DISSERTATION

CHARACTERIZING THE TARGET OF IVERMECTIN, THE GLUTAMATE-GATED CHLORIDE CHANNEL, AND OTHER INSECTICIDE TARGETS AS CANDIDATE ANTIGENS FOR AN ANTI-MOSQUITO VACCINE

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2015

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ABSTRACT

CHARACTERIZING THE TARGET OF IVERMECTIN, THE GLUTAMATE-GATED CHLORIDE CHANNEL, AND OTHER INSECTICIDE TARGETS AS CANDIDATE ANTIGENS FOR AN ANTI-MOSQUITO VACCINE

The latest WHO World Malaria Report estimates that, in 2013, there were 198 million cases worldwide causing 584,000 malaria-related deaths. Current malaria control programs primarily target malaria vectors through the use of long lasting insecticide treated bed nets and indoor residual spraying of pyrethroid-based insecticides. However, pyrethroid resistance is becoming widespread in many *An. gambiae* populations across Africa (Ranson et al., 2011; Trape et al., 2011).

Out of recent efforts to find new vector-targeting interventions with novel modes of action, the endectocide ivermectin (IVM) has arisen as a new candidate to control malaria transmission. IVM, when imbibed by vectors from host-treated blood meals, has proven to efficiently kill or disable *An. gambiae* s.s. both in the lab and the field (Kobylinski et al., 2010; Sylla et al., 2010). More recently, IVM mass drug administrations in multiple locations across west Africa have been shown to temporarily reduce the proportion of *P. falciparum*-infected *An. gambiae* in IVM-treated villages (Kobylinski et al., 2011; Alout et al., 2014).

The primary target of IVM is the invertebrate glutamate-gated chloride channel (GluCl) (Cully et al., 1994; Cully et al., 1996; Janssen et al., 2007; McCavera et al., 2009; Janssen et al., 2010; Moreno et al., 2010). The purpose of the first chapter of this thesis was to characterize GluCl from *An. gambiae* in order to understand the physiological role of GluCl and how IVM may be affecting mosquito physiology. Cloning of the *An. gambiae* GluCl (AgGluCl) revealed

unique splicing sites and products not previously predicted. We expressed AgGluCl clones in *Xenopus laevis* oocytes to measure its electrophysiological activity in response to glutamate and IVM. We also examined AgGluCl isoform-specific transcript levels across different tissues, ages, blood feeding status and gender and GluCl tissue expression in adult *An. gambiae*.

Given that GluCl can be targeted by drugs found in a blood meal and that GluCl is not expressed in mammals, we wanted to test the efficacy of AgGluCl as a candidate mosquitocidal vaccine antigen. We administered a polyclonal anti-AgGluCl immunoglobulin G (anti-AgGluCl IgG) to *An. gambiae* mosquitoes through a blood meal or directly into the hemocoel by intrathoracic injections and found it significantly reduced *An. gambiae* survivorship. By co-administering anti-AgGluCl IgG with a known GluCl agonist, IVM, we discovered anti-AgGluCl IgG reverses the mosquitocidal effects of IVM.

Our results describing the mosquitocidal properties of anti-AgGluCl IgG suggest that other neuronal proteins could be used as candidate antigens for a mosquitocidal vaccine. The *An. gambiae* GABA-gated chloride channel (resistance to dieldrin; AgRDL) is another member of the cys-loop ligand-gated ion channels with a similar structure and physiological function to AgGluCl. The *An. gambiae* voltage-gated sodium channel (AgVGSC) is the target of dichlorodiphenyltrichloroethane (DDT) and the pyrethroid class of insecticides (Soderlund and Bloomquist, 1989). VGSCs are also the target of multiple classes of spider, scorpion and snail toxins, demonstrating that peptides binding to VGSC extracellular residues can affect channel function (Nicholson, 2007; King et al., 2008; Stevens et al., 2011; Klint et al., 2012). Preliminary results shows that IgG targeting AgRDL or AgVGSC similarly reduce *An. gambiae* survivorship.

Finally we tested anti-AgGluCl IgG against *A. aegypti* and *C. tarsalis* to see if this strategy has broad potential across both Anopheline and Culicine mosquitoes. However, blood meals

containing anti-AgGluCl IgG had no effect on *A. aegypti* or *C. tarsalis* survivorship. We determined that this was due to a barrier in antibody translocation from the blood meal to the hemolymph. Since the IgG target, AgGluCl, is only expressed in the hemocoel, antibody translocation was required for mosquito toxicity.

ACKNOWLEDGEMENTS

I thank the Molecular, Cellular & Integrative Neurosciences Program for the opportunity to develop a collaborative project across labs in the Departments of Biomedical Sciences and Microbiology, Immunology and Pathology. I thank Drs. Foy, Partin, Vigh and Tsunoda for their mentorship and guidance. I thank Dr. Christopher Snow and Lucas Price for their assistance on crystal structure modelling and analysis. I thank Dr. Leslie Stone-Roy, Dr. Haoues Alout, Meg Gray, Dr. Jonathan Harms and the late Ines Marques da Silva for their laboratory training. I thank Chilinh Nguyen, Tim Burton, Wojtek Kuklinski, Jon Seaman, Jasmine Donkoh, Amanda Dudek and Sara Monahan for their laboratory assistance. I thank Dr. Roch Dabiré and the Burkina Faso Ministry of Health for their assistance and support in facilitating our field work in Burkina Faso. I thank the villages of Diarkadougou and Bougouriba for allowing and supporting our field work. Finally, I thank Alyssa Meyers and the rest of my family and friends for their continued support.

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Chapter 1: Literature Review

1.1: International goals of malaria reduction

Malaria is a complex disease caused by four different *Plasmodium* parasites and transmitted by approximately 60 Anopheline mosquito species (Walker and Lynch, 2007). Malaria control is a top priority on the international health agenda. The Millennium Development Goals put forth by the United Nations in 2000 broadly aimed to "halt and begin to reverse the incidence of malaria by 2015." Malaria reduction is also the focus of the World Health Assembly and Roll Back Malaria. In 2005 the World Health Assembly targeted a 75% reduction in malaria cases and deaths by 2015. In 2008, these objectives were enhanced to a more ambitious goal of reducing malaria deaths to near zero by 2015. To achieve these goals, increased funding and targeted strategies have been put into three main foci: combating malaria transmission, chemoprevention and case management. Driven by the initiatives set forth by multiple international organizations, there have been major reductions in malaria cases and deaths since 2000. In that time, malaria cases have decreased by 26% and malaria-related deaths have decreased by 47% worldwide (World Malaria Report, 2014).

Though meaningful progress has been made towards malaria reduction, the malaria burden remains prevalent worldwide. In 2013, an estimated 3.3 billion people were at risk for being infected by malaria. Though effective drugs have been developed to treat malaria parasite infections, an estimated 128 million cases of malaria occurred in 2013 leading to 584,000 deaths. The majority of the malaria burden occurs in Africa which accounