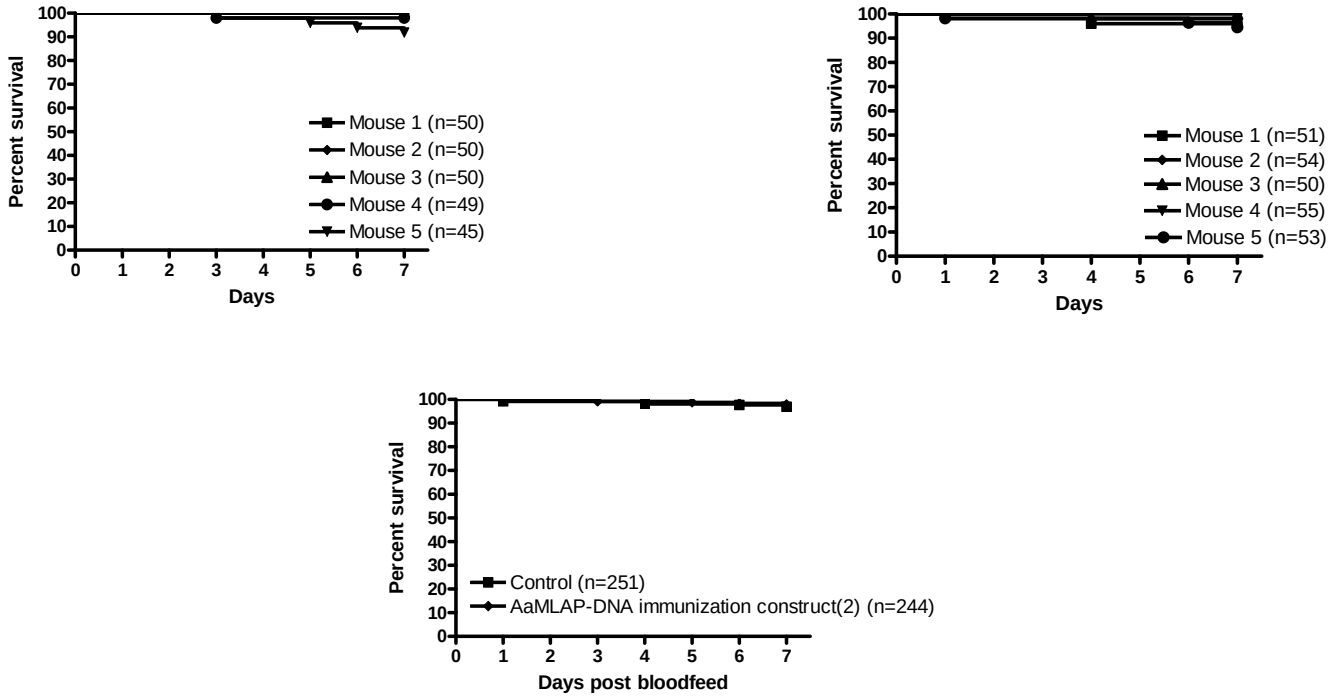


Figure 2.5: Survival of mosquitoes after blood feeding on mice immunized (i.m) with the AaMLAP-DNA-immuzation construct(2). Two weeks following the final immunization, each mouse from the experimental and control group was fed upon by *Ae. aegypti*. Percent survival of mosquitoes blood fed on each mouse that had been immunized with (A) pCDNA3.1(+) plasmid only + mIL-12 (B) AaMLAP-DNA immunization construct(2) are shown. Within group comparisons of mosquito mortality by mouse revealed no significant differences, and data were pooled (C). No significant difference ($P>0.05$) in mosquito mortality was observed between mosquitoes that fed on control immunized mice and mice immunized with the AaMLAP-DNA immunization construct(2).

Expression of recombinant AaMLAP protein in the pBAD expression system.

The AaMLAP protein is approximately 40 kilodaltons (kDa) in size (Cho and Raikhel 1992). The polyhistidine tag and V5 epitope increase the size of the protein by 16 kDa. Therefore, the expected size of the recombinant AaMLAP protein, expressed in the pBAD expression system, is approximately 56 kDa. Expression of the recombinant AaMLAP protein in bacterial cultures was confirmed via Coomassie brilliant blue staining and Western blot. A 56 kDa protein was observed in bacterial lysates from cultures that had been treated with L-ara, but was not observed in the control bacterial lysate that was not treated with L-ara (Figure 2.6A). A Western blot using the anti-V5-HRP antibody revealed that a 56 kDa protein containing a V5 epitope was expressed in all L-ara treated cultures, except at the lowest concentration tested (0.00002%). No protein containing a V5 epitope was detected in the non-induced control culture (Figure 2.6B).

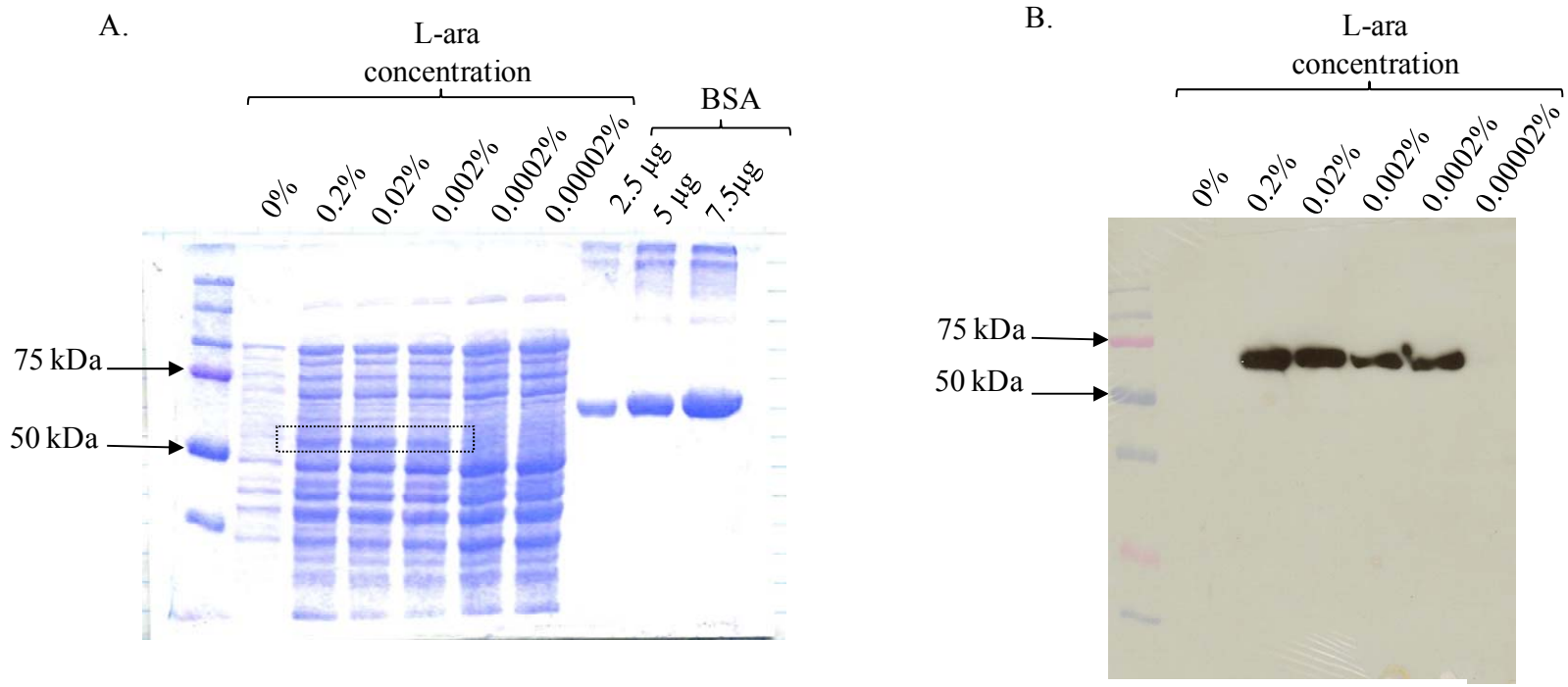


Figure 2.6. Coomassie brilliant blue staining and Western blotting analysis of recombinant AaMLAP protein expressed with the pBAD expression system. *E. coli* cells transformed with the AaMLAP-pBAD plasmid were induced with varying concentrations of L-ara. Cell lysates were prepared, and 20 µl from each lysate was loaded onto 10% SDS-PAGE gels, which were stained with Coomassie brilliant blue (A) or probed with an anti-V5 antibody via Western blotting analysis (B). Known quantities of BSA (2.5 µg , 5.0 µg, 7.5 µg) were included in Coomassie brilliant blue staining to allow for relative quantitation of recombinant AaMLAP. The Coomassie brilliant blue-stained recombinant protein is shown in the box.

Mosquitocidal bioassay of the recombinant AaMLAP protein immunization regimen.

To evaluate whether immunization with the AaMLAP polyacrylamide gel slurry (described above) could elicit a mosquitocidal response, mice were injected (s.c.), either three or four times. Control immunizations were administered by injecting mice (s.c.), either three or four times, with the polyacrylamide-BSA gel slurry (described above).

Mosquitocidal bioassay of mice immunized three times with the polyacrylamid-AaMLAP slurry: Analysis of mosquito survival, by mouse, within treatment groups did not reveal significant differences in either the control group (Figure 2.7A) or the group which received three injections of the polyacrylamide-AaMLAP gel slurry (Figure 2.7B). Mosquito survival data within treatment groups were pooled and analyzed. After seven days, the percentage of mosquitoes surviving after feeding on mice injected three times with the polyacrylamide-AaMLAP gel slurry was significantly reduced ,1.11-fold, relative to control-immunized mice (P=0.034) (Figure 2.7C).

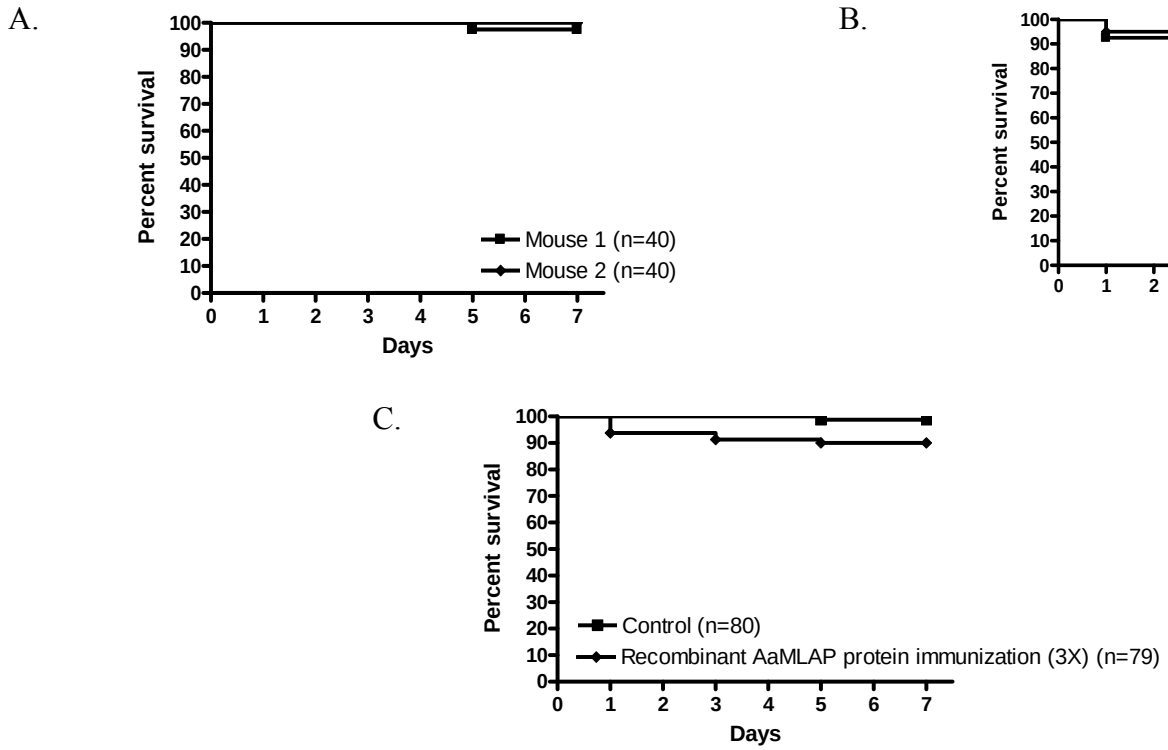


Figure 2.7: Survival of mosquitoes after blood feeding on mice immunized three times (s.c.) with the recombinant AaMLA_-polyacrylamide gel slurry. Two weeks following the final immunization, all mice were fed upon by *Ae. aegypti.* Percent survival of mosquitoes blood fed on each mouse that had been immunized three times with the polyacrylamide BSA gel slurry (A) or the AaMLAP-polyacrylamide gel slurry (B). Within group comparisons of mosquito mortality by mouse revealed no significant differences, and data were pooled. Mortality of mosquitoes that fed upon mice immunized with the recombinant AaMLAP protein was significantly greater (P=0.034) than mortality observed in mosquitoes fed on control immunized mice (C). n= the total number of blood fed mosquitoes.

Mosquitocidal bioassay of mice immunized four times with the polyacrylamid-AaMLAP slurry: Analysis of mosquito survival, by mouse, within the group of mice immunized four times (s.c.) with the polyacrylamide AaMLAP gel slurry did not reveal significant differences in mosquito survival among mice (Figure 2.8B). Analysis of mosquito survival, by mouse, within the control group revealed that mosquito survival after blood feeding on control mouse-1 was significantly lower than control mouse-2 and control mouse-3 ($P<0.001$) (Figure 2.8A). Mosquito survival after feeding on control mouse-1 was also significantly lower than each of the mice immunized with the AaMLAP polyacrylamide gel slurry ($P<0.05$), and was therefore treated as an outlier and removed from analysis. Mosquito survival data within groups were pooled and analyzed. After seven days, the percentage of mosquitoes surviving after feeding on mice injected four times with the polyacrylamide-AaMLAP gel slurry was significantly reduced,1.08-fold, relative to control-immunized mice ($P=0.031$) (Figure 2.8C).

The cumulative percent mortality of mosquitoes fed on mice immunized three times or four times with the polyacrylamide gel-AaMLAP protein slurry was 1.11-fold and 1.08-fold lower, respectively, compared to the corresponding control groups. The mosquitocidal bioassays for the two immunization regimens were completed using different cohorts of mosquitoes, and were not completed concurrently. Thus, differences in mosquito survival between the two different immunization regimens were not subjected to statistical analysis.

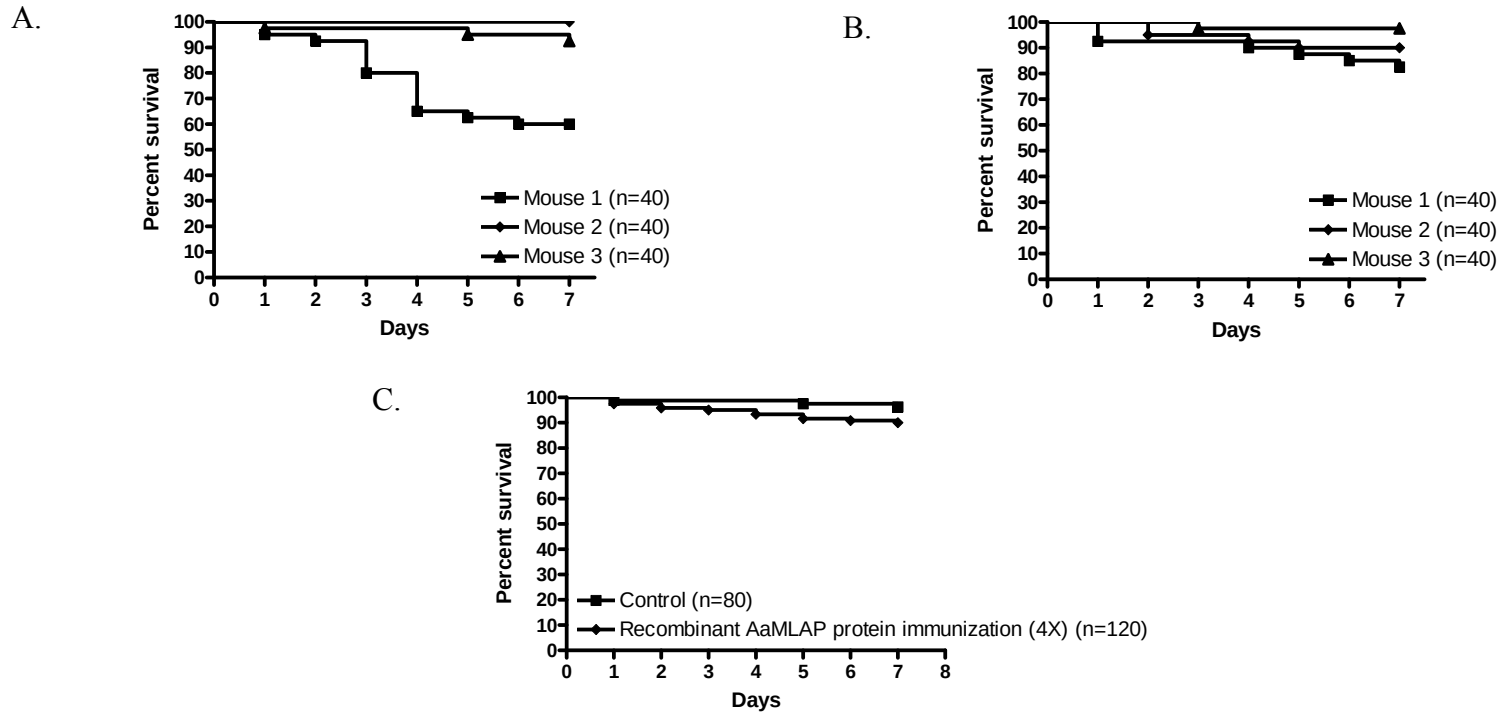


Figure 2.8: Survival of mosquitoes after blood feeding on mice immunized four times (s.c.) with the recombinant AaMLAP polyacrylamide gel slurry. Two weeks following the final immunization, all mice were fed upon by *Ae. aegypti.* Percent survival of mosquitoes blood fed on each mouse that had been immunized four times with the polyacrylamide BSA slurry (A) or the polyacrylamide AaMLAP gel surry (B). In the control immunized group, mosquito mortality after feeding on mouse 1 was significantly different than control mice 1 and 2 ($P<0.001$), and was omitted from cumulative analysis. Within group comparisons of AaMLAP immunized mice revealed no significant differences in mosquito mortality, by mouse. Mortality of mosquitoes that fed upon mice immunized against AaMLAP was significantly lower ($P=0.031$) than mortality observed in mosquitoes fed on control immunized mice 2 and 3 (C). n= the total number of blood fed mosquitoes.

Detection of AaMLAP antibody from immunized mice.

The AaMLAP protein isolated from *Ae. aegypti* mosquitoes has a molecular mass of 40 kDa (Cho and Raikhel 1992). Western blot analysis using whole mosquito homogenate did not detect anti-AaMLAP antibodies in the pre-immune serum pool or in post-immune serum pools from any of the immunization regimens. No cross-reactivity was observed between the *S. japonicum* cathepsin D anti-sera and the mosquito homogenate (Figure 2.10).

Efforts to remove anti-*E.coli* antibodies from the post-immune serum harvested from mice immunized with the AaMLAP polyacrylamide gel slurry were unsuccessful. When post-immune serum from mice immunized with the AaMLAP polyacrylamide gel slurry was probed against *E. coli* cell lysate from non-transformed *E. coli* cells, a band at approximately 56 kDa was observed (Figure 2.11A).

To determine if post-immune serum from any of the immunization regimens would react with the recombinant AaMLAP protein produced in the pBAD expression system, serum was probed against a bacterial lysate prepared from *E. coli* cells expressing the recombinant AaMLAP protein. An anti-V5 antibody was used to confirm expression of the protein. Post-immune serum from mice immunized with the AaMLAP polyacrylamide gel slurry detected a band of similar molecular weight to that of the positive control. Post-immune serum from mice immunized (i.m.) with the AaMLAP-DNA immunization construct(1) and AaMLAP-DNA immunization construct(2) yielded a band at approximately 25 kDa, which was not the in the molecular weight range of the AaMLAP protein (Figure 2.11B). The *S. japonicum* cathepsin-D antisera yielded a band of approximately 60 kDa, which was also not in the molecular weight range of the

AaMLAP protein (Figure 2.11B). Post-immune serum from i.d. immunization with the AaMLAP-DNA immunization construct(1) and immunization from the AaMLAP-prime-alphavirus boost immunization regimen did not yield distinct bands of any size (Figure 2.11B).

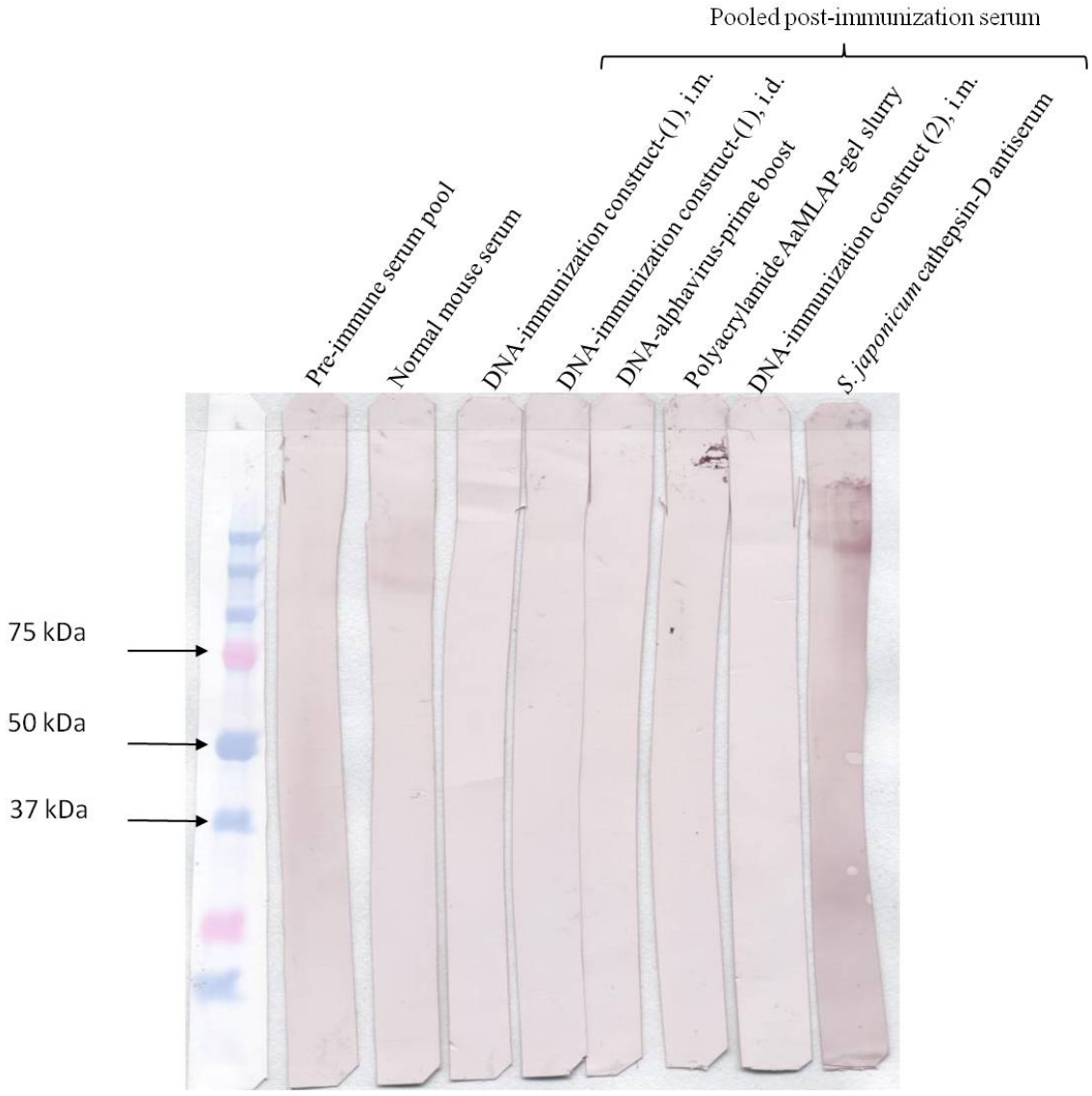


Figure 2.10. Reactivity of pre-and post-immune sera collected from immunized mice with whole mosquito homogenate. Pre- and post-immune mouse serum and the *S. japonicum* cathepsin-D antiserum were probed against *Ae. aegypti* mosquito homogenate prepared from 42-hour post-blood fed mosquitoes.

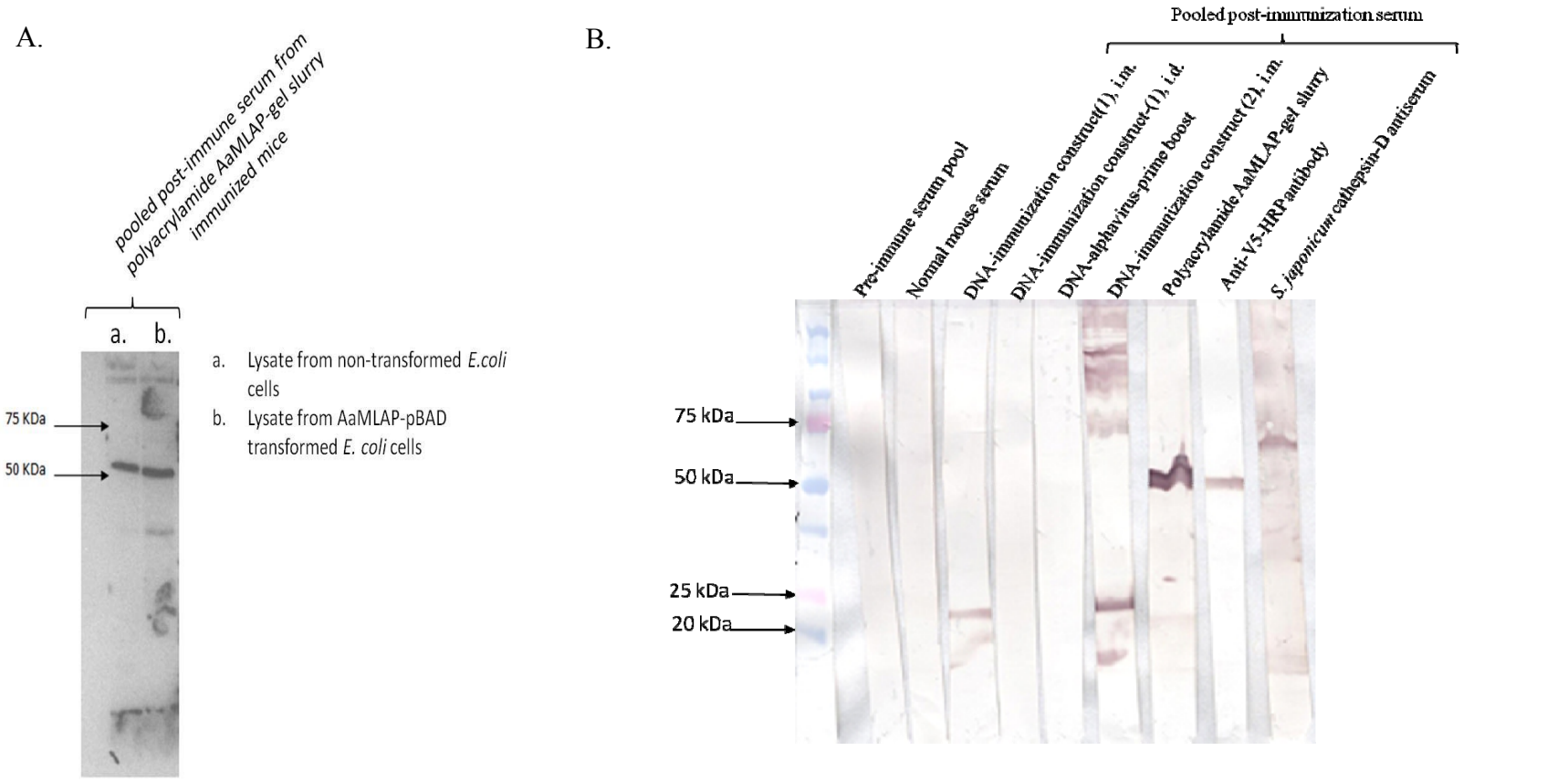


Figure 2.11. Reactivity of pre-and post-immune sera collected from immunized mice with bacterial lysate. Post-immune serum from all mice immunized with the polyacrylamide AaMLAP gel slurry was probed against *E. coli* lysate prepared from cells that had not been transformed with the AaMLAP-pBAD plasmid (A). Pre- and post-immune mouse and the *S. japoinicum* cathepsin-D antiserum were probed against bacterial lysate prepared from *E.coli* cells that were expressing the AaMLAP protein. Expression of the recombinant AaMLAP protein was verified by probing the lysate with a commercial anti-V5 antibody as a positive control (B).

Survival of *Ae. aegypti* mosquitoes after imbibing AgGluCl antiserum.

Twenty mosquitoes blood fed on blood containing the AgGluCl antiserum, however only one mosquito blood fed on the blood containing the pre-immune rabbit serum, and two mosquitoes blood fed on the blood containing normal rabbit serum. After seven days, no mortality was seen in any of the mosquitoes which had imbibed a blood meal containing the AgGluCl antiserum or in mosquitoes which had imbibed blood containing normal rabbit serum. The only mosquito which had imbibed blood containing the pre-immune rabbit serum died two days after blood feeding. Due to exceptionally low sample sizes in the control groups, survival data from the groups were not subjected to statistical analysis.

Discussion.

Mosquitocidal immunity has been demonstrated numerous times (Alger and Cabrera 1972, Hatfield 1988, Almeida and Billingsley 1998, 1999, Lal et al. 2001, Almeida and Billingsley 2002, Foy et al. 2003), yet only two specific mosquitocidal antigens, the *Anopheles gambiae* Mucin-1 protein (Foy et al. 2003), and the *Aedes albopictus* subolisin (Canales et al. 2009) have been described. If mosquitocidal vaccines are ever to become a possible strategy for mosquito-borne disease, specific mosquitocidal antigens must first be identified. The purpose of this research was to determine if the AaMLAP and AaGluCl anion channel could be immunologically targeted to induce mosquitocidal activity. While we did observe a decrease in mosquito survival after feeding on certain AaMLAP-immunized mice, it remains unclear whether or not the AaMLAP is a mosquitocidal target.

A reduction in *Ae. aegypti* mosquito survival was observed in three different experiments where mosquitoes had blood fed on mice that had been immunized (i.m. and i.d.) with the AaMLAP-DNA immunization construct(1). These results are especially surprising because we discovered a deletion in the start codon in the AaMLAP-pCDNA3.1(+) plasmid included in this immunization construct. Examination of alternate open reading frames in the DNA plasmid revealed numerous other start codons (5'ATG 3'), which could have resulted in expression of a truncated AaMLAP protein or multiple other polypeptides. We could have characterized any of the putative polypeptides and evaluated their ability to induce mosquitocidal immunity in mice, however, the reduction in mosquito survival was minimal, and we did not further explore these putative polypeptides. It is unlikely that co-immunization with the pCDNA3.1(+) and mIL-12 plasmids is what resulted in a mosquitocidal immune response, as mosquito survival in control-immunized mice was not similarly reduced.

Given the incomplete start codon in the AaMLAP-pCDNA3.1(+) plasmid, it is not surprising that serum collected from mice immunized with the AaMLAP-DNA immunization construct(1) did not yield a band within the expected molecular weight range (40 kDa) when probed against mosquito homogenate or bacterial lysate containing the recombinant protein expressed from the pBAD-AaMLAP plasmid. However, an important caveat is that we did not have a positive control antiserum, thus we cannot conclude that the failure to detect antibody was not due to experimental error. We included the *S. japonicum* cathepsin-D antiserum in western blotting analysis to determine if this serum could act as a positive control, however, it also failed to yield a band within the expected molecular weight range of the AaMLAP protein. These

findings may not be extraordinarily surprising, as post-immune serum from mice immunized against the *S. japonicum* cathepsin-D aspartic protease failed to cross-react with homogenates prepared from *S. mansonii* (Chlichlia et al. 2002).

No reduction was seen in mosquito survival after feeding on mice that had been immunized via the AaMLAP-DNA: prime-alphavirus-boost immunization regimen. These results are somewhat surprising, as the DNA immunization plasmid used in the AaMLAP-DNA:prime-alphavirus-boost immunization regimen was the same plasmid used to generate the AaMLAP-DNA immunization(1) construct. One possible explanation for the lack of mosquitocidal response is that the SINV virions encoded in its subgenomic promoter were stronger immunogens than AaMLAP-DNA immunization construct(1). If the mouse immune response was primarily directed against SINV antigens, then such a response could have had a dilution effect on the effector of mosquitocidal immunity seen in the previous experiments. We made an attempt to quantify SINV antibody titers from all mice by a plaque-reduction neutralization test, however sufficient quantities of post-immune serum to optimize and trouble-shoot the assay were not available, therefore accurate titers could not be determined.

Post-immune serum from mice immunized with the AaMLAP-prime-alphavirus boost immunization regimen did not react with either the mosquito homogenate or bacterial lysate containing the recombinant protein expressed from the AaMLAP-pBAD plasmid. Although alphavirus vectors are known to be high-level transient expression vectors (Atkins et al. 2008), we did not have a known anti-AaMLAP antiserum, and could not determine whether the AaMLAP protein was expressed by the alphavirus vector. The failure of the post-immune serum to react with either the mosquito

homogenate or the recombinant protein could be a result of no or exceptionally low *in vivo* expression of the AaMLAP protein, resulting in a failure to induce an anti-AaMLAP antibody response in the mouse. Alternative explanations could be poor immunogenicity of the AaMLAP protein, or failure of mouse anti-AaMLAP antibodies to recognize the non-glycosylated protein produced in from the bacterial expression system, or experimental error in the western blotting procedure.

No reduction in mosquito survival was observed in mosquitoes that had fed on mice immunized (i.m.) with the AaMLAP-DNA immunization construct(2). Post-immune mouse serum did not detect protein corresponding to the expected molecular weight of AaMLAP in either the whole mosquito homogenate or in bacterial lysate that had been induced to express recombinant protein from the pBAD-AaMLAP plasmid. Again, given the lack of positive-control antiserum, we cannot conclude that anti-MLAP antibody was not generated in response to immunization.

Using the pBAD/Thio-TOPO expression system, a recombinant protein containing a polyhistidine tag and V5 epitope was produced from the pBAD-AaMLAP plasmid. When accounting for the polyhistidine tag and V5 epitope, the recombinant protein detected by the anti-V5-HRP antibody was the expected size of the AaMLAP protein. Efforts to purify the protein yielded quantities that were too low to detect via colorimetric or spectrophotometric assays, thus mice were immunized with the polyacrylamide AaMLAP gel slurry. In retrospect, a more appropriate control polyacrylamide gel slurry immunization would have been an excised SDS-PAGE gel strip from a non-transformed bacterial lysate, corresponding to the same molecular weight as the excised recombinant protein. The polyacrylamide-BSA gel slurry did not

account for any bacterial antigens that were co-immunized with the recombinant protein, and it is possible that bacterial antigens in the mosquito midgut could have complexed with anti-*E. coli* antibodies ingested by the mosquito after feeding on immunized mice. Antibody-antigen complexes have been shown to cause blockages in the gut of the sheep blowfly larva, *Lucillia cuprina*, that result in death of the insect (Casu et al. 1997). Without the proper control in our experiments, we cannot rule out the possibility that the reduction in survival seen in mosquitoes that blood fed on the polyacrylamide-AaMLAP gel slurry immunized mice was not due to unintended immunological targeting of endosymbiotic bacteria in the mosquito midgut.

We were unable to definitively demonstrate that any of the immunization regimens induced the production of AaMLAP antibodies in mice. The lack of antibody production could be due to low immunogenicity of the AaMLAP immunization constructs, poor *in vivo* expression of AaMLAP from the pCDNA3.1(+) and AaMLAP SINV expression vectors, or error in the western blotting procedures used to probe for anti-AaMLAP antibody. Ultimately, the experiments outlined above do not clearly define or eliminate the AaMLAP as a mosquitocidal antigen. Given the lack of a positive control antiserum or purified AaMLAP protein, we decided to evaluate the GluCl anion channel as a potential mosquitocidal antigen.

The GluCl anion channel is the target of the anthelmintic drug, ivermectin. Numerous studies have shown that when ivermectin is imbibed in a bloodmeal, mosquito survival is reduced (Pampiglioni et al. 1985, Iakubovich et al. 1989a, Tesh and Guzman 1990, Cartel et al. 1991, Focks 1991, Focks et al. 1991, Mahmood et al. 1991, Gardner et al. 1993, Bockarie et al. 1999, Foley et al. 2000, Fritz et al. 2009, Kobylinski et al. 2010,

Sylla et al. 2010, Kobylinski et al. 2011). We hypothesized that if *Ae. aegypti* mosquitoes imbibed a blood meal containing high-titer, GluCl antiserum, that survival of the mosquitoes would be reduced. After feeding on a bloodmeal containing antiserum against the extracellular domain of the *An. gambiae* GluCl anion channel, we did not observe any reduction in mosquito survival. Data from this experiment was not subjected to statistical analysis due to low feeding rates in the control-fed mosquitoes. However, given that survival of mosquitoes after feeding on the GluCl antiserum was not affected, and that we had a limited supply of the antiserum, we did not repeat the experiment. Clearly one weakness in this experimental design is the use of heterologous antisera; however the extracellular domains of the *An. gambiae* and *Ae. aegypti* share a 90% amino acid identity. One possible explanation for the lack of mortality observed in *Ae. aegypti* after blood feeding on the GluCl antiserum could be that antigenic variation between the anion channels of the two mosquitoes prevented the antiserum from binding to the *Ae. aegypti* GluCl receptor. Another possible explanation is that the GluCl antiserum was raised against the extracellular domain of the GluCl anion channel, rather than the whole anion channel protein. In *Caenorhabditis elegans,* ivermectin integrates between the transmembrane domains of the GluCl channel subunit proteins, thereby sterically opening the GluCl channel (Hibbs and Gouaux 2011). It would be intriguing to determine if antibody raised against the transmembrane domains of the GluCl channel subunit proteins would mimic the effects of orally imbibed ivermectin on mosquito survival. Ultimately, we were not able to rule out the GluCl anion channel as a mosquitocidal antigen.

Mathematical modeling suggest that, if developed and deployed, mosquitocidal vaccines could be effective in decreasing the transmission of mosquito-borne pathogens such as DENV, CHIKV and YFV (Billingsley et al. 2008). Ultimately, if mosquitocidal vaccines are ever to be used as a tool to decrease the transmission of mosquito-borne pathogens, specific mosquitocidal antigens must first be identified.

CHAPTER 3: THE EFFECT OF ANTHELMENTICS ON ADULT SURVIVORSHIP FECUNDITY AND EGG HATCH RATE IN *AEDES AEGYPTI* (DIPTERA: CULICIDAE)

Introduction.

Globally, human populations are routinely treated via mass drug administration (MDA) with anthelmintics for the control of numerous nematode parasites. Ivermectin (IVM), diethylcarbamazine (DEC), and albendazole (ALB) are all currently administered via MDA for the control of onchocerciasis and lymphatic filariasis (Hotez 2007, Ottesen et al. 2008). Pyrantel (PYL) is distributed for control of numerous human nematode infections, including hookworm, roundworm and whipworm infections (Reddy et al. 2007). Amid concerns over the emergence of drug-resistant parasites (Osei-Atweneboana et al.) and reports of serious adverse events following MDA treatment of individuals co-infected with lymphatic filariasis and *Loa loa* (Kamgno et al. 2009), there has been a call for alternative MDA drugs and regimens (Bockarie and Deb 2010). Moxidectin (MOX) and selamectin (SEL) are anthelmintic drugs frequently used in veterinary medicine, but may eventually hold promise in augmenting current MDA regimens. Clinical trials are underway to evaluate the efficacy of MOX for the control onchocerciasis in human populations (Siva 2009).

There is considerable geographic overlap of locales treated by MDA and areas endemic for mosquito-borne diseases transmitted by *Aedes aegypti*. *Ae. aegypti* is an anthropophilic mosquito and feeds frequently and nearly exclusively on humans (Scott et al. 2000b). Given the considerable geographic overlap of human populations treated via MDA with the habitat of *Ae. aegypti*, it is reasonable to expect that *Ae. aegypti* will imbibe anthelmintic drugs and their metabolites circulating in human blood. Models have clearly shown that even modest reductions in the daily probability of mosquito survival can dramatically reduce mosquito vectorial capacity (Black and Moore 2005,

Billingsley et al. 2008), and numerous studies have demonstrated that IVM is able to reduce the survival of adult mosquitoes (Pampiglioni et al. 1985, Iakubovich et al. 1989b, Tesh and Guzman 1990, Cartel et al. 1991, Jones et al. 1992, Gardner et al. 1993, Bockarie et al. 1999, Foley et al. 2000, Fritz et al. 2009, Sylla et al. 2010, Kobylinski 2011). In the field, MDA of IVM reduced the survival of field caught *Anopheles* mosquitoes (Bockarie et al. 1999, Sylla et al. 2010), and may also reduce malaria parasite transmission (Kobylinski 2011).

While a large body of literature describing the effects of IVM on mosquitoes has been published, comparatively fewer reports evaluating the effects of other anthelmintic drugs on mosquitoes have been published. At present, the effects of anthelmintics (other than IVM) have largely concentrated on *Anopheles* spp. (Kobylinski et al. 2010, Butters submitted), and there is one report that suggests MDA of DEC may reduce the survival of *Ae. polynesiensis* (Cartel et al. 1991). With the exception of IVM, the effects of anthelmintic drugs have not yet been evaluated in *Ae. aegypti.*

The experiments described in this chapter employed an *in vitro* blood feeding assay to compare the effects of IVM, MOX, SEL, DEC, PYL, and albendazole sulfoxide (ALB SOx), the primary metabolite of ALB (Mathew 2007), on *Ae. aegypti* mosquitoes. The concentration of each drug required to kill 50% of adults ($LC_{50(adult)}$) was calculated by feeding multiple concentrations of the drug to mosquitoes. Drugs that induced adult mortality were further evaluated for their effect on mosquito fecundity and egg hatch rate.

Methods and Materials.

Mosquitoes. The *Ae. aegypti* Rexville D strain (RexD), origin Puerto Rico, was used in this study. Mosquitoes were reared at 28°C ± 2°C, and 80% humidity under a photoperiod of 14:10 (L:D). Larvae were raised in 28 L containers filled with approximately 15 L of tap water. Larval density was maintained at 500-600 mosquito larvae per container in order to ensure uniform development and size. Mosquito larvae were fed a diet of ground Tetramin® fish food mixed with ground mouse food. Adult mosquitoes were provided with water and raisins as a sugar source *ad libitum.*

Drugs. Powdered formulations of IVM, DEC and PYL (as pyrantel pamoate) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A powdered formulation of ALB SOx was purchased from WITEGA (Berlin, Germany). A powdered formulation of SEL was kindly provided by Pfizer, Inc. (Groton, CT, USA). All powders were dissolved in dimethylsulfoxide (DMSO) to a concentration of 10 mg/ml. Multiple aliquots of each drug were stored, frozen, at -20°C. A fresh aliquot was used for each blood feed. Moxidectin is not available in a powder form, and was thus purchased as the 10 mg/ml commercially available solution, Cydectin® (Fort Dodge Animal Health, Fort Dodge, IA, USA). The Cydectin was maintained at 4°C in a light proof container, according to the manufacturer's storage instructions.

***In vitro* blood feeds.** Human blood was used for all *in vitro* blood feeds. Blood was drawn from a human volunteer into 3.2% sodium citrate blood collection tubes by a phlebotomist at the Colorado State University Health Network medical clinic in Fort Collins, Colorado. Blood was drawn from the same volunteer for all of the experiments described, and was no more than one week post-draw at the time of the blood feed. All

drugs were serially diluted into phosphate buffered saline (PBS) to a concentration 10 times (10X) greater than the final desired concentration. The 10X drug-PBS solutions were diluted 1:10 into blood to reach the final concentrations provided to mosquitoes. For all drug feeds, a drug-free control group was included. At the time of blood feed, mosquitoes were 3-5 days post-emergence. Twenty-four hours prior to the blood feed, adult mosquitoes were placed into 4 L plastic containers. Mosquitoes were starved of sugar and water for 12 and three hours, respectively. Glass membrane feeders (Lillie, Glass Blowers, Smyrna, GA, USA) were covered by securing hog sausage casing to the feeder with a rubber band, and were then heated and maintained at 37°C using a heated water circulator. Mosquitoes were allowed to feed for 30 minutes. After the feed, mosquitoes were cold-anesthetized in a refrigerator, placed onto a glass Petri dish maintained on ice and sorted for the presence of a visible blood meal. Only fully engorged mosquitoes were retained for survival analysis, fecundity and hatch rate studies.

$LC_{50(adult)}$ determination. The concentration of drug required to kill 50% of adults ($LC_{50(adult)}$) was determined by feeding each drug at multiple concentrations to mosquitoes, and all concentrations are reported in Figure 3.1. Control mosquitoes, included in all experiments, were offered a blood meal containing 10% PBS. The $LC_{50(adult)}$ for each drug was determined from three experimental replicates using a non-linear mixed model with probit analysis (Proc probit, Proc nlmixed), (Kobylinski et al. 2010).

Estimation of fecundity. The effects of IVM, MOX and SEL on the fecundity of *Ae. aegypti* were assessed by feeding each drug at multiple concentrations to mosquitoes (Figure 3.2). For IVM and SEL, control mosquitoes were fed on blood containing DMSO

which had been diluted in PBS. The concentration of DMSO in the blood meal offered to control mosquitoes was equivalent to the concentration of DMSO contained the highest concentration of drug fed to experimental mosquitoes. Moxidectin was provided as a suspension in an unknown carrier, and control mosquitoes were fed on blood containing PBS only. Following the blood feed, mosquitoes were sorted for the presence of a blood meal (as described above), and five mosquitoes for each concentration of each drug were placed into 500 ml containers. Control mosquitoes were included in all experiments, and were selected to be of similar body and blood meal size. A 10 ml oviposition cup lined with a paper towel was filled with approximately 8 ml of water and placed into each container. Containers were covered with organdy fabric, and mosquitoes had access to raisins as a sugar source. Two days following the blood feed, surviving mosquitoes were counted and recorded. Five days post-blood feed, mosquitoes were anesthetized using carbon dioxide, and the oviposition cup was removed. Eggs laid on the water surface were collected by filtering the water through a coffee filter. Eggs were allowed to dry inside of a plastic container covered with organdy fabric, which was maintained in the insectary for three days. Eggs were counted using a stereoscopic dissecting microscope. The number of eggs laid per female mosquito was estimated by dividing the total number of eggs by the number of surviving mosquitoes at two days post-blood feed. Eggs were then placed into plastic bags and maintained in the insectary for five additional days. The effects of PYL, ALB SOx and DEC on mosquito fecundity were not evaluated due to the complete failure of these drugs to cause mortality in adult mosquitoes (see below).

Hatch rate determination. The effect of a maternal blood meal containing IVM, MOX and SEL on the hatch rate of eggs was assessed by submerging a subset of 50-60

eggs (obtained as described above) in 500 ml of tap water. To minimize error associated with installment hatching (Gillett et al. 1977), water was de-oxygenated by bubbling nitrogen gas into the hatch container for 10 minutes. Hatch cups were maintained in the insectary and three days after hatching all larvae were counted. The number of eggs that failed to hatch was calculated by subtracting the number of larvae counted from the number of eggs submerged in water. The concentration of drug contained in a maternal blood meal that prevented 50% of eggs from hatching ($LC_{50(hatch)}$) was estimated using the NLM described above.

Statistical analysis. The effect of IVM, MOX and SEL on the fecundity of *Ae. aegypti* RexD mosquitoes was assessed using a one-way ANOVA, and the mean number of eggs laid at each of the concentrations of IVM fed to mosquitoes was compared with the corresponding control group. The significance level was set at $P < 0.05$, and statistical analysis was carried out using PROC GLM and least squares means procedure (SAS version 9.2, SAS Institute, Cary, NC, USA).

The effect of IVM, MOX and SEL on the egg hatch rate was assessed for each mosquito strain by comparing the hatch rate from each concentration to the corresponding control. Data from all three replicates were pooled and then analyzed using Fisher's Exact test using R (http://www.r-project.org/).

Results.

$LC_{50(adult)}$ determination. Ivermectin, MOX and SEL all reduced the survival of adult *Ae. aegypti*. No reduction in adult mosquito survival was seen at the concentrations tested for DEC, PYL, or ALB SOx (Figure 3.1). The $LC_{50(adult)}$ estimates and

corresponding 95% fiducial limits are reported in Table 3.1. Ivermectin had the lowest $LC_{50(adult)}$, followed by SEL, and finally MOX.

Fecundity of *Ae. aegypti* following a blood meal containing anthelmintic drugs. A one-way ANOVA revealed that the fecundity of *Ae. aegypti* was significantly affected following ingestion of IVM in a blood meal ($F = 32.89$; $df = 7, 23$; $P < 0.001$). A significant reduction in the mean number of eggs laid was seen at 800, 400, 200, 100, 75 and 50 ng/ml concentrations of IVM (Figure 3.2a). At the concentrations tested, significant reductions in fecundity were not observed with SEL or MOX (Figure 3.2a-b).

Hatch rate of *Ae. aegypti* following a blood meal containing anthelmintic drugs. The hatch rate of eggs was reduced following a maternal blood meal containing IVM, MOX and SEL. Hatch rates for each drug, by concentration, are reported in Figure 3.3. Maternal blood meals containing IVM at concentrations of 400, 200, 100, and 75 ng/ml resulted in a complete failure of eggs to hatch, and at 50 and 35 ng/ml the hatch rate was significantly less than the corresponding PBS-DMSO control (Figure 3.3a). Maternal blood meals containing SEL at all concentrations tested significantly reduced the egg hatch rate when compared to the corresponding PBS-DMSO control (Figure 3.3b). Maternal blood meals containing MOX significantly reduced the egg hatch rate at all but the lowest concentration tested (Figure 3.3c). The $LC_{50(hatch)}$ estimate for IVM was 38.0 ng/ml (35.8, 39.9), (Table 3.1). Due to the limited effects of the experimental drug concentrations on the hatch rates of eggs, accurate estimates of the $LC_{50(hatch)}$ for SEL and MOX could not be made using the NLM.

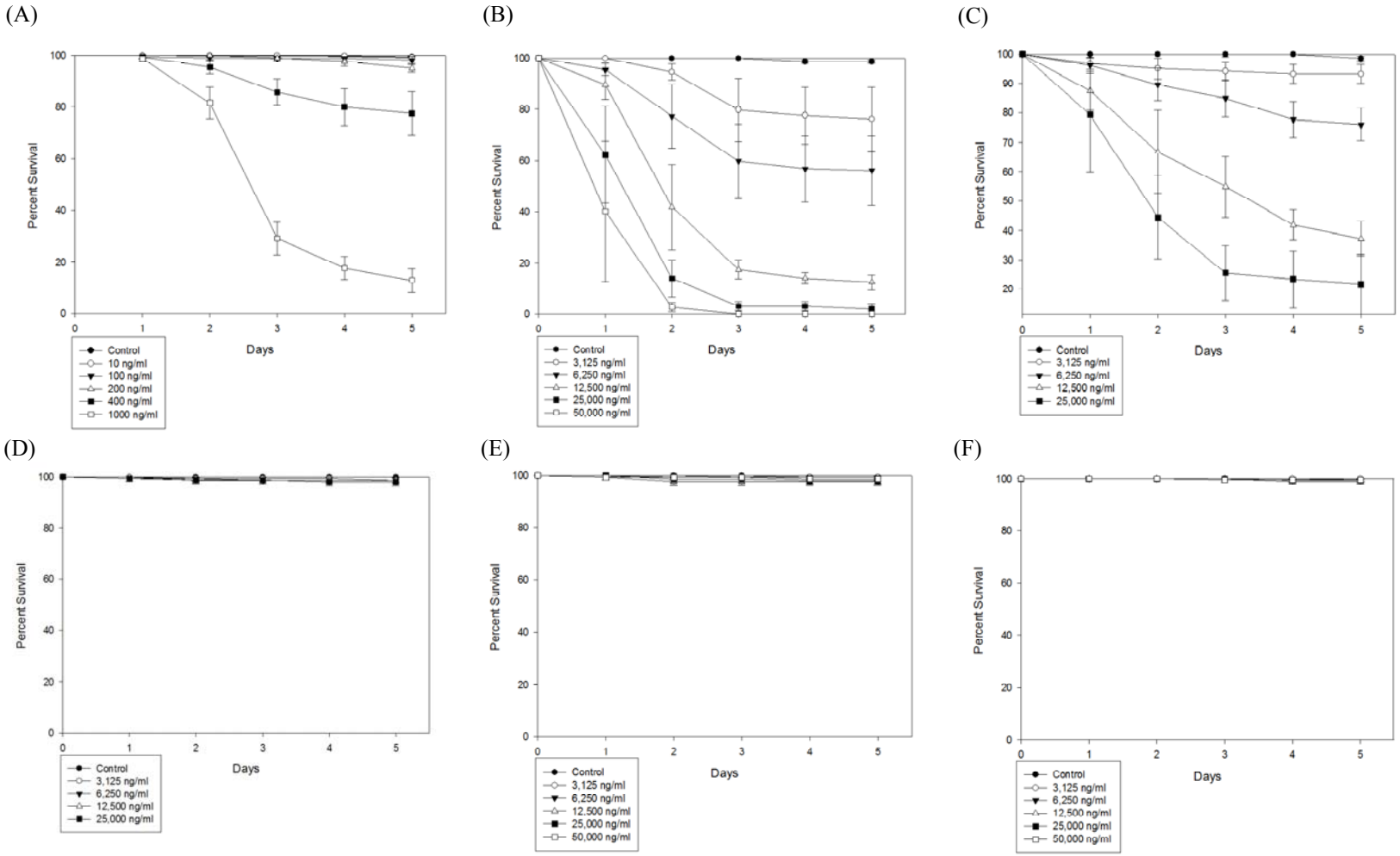


Figure 3.1. Percent survival of *Ae. aegypti* (RexD) mosquitoes following blood meals containing different anthelmintic drugs. (A) IVM (B) SEL (C) MOX (D) DEC (E) PYL (F) ALB SOx.

Table 3.1. Effect of imbibed anthelmintic drugs on adult survival and hatch rate of *Ae. aegypti*

Drug	$LC_{50(adult)}$ 95% fiducial limits *n*	$LC_{50(hatch)}$ 95% fiducial limits *n*
IVM	601.3 (506.7, 712.9) *n* = 1,669	38.0 (35.8, 39.9) *n* = 1,125
SEL	5,958 (4,464, 7,531) *n* = 658	*n.d.**
MOX	11,277 (8,668, 14,808) *n* = 820	*n.d.**
DEC	*n.d.* *n* = 906	*n.e.*
PYL	*n.d.* *n* = 924	*n.e.*
ALB SOx	*n.d.* *n* = 1,106	*n.e.*

n :the number of individuals used to estimate the LC_{50}

n.d.: value could not be determined due to no mortality observed at experimental concentrations, and * a high hatch rate in all experimental concentrations

n.e.: not estimated

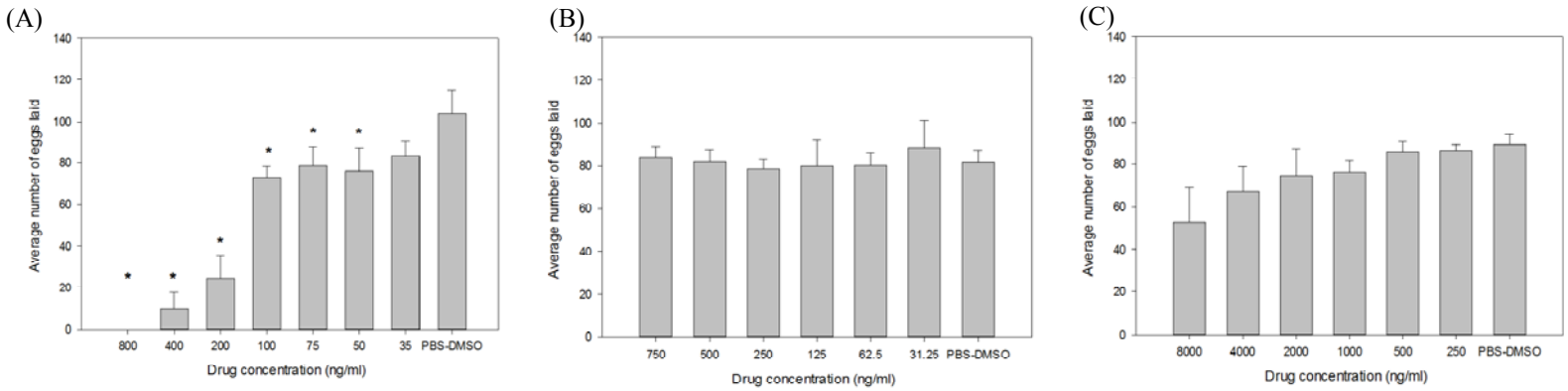


Figure 3.2. Fecundity of *Ae. aegypti* (RexD) mosquitoes following blood meals containing different anthelmintic drugs. (A) IVM (B) SEL (C) MOX. Significant reductions in fecundity, relative to control, are denoted with an *.

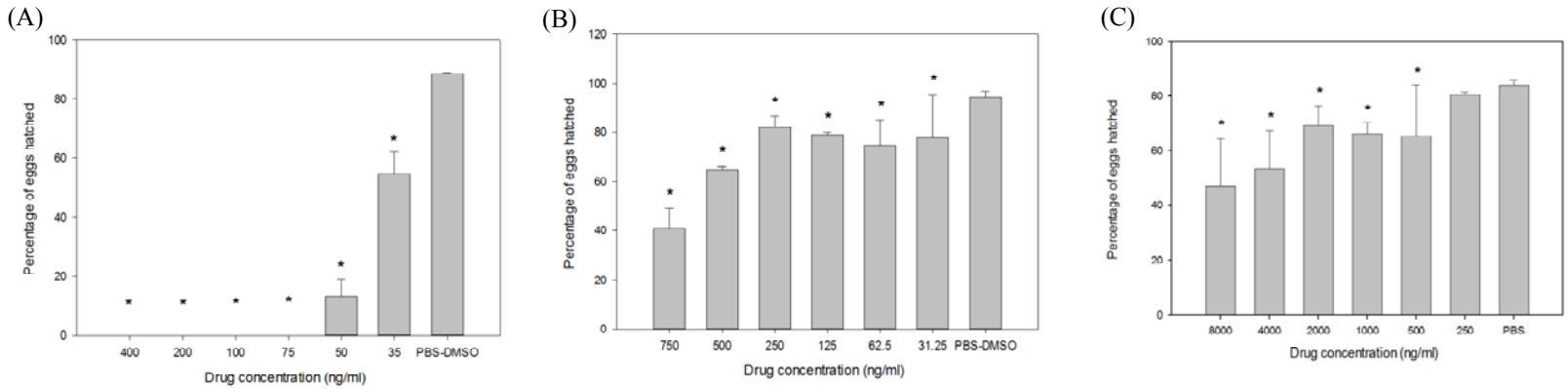


Figure 3.3. Percent hatch rate of *Ae. aegypti* (RexD) mosquito eggs following a maternal blood meals containing different anthelmintic drugs.

(A) IVM (B) SEL (C) MOX. Significant reductions, relative to control, in the percentage of eggs hatched are denoted with an *.

Discussion.

Three outcomes (adult survival, fecundity and hatch rate) were used to evaluate the susceptibility of *Ae. aegypti* RexD to different concentrations of six anthelmintic drugs, when administered through a blood meal. With the exception of MOX and SEL, all of the drugs evaluated in this study are currently distributed world-wide via MDA for the control of human nematode parasites (Hotez 2007, Reddy et al. 2007, Ottesen et al. 2008). Moxidectin, was included in this study, as it is currently being evaluated as a drug that could be used in addition to IVM for the control of onchocerciasis (Siva 2009). Selamectin, although only licensed for veterinary use can be applied topically for the control of endo and ecto parasites of companion animals (Bishop et al. 2000). All MDA regimens are currently given orally, however, a topical application could be an attractive means of administering MDA, thus we also included SEL in our study.

Others have reported on the effects of IVM on *Ae. aegypti* (Pampiglioni et al. 1985, Tesh and Guzman 1990, Mahmood et al. 1991, Focks et al. 1995, Kobylinski et al. 2010), but this is the first study to comparatively evaluate the efficacy of multiple anthelmintics in *Ae. aegypti.* The *in vitro* feeding strategy employed in this study allowed for consistent administration of the different anthelmintic drugs, thus allowing for direct comparisons of the effect of the drugs on adult survivorship, mosquito fecundity and hatch rate. Of all the drugs tested, only the macrocyclic lactones (IVM and SEL) and the closely related milbemycin, MOX, significantly affected the survival of *Ae. aegypti* when imbibed in a blood meal.

In light of the recent clinical trials of MOX for the control of onchocerciasis, we also evaluated MOX for its ability to reduce the survival of adult *Ae. aegypti* mosquitoes.

The $LC_{50(adult)}$ of MOX was 11,277 ng/ml, which is approximately 38 times higher than the mean maximal serum concentration of MOX (296 ng/ml) in human volunteers following a 36 mg, orally ingested dose (Cotreau et al. 2003). These findings, coupled with the observation of adverse side effects at high doses indicate that MOX is unlikely to be effective in controlling pathogens transmitted by *Ae. aegypti*. Selamectin has not yet been evaluated for tolerability or pharmacokinetics in humans, however the $LC_{50(adult)}$ of SEL in *Ae. aegypti* RexD was 5,958 ng/ml. Even if such serum concentrations could be safely reached in humans, it would likely require a prohibitively expensive dose.

Ivermectin, MOX and SEL are all thought to exhibit insecticidal activity by allosterically agonizing the glutamate-gated chloride (GluCl) anion channels. Binding of the drug to the channel causes an increased permeability of the channel to chloride ions, ultimately leading to hyperpolarization of the nerve-cell membrane culminating in flaccid paralysis and death of the insect (Cleland 1996, Wolstenholme and Rogers 2005). Despite significant structural similarities (reviewed in Chapter 1) between IVM, MOX and SEL, the efficacy of the drugs in inducing adult mortality was exceptionally variable. The most significant structural difference between IVM, MOX and SEL is the substituent found at the 13-position in the macrolide ring. Moxidectin has no substituent at the 13-position, SEL has an oleandrosyloxy subunit and IVM has a bisoleandrosyloxy subunit. The crystal structure of IVM bound to the GluCl channel in *Caenorhabditis elegans* shows that IVM integrates between the transmembrane domains of the channel subunit proteins, thereby opening the channel (Hibbs and Gouaux 2011). Given that all of these drugs are allosteric agonists of the GluCl receptor, one possible explanation for the large amount of variability we observed in adult mortality could be that the size and chemical

properties of the substituent at the 13-position in the macrolide ring. If the substituent at the 13-position in the macrolide ring alters the interaction of a macrocyclic lactone with the GluCl channel transmembrane domains, then macrocyclic lactones will vary in their efficacy in inducing mortality because the degree to which the channel is opened depends on the size and chemical properties of the substituent. Of IVM, MOX and SEL, IVM has the largest substituent followed by SEL and finally MOX, suggesting that the size of the substituent may play some role in the endectocide efficacy of the drug. The research presented here highlights the importance of elucidating the mechanism for the variability between the different macrocyclic lactones and milbimycins in their efficacy. Such knowledge could assist in the development of novel and more potent endectocide compounds.

Diethylcarbamazine, PYL and ALB SOx had no effect on the survival of adult *Ae. aegypti*. Albendazole sulfoxide, the primary metabolite of albendazole, disrupts microtubule assembly, and PYL is a nicotinic acetylcholine-gated receptor agonist (Martin 1997). The precise mode of action for DEC remains unclear, however it is established that DEC requires components from the vertebrate host for successful elimination of microfiliarial parasites (Hawking et al. 1948, McGarry et al. 2005). Hawking et al., 1948 observed that microfilarial parasites were rapidly eliminated from cotton rats following *in vivo* treatment with DEC, however the microfilaria were not susceptible to the drug *in vitro*. Further, the survival of *Aedes polynesiensis* was significantly reduced in response to feeding on human patients that had been treated with DEC (Cartel et al., 1991). While we did not observe a reduction in *Ae. aegypti* survival following a blood meal containing DEC, our methods employed an *in vitro* feeding assay,

therefore the question of whether *Ae. aegypti* would experience reduced survival after feeding directly on a DEC-treated human remains to be answered. With the exception of Africa, DEC remains the primary treatment for lymphatic filariasis world-wide. Clearly, the question of whether a blood meal from a patient treated with DEC can reduce the survival of *Ae. aegypti* should be further explored.

We also investigated the effect of sublethal concentrations of IVM, MOX and SEL on the fecundity and hatch rate of *Ae. aegypti* RexD. Due to their lack of effect on adult survival, DEC, PYL and ALB SOx were not further evaluated. Ivermectin significantly decreased the fecundity of mosquitoes that ingested different concentrations of the drug, and these data are consistent with the findings of other reports of IVM in *Ae. aegypti* (Tesh and Guzman 1990, Mahmood et al. 1991). At the concentrations evaluated, MOX and SEL had no significant effect on the fecundity of *Ae. aegypti*. The hatch rate of *Ae. aegypti* RexD was significantly reduced following a maternal blood meal containing IVM, MOX and SEL. While IVM has been shown to reduce the fecundity and hatch rate of eggs following a sublethal maternal blood meal (Tesh and Guzman 1990, Mahmood et al. 1991), this is the first report to show that of MOX and SEL, albeit at exorbitantly high concentrations, can reduce the hatch rate of eggs following a maternal blood meal containing the drugs. Fritz et al. demonstrated that hatchability of *Anopheles gambiae* eggs was not affected following a blood meal from bulls injected with a 600 µg/kg dose of MOX (Fritz et al. 2009), and these observations are likely due to the fact that the concentration of MOX imbibed was not high enough to affect the hatch rate of eggs. The $LC_{50(hatch)}$ of IVM was 38.0 ng/ml in the *Ae. aegypti* RexD strain. The $LC_{50(hatch)}$ for SEL and MOX could not be accurately determined due to

the limited lethality at the experimental concentrations. Additional experiments to pinpoint the $LC_{50(hatch)}$ were not carried out as it is unlikely that these endectocides will be effective in reducing transmission of mosquito-borne diseases by *Ae. aegypti.*

Of all the drugs that reduced mosquito survivorship, IVM was the most potent. Interestingly, our estimates for the $LC_{50(adult)}$ and $LC_{50(hatch)}$ of IVM for the *Ae. aegypti* RexD (601.3 ng/ml and 38.0 ng/ml, respectively) strain are noticeably higher than those observed by Tesh and Guzman in the *Ae. aegypti* Rock strain ($LC_{50(adult)}$ = 126 ng/ml, $LC_{50(hatch)}$ = 3.4 ng/ml). Due to different experimental methods, our results cannot be directly compared, nonetheless, the large difference in the LC_{50} estimates is notable. Following ingestion of a MDA dose of IVM (150 µg/kg), the mean maximal concentration of IVM in human venous plasma is approximately 46 ng/ml. While the $LC_{50(adult)}$ of IVM calculated in both strains is far greater than the serum concentrations expected in human plasma, the question of whether other strains of *Ae. aegypti* may be more susceptible to IVM arises, and this is an area that should be further explored.

CHAPTER 4: THE EFFECT OF IVERMECTIN IN SEVEN DIFFERENT STRAINS OF *AEDES AEGYPTI* (DIPTERA: CULICIDAE), AND ESTIMATION OF REALIZED HERITABILITY OF RESISTACE TO IVERMECTIN IN A GENETICALLY DIVERSE LABORATORY STRAIN.

Introduction.

In the last 30 years, a number of *in vitro* and *in vivo* studies have demonstrated that, when imbibed in a blood meal, ivermectin (IVM) causes a significant reduction in adult female mosquito survival and fecundity, and decreased egg hatch rate (Pampiglioni et al. 1985, Iakubovich et al. 1989a, Tesh and Guzman 1990, Cartel et al. 1991, Focks 1991, Focks et al. 1991, Mahmood et al. 1991, Gardner et al. 1993, Bockarie et al. 1999, Foley et al. 2000, Fritz et al. 2009). Ivermectin, a macrocyclic lactone, is a broad-spectrum drug which is widely used for the treatment of a number of parasitic infections, including the control of onchocerciasis and lymphatic filariasis. Mass drug administration (MDA) of IVM through the African Programme for Onchocerciasis Control and the Global Program for the Elimination of Lymphatic Filariasis distributes IVM to over 80 million people annually across the globe (Amazigo 2008, Ottesen et al. 2008). There is considerable geographic overlap of locales treated by MDA with IVM and areas endemic for mosquito-borne diseases such as malaria and dengue. It has been proposed that in addition to controlling nematode infections, more frequent MDA could also be effective in controlling mosquito-borne diseases (Wilson 1993, Kobylinski et al. 2010, Foy et al. 2011, Kobylinski et al. 2011).

A number of reports illustrate that MDA of IVM is effective in reducing the survivorship of adult mosquitoes (Cartel 1991, Bockarie 1999, Sylla 2010). Two field-based studies have demonstrated that MDA using IVM is significantly associated with the reduction in survivorship of adult field-caught mosquitoes (Bockarie et al. 1999, Sylla et al. 2010). Mathematical models have shown that even modest reductions in the daily probability of mosquito survival may have a significant impact on the transmission of

mosquito-borne disease such as dengue and malaria (Garrett-Jones 1964, Billingsley et al. 2008, Sylla et al. 2010) Further, MDA of IVM is effective in disrupting malaria parasite transmission (Kobylinski 2011).

Despite a growing body of literature on the effects of IVM in mosquitoes, the variation in susceptibility to IVM among strains of a mosquito species has not been investigated, nor have insecticide resistant strains been evaluated for IVM cross-resistance, or whether IVM resistance may develop in a mosquito following successive blood meals containing IVM. Multiple laboratory strains of *Aedes aegypti* are available and easily maintained in the lab, thus we chose *Ae. aegypti* as a model to study the variation in susceptibility to IVM imbibed in a blood meal, and to attempt to select for IVM resistance. To assess the variation in susceptibility to IVM, seven strains of *Ae. aegypti,* including three laboratory-selected permethrin resistant strains, were administered blood meals containing IVM through an artificial membrane feeding system. The concentrations of IVM which affected adult survivorship, fecundity and hatch rate were evaluated and compared among all seven mosquito lines. To assess the likelihood of the development for IVM resistance, a genetically diverse laboratory strain (GDLS) was constructed (Wise de Valdez et al., 2010) and subjected to three successive rounds of selection with IVM, and the realized heritability (h^2) was calculated.

Materials and Methods

Mosquitoes. The Solidaridad (SLD) strain originated from Mexico was collected as previously described (Flores et al. 2006), and the Iquitos strain (IQT) originating from Iquitos, Peru were from a lab colony maintained by Dr. Amy Morrison. The SLD and IQT strains were used to generate permethrin-resistant laboratory strains of mosquitoes

(SLD-PR and IQT-PR, respectively), as described elsewhere (Saavedra-Rodriguez 2011). The Isla Mujeres strain (IMU-PR), was collected from the field and exhibited high levels of pyrethroid and temephos resistance without laboratory selection (Saavedra-Rodriguez et al. 2008). The genetically diverse laboratory strain (GDLS) was constructed as described (Wise de Valdez et al. 2010). The standard laboratory reference strain, New Orleans (NO), was kindly provided by the Center for Disease Control and Prevention (CDC), Atlanta, GA, USA. All of the mosquitoes were reared at 28°C ± 2°C, 80% humidity under a photoperiod of 14:10 (L:D). Larvae were raised in 28 L containers filled with approximately 15 L of tap water. Larval density was maintained at 500-600 mosquito larvae per container in order to ensure uniform development and size. Mosquito larvae were fed a diet of ground Tetramin® fish food mixed with ground mouse food. Adult mosquitoes were provided with water and raisins as a sugar source *ad libitum.*

Ivermectin. A powdered formulation of Ivermectin (IVM) was obtained from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethylsulfoxide (DMSO) to a concentration of 10 mg/ml. Multiple aliquots were stored, frozen, at -20°C. A fresh aliquot was used for each blood feed.

***In vitro* blood feeds.** Human blood was used for all *in vitro* blood feeds. Blood was drawn from a human volunteer into 3.2% sodium citrate blood collection tubes by a phlebotomist at the Colorado State University Health Network Medical Clinic in Fort Collins, Colorado. Blood was drawn from the same volunteer for all of the experiments described, and was no more than one week post-draw at the time of the blood feed. Ivermectin diluted in DMSO was thawed, and then serially diluted into phosphate

buffered saline (PBS) to a concentration 10 times greater than the final concentration desired, which was finally diluted 1:10 into blood to reach the final concentrations provided to mosquitoes. Control mosquitoes were fed PBS containing a concentration of DMSO equivalent to the highest concentration fed to experimental mosquitoes. At the time of blood feed, mosquitoes were 3-5 days post-emergence. Twenty-four hours prior to the blood feed, adult mosquitoes were placed into 4 L plastic containers. Mosquitoes were starved of sugar and water for 12 and three hours, respectively, prior to the blood feed. Glass membrane feeders (Lillie, Glass Blowers, Smyrna, GA) were covered by securing hog sausage casing to the feeder with a rubber band, and then heated to 37°C with a heated water circulator. Mosquitoes were allowed to feed for 30 minutes. After the feed, mosquitoes were cold-anesthetized in a refrigerator, placed onto a glass Petri dish maintained on ice and sorted for the presence of a blood meal. Only fully engorged mosquitoes were retained for survival analysis, fecundity and embryo survival studies.

$LC_{50(adult)}$ determination. The concentration of IVM required to kill 50% of adults ($LC_{50(adult)}$) was determined by feeding the following concentrations of the drug to mosquitoes: 800, 400, 200, 100, 75, 50, 35 and 0 ng/ml. For each of the mosquito strains, the $LC_{50(adult)}$ was determined from three experimental replicates using a non-linear mixed model with probit analysis (NLM) (Kobylinski et al. 2010). For each mosquito strain, the fit of the model with the experimental data was checked by plotting cumulative mortality from all replicates against the experimental concentration, and overlaying the resulting curve with a plot of the probit mortality versus IVM concentration calculated from the NLM.

Estimation of fecundity. A subset of five fully engorged females were reserved from each of the blood feeds. Mosquitoes of similar body and blood meal size were placed into 500 ml ice cream containers. A 10 ml oviposition cup lined with a paper towel was filled with approximately 8 ml of water and placed into each container. Ice cream containers were covered with organdy fabric, and mosquitoes had access to raisins as a sugar source. Two days following the blood feed, surviving mosquitoes were counted and recorded. Five days post-blood feed, mosquitoes were anesthetized using carbon dioxide, and the oviposition cup was removed. Eggs laid on the water surface were collected by filtering the water through a coffee filter. Eggs were allowed to dry inside of a plastic container covered with organdy fabric which was maintained in the insectary for three days. Eggs were counted using a stereoscopic dissecting microscope. The number of eggs laid per female mosquito was estimated by dividing the total number of eggs by the number of surviving mosquitoes at two days post-blood feed. Eggs were then placed into plastic bags and maintained in the insectary for five additional days to allow for larvae development.

$LC_{50(hatch)}$ determination. The ability of IVM in a maternal blood meal to prevent 50% of eggs from hatching ($LC_{50(hatch)}$) was assessed by submerging a subset of 50-60 eggs from mosquitoes fed each different concentration of IVM in 500 ml of water. To minimize installment hatching (Gillett et al. 1977), water was de-oxygenated by bubbling nitrogen gas into the hatch container for 10 minutes. Hatch cups were maintained in the insectary, and three days after hatching all larvae were counted. The number of eggs that failed to hatch was calculated by subtracting the number of larvae counted from the number of eggs submerged in water. The $LC_{50(hatch)}$ for each mosquito

strain was calculated using the NLM described above. The fit of the NLM for each of the mosquito strains was checked as described above.

Selection for resistance to IVM in the *Ae. aegypti* GDLS. We did not select for IVM resistance in adult mosquitoes because concentrations of IVM that significantly reduce adult survival have been reported to result in drastic reductions in mosquito fecundity and hatch rate, and production of successive generations would not be possible. Thus, we chose to evaluate whether or not the $LC_{25(hatch)}$ of IVM would increase in response selection pressure applied during three successive generations. Due to a limited number of eggs and the high variation in hatch rate observed between individual replicates, the median $LC_{25(hatch)}$ value of the IQT, SLD and NO strains (48 ng/ml) was used for the initial round of selection.

Three successive rounds of selection with IVM were carried out. First generation GDLS eggs were used as the progenitor strain (designated GDLS-P_1). The first IVM-selected generation was designated GDLS-$F^{IVM}{}_1$, followed by GDLS-$F^{IVM}{}_2$, and finally GDLS-$F^{IVM}{}_3$. To generate the GDLS-$F^{IVM}{}_1$, approximately 2,000 GDLS-P eggs were hatched, reared to adults and then fed a blood meal containing IVM at a concentration of 48 ng/ml. Mosquitoes were sorted for the presence of a blood meal as described above. Fully engorged mosquitoes were placed into flight cages containing four 200 ml oviposition containers which were lined with a paper towel and filled with approximately 180 ml of tap water. The GDLS-$F^{IVM}{}_1$ eggs were collected five days after the blood feed, placed into a covered container maintained in the insectary, and allowed to dry for seven days. A subset of GDLS-$F^{IVM}{}_1$ eggs were then hatched, reared to adults and used to estimate the $LC_{25(hatch)}$ using the same bioassay and NLM used to estimate the

$LC_{50(hatch)}$ as described above. After the $LC_{25(hatch)}$ for the GDLS-F^{IVM}_1 was determined, the remainder of GDLS-F^{IVM}_1 eggs were then counted and submerged in approximately 500 ml of tap water. Water was de-oxygenated as described in the previous section. Three days after hatching, all larvae were counted. The proportion of viable eggs was calculated by dividing the total number of larvae counted by the total number of eggs submerged. The resulting GDLS-F^{IVM}_1 larvae were then reared to adults and fed a blood meal containing a concentration of IVM equivalent to the corresponding $LC_{25(hatch)}$ estimate for the GDLS-F^{IVM}_1. Mosquitoes were sorted for the presence of a blood meal and transferred to a flight cage, allowing for oviposition of the GDLS-F^{IVM}_2 generation. The $LC_{25(hatch)}$ of the GDLS-F^{IVM}_2 generation was then determined by bioassay, and the GDLS-F^{IVM}_3 generation was produced according to the same procedure. Selection-free, generation-matched control groups were maintained by feeding mosquitoes blood meals containing PBS with DMSO added at a concentration equivalent to that of the IVM-treated groups. Selection free generations are denoted as GDLS-F^{PBS}_1, followed by GDLS-F^{PBS}_2, and finally GDLS-F^{PBS}_3.

Estimation of realized heritability. To estimate the additive genetic variance for development of IVM resistance in the GDLS mosquitoes, the realized heritability (h^2) was estimated according to the methods described by Falconer and Mackay (Falconer 1996). Realized heritability h^2 is estimated as the slope of a linear regression of the cumulative response to selection across generations on cumulative selection differential, and is given by the equation $h^2 = R/S$ (where R is the cumulative response to selection and S is the cumulative selection differential). The cumulative response to selection (R) was calculated by summing the estimated response to selection at each generation over

the three successive generations. The selection differential for each generation, denoted (*R'*) was estimated as

$$R' = [\ln(\text{generation } LC_{25(\text{hatch})}) - \ln(\text{initial } LC_{25(\text{hatch})})]$$

where the generation $LC_{25(\text{hatch})}$ is the $LC_{25(\text{hatch})}$ at each generation following selection, and the initial $LC_{25(\text{hatch})}$ is the $LC_{25(\text{hatch})}$ of the parental generation prior to initiating selection. The cumulative selection (*S*) differential was calculated by summing the estimated selection differential at each generation over the successive generations (denoted $S'_{.}$) The selection differential for each generation (*S'*) was estimated as

$$S' = \sigma i$$

where *i* is the intensity of selection and σ is the standardized phenotypic variation. Values for the intensity of selection (*i*) corresponding to the proportion of survivors at each generation were obtained from Falconer and Mackay (Appendix A). Values for the standardized phenotypic variation at each generation (σ) were estimated as

$$\sigma = 1/\text{slope from the regression analysis}$$

where the slope from the regression analysis corresponds to the slope of the regression line from the NLM used to calculate the $LC_{25(\text{hatch})}$ at each generation.

Statistical analysis. The $LC_{50(\text{adult})}$ and $LC_{50(\text{hatch})}$ estimates were compared among experimental replicates. Data from all seven strains was compared using a one-way ANOVA followed by multiple pairwise comparisons. Statistical analysis was carried out using PROC GLM and least squares means (SAS version 9.2, SAS Institute, Cary, NC).

Resistance ratios for the $LC_{50(adult)}$ and $LC_{50(hatch)}$ were calculated relative to the susceptible NO strain.

The effect of IVM on the fecundity for each mosquito line was assessed using a one-way ANOVA, and the mean number of eggs laid at each of the concentrations of IVM fed to mosquitoes was compared with the PBS-DMSO control group. The significance level was set at $P < 0.05$, and statistical analysis was carried out using PROC GLM and least squares means procedure (SAS version 9.2, SAS Institute, Cary, NC).

The effect of each IVM concentration on the egg hatch rate was assessed for each mosquito strain by comparing the hatch rate from each concentration to the corresponding PBS-DMSO control. Data from all three replicates were pooled and then analyzed using a Fisher's Exact Test (http://www.r-project.org/).

Results

Adult LC_{50} determination. The $LC_{50(adult)}$ estimates and the corresponding 95% fiducial limits are reported in Table 4.1 and depicted in Figure 4.1. Ivermectin reduced the survivorship of all strains of adult *Ae. aegypti* mosquitoes. Calculated $LC_{50(adult)}$ IVM estimates across all mosquito lines ranged from 187 ng/ml to 576 ng/ml. The $LC_{50(adult)}$ estimates of IVM for the IMU-PR, IQT-PR and SLD-PR strains differed significantly from the NO standard reference strain ($P < 0.05$) The SLD and IQT strains did not differ from the NO standard reference strain ($P > 0.05$). The GDLS did not differ significantly from any of the other mosquito strains. Pairwise comparisons of strains IQT-PR and SLD-PR with their respective selection free strains revealed significant differences in

$LC_{50(adult)}$ estimates. The fit of the NLM for the $LC_{50(adult)}$ estimates for each mosquito strain is presented in Figure 4.2.

Effect of IVM on hatch rate. The $LC_{50(hatch)}$ estimates and the corresponding 95% fiducial limits are reported in Table 4.1, and depicted in Figure 4.3. The hatch rate of eggs from all mosquito strains was reduced following a maternal blood meal containing IVM. Calculated $LC_{50(hatch)}$ IVM estimates across all mosquito lines ranged from 43 ng/ml to 334 ng/ml. Hatch rates for each concentration of IVM recorded by mosquito strain are presented in Table 4.2. The IMU-PR mosquito strain was the only strain that differed significantly from the susceptible NO strain. Pairwise comparisons of strains IQT-PR and SLD-PR with their respective selection-free strains did not reveal significant differences. The fit of the NLM for the $LC_{50(hatch)}$ estimates for each mosquito strain is presented in Fig 4.4.

Table 4.1: Effect of imbibed ivermectin on adult survival and hatch rate of seven different *Ae. aegypti* strains

$LC_{50(adult)}$						$LC_{50(hatch)}$				
Strain	IVM conc. (ng/ml)	95 % fiducial limits	RR*	Slope ± SE	Intercept ± SE	IVM conc. (ng/ml)	95 % fiducial limits	RR*	Slope ± SE	Intercept ± SE
IMU-PR	576	(458.80, 722.96)	3.08	6.19 ± 0.48	-11.58 ± 3.07	334	(3.64, 645.12)	7.61	6.69 ± 0.87	-10.84 ±5.14
SLD-PR	536	(402.11,822.64)	2.87	3.63 ± 0.31	-8.16 ± 1.89	60	(43.87, 76.83)	1.38	4.66 ± 0.32	-6.32 ± 1.39
IQT-PR	487	(397.36,605.13)	2.60	5.29 ± 0.32	-10.3 ± 1.97	125	(79.96, 178.23)	2.84	2.44 ± 0.16	-4.28 ± 0.84
GDLS	355	(297.04, 424.66)	1.90	4.06 ± 0.18	-8.21 ± 1.04	110	(75.65, 156.59)	2.52	2.66 ± 0.17	-4.59 ± 0.86
IQT	284	(242.93, 331.32)	1.52	4.31 ± 0.17	-8.28 ± 0.99	75	(57.28, 92.43)	1.72	2.23 ± 0.17	-4.59 ± 0.86
SLD	284	(242.03, 333.89)	1.52	4.10 ± 0.15	-7.9 ± 0.8	52	(40.44, 68.54)	1.20	10.18 ± 0.55	-9.21 ± 2.27
NO	187	(162.79, 215.75)	–	5.10 ± 0.15	-8.5 ± 0.81	43	(34.80, 50.95)	–	10.49 ± 0.43	-8.91 ± 1.73

* with respect to NO

Table 4.2. Hatch rate of seven different *Ae. aegypti* strains following a maternal blood meal containing ivermectin

Mosquito strain	Conc. of ivermectin fed to female mosquito, ng/ml							
	800	400	200	100	75	50	35	PBS-DMSO
IMU-PR	**4.2 ± 4.2**	**34.0 ± 31.1**	**77.0 ± 9.0**	**83.0 ± 9.4**	**98.0 ± 2.0**	**78.5 ± 14.8**	92.7 ± 3.4	95.3 ± 2.7
SLD-PR	**0**	**1.7 ± 1.7**	**0**	**16.6 ± 8.9**	**47.2 ± 9.6**	**55.1 ± 10.6**	**76.7 ± 11.8**	95.3 ± 2.7
IQT-PR	**0**	**6.1 ± 4.0**	**43.6 ± 14.0**	**68.5 ± 1.5**	**58.3 ±13.0**	**70.3 ± 8.7**	**76.1 ± 4.5**	94.6 ± 2.8
GDLS	**0**	**16.7 ± 11.2**	**22.7 ± 16.2**	**39.2 ± 12.4**	**70.3 ± 13.9**	**80.1 ± 4.2**	**81.2 ± 4.2**	96.0 ± 4.0
IQT	–	**0.7 ± 0.7**	**0**	**34.7 ± 19.1**	**44.9 ± 5.1**	**81.0 ± 4.2**	**82.2 ± 10.2**	96.8 ± 3.2
SLD	–	**0**	**0**	**16.3 ± 16.3**	**12.6 ± 0.7**	**37.3 ± 19.3**	**93.9 ± 3.0**	98.7 ± 1.3
NO	–	–	**0**	**12 ± 7.6**	**32.7 ± 16.5**	**32.7 ± 16.5**	**55.2 ± 1.7**	82.5 ± 1.3

Numbers correspond to the percentage of larvae hatching from eggs ± SEM. Numbers denoted in bold font correspond with an overall significant reduction in hatch rate are denoted in bold font (Chi-square test for significance $P<0.05$).
– denotes a conc. where no mosquitoes survived to lay eggs

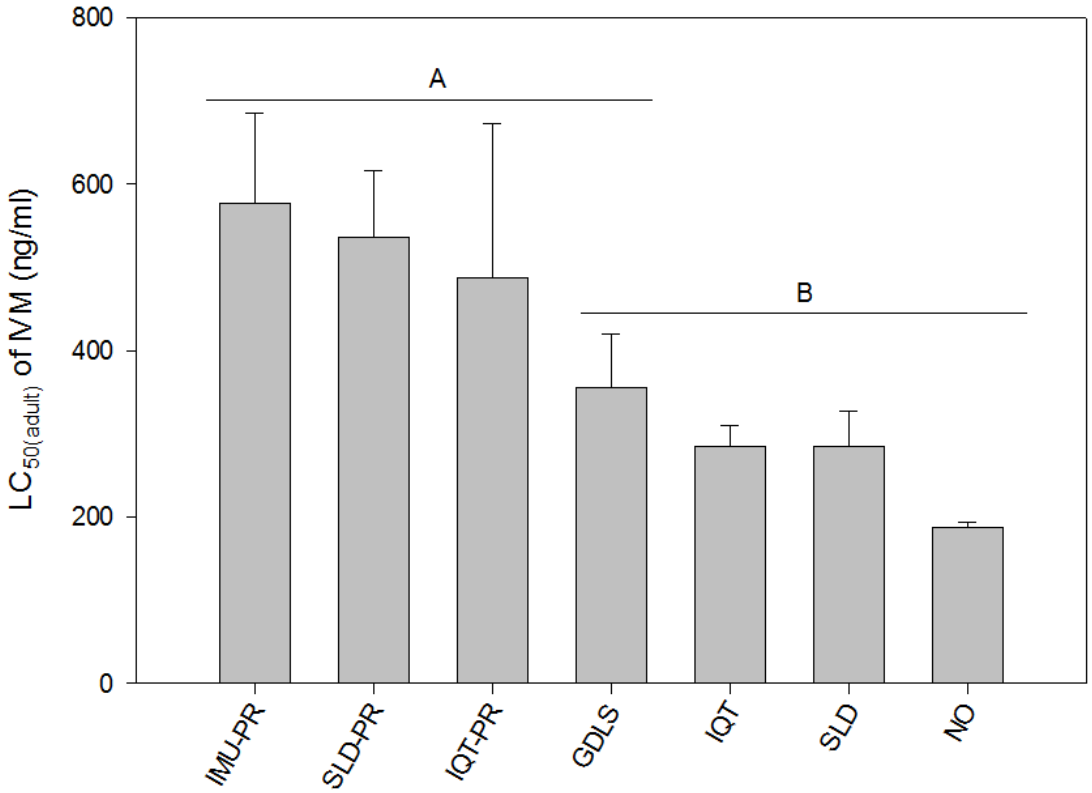


Figure 4.1: Comparison of $LC_{50(adult)}$ for IVM between seven strains of *Ae. aegypti.* Error bars indicate experiment-wise standard error of the mean LC_{50} value of three replicate experiments. Strains that did not differ significantly in the $LC_{50(hatch)}$ for IVM are denoted with the same letter ($\alpha = 0.05$; Least Squares Means procedure).

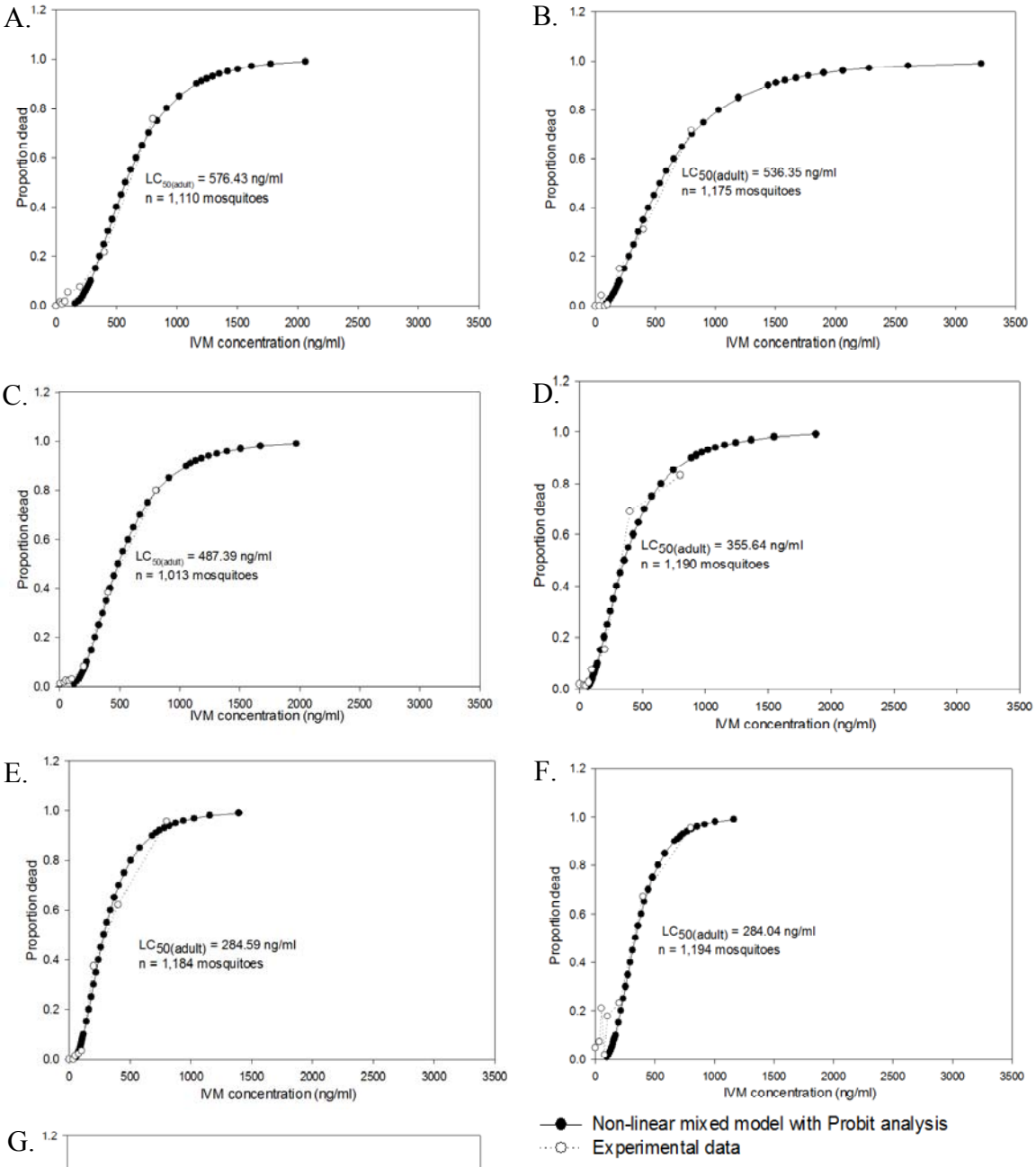


Figure 4.2: Fit of NLM to calculate $LC_{50(adult)}$ estimates for orally ingested IVM in seven strains of *Ae. aegypti.* A plot of the observed mortality versus the IVM concentration imbibed by mosquitoes was overlaid with a plot of the probit mortality versus predicted IVM concentration from the NLM. (A) IMU-PR. (B) SLD-PR. (C) IQT-PR. (D) GDLS. (E) IQT. (F) SLD. (G) NO.

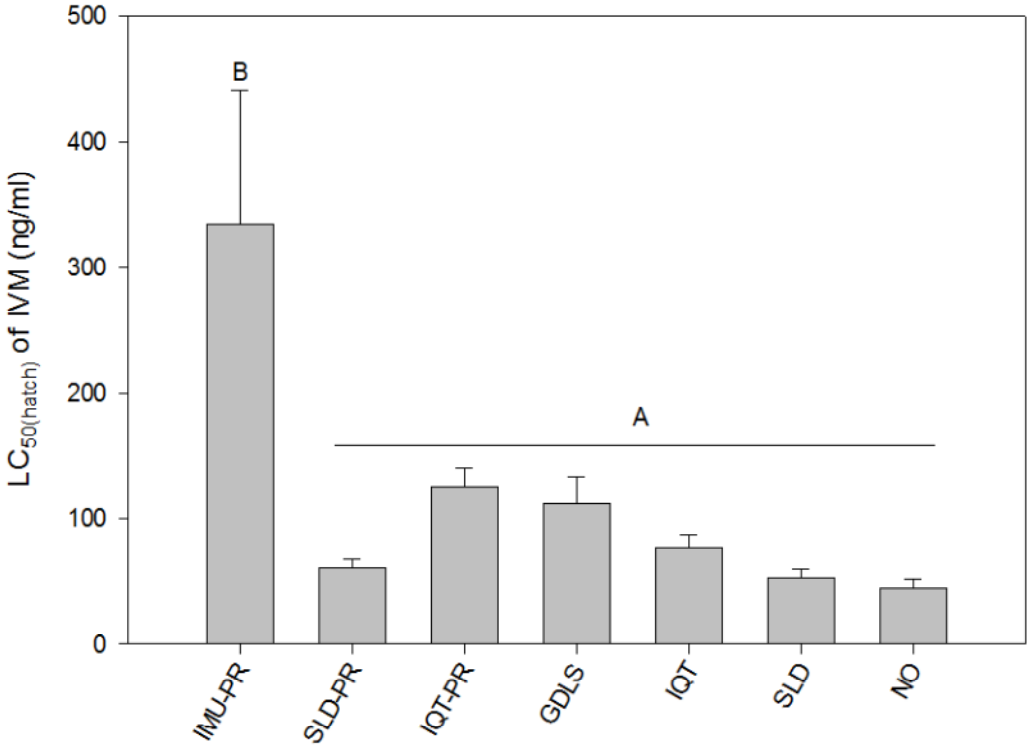


Figure 4.3: Comparison of $LC_{50(hatch)}$ for IVM between seven strains of *Ae. aegypti.* Error bars indicate experiment-wise standard error of the mean LC_{50} value from three replicate experiments. Strains that did not differ significantly in the $LC_{50(hatch)}$ for IVM are denoted with the same letter ($\alpha = 0.05$; Least Squares Means procedure).

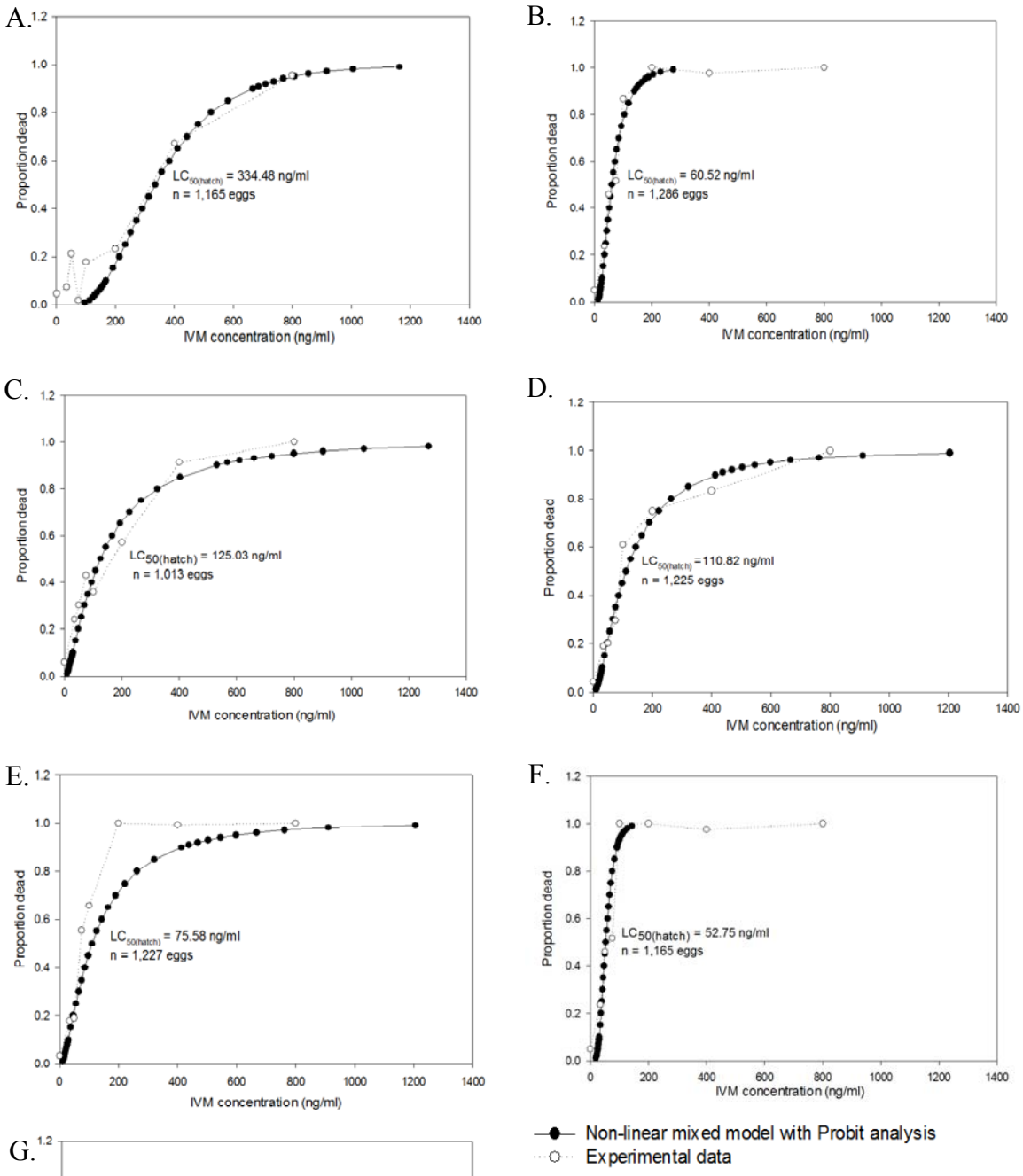


Figure 4.4: Fit of NLM to calculate $LC_{50(hatch)}$ estimates for orally ingested IVM in seven strains of *Ae. aegypti*. A plot of the observed mortality versus the IVM concentration imbibed by mosquitoes was overlaid with a plot of the probit mortality versus predicted IVM concentration from the NLM. (A) IMU-PR. (B) SLD-PR. (C) IQT-PR. (D) GDLS. (E) IQT. (F) SLD. (G) NO.

Fecundity of *Ae. aegypti* mosquitoes following a blood meal containing IVM. A two-way ANOVA revealed that IVM concentration had a significant effect on the average number of eggs laid per female mosquito ($F = 32.89$; df =7, 112; $P < 0.001$), and that the mean number of eggs laid per female differed significantly among mosquito strains ($F = 4.487$; df = 6,112; $P < 0.001$). There was no significant interaction effect between IVM concentration and mosquito strain ($F=0.780$, df = 42, 112, $P =0.819$). Because the average number of eggs laid per female differed significantly among mosquito strains, we analyzed the effects of IVM on the mean number of eggs produced per female for each mosquito strain separately (Table 4.3). A significant reduction in the mean number of eggs laid by the IQT-PR and the SLD-PR strains was seen only at the 800 and 400 ng/ml concentrations of IVM. In the corresponding selection free strains, no mosquitoes survived to oviposit after feeding on IVM at a concentration of 800 ng/ml, and significant reductions in the mean number of eggs laid were seen following a blood meal containing 400 and 200 ng/ml of IVM (Table 4.3).

Correlation between LC_{50} of permethrin and $LC_{50(adult)}$ of IVM. We observed a significant correlation between the LC_{50} for permethrin (measured in µg/bottle) and the LC_{50} for IVM (Pearson's product-moment correlation squared = 0.97, $P = 0.002$). The LC_{50} estimates for each of the mosquito strains are published elsewhere (Saavedra-Rodriguez et al. 2007, Saavedra-Rodriguez 2011). A plot of the LC_{50} of permethrin versus the $LC_{50(adult)}$ for IVM is shown in Fig 4.5. The GDLS was omitted from correlation analysis because the LC_{50} for permethrin was not available for this strain.

Table 4.3. Fecundity of varied strains of *Ae. aegypti* following ingestion of a blood meal containing ivermectin

Mosquito strain	Conc. of ivermectin fed to female mosquito, ng/ml 800	400	200	100	75	50	35	PBS-DMSO
IMU-PR	**11.2 ± 10.4**	**54.39 ± 33.6**	**60.5 ± 11.4**	117.3 ± 22.1	120.1 ±11.5	111.7 ± 13.0	**70.6 ± 8.5**	128.1 ± 21.0
SLD-PR	**38 ± 38**	**30.8 ± 10.1**	122.7 ± 48.4	105.6 ± 5.1	113.5 ± 3.2	133.1 ± 4.1	112.5 ± 4.7	122.4 ± 17.4
IQT-PR	**5.3 ± 5.3**	**34.6 ± 26.7**	90.1 ± 13.0	91.0 ± 12.8	85.6 ± 28.1	113.9 ± 16.9	103.3 ± 12.0	125.0 ±6.5
GDLS	**1.8 ± 1.8**	**28.4 ± 14.3**	53.4 ± 18.0	64.4 ± 12.1	83.6 ± 22.2	97.0 ± 11.6	92.4 ± 10.9	88.9 ± 5.1
IQT	–	**7.9 ±7.9**	**18.8 ± 12.7**	92.7 ± 30.0	81.3 ± 8.4	99.1 ± 12.1	90.9 ± 12.1	77.3 ± 21.1
SLD	–	**9.3 ± 9.3**	**39.6 ± 28.3**	81.0 ± 21.5	92.7 ± 14.0	134.7 ± 25.1	126.4 ± 26.7	118.7 ± 12.8
NO	–	–	**59.3 ± 26.7**	65.2 ± 37.8	81.9 ± 17.3	98.9 ± 14.2	105.5 ± 7.2	118.4 ± 25.7

Numbers correspond to the mean number of eggs ± SEM. For each strain of mosquito, the mean number of eggs laid by mosquitoes at each concentration of IVM was compared to the corresponding control. Significant reductions in fecundity are denoted in bold font (Chi-square test for significance ($P < 0.05$)).

– denotes a conc. where no mosquitoes survived to lay eggs

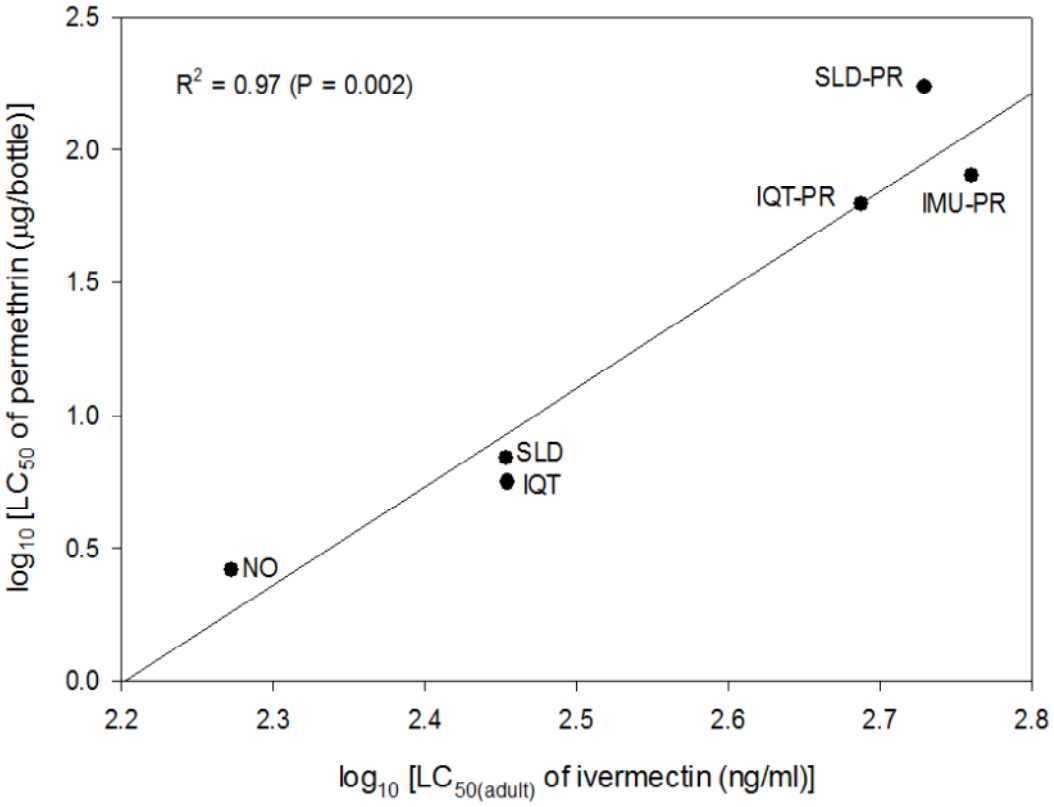


Figure 4.5: Regression of LC_{50} of permethrin against the $LC_{50(adult)}$ of IVM in six strains of *Ae. aegypti.*

Correlation between $LC_{50(adult)}$ and $LC_{50(hatch)}$ of IVM. The correlation observed between the $LC_{50(adult)}$ and $LC_{50(hatch)}$ of IVM was not significant (Pearson's product-moment correlation = 0.70, P = 0.08).

Estimation of realized heritability of resistance to IVM in the GDLS. The $LC_{25(hatch)}$ for each generation of the IVM-selected GDLS and selection-free GDLS controls are reported in Table 4.4. The $LC_{25(hatch)}$ of the GDLS-F^{IVM}_3 and the GDLS-F^{PBS}_3 increased from 55 ng/ml to 69 ng/ml and 85 ng/ml, respectively (Table 4.5). The cumulative response to selection (*R*) was -0.39 in the IVM-selected GDLS and was 0.89 in the selection-free control. The cumulated selection differential (*S)* was 0.83 in the IVM-selected GDLS and was 0.43 in the selection free controls. Realized heritability (h^2) estimates were estimated using the slope of the regression line of the cumulative response to selection versus the cumulated selection differential, and were -0.37 in the IVM-selected GDLS, and 2.08 for the selection free control (Table 4.5).

Table 4.4: Estimation of $LC_{25(hatch)}$ of IVM in successive generations of IVM-selected and non-selected control *Ae. aegypti* GDLS mosquitoes

	GDLS-IVM Selected			GDLS- Selection Free Control		
Generation	Egg Mortality (%)	No. viable larvae/ Total eggs submerged	$LC_{25(hatch)}$ IVM (ng/ml)[a]	Egg Mortality (%)	No. viable larvae/ Total eggs submerged	$LC_{25(hatch)}$ IVM (ng/ml)[a]
P	24	760/1000	55.48	10.6	894/1000	55
F_1	18.4	872/1069	101.96	14.7	891/1045	35
F_2	64.2	1372/3828	70.86	25	1289/1719	75
F_3	25	750/1125	69.41	20	237/1186	85

a- 95% fiducial limits are not reported because estimates for the $LC_{25(hatch)}$ in generations F_1 through F_3 are from one experimental replicate.

Table 4.5: Estimation of realized heritability (h^2) of IVM resistance in *Ae. aegypti* GDLS. Parent generation to F_3

Treatment group	Estimate of mean response per generation					Estimate of mean selective differential per generation				
	Initial $LC_{25(hatch)}$ (ng/ml)	Final $LC_{25(hatch)}$ (ng/ml)	R	*p*	*i*	Initial Slope ± SE	Final Slope ± SE	σ	S	h^2
GDLS-IVM	55.48	69.41	-0.39	0.67	0.57	2.66 ± 0.17	5.53 ± 0.17	0.18	0.83	-0.37
GDLS-Free	55.48	85.80	0.89	0.80	0.43	2.66 ± 0.17	11.88 ± 0.26	0.40	0.43	2.08

Discussion

Three outcomes (adult survival, fecundity and hatch rate) were used to evaluate the susceptibility of seven different lines of *Ae. aegypti* mosquitoes to different concentrations of IVM contained in a blood meal. While we and others have reported on the effects of IVM on *Ae. aegypti* (Pampiglioni et al. 1985, Tesh and Guzman 1990, Mahmood et al. 1991, Focks et al. 1995, Kobylinski et al. 2010), this is the first analysis of variation in IVM susceptibility within a species. This is also the first study to determine the $LC_{50(adult)}$ and $LC_{50(hatch)}$ of IVM in mosquitos with known insecticide resistance.

We chose to use *Ae. aegypti* as a model in the present study due to our possession of multiple different mosquito strains that have been previously characterized, including the GDLS and three permethrin-resistant mosquito strains, two of which we also possessed their parent non-selected strains. An advantage to using *Ae. aegypti* is that oogenesis is completed after one blood meal, whereas *Anopheles gambiae* has been shown to visually require more than one blood meal for completion of oogenesis (Fernandes and Briegel 2005). Since effects of IVM on fecundity and hatch rate are temporary, and diminish after a second blood meal that does not contain IVM (Tesh and Guzman 1990), *Ae aegypti* is an attractive model for assessing the variability of the effects of IVM on mosquito fertility, egg development and viability.

The *in vitro* feeding strategy employed in this study allowed for the consistent administration of varied concentrations of IVM to all of the mosquito strains, thus allowing for direct comparisons of the $LC_{50(adult)}$ and $LC_{50(hatch)}$ for IVM among the

different strains. Following oral ingestion of IVM in blood meal, we observed a large degree of variation in the $LC_{50(adult)}$ of IVM among strains.

The $LC_{50(adult)}$ estimates for all of the laboratory-selected permethrin-resistant mosquito lines (IMU-PR, SLD-PR and IQT-PR) were significantly higher than the standard laboratory reference strain (NO). A selection-free line of IMU could not be maintained in the laboratory; therefore a contrast between the IMU-PR strain and the corresponding selection-free strain could not be made. However, the $LC_{50(adult)}$ for IVM in the SLD-PR and IQT-PR strains differed significantly from the corresponding selection-free strains, and the LC_{50} of permethrin was positively correlated with the $LC_{50(adult)}$ for IVM. Collectively, these results indicate that a cross-resistance mechanism could be responsible for the increased tolerance to IVM by the IMU-PR, SLD-PR and IQT-PR *Ae. aegypti* mosquito strains. These results were indeed quite surprising and unexpected.

Ivermectin is an allosteric agonist of glutamate-gated chloride (GluCl) anion channels. In parasitic worms, IVM binds the GluCl receptor causing an increased permeability to chloride ions, which then leads to hyperpolization of the nerve-cell membrane, leading to flaccid paralysis and death of the parasite (Cleland 1996, Wolstenholme and Rogers 2005). However, they pyrethroids delay the normal closing of voltage-gated sodium channels of arthropods, resulting in depolarization of nerve-cell membranes ultimately leading to excessive neuroexcitation and death (Soderlund and Bloomquist 1989). Given the disparate modes of action and target sites for these two compounds, cross-resistance is more likely due to metabolic mechanisms. Permethrin-induced cross-resistance to abamectin, a macrocyclic lactone differing from IVM only by

the presence of a double-bond, has been reported in house flies (Scott 1989, Geden et al. 1992) and German cockroaches (Scott 1991). In one study, permethrin-resistant *Musca domestica* were observed to have a 25-fold increased cross-resistance to abamectin that was temporarily suppressed by the mixed-function oxidase inhibitor, piperonyl butoxide (Scott 1989). Reports of permethrin-induced cross-resistance to avermectin are conflicting. Others have reported that permethrin-resistant house flies (Roush and Wright 1986b) and permethrin-resistant German cockroaches (Cochran 1990) are fully susceptible to avermectin, and recently permethrin-resistant head lice were shown to be susceptible to IVM (Strycharz et al. 2008). In all of these studies, avermectin or IVM was applied topically, which differs from our methods in which IVM was orally imbibed.

While it is possible that the increased tolerance to IVM observed in any one of the permethrin-resistant *Ae. aegypti* mosquito strains is an artifact of laboratory-selection, it is interesting that adult mosquitoes of all three permethrin-resistant strains were approximately two-fold less susceptible to the effects of IVM than any of the permethrin-susceptible lines. Pyrethroid resistance in field populations of mosquitoes is well documented (Santolamazza et al. 2008, Garcia et al. 2009). In light of the recent report and models that demonstrate MDA of IVM can disrupt the transmission of human malaria parasites (Sylla et al. 2010, Kobylinski 2011), the question of whether pyrethroid resistance can result in cross-resistance to IVM is clearly an area that needs to be further explored.

The hatch rate of all mosquito strains was decreased following a maternal blood meal containing IVM. These data are consistent with the findings of other reports of IVM in *Ae. aegypti* (Tesh and Guzman 1990, Mahmood et al. 1991), however the

$LC_{50(hatch)}$ of IVM for all of the mosquito strains we evaluated are notably higher than the previous reports. Tesh and Guzman (1990) reported a $LC_{50(hatch)}$ of 3.4 ng/ml using the Rock strain.

With the exception of the IMU-PR strain, no significant differences among strains were observed with respect to the $LC_{50(hatch)}$ of IVM. These results are strikingly different from those for the $LC_{50(adult)}$ estimates, where all permethrin-resistant strain estimates were significantly higher than standard susceptible strain. In addition, there was no correlation between the $LC_{50(adult)}$ and $LC_{50(hatch)}$ of IVM. Collectively, these results suggest that IVM induces adult mortality and decreases the hatch rate of eggs through different mechanisms.

Mahmood et al (1991) blood-fed *Ae. aegypti* mosquitoes on sublethal concentrations of IVM, and also observed a large decrease in hatch rate of the eggs from treated mosquitoes. Many of the un-hatched eggs contained live larvae that failed to hatch despite multiple submersions in water (Mahmood et al. 1991). In the same report, the authors propose that residual amounts of IVM may be deposited in the egg and prevent eclosion. Such a mechanism could explain why we did not see a large variations in the $LC_{50(hatch)}$ of the mosquito strains. Our experiments were not designed to elucidate the mechanism through which IVM interferes with egg hatching, but clearly this is an area that should be further explored.

The maximal concentrations of IVM found in human venous plasma following a standard MDA dose of IVM (150 µg/kg) ranges from 9-75 ng/ml, with a mean maximal concentration of approximately 46 ng/ml (Elkassaby 1991). The $LC_{50(adult)}$ of IVM in *Ae. aegypti* reported here and elsewhere (Tesh and Guzman 1990, Kobylinski et al. 2010) are

far greater than the serum concentrations expected in humans following ingestion of the 150 µg/kg dose of IVM typically used in MDA (Elkassaby 1991), thus it is unlikely that MDA administration of IVM will be effective in controlling diseases transmitted by *Ae. aegypti*. Nonetheless, *Ae. aegypti* may prove to be a useful laboratory model for studying the mechanisms of IVM-induced pathology in the mosquito as well as potential mechanisms of resistance that could develop in the mosquito.

In addition to characterizing the susceptibility of seven different *Ae. aegypti* mosquito lines to IVM, we also attempted to select for resistance to IVM in the GDLS. While several reports of IVM resistance in other organisms have been published (Rugg 1998, Kane et al. 2000, Blackhall et al. 2003, Currie et al. 2004, Soutello et al. 2007, Klafke et al.), to the best of our knowledge there are no reports of IVM resistance in mosquitoes resulting from either field or laboratory selection. Given the considerable promise that MDA of IVM has for reducing the transmission of mosquito-borne disease (Sylla et al. 2010, Foy 2011, Kobylinski 2011), we sought to investigate whether resistance to IVM could be selected for in a mosquito with a genetically diverse background.

After three successive generations of selection, there was no evidence of a cumulative response to selection in the GDLS-IVM selected mosquitoes (r = -0.39). Interestingly, a strong cumulative response to selection (r= 0.89) was observed in the selection-free, generation-matched control GDLS population. Prior to initiating the selection experiments, we anticipated that the $LC_{25(hatch)}$ estimates for IVM would fluctuate across generations, as seen in Table 4.4. Such fluctuations are typical in artificial selection experiments, and the causes are presumably due to random genetic

drift, sampling error, and environmental variance (Falconer 1996). The generation-matched, selection-free control was included in this study to assess to what degree environmental variance contributed to the fluctuations in the $LC_{25(hatch)}$ between generations; however, due to the unexpected increase in the $LC_{25(hatch)}$ values in the non-selected line, this was not possible. It is important to note that both the GDLS-IVM selected population and the selection-free control population were each subject to the effects of random genetic drift, and random genetic drift is a likely explanation for the strong response to selection seen in the selection-free control.

Given the recent suggestion that IVM may serve as a potent tool in reducing the incidence of mosquito-borne disease, (Sylla et al. 2010, Foy 2011, Kobylinski 2011) we chose to use the *Ae. aegypti* GDLS as a model to study whether or not IVM resistance could rapidly develop in a genetically diverse mosquito population when IVM was repeatedly ingested through a blood meal. While we did not see a response to selection to IVM in the GDLS-IVM selected mosquitoes, it is premature to conclude that resistance to IVM is unlikely to develop in *Ae. aegypti* in response to the drug being imbibed in a blood meal. We only carried out our selection experiments for three generations. Assuming that an IVM-resistance allele or alleles are present in the GDLS, it is possible that a response to selection would have been observed in later generations if we had continued the selection process through successive generations, especially if resistance is conferred by a recessive allele.

In summary, we found that in *Ae. aegypti,* adult survival following ingestion of IVM in a blood meal varies largely by mosquito strain, whereas the effect of a maternal blood meal on the ability of eggs to hatch varies among strains to a far lesser extent. We

did not see a response to selection for IVM resistance in the *Ae. aegypti* GDLS, however our results support that cross-resistance to IVM may develop in permethrin-resistant *Ae. aegypti* mosquitoes. Clearly these are areas that necessitate additional research. One possible approach to investigating the ability to laboratory select a mosquito strain for IVM resistance would be to cross an IVM-resistant strain (such as the IMU-PR) with an IVM-susceptible strain (such as the NO), and then select the hybrid population for IVM resistance.

CHAPTER 5: SUMMARY AND FUTURE DIRECTIONS

The future of the control of mosquito-borne diseases will require the integration of novel methods to augment current vector control efforts. Two possibilities for novel control methods could include the development and deployment of anti-vector vaccines or the administration of anthelmintic drugs to human populations that are fed upon by vector mosquitoes.

The field of anti-mosquito immunity is still in its infancy, and has been largely ignored and unexplored. The development and commercialization of two vaccines against the tick *Boophilus microplus* demonstrates that anti-vector immunity is not only possible, but is also highly effective in decreasing the burden of these ectoparasites in cattle (de la Fuente et al. 1999, Willadsen 1999, de la Fuente et al. 2008). The experiments presented in this dissertation attempted to evaluate the *Aedes aegypti* mosquito lysosomal aspartic protease (AaMLAP) and a mosquito glutamate gated chloride anion channel as potential mosquitocidal antigens. To evaluate AaMLAP as a potential antigen, we immunized mice with a number of immunization preparations, using a number of immunization regimens. The recombinant cDNA immunization construct was chosen because of the ease of inserting the AaMLAP gene into the pCDNA3.1(+) immunization vector. Further, the cDNA immunization strategy employed in the studies described here has been effective in eliciting consistent mosquitocidal responses, in mice, against *Anopheles gambiae* midgut cDNA (Foy et al. 2003). We were unable to induce a mosquitocidal response in mice through immunization with either the AaMLAP cDNA immunization construct or a recombinant AaMLAP-SINV expression vector, and possible reasons are discussed in Chapter 2.

Efforts to generate a purified source of recombinant AaMLAP protein were unsuccessful, and thus the mosquitocidal response elicited by mice immunized with the crude AaMLAP recombinant protein are difficult to interpret. Without a source of purified AaMLAP, it was difficult to move forward in evaluating AaMLAP as a potential mosquitocidal antigen. We therefore chose to investigate the effect of orally imbibed antiserum generated against the glutamate-gated chloride (GluCl) anion channel due to numerous studies which show oral ingestion of ivermectin (thought to be a GluCl agonist) causes a reduction in mosquito survival (Pampiglioni et al. 1985, Iakubovich et al. 1989a, Tesh and Guzman 1990, Cartel et al. 1991, Focks 1991, Focks et al. 1991, Mahmood et al. 1991, Gardner et al. 1993, Bockarie et al. 1999, Foley et al. 2000, Fritz et al. 2009, Kobylinski et al. 2010, Sylla et al. 2010, Kobylinski et al. 2011). We did not see a reduction in mosquito survival after feeding on high titer GluCl antiserum and did not pursue the GluCl anion channel further as a mosquitocidal target for *Ae. aegypti.*

The difficulties encountered in evaluating the AaMLAP and GluCl anion channel as potential mosquitocidal antigens highlight the bottleneck in the discovery and development of anti-vector vaccines. Ultimately, the key to the development of any anti-vector vaccine is identification of specific target antigens. Identifying the tick antigen, *Bm*86, the basis for the TickGARD and Gavac vaccines took ten years, requiring the fractionation of one kilogram of ticks, several rounds of immunizations and tick challenges (Willadsen 2004). Such methods are painstakingly slow, and when one factors the cost of experimental animals, are exceptionally expensive. If mosquitocidal vaccines are to someday become a reality, high throughput antigen screening methods must first be developed.

Numerous studies have clearly demonstrated that when imbibed in a blood meal, certain anthelmintic drugs can elicit mosquitocidal responses (Pampiglioni et al. 1985, Iakubovich et al. 1989b, 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, Foley et al. 2000, Fritz et al. 2009, Chaccour et al. 2010, Sylla et al. 2010). The research presented here provides a detailed and comparative evaluation of several anthelmintic drugs, including: ivermectin, moxidectin, selamectin, DEC, albendazole-sulfoxide and pyrantel for their ability to reduce survival of adult *Ae. aegypti* mosquitoes. The results presented here and other published reports (Tesh and Guzman 1990, Focks et al. 1991, Mahmood et al. 1991) show that MDA of drugs frequently used for the control of nematode parasites are unlikely to impact the transmission of pathogens vectored by *Ae. aegypti*. Nonetheless, our results highlight important questions that warrant further investigation.

Of all the drugs tested, ivermectin was the most effective in reducing adult mosquito survival. It is interesting that ivermectin, moxidectin and selamectin differed so greatly in their ability to reduce adult mosquito survival, as they are structurally similar compounds. The mechanism for the difference in drug potency between these related compounds is unknown, but should be investigated. An understanding of the underlying biochemical mechanism could be used to design more potent anthelmintic derivatives effective in targeting *Ae. aegypti* or other ivermectin-refractory mosquito genera such as *Culex* spp. mosquitoes (Tesh and Guzman 1990, Chandre and Hougard 1999).

Although it is believed that ivermectin exerts insecticidal activity by agonizing the GluCl anion channel, a precise mechanism has not been proposed in any mosquito

species. In analyzing the data presented here and in other published reports, it is clear that *Anopheles* mosquitoes are far more susceptible to orally-imbibed ivermectin than *Ae. aegypti.* The mechanism for the difference between these two mosquito species is not known, however the remarkable difference in susceptibility between the two genera should be further investigated. Elucidating the mechanism responsible for the difference in ivermectin susceptibility between the genera could aid in engineering more potent endectocide drugs, but also promises considerable opportunity for increasing an understanding of the mechanism of action for ivermectin in mosquitoes as well as shedding light on many other aspects of mosquito physiology.

Aedes aegypti may prove to be a useful laboratory model for investigating the effects of orally imbibed ivermectin over successive generations. Recent field studies indicate that if administered on a monthly basis, ivermectin could have considerable potential for reducing malaria transmission by *Anopheles* mosquitoes in the field (Sylla et al. 2010, Foy et al. 2011, Kobylinski et al. 2011). There are many important questions to address, however, prior to implementing such an approach. Questions such as whether such frequent administrations would select for ivermectin resistance in mosquitoes or nematode parasites currently treated with ivermectin must be first be answered. For reasons already discussed, *Ae. aegypti* is exceptionally easy to rear and propagate in the laboratory. The data presented in this dissertation argues for utilizing *Ae. aegypti* as a model to help begin investigating phenomena such as the existence of cross-resistance or development of resistance to ivermectin in a mosquito species.

After three successive generations of selection with ivermectin, we did not see any evidence for the development of ivermectin resistance in *Ae. aegypti.* These results

should not be interpreted to mean that resistance is unlikely to develop in field populations of mosquitoes if ivermectin were to be administered to human populations more frequently. We and others have shown that ivermectin imbibed by mosquitoes in a blood meal not only affects adult survival, but also affects the fecundity, hatch rate and larval survival (Focks et al. 1991, Mahmood et al. 1991, Gardner et al. 1993, Fritz et al. 2009). Further, the effects of ivermectin on hatch rate and larval survival appear to occur at exceptionally low concentrations. In field studies, a single MDA dose of ivermectin decreased adult *Anopheles* mosquito survival for six days after administration (Sylla et al. 2010). Sub-lethal effects of ivermectin MDA on fecundity, egg hatch rate and offspring survival could potentially extend for a greater period of time, thereby exerting selection pressure at all stages of the mosquito life cycle, with the exception being on the adult male mosquito. Currently, ivermectin MDA is administered once or twice yearly in regions throughout the tropics and subtropics for the control and treatment of onchocerciasis and lymphatic filariasis. Given the short half life of ivermectin, the current regimen is unlikely to result in ivermectin resistance in mosquitoes biting on treated people; however, careful consideration and additional laboratory experimentation determining whether or not resistance to ivermectin can be selected for in mosquitoes, including *Anopheles* spp. should be conducted.

REFERENCES

Abro, G. H., R. A. Dybas, A. S. J. Green, and D. J. Wright. 1988. Toxicity of Avermectin B1 against a susceptible laboratory strain and an insecticide-resistant strain of *Plutella xylostella* (Lepidoptera: Plutellidae). J. Econ. Entomol. 81: 1575-1580.

Addiss, D. G., M. L. Eberhard, P. J. Lammie, W. L. Hitch, and H. C. Spencer. 1991. Tolerance of single high-dose ivermectin for treatment of lymphatic filariasis. Trans R Soc Trop Med Hyg 85: 265-6.

Addiss, D. G., M. J. Beach, T. G. Streit, S. Lutwick, F. H. LeConte, J. G. Lafontant, A. W. Hightower, and P. J. Lammie. 1997. Randomised placebo-controlled comparison of ivermectin and albendazole alone and in combination for Wuchereria bancrofti microfilaraemia in Haitian children. Lancet 350: 480-4.

Alger, N. E., and E. J. Cabrera. 1972. An increase in death rate of Anopheles stephensi fed on rabbits immunised with mosquito antigen. J Econ Entomol 65: 165-168.

Ali, A., and J. K. Nayar. 1985. Activity of an avermectin insecticide, Abamectin (MK-936), against mosquitoes and chironomid midges in the laboratory. J Am Mosq Control Assoc 1: 384-6.

Almeida, A. P., and P. F. Billingsley. 1998. Induced immunity against the mosquito Anopheles stephensi Liston (Diptera: Culicidae): effects on mosquito survival and fecundity. Int J Parasitol 28: 1721-31.

Almeida, A. P., and P. F. Billingsley. 1999. Induced immunity against the mosquito Anopheles stephensi: reactivity characteristics of immune sera. Med Vet Entomol 13: 53-64.

Almeida, A. P., and P. F. Billingsley. 2002. Induced immunity against the mosquito Anopheles stephensi (Diptera: Culicidae): effects of cell fraction antigens on survival, fecundity, and plasmodium berghei (Eucoccidiida: Plasmodiidae) transmission. J Med Entomol 39: 207-14.

Amazigo, U. 2008. The African Programme for Onchocerciasis Control (APOC). Ann Trop Med Parasitol 102 Suppl 1: 19-22.

Anderson, J., and H. Fuglsang. 1978. Further studies on the treatment of ocular onchocerciasis with diethylcarbamazine and suramin. British Journal of Opthalmology 62: 450-457.

Arena, J. P., K. K. Liu, P. S. Paress, J. M. Schaeffer, and D. F. Cully. 1992. Expression of a glutamate-activated chloride current in Xenopus oocytes injected with Caenorhabditis elegans RNA: evidence for modulation by avermectin. Brain Res Mol Brain Res 15: 339-48.

Argentine, J. A., and J. M. Clark. 1990. Selection for abamectin resistance in Colorado potato beetle (Coleoptera: Chrysomeliadae). Pestic. Sci 28: 17-24.

Argentine, J. A., M. J. Clark, and H. Lin. 1992. Genetics and biochemical mechanisms of abamectin resistance in two isogenic strains of Colorado Potato Beetle. Pesticide biochemistry and physiology 44: 191-207.

Atkins, G. J., M. N. Fleeton, and B. J. Sheahan. 2008. Therapeutic and prophylactic applications of alphavirus vectors. Expert Rev Mol Med 10: e33.

Awadzi, K., G. Edwards, N. O. Opoku, A. E. Ardrey, S. Favager, E. T. Addy, S. K. Attah, L. K. Yamuah, and B. T. Quartey. 2004a. The safety, tolerability and pharmacokinetics of levamisole alone, levamisole plus ivermectin, and levamisole plus albendazole, and their efficacy against Onchocerca volvulus. Ann Trop Med Parasitol 98: 595-614.

Awadzi, K., S. K. Attah, E. T. Addy, N. O. Opoku, B. T. Quartey, J. K. Lazdins-Helds, K. Ahmed, B. A. Boatin, D. A. Boakye, and G. Edwards. 2004b. Thirty-month follow-up of sub-optimal responders to multiple treatments with ivermectin, in two onchocerciasis-endemic foci in Ghana. Ann Trop Med Parasitol 98: 359-70.

Awadzi, K., D. A. Boakye, G. Edwards, N. O. Opoku, S. K. Attah, M. Y. Osei-Atweneboana, J. K. Lazdins-Helds, A. E. Ardrey, E. T. Addy, B. T. Quartey, K. Ahmed, B. A. Boatin, and E. W. Soumbey-Alley. 2004c. An investigation of persistent microfilaridermias despite multiple treatments with ivermectin, in two onchocerciasis-endemic foci in Ghana. Ann Trop Med Parasitol 98: 231-49.

Aziz, M. A., S. Diallo, I. M. Diop, M. Lariviere, and M. Porta. 1982a. Efficacy and tolerance of ivermectin in human onchocerciasis. Lancet 2: 171-3.

Aziz, M. A., S. Diallo, M. Lariviere, I. M. Diop, I. M. Porta, and P. Gaxotte. 1982b. Ivermectin in onchocerciasis. Lancet 2: 1456-7.

Babu, B. V., and K. Satyanarayana. 2003. Factors responsible for coverage and compliance in mass drug administration during the programme to eliminate lymphatic filariasis in the East Godavari District, South India. Trop Doct 33: 79-82.

Babu, B. V., and S. K. Kar. 2004. Coverage, compliance and some operational issues of mass drug administration during the programme to eliminate lymphatic filariasis in Orissa, India. Trop Med Int Health 9: 702-9.

Barrett, A. J., Rawlings, D., and Woessner, J.F.. [ed.] 1998. Handbook of Proteolytic Enzymes. Academic Press, Oxford.

Basanez, M. G., S. D. Pion, E. Boakes, J. A. Filipe, T. S. Churcher, and M. Boussinesq. 2008. Effect of single-dose ivermectin on Onchocerca volvulus: a systematic review and meta-analysis. Lancet Infect Dis 8: 310-22.

Benz, G. W., R. A. Roncalli, and S. J. Gross. 1984. Use of ivermectin in cattle, sheep, goats and swine, pp. 215-229. *In* W. C. Campbell [ed.], Ivermectin and Abamectin. Springer, New York.

Besansky, N., C. A. Hill, and C. Costantini. 2004. No accounting for tast: host preference in malaria vectors. Trends Parasitol 20: 250-251.

Billingsley, P. F., B. Foy, and J. L. Rasgon. 2008. Mosquitocidal vaccines: a neglected addition to malaria and dengue control strategies. Trends Parasitol 24: 396-400.

Bishop, B. F., C. I. Bruce, N. A. Evans, A. C. Goudie, K. A. Gration, S. P. Gibson, M. S. Pacey, D. A. Perry, N. D. Walshe, and M. J. Witty. 2000. Selamectin: a novel broad-spectrum endectocide for dogs and cats. Vet Parasitol 91: 163-76.

Black, W. C. I., and C. G. Moore. 2005. Population Biology as a Tool to Study Vector-Borne Diseases, pp. 187-206. *In* W. C. Marquardt [ed.], Biology of Disease Vectors, Second ed. Elsevier Academic Press, San Diego, CA.

Blackhall, W. J., R. K. Prichard, and R. N. Beech. 2003. Selection at a gamma-aminobutyric acid receptor gene in Haemonchus contortus resistant to avermectins/milbemycins. Mol Biochem Parasitol 131: 137-45.

Blackhall, W. J., H. Y. Liu, M. Xu, R. K. Prichard, and R. N. Beech. 1998. Selection at a P-glycoprotein gene in ivermectin- and moxidectin-selected strains of Haemonchus contortus. Mol Biochem Parasitol 95: 193-201.

Boatin, B. 2008. The Onchocerciasis Control Programme in West Africa (OCP). Ann Trop Med Parasitol 102 Suppl 1: 13-7.

Boatin, B. A., and F. O. Richards, Jr. 2006. Control of onchocerciasis. Adv Parasitol 61: 349-94.

Bockarie, M. J., and R. M. Deb. 2010. Elimination of lymphatic filariasis: do we have the drugs to complete the job? Curr Opin Infect Dis 23: 617-20.

Bockarie, M. J., E. M. Pedersen, G. B. White, and E. Michael. 2009. Role of vector control in the global program to eliminate lymphatic filariasis. Annu Rev Entomol 54: 469-87.

Bockarie, M. J., J. L. Hii, N. D. Alexander, F. Bockarie, H. Dagoro, J. W. Kazura, and M. P. Alpers. 1999. Mass treatment with ivermectin for filariasis control in Papua New Guinea: impact on mosquito survival. Med Vet Entomol 13: 120-3.

Bogitsh, B. J., K. F. Kirschner, and J. P. Rotmans. 1992. Schistosoma japonicum: immunoinhibitory studies on hemoglobin digestion using heterologous antiserum to bovine cathepsin D. J Parasitol 78: 454-9.

Boussinesq, M. 2006. Loiasis. Ann Trop Med Parasitol 100: 715-31.

Boussinesq, M., J. Gardon, N. Gardon-Wendel, J. Kamgno, P. Ngoumou, and J. P. Chippaux. 1998. Three probable cases of Loa loa encephalopathy following ivermectin treatment for onchocerciasis. Am J Trop Med Hyg 58: 461-9.

Brindley, P. J., B. H. Kalinna, J. Y. Wong, B. J. Bogitsh, L. T. King, D. J. Smyth, C. K. Verity, G. Abbenante, R. I. Brinkworth, D. P. Fairlie, M. L. Smythe, P. J. Milburn, H. Bielefeldt-Ohmann, Y. Zheng, and D. P. McManus. 2001. Proteolysis of human hemoglobin by schistosome cathepsin D. Mol Biochem Parasitol 112: 103-12.

Brown, A., J. M. Burleigh, E. E. Billett, and D. I. Pritchard. 1995. An initial characterization of the proteolytic enzymes secreted by the adult stage of the human hookworm Necator americanus. Parasitology 110 (Pt 5): 555-63.

Burg, R. W., B. M. Miller, E. E. Baker, J. Birnbaum, S. A. Currie, R. Hartman, Y. L. Kong, R. L. Monaghan, G. Olson, I. Putter, J. B. Tunac, H. Wallick, E. O. Stapley, R. Oiwa, and S. Omura. 1979. Avermectins, new family of potent anthelmintic agents: producing organism and fermentation. Antimicrob Agents Chemother 15: 361-7.

Bush, A. O., J. C. Fernandez, G. W. Esch, and J. R. Seed. 2001. Nematoda: the roundworms, pp. 190-192, Parasitism: The diversity and ecology of animal parasites. Cambridge University Press.

Butters, M. P., Kobylinski, K.C., Deus, K.M., Marques da Silva, I., Gray, M., Sylla, M., and Foy, B.D.. submitted. Comparative evaluation of systemic veterinary drugs for their effects against *Anopheles gambiae*. Acta Tropica.

Campanhola, C., and F. W. Plapp. 1989. Toxicity and synergism of insecticides agaisnt susceptible and pyrethroid resistant neonate larvae and adults of the tobacco budworm (Lepidoptera: Noctuidae). J. Econ. Entomol. 82: 1495-1501.

Canales, M., A. Enriquez, E. Ramos, D. Cabrera, H. Dandie, A. Soto, V. Falcon, M. Rodriguez, and J. de la Fuente. 1997. Large-scale production in Pichia pastoris of the recombinant vaccine Gavac against cattle tick. Vaccine 15: 414-22.

Canales, M., V. Naranjo, C. Almazan, R. Molina, S. A. Tsuruta, M. P. Szabo, R. Manzano-Roman, J. M. Perez de la Lastra, K. M. Kocan, M. I. Jimenez, J. Lucientes, M. Villar, and J. de la Fuente. 2009. Conservation and immunogenicity of the mosquito ortholog of the tick-protective antigen, subolesin. Parasitol Res 105: 97-111.

Cartel, J. L., Y. Sechan, A. Spiegel, L. Nguyen, P. Barbazan, P. M. Martin, and J. F. Roux. 1991. Cumulative mortality rates in Aedes polynesiensis after feeding on polynesian Wuchereria bancrofti carriers treated with single doses of ivermectin, diethylcarbamazine and placebo. Trop Med Parasitol 42: 343-5.

Carter, G. T., J. A. Nietsche, M. R. Hertz, D. R. Williams, M. M. Siegel, G. O. Morton, J. C. James, and D. B. Borders. 1988. LL-F28249 antibiotic complex: a new family of antiparasitic macrocyclic lactones. Isolation, characterization and structures of LL-F28249 alpha, beta, gamma, lambda. J Antibiot (Tokyo) 41: 519-29.

Castle, T., M. Amador, S. Rawlins, J. P. Figueroa, and P. Reiter. 1999. Absence of impact of aerial malathion treatment on Aedes aegypti during a dengue outbreak in Kingston, Jamaica. Rev Panam Salud Publica 5: 100-5.

Casu, R., C. Eisemann, R. Pearson, G. Riding, I. East, A. Donaldson, L. Cadogan, and R. Tellam. 1997. Antibody-mediated inhibition of the growth of larvae from an insect causing cutaneous myiasis in a mammalian host. Proc Natl Acad Sci U S A 94: 8939-44.

Chabala, J. C., H. Mrozik, R. L. Tolman, P. Eskola, A. Lusi, L. H. Peterson, M. F. Woods, M. H. Fisher, W. C. Campbell, J. R. Egerton, and D. A. Ostlind. 1980. Ivermectin, a new broad-spectrum antiparasitic agent. J Med Chem 23: 1134-6.

Chaccour, C., J. Lines, and C. J. Whitty. 2010. Effect of ivermectin on Anopheles gambiae mosquitoes fed on humans: the potential of oral insecticides in malaria control. J Infect Dis 202: 113-6.

Chamberlain, W. F. 1982. Evaluation of toxicants against the secondary screwworm. Insectic. Acaric. Tests 7: 255.

Chandre, F., and J. M. Hougard. 1999. [Systemic action of ivermectin on Culex quinquefasciatus and Simulium squamosum]. Bull Soc Pathol Exot 92: 71-2.

Chavasse, D. C., R. J. Post, P. A. Lemoh, and J. A. Whitworth. 1992. The effect of repeated doses of ivermectin on adult female Onchocerca volvulus in Sierra Leone. Trop Med Parasitol 43: 256-62.

Chippaux, J. P., M. Boussinesq, J. Gardon, N. Gardon-Wendel, and J. C. Ernould. 1996. Severe adverse reaction risks during mass treatment with ivermectin in loiasis-endemic areas. Parasitol Today 12: 448-50.

Chlichlia, K., M. Bahgat, V. Schirrmacher, and A. Ruppel. 2002. Species-restricted antibody response against a DNA-construct coding for aspartic proteinase from Schistosoma japonicum. Parasitol Res 88: 368-75.

Cho, W. L., and A. S. Raikhel. 1992. Cloning of cDNA for mosquito lysosomal aspartic protease. Sequence analysis of an insect lysosomal enzyme similar to cathepsins D and E. J Biol Chem 267: 21823-9.

Cho, W. L., T. S. Dhadialla, and A. S. Raikhel. 1991. Purification and characterization of a lysosomal aspartic protase with cathepsin D activity from the mosquito. Insect Biochem Mol Biol 21: 165-176.

Chosidow, O., B. Giraudeau, J. Cottrell, A. Izri, R. Hofmann, S. G. Mann, and I. Burgess. 2010. Oral ivermectin versus malathion lotion for difficult-to-treat head lice. N Engl J Med 362: 896-905.

Clark, D. V., M. P. Mammen, Jr., A. Nisalak, V. Puthimethee, and T. P. Endy. 2005. Economic impact of dengue fever/dengue hemorrhagic fever in Thailand at the family and population levels. Am J Trop Med Hyg 72: 786-91.

Cleland, T. A. 1996. Inhibitory glutamate receptor channels. Mol Neurobiol 13: 97-136.

Cochran, D. G. 1990. Efficacy of abamectin fed to German cockroaches (Dictyoptera: Blattellidae) resistant to pyrethroids. J Econ Entomol 83: 1243-5.

Collins, K. 2004. Profitable gifts: a history of the Merck Mectizan donation program and its implications for international health. Perspect Biol Med 47: 100-9.

Cotreau, M. M., S. Warren, J. L. Ryan, L. Fleckenstein, S. R. Vanapalli, K. R. Brown, D. Rock, C. Y. Chen, and U. S. Schwertschlag. 2003. The antiparasitic moxidectin: safety, tolerability, and pharmacokinetics in humans. J Clin Pharmacol 43: 1108-15.

Coulaud, J. P., M. Lariviere, M. C. Gervais, P. Gaxotte, A. Aziz, A. M. Deluol, and J. Cenac. 1983. [Treatment of human onchocerciasis with ivermectin]. Bull Soc Pathol Exot Filiales 76: 681-8.

Cully, D. F., P. S. Paress, K. K. Liu, J. M. Schaeffer, and J. P. Arena. 1996a. Identification of a Drosophila melanogaster glutamate-gated chloride channel sensitive to the antiparasitic agent avermectin. J Biol Chem 271: 20187-91.

Cully, D. F., H. Wilkinson, D. K. Vassilatis, A. Etter, and J. P. Arena. 1996b. Molecular biology and electrophysiology of glutamate-gated chloride channels of invertebrates. Parasitology 113 Suppl: S191-200.

Cully, D. F., D. K. Vassilatis, K. K. Liu, P. S. Paress, L. H. Van der Ploeg, J. M. Schaeffer, and J. P. Arena. 1994. Cloning of an avermectin-sensitive glutamate-gated chloride channel from Caenorhabditis elegans. Nature 371: 707-11.

Cupp, E. W., A. O. Ochoa, R. C. Collins, F. R. Ramberg, and G. Zea. 1989. The effect of multiple ivermectin treatments on infection of Simulium ochraceum with Onchocerca volvulus. Am J Trop Med Hyg 40: 501-6.

Currie, B. J., P. Harumal, M. McKinnon, and S. F. Walton. 2004. First documentation of in vivo and in vitro ivermectin resistance in Sarcoptes scabiei. Clin Infect Dis 39: e8-12.

de Azambuja, P., J. E. Gomes, F. Lopes, and E. S. Garcia. 1985. Efficacy of ivermectin against the bloodsucking insect, Rhodnius prolixus (Hemiptera, Triatominae). Mem Inst Oswaldo Cruz 80: 439-42.

de la Fuente, J., K. M. Kocan, C. Almazan, and E. F. Blouin. 2008. Targeting the tick-pathogen interface for novel control strategies. Front Biosci 13: 6947-56.

de la Fuente, J., M. Rodriguez, C. Montero, M. Redondo, J. C. Garcia-Garcia, L. Mendez, E. Serrano, M. Valdes, A. Enriquez, M. Canales, E. Ramos, O. Boue, H. Machado, and R. Lleonart. 1999. Vaccination against ticks (Boophilus spp.): the experience with the Bm86-based vaccine Gavac. Genet Anal 15: 143-8.

de la Fuente, J., M. Rodriguez, M. Redondo, C. Montero, J. C. Garcia-Garcia, L. Mendez, E. Serrano, M. Valdes, A. Enriquez, M. Canales, E. Ramos, O. Boue, H. Machado, R. Lleonart, C. A. de Armas, S. Rey, J. L. Rodriguez, M. Artiles, and L. Garcia. 1998. Field studies and cost-effectiveness analysis of vaccination with Gavac against the cattle tick Boophilus microplus. Vaccine 16: 366-73.

De Sole, G., K. Awadzi, J. Remme, K. Y. Dadzie, O. Ba, J. Giese, M. Karam, F. M. Keita, and N. O. Opoku. 1989a. A community trial of ivermectin in the onchocerciasis focus of Asubende, Ghana. II. Adverse reactions. Trop Med Parasitol 40: 375-82.

De Sole, G., J. Remme, K. Awadzi, S. Accorsi, E. S. Alley, O. Ba, K. Y. Dadzie, J. Giese, M. Karam, and F. M. Keita. 1989b. Adverse reactions after large-scale treatment of onchocerciasis with ivermectin: combined results from eight community trials. Bull World Health Organ 67: 707-19.

Dent, J. A., M. M. Smith, D. K. Vassilatis, and L. Avery. 2000. The genetics of ivermectin resistance in Caenorhabditis elegans. Proc Natl Acad Sci U S A 97: 2674-9.

Dollery, C. T. 1999. Albendazole, pp. A51-A53, Therapeutic Drugs. Churchill Livingstone, Edingurg.

Dreyer, G., M. L. Pires, L. D. de Andrade, E. Lopes, Z. Medeiros, J. Tenorio, A. Coutinho, J. Noroes, and J. Figueredo-Silva. 1994. Tolerance of diethylcarbamazine by microfilaraemic and amicrofilaraemic individuals in an endemic area of Bancroftian filariasis, Recife, Brazil. Trans R Soc Trop Med Hyg 88: 232-6.

Dukuly, Z. D., M. Pacque, A. Nara, H. R. Taylor, P. N. Williams, and B. M. Greene. 1990. A prospective study in high risk subjects of electrocardiographic changes with ivermectin. Trop Med Parasitol 41: 73-4.

Dunyo, S. K., F. K. Nkrumah, and P. E. Simonsen. 2000a. A randomized double-blind placebo-controlled field trial of ivermectin and albendazole alone and in combination for the treatment of lymphatic filariasis in Ghana. Trans R Soc Trop Med Hyg 94: 205-11.

Dunyo, S. K., F. K. Nkrumah, and P. E. Simonsen. 2000b. Single-dose treatment of Wuchereria bancrofti infections with ivermectin and albendazole alone or in combination: evaluation of the potential for control at 12 months after treatment. Trans R Soc Trop Med Hyg 94: 437-43.

Edwards, G., A. Dingsdale, N. Helsby, M. L. Orme, and A. M. Breckenridge. 1988. The relative systemic availability of ivermectin after administration as capsule, tablet, and oral solution. Eur J Clin Pharmacol 35: 681-4.

Egerton, J. R., D. A. Ostlind, L. S. Blair, C. H. Eary, D. Suhayda, S. Cifelli, R. F. Riek, and W. C. Campbell. 1979. Avermectins, new family of potent

anthelmintic agents: efficacy of the B1a component. Antimicrob Agents Chemother 15: 372-8.

Egerton, J. R., J. Birnbaum, L. S. Blair, J. C. Chabala, J. Conroy, M. H. Fisher, H. Mrozik, D. A. Ostlind, C. A. Wilkins, and W. C. Campbell. 1980. 22, 23--dihydroavermectin B1, a new broad-spectrum antiparasitic agent. Br Vet J 136: 88-97.

Ekenna, O., J. O. Chikwem, I. Mohammed, and S. O. Durojaiye. 2010. Epidemic yellow fever in Borno State of Nigeria: characterisation of hospitalised patients. West Afr J Med 29: 91-7.

Elkassaby, M. H. 1991. Ivermectin uptake and distribution in the plasma and tissue of Sudanese and Mexican patients infected with Onchocerca volvulus. Trop Med Parasitol 42: 79-81.

Falconer, D. S., Makay, T.F.C. 1996. Selection: The response and its prediction, pp. 184-204, Introduction to Quantitative Genetics. Addison Wesley Longman Limited, Essex, England.

Fernandes, L., and H. Briegel. 2005. Reproductive physiology of Anopheles gambiae and Anopheles atroparvus. J Vector Ecol 30: 11-26.

Fine, P. E. M. 1981. Epidemiological principles of vector-mediated transmission. *In* B. F. E. J.J. McKelvey, and K. Maramorosch [ed.], Vectors of Disease Agents. Prager Scientific, New York.

Flores, A. E., J. S. Grajales, I. F. Salas, G. P. Garcia, M. H. Becerra, S. Lozano, W. G. Brogdon, W. C. t. Black, and B. Beaty. 2006. Mechanisms of insecticide resistance in field populations of Aedes aegypti (L.) from Quintana Roo, Southern Mexico. J Am Mosq Control Assoc 22: 672-7.

Focks, D. A. 1991. Impact of minor reductions in adult and larval survival, fecundity and hatch on the population dynamics of Psorophora columbiae: a simulation study. J Am Mosq Control Assoc 7: 476-80.

Focks, D. A., R. E. McLaughlin, and S. B. Linda. 1991. Effects of ivermectin (MK-933) on the reproductive rate of Aedes aegypti (Diptera: Culicidae). J Med Entomol 28: 501-5.

Focks, D. A., E. Daniels, D. G. Haile, and J. E. Keesling. 1995. A simulation model of the epidemiology of urban dengue fever: literature analysis, model development, preliminary validation, and samples of simulation results. Am J Trop Med Hyg 53: 489-506.

Foley, D. H., J. H. Bryan, and G. W. Lawrence. 2000. The potential of ivermectin to control the malaria vector Anopheles farauti. Trans R Soc Trop Med Hyg 94: 625-8.

Forrester, J. E., J. C. Bailar, 3rd, S. A. Esrey, M. V. Jose, B. T. Castillejos, and G. Ocampo. 1998. Randomised trial of albendazole and pyrantel in symptomless trichuriasis in children. Lancet 352: 1103-8.

Forrester, S. G., R. K. Prichard, and R. N. Beech. 2002. A glutamate-gated chloride channel subunit from Haemonchus contortus: expression in a mammalian cell line, ligand binding, and modulation of anthelmintic binding by glutamate. Biochem Pharmacol 63: 1061-8.

Foy, B. D. 2005- personal communication. Cloning of the *Aedes aegypti* lysosomal aspartic protease into the pCDNA3.1(+) plasmid.

Foy, B. D., G. F. Killeen, T. Magalhaes, and J. C. Beier. 2002. Immunological targeting of critical insect antigens. Am Entomol 48: 150-163.

Foy, B. D., K. C. Kobylinski, I. M. Silva, J. L. Rasgon, and M. Sylla. 2011. Endectocides for malaria control. Trends Parasitol.

Foy, B. D., K. M. Myles, D. J. Pierro, I. Sanchez-Vargas, M. Uhlirova, M. Jindra, B. J. Beaty, and K. E. Olson. 2004. Development of a new Sindbis virus transducing system and its characterization in three Culicine mosquitoes and two Lepidopteran species. Insect Mol Biol 13: 89-100.

Foy, B. D., T. Magalhaes, W. E. Injera, I. Sutherland, M. Devenport, A. Thanawastien, D. Ripley, L. Cardenas-Freytag, and J. C. Beier. 2003. Induction of mosquitocidal activity in mice immunized with Anopheles gambiae midgut cDNA. Infect Immun 71: 2032-40.

Foy, B. D., Kobylinski, K.C., Marques da Silva, I., Rasgon, J.L., Sylla, M. 2011. Endectocides for malaria control. Trends Parasitol article in press.

Fritz, M. L., P. Y. Siegert, E. D. Walker, M. N. Bayoh, J. R. Vulule, and J. R. Miller. 2009. Toxicity of bloodmeals from ivermectin-treated cattle to Anopheles gambiae s.l. Ann Trop Med Parasitol 103: 539-47.

Garcia, G. P., A. E. Flores, I. Fernandez-Salas, K. Saavedra-Rodriguez, G. Reyes-Solis, S. Lozano-Fuentes, J. Guillermo Bond, M. Casas-Martinez, J. M. Ramsey, J. Garcia-Rejon, M. Dominguez-Galera, H. Ranson, J. Hemingway, L. Eisen, and I. W. Black. 2009. Recent rapid rise of a permethrin knock down resistance allele in Aedes aegypti in Mexico. PLoS Negl Trop Dis 3: e531.

Gardner, K., M. V. Meisch, C. L. Meek, and W. S. Biven. 1993. Effects of ivermectin in canine blood on Anopheles quadrimaculatus, Aedes albopictus and Culex salinarius. J Am Mosq Control Assoc 9: 400-2.

Gardon, J., N. Gardon-Wendel, N. Demanga, J. Kamgno, J. P. Chippaux, and M. Boussinesq. 1997. Serious reactions after mass treatment of onchocerciasis with ivermectin in an area endemic for Loa loa infection. Lancet 350: 18-22.

Garrett-Jones, C. 1964. Prognosis for Interruption of Malaria Transmission through Assessment of the Mosquito's Vectorial Capacity. Nature 204: 1173-5.

Geden, C. J., D. A. Rutz, J. G. Scott, and S. J. Long. 1992. Susceptibility of house flies (Diptera: Muscidae) and five pupal parasitoids (Hymenoptera: Pteromalidae) to abamectin and seven commercial insecticides. J Econ Entomol 85: 435-40.

Georghiou, G. P. 1969. Parasitological review. Genetics of resistance to insecticides in houseflies and mosquitoes. Exp Parasitol 26: 224-55.

Gillett, J. D., E. A. Roman, and V. Phillips. 1977. Erratic hatching in Aedes eggs: a new interpretation. Proc R Soc Lond B Biol Sci 196: 223-32.

Gould, L. H., M. S. Osman, E. C. Farnon, K. S. Griffith, M. S. Godsey, S. Karch, B. Mulenda, A. El Kholy, F. Grandesso, X. de Radigues, M. E. Brair, S. Briand, S. M. El Tayeb el, E. B. Hayes, H. Zeller, and W. Perea. 2008. An outbreak of yellow fever with concurrent chikungunya virus transmission in South Kordofan, Sudan, 2005. Trans R Soc Trop Med Hyg 102: 1247-54.

Graham, D., F. Pfeiffer, and H. Betz. 1982. Avermectin B1a inhibits the binding of strychnine to the glycine receptor of rat spinal cord. Neurosci Lett 29: 173-6.

Gubler, D. J. 1998. Dengue and dengue hemorrhagic fever. Clin Microbiol Rev 11: 480-96.

Gubler, D. J. 2002. The global emergence/resurgence of arboviral diseases as public health problems. Arch Med Res 33: 330-42.

Gubler, D. J. 2004. The changing epidemiology of yellow fever and dengue, 1900 to 2003: full circle? Comp Immunol Microbiol Infect Dis 27: 319-30.

Gubler, D. J., and M. Meltzer. 1999. Impact of dengue/dengue hemorrhagic fever on the developing world. Adv Virus Res 53: 35-70.

Guzman, M. G., S. B. Halstead, H. Artsob, P. Buchy, J. Farrar, D. J. Gubler, E. Hunsperger, A. Kroeger, H. S. Margolis, E. Martinez, M. B. Nathan, J. L. Pelegrino, C. Simmons, S. Yoksan, and R. W. Peeling. 2010. Dengue: a continuing global threat. Nat Rev Microbiol 8: S7-16.

Gyapong, J. O., V. Kumaraswami, G. Biswas, and E. A. Ottesen. 2005. Treatment strategies underpinning the global programme to eliminate lymphatic filariasis. Expert Opin Pharmacother 6: 179-200.

Habbema, J. D., E. S. Alley, A. P. Plaisier, G. J. van Oortmarssen, and J. H. Remme. 1992. Epidemiological modelling for onchocerciasis control. Parasitol Today 8: 99-103.

Harrington, L. C., J. D. Edman, and T. W. Scott. 2001. Why do female Aedes aegypti (Diptera: Culicidae) feed preferentially and frequently on human blood? J Med Entomol 38: 411-22.

Hatfield, P. R. 1988. Anti-mosquito antibodies and their effects on feeding, fecundity and mortality of Aedes aegypti. Med Vet Entomol 2: 331-338.

Hawking, F., P. Sewell, and J. P. Thurston. 1948. Mode of action of hetrazan in filariasis. Lancet 2: 730.

Helmy, H., G. J. Weil, A. S. Ellethy, E. S. Ahmed, M. E. Setouhy, and R. M. Ramzy. 2006. Bancroftian filariasis: effect of repeated treatment with diethylcarbamazine and albendazole on microfilaraemia, antigenaemia and antifilarial antibodies. Trans R Soc Trop Med Hyg 100: 656-62.

Hibbs, R. E., and E. Gouaux. 2011. Principles of activation and permeation in an anion-selective Cys-loop receptor. Nature 474: 54-60.

Holden-Dye, L., and R. J. Walker. 1990. Avermectin and avermectin derivatives are antagonists at the 4-aminobutyric acid (GABA) receptor on the somatic muscle cells of Ascaris; is this the site of anthelmintic action? Parasitology 101 Pt 2: 265-71.

Hooper, P. J., M. H. Bradley, G. Biswas, and E. A. Ottesen. 2009. The Global Programme to Eliminate Lymphatic Filariasis: health impact during its first 8 years (2000-2007). Ann Trop Med Parasitol 103 Suppl 1: S17-21.

Hotez, P. J. 2007. Control of onchocerciasis--the next generation. Lancet 369: 1979-80.

Hotez, P. J., D. H. Molyneux, A. Fenwick, J. Kumaresan, S. E. Sachs, J. D. Sachs, and L. Savioli. 2007. Control of neglected tropical diseases. N Engl J Med 357: 1018-27.

Hotez, P. J., J. Ashcom, Z. Bin, J. Bethony, A. Williamson, J. M. Hawdon, F. Jianjun, A. Dobardzic, I. Rizo, J. Bolden, Q. Jin, W. Yan, R. Dobardzic, S. Chung-Debose, M. Crowell, B. Datu, A. Delaney, D. Dragonovski, Y. Jiang, L. Yueyuan, K. Ghosh, A. Loukas, W. Brandt, P. K. Russell, and B. C. Zook. 2002. Effect of vaccinations with recombinant fusion proteins on Ancylostoma caninum habitat selection in the canine intestine. J Parasitol 88: 684-90.

Huffam, S. E., and B. J. Currie. 1998. Ivermectin for Sarcoptes scabiei hyperinfestation. Int J Infect Dis 2: 152-4.

Hughes, P. B., and G. W. Levot. 1990. Toxicity of three avermectins to insecticide susceptible and resistant larvae of *Lucillia cuprina* (Wiedemann) (Diptera: Calliphoridae). J. Austral. Entomol. Soc. 29: 109-111.

Iakubovich, V., N. F. Zakharova, A. N. Alekseev, and E. A. Alekseev. 1989a. [Evaluation of the action of ivermectin on blood-sucking mosquitoes]. Med Parazitol (Mosk): 60-4.

Iakubovich, V. I., N. F. Zakharova, A. N. Alekseev, and E. A. Alekseev. 1989b. Evaluation of ivermectin on blood-sucking mosquitoes. Medicinskaya Parazitologia Moskva 3: 60-4.

James, C. E., and M. W. Davey. 2009. Increased expression of ABC transport proteins is associated with ivermectin resistance in the model nematode Caenorhabditis elegans. Int J Parasitol 39: 213-20.

Jirakanjanakit, N., S. Saengtharatip, P. Rongnoparut, S. Duchon, C. Bellec, and S. Yoksan. 2007a. Trend of temephos resistance in Aedes (Stegomyia) mosquitoes in Thailand during 2003-2005. Environ Entomol 36: 506-11.

Jirakanjanakit, N., P. Rongnoparut, S. Saengtharatip, T. Chareonviriyaphap, S. Duchon, C. Bellec, and S. Yoksan. 2007b. Insecticide susceptible/resistance status in Aedes (Stegomyia) aegypti and Aedes (Stegomyia) albopictus (Diptera: Culicidae) in Thailand during 2003-2005. J Econ Entomol 100: 545-50.

Johnston, L. A., D. H. Kemp, and R. D. Pearson. 1986. Immunization of cattle against Boophilus microplus using extracts derived from adult female ticks: effects of induced immunity on tick populations. Int J Parasitol 16: 27-34.

Jones, J. W., M. V. Meisch, C. L. Meek, and W. S. Bivin. 1992. Lethal effects of ivermectin on Anopheles quadrimaculatus. J Am Mosq Control Assoc 8: 278-80.

Jonsson, N. N., A. L. Matschoss, P. Pepper, P. E. Green, M. S. Albrecht, J. Hungerford, and J. Ansell. 2000. Evaluation of tickGARD(PLUS), a novel vaccine against Boophilus microplus, in lactating Holstein-Friesian cows. Vet Parasitol 88: 275-85.

Jupp, P. G., and B. M. McIntosh. 1988. Chikungunya virus disease. *In* T. P. Monath [ed.], The arboviruses: epidemiology and ecology. CRC Press, Boca Raton, FL.

Kale, O. O. 1977. A comparative trial of the anthelminthic efficacy of pyrantel pamoate (Combantrin) and thiabendazole (Mintezol). Afr J Med Med Sci 6: 89-93.

Kamgno, J., J. Gardon, and M. Boussinesq. 2000. [Analysis of the prevention of post-ivermectin Loa loa encephalopathy by administration of initial low dose]. Med Trop (Mars) 60: 275-7.

Kamgno, J., S. D. Pion, C. D. Mackenzie, B. Thylefors, and M. Boussinesq. 2009. Loa loa microfilarial periodicity in ivermectin-treated patients: comparison between those developing and those free of serious adverse events. Am J Trop Med Hyg 81: 1056-61.

Kane, N. S., B. Hirschberg, S. Qian, D. Hunt, B. Thomas, R. Brochu, S. W. Ludmerer, Y. Zheng, M. Smith, J. P. Arena, C. J. Cohen, D. Schmatz, J. Warmke, and D. F. Cully. 2000. Drug-resistant Drosophila indicate glutamate-gated chloride channels are targets for the antiparasitics nodulisporic acid and ivermectin. Proc Natl Acad Sci U S A 97: 13949-54.

Kariuki Njenga, M., L. Nderitu, J. P. Ledermann, A. Ndirangu, C. H. Logue, C. H. Kelly, R. Sang, K. Sergon, R. Breiman, and A. M. Powers. 2008. Tracking epidemic Chikungunya virus into the Indian Ocean from East Africa. J Gen Virol 89: 2754-60.

Kass, I. S., C. C. Wang, J. P. Walrond, and A. O. Stretton. 1980. Avermectin B1a, a paralyzing anthelmintic that affects interneurons and inhibitory motoneurons in Ascaris. Proc Natl Acad Sci U S A 77: 6211-5.

Keiser, P. B., S. M. Reynolds, K. Awadzi, E. A. Ottesen, M. J. Taylor, and T. B. Nutman. 2002. Bacterial endosymbionts of Onchocerca volvulus in the pathogenesis of posttreatment reactions. J Infect Dis 185: 805-11.

Kemp, D. H., R. I. Agbede, L. A. Johnston, and J. M. Gough. 1986. Immunization of cattle against Boophilus microplus using extracts derived from adult female ticks: feeding and survival of the parasite on vaccinated cattle. Int J Parasitol 16: 115-20.

Kerboeuf, D., F. Guegnard, and Y. L. Vern. 2003a. Detection of P-glycoprotein-mediated multidrug resistance against anthelmintics in Haemonchus contortus using anti-human mdr1 monoclonal antibodies. Parasitol Res 91: 79-85.

Kerboeuf, D., W. Blackhall, R. Kaminsky, and G. von Samson-Himmelstjerna. 2003b. P-glycoprotein in helminths: function and perspectives for anthelmintic treatment and reversal of resistance. Int J Antimicrob Agents 22: 332-46.

Kipp, W., J. Bamhuhiiga, T. Rubaale, and D. W. Buttner. 2003. Adverse reactions to ivermectin treatment in Simulium neavei-transmitted onchocerciasis. Am J Trop Med Hyg 69: 621-3.

Klafke, G. M., T. A. de Albuquerque, R. J. Miller, and T. T. Schumaker. 2010. Selection of an ivermectin-resistant strain of Rhipicephalus microplus (Acari: Ixodidae) in Brazil. Vet Parasitol 168: 97-104.

Klager, S. L., J. A. Whitworth, and M. D. Downham. 1996. Viability and fertility of adult Onchocerca volvulus after 6 years of treatment with ivermectin. Trop Med Int Health 1: 581-9.

Klei, T. R., and B. J. Torbert. 1980. Efficacy of ivermectin (22,23-dihydroavermectin B1) against gastro-intestinal parasites in ponies. Am. J. Vet. Res. 41: 1747-1750.

Kobylinski, K. C., M. Sylla, P. L. Chapman, M. D. Sarr, and B. D. Foy. 2011. Ivermectin mass drug administration to humans disrupts malaria parasite transmission in senegalese villages. Am J Trop Med Hyg 85: 3-5.

Kobylinski, K. C., K. M. Deus, M. P. Butters, T. Hongyu, M. Gray, I. M. da Silva, M. Sylla, and B. D. Foy. 2010. The effect of oral anthelmintics on the survivorship and re-feeding frequency of anthropophilic mosquito disease vectors. Acta Trop 116: 119-26.

Kobylinski, K. C., Sylla, M., Chapman, P., Sarr, M.D., Foy, B.D. 2011. Short Report: Ivermectin Mass Drug Administration to Humans Disrupts Malaria Parasite Transmission in Senegalese Villages. American Journal of Tropical Medicine and Hygeine article in press.

Kouri, G. P., M. G. Guzman, J. R. Bravo, and C. Triana. 1989. Dengue haemorrhagic fever/dengue shock syndrome: lessons from the Cuban epidemic, 1981. Bull World Health Organ 67: 375-80.

Krautmann, M. J., M. J. Novotny, K. De Keulenaer, C. S. Godin, E. I. Evans, J. W. McCall, C. Wang, T. G. Rowan, and A. D. Jernigan. 2000. Safety of selamectin in cats. Vet Parasitol 91: 393-403.

Labuda, M., A. R. Trimnell, M. Lickova, M. Kazimirova, G. M. Davies, O. Lissina, R. S. Hails, and P. A. Nuttall. 2006. An antivector vaccine protects against a lethal vector-borne pathogen. PLoS Pathog 2: e27.

Lal, A. A., P. S. Patterson, J. B. Sacci, J. A. Vaughan, C. Paul, W. E. Collins, R. A. Wirtz, and A. F. Azad. 2001. Anti-mosquito midgut antibodies block development of Plasmodium falciparum and Plasmodium vivax in multiple species of Anopheles mosquitoes and reduce vector fecundity and survivorship. Proc Natl Acad Sci U S A 98: 5228-33.

Langley, P. A., and J. M. Roe. 1984. Ivermectin as a possible control agent for tsetse fly, *Glossina morsitans.* Entomol. Exp Appl 36: 137-143.

Lawrence, G., J. Leafasia, J. Sheridan, S. Hills, J. Wate, C. Wate, J. Montgomery, N. Pandeya, and D. Purdie. 2005. Control of scabies, skin sores and haematuria in children in the Solomon Islands: another role for ivermectin. Bull World Health Organ 83: 34-42.

Le Jambre, L. F., J. H. Gill, I. J. Lenane, and P. Baker. 2000. Inheritance of avermectin resistance in Haemonchus contortus. Int J Parasitol 30: 105-11.

Leontsini, E., E. Gil, C. Kendall, and G. G. Clark. 1993. Effect of a community-based Aedes aegypti control programme on mosquito larval production sites in El Progreso, Honduras. Trans R Soc Trop Med Hyg 87: 267-71.

Lloyd, L. S. 2003. Best practices for dengue prevention and control in the Americas, pp. 71, Environmental Health Project/UnitedStates Agency for International Development (USAID).

Lloyd, L. S., P. Winch, J. Ortega-Canto, and C. Kendall. 1994. The design of a community-based health education intervention for the control of Aedes aegypti. Am J Trop Med Hyg 50: 401-11.

Mackenzie, C. D., T. G. Geary, and J. A. Gerlach. 2003. Possible pathogenic pathways in the adverse clinical events seen following ivermectin administration to onchocerciasis patients. Filaria J 2 Suppl 1: S5.

Mackenzie, J. S., D. J. Gubler, and L. R. Petersen. 2004. Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. Nat Med 10: S98-109.

Mahmood, F., L. L. Walters, H. Guzman, and R. B. Tesh. 1991. Effect of ivermectin on the ovarian development of Aedes aegypti (Diptera: Culicidae). J Med Entomol 28: 701-7.

Marquardt, W. C., R. S. Demaree, and R. B. Grieve. 2000. Filarioid and other spirurid nematodes, pp. 453-471, Parasitology and Vector Biology. Academic Press, San Diego, CA.

Martin, R. J. 1997. Modes of action of anthelmintic drugs. Vet J 154: 11-34.

Mathew, N., and Kalyanasundaram, M. 2007. Antifilarial agents. Expert Opin. Ther. Pat 17: 767-789.

McGarry, H. F., L. D. Plant, and M. J. Taylor. 2005. Diethylcarbamazine activity against Brugia malayi microfilariae is dependent on inducible nitric-oxide synthase and the cyclooxygenase pathway. Filaria J 4: 4.

McIntire, S. L., E. Jorgensen, and H. R. Horvitz. 1993. Genes required for GABA function in Caenorhabditis elegans. Nature 364: 334-7.

Miller, J. A., D. D. Oehler, A. J. Siebernaler, and S. E. Kunz. 1986. Effect of ivermectin on survival and fecundity of horn flies and stable flies (Diptera: Muscidae). J. Econ. Entomol. 79: 1564-1569.

Minarowska, A., A. Karwowska, and M. Gacko. 2009. Quantitative determination and localization of cathepsin D and its inhibitors. Folia Histochem Cytobiol 47: 153-77.

Monath, T. P., and F. X. Heinz. 1996. Flaviviruses, pp. 961-1034. *In* D. M. K. B.N. Fields, P.M. Howley [ed.], Fields Virology. Lippincott-Raven, Philadelphia.

Morales, M. E., G. Rinaldi, G. N. Gobert, K. J. Kines, J. F. Tort, and P. J. Brindley. 2008. RNA interference of Schistosoma mansoni cathepsin D, the apical enzyme of the hemoglobin proteolysis cascade. Mol Biochem Parasitol 157: 160-8.

Myles, K. M., D. J. Pierro, and K. E. Olson. 2003. Deletions in the putative cell receptor-binding domain of Sindbis virus strain MRE16 E2 glycoprotein reduce midgut infectivity in Aedes aegypti. J Virol 77: 8872-81.

Nakagawa, K., Y. Tsukamoto, K. Sato, and A. Torikata. 1995. Microbial conversion of milbemycins: oxidation of milbemycin A4 and related compounds at the C-25 ethyl group by Circinella umbellata and Absidia cylindrospora. J Antibiot (Tokyo) 48: 831-7.

Njue, A. I., J. Hayashi, L. Kinne, X. P. Feng, and R. K. Prichard. 2004. Mutations in the extracellular domains of glutamate-gated chloride channel alpha3 and beta subunits from ivermectin-resistant Cooperia oncophora affect agonist sensitivity. J Neurochem 89: 1137-47.

Noden, B. H., J. A. Vaughan, M. S. Imbrahim, and J. C. Beier. 1995. An immunological factor that effects Anopheles gambiae survival. J Amer Mosquito Contr Assn. 11: 45-49.

Novotny, M. J., M. J. Krautmann, J. C. Ehrhart, C. S. Godin, E. I. Evans, J. W. McCall, F. Sun, T. G. Rowan, and A. D. Jernigan. 2000. Safety of selamectin in dogs. Vet Parasitol 91: 377-91.

Olsen, R. W., and A. J. Tobin. 1990. Molecular biology of GABAA receptors. Faseb J 4: 1469-80.

Olson, K. E., K. M. Myles, R. C. Seabaugh, S. Higgs, J. O. Carlson, and B. J. Beaty. 2000. Development of a Sindbis virus expression system that efficiently expresses green fluorescent protein in midguts of Aedes aegypti following per os infection. Insect Mol Biol 9: 57-65.

Osei-Atweneboana, M. Y., J. K. Eng, D. A. Boakye, J. O. Gyapong, and R. K. Prichard. 2007. Prevalence and intensity of Onchocerca volvulus infection and efficacy of ivermectin in endemic communities in Ghana: a two-phase epidemiological study. Lancet 369: 2021-9.

Osei-Atweneboana, M. Y., K. Awadzi, S. K. Attah, D. A. Boakye, J. O. Gyapong, and R. K. Prichard. Phenotypic evidence of emerging ivermectin resistance in Onchocerca volvulus. PLoS Negl Trop Dis 5: e998.

Osei-Atweneboana, M. Y., K. Awadzi, S. K. Attah, D. A. Boakye, J. O. Gyapong, and R. K. Prichard. 2011. Phenotypic evidence of emerging ivermectin resistance in Onchocerca volvulus. PLoS Negl Trop Dis 5: e998.

Ostlind, D. A., S. Cifelli, and R. Lang. 1979. Insecticidal activity of the anti-parasitic avermectins. Vet Rec 105: 168.

Ottesen, E. A., M. M. Ismail, and J. Horton. 1999. The role of albendazole in programmes to eliminate lymphatic filariasis. Parasitol Today 15: 382-6.

Ottesen, E. A., B. O. Duke, M. Karam, and K. Behbehani. 1997. Strategies and tools for the control/elimination of lymphatic filariasis. Bull World Health Organ 75: 491-503.

Ottesen, E. A., P. J. Hooper, M. Bradley, and G. Biswas. 2008. The global programme to eliminate lymphatic filariasis: health impact after 8 years. PLoS Negl Trop Dis 2: e317.

Pacque, M., B. Munoz, G. Poetschke, J. Foose, B. M. Greene, and H. R. Taylor. 1990a. Pregnancy outcome after inadvertent ivermectin treatment during community-based distribution. Lancet 336: 1486-9.

Pacque, M., B. Munoz, B. M. Greene, A. T. White, Z. Dukuly, and H. R. Taylor. 1990b. Safety of and compliance with community-based ivermectin therapy. Lancet 335: 1377-80.

Pacque, M. C., Z. Dukuly, B. M. Greene, B. Munoz, E. Keyvan-Larijani, P. N. Williams, and H. R. Taylor. 1989. Community-based treatment of onchocerciasis with ivermectin: acceptability and early adverse reactions. Bull World Health Organ 67: 721-30.

Paiement, J., R. K. Prichard, and P. Ribeiro. 1999. Haemonchus contortus: characterization of a glutamate binding site in unselected and ivermectin-selected larvae and adults. Exp Parasitol 92: 32-9.

Pampiglioni, S., G. Majori, G. Petrangeli, and R. Romi. 1985. Avermectins, MK-933 and MK-936, for mosquito control. Trans R Soc Trop Med Hyg 79: 797-9.

Paul, A. J., W. J. Tranquilli, R. L. Seward, K. S. Todd, Jr., and J. A. DiPietro. 1987. Clinical observations in collies given ivermectin orally. Am J Vet Res 48: 684-5.

Pennington, J., and M. Wells. 2005. The Adult Midgut: Structure and Function, pp. 289-295. *In* W. Marquardt [ed.], Biology of Disease Vectors, 2nd ed. Elsevier Academic Press.

Pethuan, S., N. Jirakanjanakit, S. Saengtharatip, T. Chareonviriyaphap, D. Kaewpa, and P. Rongnoparut. 2007. Biochemical studies of insecticide resistance in Aedes (Stegomyia) aegypti and Aedes (Stegomyia) albopictus (Diptera: Culicidae) in Thailand. Trop Biomed 24: 7-15.

Pipano, E., E. Alekceev, F. Galker, L. Fish, M. Samish, and V. Shkap. 2003. Immunity against Boophilus annulatus induced by the Bm86 (Tick-GARD) vaccine. Exp Appl Acarol 29: 141-9.

Plaisier, A. P., E. S. Alley, B. A. Boatin, G. J. Van Oortmarssen, H. Remme, S. J. De Vlas, L. Bonneux, and J. D. Habbema. 1995. Irreversible effects of ivermectin on adult parasites in onchocerciasis patients in the Onchocerciasis Control Programme in West Africa. J Infect Dis 172: 204-10.

Pourgholami, M. H., M. Szwajcer, M. Chin, W. Liauw, J. Seef, P. Galettis, D. L. Morris, and M. Links. 1998. Phase I clinical trial to determine maximum tolerated dose of oral albendazole in patients with advanced cancer. Cancer Chemother Pharmacol 65: 597-605.

Powers, A. M., and C. H. Logue. 2007. Changing patterns of chikungunya virus: re-emergence of a zoonotic arbovirus. J Gen Virol 88: 2363-77.

Ramasamy, M. S., R. Ramasamy, B. H. Kay, and C. Kidson. 1988. Anti-mosquito antibodies decrease the reproductive capacity of Aedes aegypti. Med Vet Entomol. 2: 87-93.

Ramasamy, M. S., L. Raschid, K. A. Srikrishnaraj, and R. Ramasamy. 1996. Antimidgut antibodies inhibit peritrophic membrane formation in the posterior midgut of Anopheles tessellatus (Diptera: Culicidae). J Med Entomol 33: 162-4.

Ramasamy, M. S., K. A. Srikrishnaraj, S. Wijekoone, L. S. Jesuthasan, and R. Ramasamy. 1992. Host immunity to mosquitoes: effect of antimosquito antibodies on Anopheles tessellatus and Culex quinquefasciatus (Diptera: Culicidae). J Med Entomol 29: 934-8.

Ramzy, R. M., M. El Setouhy, H. Helmy, E. S. Ahmed, K. M. Abd Elaziz, H. A. Farid, W. D. Shannon, and G. J. Weil. 2006. Effect of yearly mass drug administration with diethylcarbamazine and albendazole on bancroftian filariasis in Egypt: a comprehensive assessment. Lancet 367: 992-9.

Reddy, M., S. S. Gill, S. R. Kalkar, W. Wu, P. J. Anderson, and P. A. Rochon. 2007. Oral drug therapy for multiple neglected tropical diseases: a systematic review. Jama 298: 1911-24.

Rezza, G., L. Nicoletti, R. Angelini, R. Romi, A. C. Finarelli, M. Panning, P. Cordioli, C. Fortuna, S. Boros, F. Magurano, G. Silvi, P. Angelini, M. Dottori, M. G. Ciufolini, G. C. Majori, and A. Cassone. 2007. Infection with chikungunya virus in Italy: an outbreak in a temperate region. Lancet 370: 1840-6.

Rigau-Perez, J. G., D. J. Gubler, G. G. Clark, P. Reiter, E. J. Sanders, and M. Valdes. 1998. Dengue and dengue hemorrhagic fever. Clin Microbiol Rev 11: 480-96.

Robinson, M. C. 1955. An epidemic of virus disease in Southern Province, Tanganyika Territory, in 1952-53. I. Clinical features. Trans R Soc Trop Med Hyg 49: 28-32.

Robles, R. 1917. Enfermedad nueva en Guatemala. La Juventud Medica 17: 97-115.

Rodriguez, M., C. L. Massard, A. H. da Fonseca, N. F. Ramos, H. Machado, V. Labarta, and J. de la Fuente. 1995a. Effect of vaccination with a recombinant Bm86 antigen preparation on natural infestations of Boophilus microplus in grazing dairy and beef pure and cross-bred cattle in Brazil. Vaccine 13: 1804-8.

Rodriguez, M., M. L. Penichet, A. E. Mouris, V. Labarta, L. L. Luaces, R. Rubiera, C. Cordoves, P. A. Sanchez, E. Ramos, A. Soto, and et al. 1995b. Control of Boophilus microplus populations in grazing cattle vaccinated with a recombinant Bm86 antigen preparation. Vet Parasitol 57: 339-49.

Roncalli, R. A. 1984. Efficacy of ivermectin against *Oestrus ovis* in sheep. Vet Med Small Anim. Clinic. 79: 1095-1097.

Rosenbaum, J., M. B. Nathan, R. Ragoonanansingh, S. Rawlins, C. Gayle, D. D. Chadee, and L. S. Lloyd. 1995. Community participation in dengue prevention and control: a survey of knowledge, attitudes, and practice in Trinidad and Tobago. Am J Trop Med Hyg 53: 111-7.

Roush, R. T., and J. E. Wright. 1986a. Abamectin toxicity to organic insecticides. J. Econ. Entomol. 79: 562-564.

Roush, R. T., and J. E. Wright. 1986b. Abamectin: toxicity to house flies (Diptera: Muscidae) resistant to synthetic organic insecticides. J Econ Entomol 79: 562-4.

Rugg, D., N. Sales, A. C. Kotze, and G. W. Levot. 1995. Susceptibility to ivermectin of pyrethroid-resistant *Lucilia cuprina* (Wiedmann) (Diptera: Calliphoridae). J. Austral. Entomol. Soc. 34: 69-70.

Rugg, D., A. C. Kotze, D. R. Thompson, and H. A. Rose. 1998. Susceptibility of laboratory-selected and field strains of *Lucilia cuprina* (Diptera: Calliphoridae) to ivermectin. J. Econ. Entomol. 91: 601-607.

Rugg, D., Kotze, A.C., Thompson, D.R., Rose, H.A. 1998. Susceptibility of laboratory-selected and field strains of the *Lucillia cuprina* (Diptera: Calliphoridae) to ivermectin. J Econ Entomol 91: 601-607

Saavedra-Rodriguez, K., C. Strode, A. Flores Suarez, I. Fernandez Salas, H. Ranson, J. Hemingway, and W. C. t. Black. 2008. Quantitative trait loci mapping of genome regions controlling permethrin resistance in the mosquito Aedes aegypti. Genetics 180: 1137-52.

Saavedra-Rodriguez, K., L. Urdaneta-Marquez, S. Rajatileka, M. Moulton, A. E. Flores, I. Fernandez-Salas, J. Bisset, M. Rodriguez, P. J. McCall, M. J. Donnelly, H. Ranson, J. Hemingway, and W. C. t. Black. 2007. A mutation in the voltage-gated sodium channel gene associated with pyrethroid resistance in Latin American Aedes aegypti. Insect Mol Biol 16: 785-98.

Saavedra-Rodriguez, K., Flores Suarez, A., Fernandez Salas, I., Strode, C., Ranson, H., Hemingway, J., Black, W.C.. 2011. Transcription of detoxification genes following permethrin selection in the mosquito *Aedes aegypti*. Submitted for publication.

Santolamazza, F., M. Calzetta, J. Etang, E. Barrese, I. Dia, A. Caccone, M. J. Donnelly, V. Petrarca, F. Simard, J. Pinto, and A. della Torre. 2008. Distribution of knock-down resistance mutations in Anopheles gambiae molecular forms in west and west-central Africa. Malar J 7: 74.

Sauerbrey, M. 2008. The Onchocerciasis Elimination Program for the Americas (OEPA). Ann Trop Med Parasitol 102 Suppl 1: 25-9.

Scott, J. G. 1989. Cross-Resistance to the Biological Insecticide Abamectin in Pyrethroid-Resistant House Flies. Pesticide biochemistry and physiology 34: 27-31.

Scott, J. G. 1991. Toxicity of abamectin and hydramethylnon to insecticide-susceptible and resistant strains of German cockroach (Dictyoptera: Blattellidae). Journal of Agricultural Entomology 8: 77-82.

Scott, T. W., P. H. Amerasinghe, A. C. Morrison, L. H. Lorenz, G. G. Clark, D. Strickman, P. Kittayapong, and J. D. Edman. 2000a. Longitudinal studies of Aedes aegypti (Diptera: Culicidae) in Thailand and Puerto Rico: blood feeding frequency. J Med Entomol 37: 89-101.

Scott, T. W., A. C. Morrison, L. H. Lorenz, G. G. Clark, D. Strickman, P. Kittayapong, H. Zhou, and J. D. Edman. 2000b. Longitudinal studies of Aedes aegypti (Diptera: Culicidae) in Thailand and Puerto Rico: population dynamics. J Med Entomol 37: 77-88.

Sergon, K., C. Njuguna, R. Kalani, V. Ofula, C. Onyango, L. S. Konongoi, S. Bedno, H. Burke, A. M. Dumilla, J. Konde, M. K. Njenga, R. Sang, and R. F. Breiman. 2008. Seroprevalence of Chikungunya virus (CHIKV) infection on Lamu Island, Kenya, October 2004. Am J Trop Med Hyg 78: 333-7.

Shoop, W. L., H. Mrozik, and M. H. Fisher. 1995. Structure and activity of avermectins and milbemycins in animal health. Vet Parasitol 59: 139-56.

Sinniah, B., P. I. Chew, and K. Subramaniam. 1990. A comparative trial of albendazole, mebendazole, pyrantel pamoate and oxantel pyrantel pamoate against soil transmitted helminthiases in school children. Tropical biomedicine 7: 129-134.

Siva, N. 2009. WHO researchers start trial on a new drug for river blindness. BMJ 339: b2755.

Soderlund, D. M., and J. R. Bloomquist. 1989. Neurotoxic actions of pyrethroid insecticides. Annu Rev Entomol 34: 77-96.

Soutello, R. G., M. C. Seno, and A. F. Amarante. 2007. Anthelmintic resistance in cattle nematodes in northwestern Sao Paulo State, Brazil. Vet Parasitol 148: 360-4.

Spradbery, J. P., R. S. Tozer, N. Drewett, and M. J. Lindsay. 1985. The efficacy of ivermectin against larvae of the screw-worm fly (*Chrysoma bezziana).* Austral. Vet. J. 62: 311-314.

Standfast, H. A., M. J. Muller, and D. D. Wilson. 1984. Mortality of *Culicoides brevitarsis* (Diptera: Ceratopogonidae) fed on cattle treated with ivermectinq. J. Econ. Entomol. 77: 419-421.

Staples, J. E., R. F. Breiman, and A. M. Powers. 2009. Chikungunya fever: an epidemiological review of a re-emerging infectious disease. Clin Infect Dis 49: 942-8.

Strycharz, J. P., K. S. Yoon, and J. M. Clark. 2008. A new ivermectin formulation topically kills permethrin-resistant human head lice (Anoplura: Pediculidae). J Med Entomol 45: 75-81.

Supavilai, P., and M. Karobath. 1981. In vitro modulation by avermectin B1a of the GABA/benzodiazepine receptor complex of rat cerebellum. J Neurochem 36: 798-803.

Sutherland, G. B., and A. B. Ewen. 1974. Fecundity decrease in mosquitoes ingesting blood from specifically sensitized mammals. J Insect Physiol 20: 655-60.

Sylla, M., K. C. Kobylinski, M. Gray, P. L. Chapman, M. D. Sarr, J. L. Rasgon, and B. D. Foy. 2010. Mass drug administration of ivermectin in south-eastern Senegal reduces the survivorship of wild-caught, blood fed malaria vectors. Malar J 9: 365.

Takiguchi, Y., H. Mishima, M. Okuda, M. Terao, A. Aoki, and R. Fukuda. 1980. Milbemycins, a new family of macrolide antibiotics: fermentation, isolation and physico-chemical properties. J Antibiot (Tokyo) 33: 1120-7.

Tang, J., and R. N. Wong. 1987. Evolution in the structure and function of aspartic proteases. J Cell Biochem 33: 53-63.

Taylor, H. R., and B. M. Greene. 1989. The status of ivermectin in the treatment of human onchocerciasis. Am J Trop Med Hyg 41: 460-6.

Taylor, H. R., M. Pacque, B. Munoz, and B. M. Greene. 1990. Impact of mass treatment of onchocerciasis with ivermectin on the transmission of infection. Science 250: 116-8.

Tesh, R. B., and H. Guzman. 1990. Mortality and infertility in adult mosquitoes after the ingestion of blood containing ivermectin. Am J Trop Med Hyg 43: 229-33.

Trager, W. 1939a. Further observations on acquired immunity of the tick *Dermacenter variabilis* Say. J Parasitol 25: 137-139.

Trager, W. 1939b. Acquired immunity to ticks. J Parasitol 25: 137-139.

Traversa, D., B. Paoletti, D. Otranto, and J. Miller. 2007. First report of multiple drug resistance in trichostrongyles affecting sheep under field conditions in Italy. Parasitol Res 101: 1713-6.

Trpis, M., J. E. Childs, D. J. Fryauff, B. M. Greene, P. N. Williams, B. E. Munoz, M. C. Pacque, and H. R. Taylor. 1990. Effect of mass treatment of a human population with ivermectin on transmission of Onchocerca volvulus by Simulium yahense in Liberia, West Africa. Am J Trop Med Hyg 42: 148-56.

Valle, M. R., L. Mendez, M. Valdez, M. Redondo, C. M. Espinosa, M. Vargas, R. L. Cruz, H. P. Barrios, G. Seoane, E. S. Ramirez, O. Boue, J. L. Vigil, H. Machado, C. B. Nordelo, and M. J. Pineiro. 2004. Integrated control of Boophilus microplus ticks in Cuba based on vaccination with the anti-tick vaccine Gavac. Exp Appl Acarol 34: 375-82.

van den Hoek, J. A., J. A. van de Weerd, T. D. Baayen, P. M. Molenaar, G. J. Sonder, I. M. van Ouwerkerk, and H. J. de Vries. 2008. A persistent problem with scabies in and outside a nursing home in Amsterdam: indications for resistance to lindane and ivermectin. Euro Surveill 13.

Verity, C. K., D. P. McManus, and P. J. Brindley. 2001. Vaccine efficacy of recombinant cathepsin D aspartic protease from Schistosoma japonicum. Parasite Immunol 23: 153-62.

Walton, S. F., J. McBroom, J. D. Mathews, D. J. Kemp, and B. J. Currie. 1999. Crusted scabies: A molecular analysis of Sarcoptes scabiei variety hominis populations from patients with repeated infestations. Clin Infect Dis 29: 1226-30.

Wamae, C. N., S. M. Njenga, B. M. Ngugi, J. Mbui, and H. K. Njaanake. 2010. Evaluation of effectiveness of diethylcarbamazine/albendazole combination in reduction of Wuchereria bancrofti infection using multiple infection parameters. Acta Trop.

White, N. J. 1997. Assessment of the pharmacodynamic properties of antimalarial drugs in vivo. Antimicrob Agents Chemother 41: 1413-22.

WHO. 1998a. Major Private Sector Partner, Merck, Welcomed to Lymphatic Fiariasis Control effort. WHO, Press Release WHO/76, Geneva.

WHO. 1998b. WHO and SmithKline Beecham to Cooperate on Elephantiasis Elimination. Press release. WHO/12, Geneva.

WHO. 2007. Scientific Working Group Report on Dengue, pp. http://apps.who.int/tdr/publications/tdr-research-publications/swg-report-dengue/pdf/swg_dengue_2.pdf, Geneva, Switzerland.

WHO. 2009. Global Programme to Eliminate Lymphatic Filariasis. Wkly Epidemiol Rec 84: 437-444.

Wijffels, G., S. Hughes, J. Gough, J. Allen, A. Don, K. Marshall, B. Kay, and D. Kemp. 1999. Peritrophins of adult dipteran ectoparasites and their evaluation as vaccine antigens. Int J Parasitol 29: 1363-77.

Wilder-Smith, A., and E. Schwartz. 2005. Dengue in travelers. N Engl J Med 353: 924-32.

Willadsen, P. 1999. Immunological control of ectoparasites: past achievements and future research priorities. Genet Anal 15: 131-7.

Willadsen, P. 2004. Anti-tick vaccines. Parasitology 129 Suppl: S367-87.

Willadsen, P., J. M. Nielsen, and G. A. Riding. 1989. Purification and properties of a novel nucleotide-hydrolysing enzyme (5'-nucleotidase) from Boophilus microplus. Biochem J 258: 79-85.

Willadsen, P., C. H. Eisemann, and R. L. Tellam. 1993. 'Concealed' antigens: expanding the range of immunological targets. Parasitol Today 9: 132-5.

Willadsen, P., P. Bird, G. S. Cobon, and J. Hungerford. 1995a. Commercialisation of a recombinant vaccine against Boophilus microplus. Parasitology 110: S43-50.

Willadsen, P., P. Bird, G. S. Cobon, and J. Hungerford. 1995b. Commercialisation of a recombinant vaccine against Boophilus microplus. Parasitology 110 Suppl: S43-50.

Williamson, A. L., P. J. Brindley, D. P. Knox, P. J. Hotez, and A. Loukas. 2003a. Digestive proteases of blood-feeding nematodes. Trends Parasitol 19: 417-23.

Williamson, A. L., P. J. Brindley, G. Abbenante, P. Prociv, C. Berry, K. Girdwood, D. I. Pritchard, D. P. Fairlie, P. J. Hotez, J. P. Dalton, and A. Loukas. 2002. Cleavage of hemoglobin by hookworm cathepsin D aspartic proteases and its potential contribution to host specificity. FASEB J 16: 1458-60.

Williamson, A. L., P. J. Brindley, G. Abbenante, B. J. Datu, P. Prociv, C. Berry, K. Girdwood, D. I. Pritchard, D. P. Fairlie, P. J. Hotez, B. Zhan, and A. Loukas. 2003b. Hookworm aspartic protease, Na-APR-2, cleaves human hemoglobin and serum proteins in a host-specific fashion. J Infect Dis 187: 484-94.

Wilson, M. L. 1993. Avermectins in arthropod vector management - prospects and pitfalls. Parasitol Today 9: 83-7.

Winch, P., L. Lloyd, M. D. Godas, and C. Kendall. 1991. Beliefs about the prevention of dengue and other febrile illnesses in Merida, Mexico. J Trop Med Hyg 94: 377-87.

Wise de Valdez, M. R., E. L. Suchman, J. O. Carlson, and W. C. Black. 2010. A large scale laboratory cage trial of Aedes densonucleosis virus (AeDNV). J Med Entomol 47: 392-9.

Wolstenholme, A. J., and A. T. Rogers. 2005. Glutamate-gated chloride channels and the mode of action of the avermectin/milbemycin anthelmintics. Parasitology 131 Suppl: S85-95.

World Health Organization, W. P. E. S. 2006. Guidlines for testing mosquito adulticides for indoor residual spraying and treatment of mosquito nets, pp. 60. World Health Organization, Geneva.

Xu, M., M. Molento, W. Blackhall, P. Ribeiro, R. Beech, and R. Prichard. 1998. Ivermectin resistance in nematodes may be caused by alteration of P-glycoprotein homolog. Mol Biochem Parasitol 91: 327-35.

Yamamoto, K. 1999. Cathepsin E and cathepsin D, pp. 59-71. *In* V. Turk [ed.], Proteases: New Perspectives. Birkhauser, Berlin.

Yao, X. L., M. J. Cowan, M. T. Gladwin, M. M. Lawrence, C. W. Angus, and J. H. Shelhamer. 1999. Dexamethasone alters arachidonate release from human epithelial cells by induction of p11 protein synthesis and inhibition of phospholipase A2 activity. J Biol Chem 274: 17202-8.

Zea-Flores, R., F. O. Richards, Jr., C. Gonzalez-Peralta, J. Castro Ramirez, G. Zea-Flores, R. C. Collins, and E. Cupp. 1992. Adverse reactions after community treatment of onchocerciasis with ivermectin in Guatemala. Trans R Soc Trop Med Hyg 86: 663-6.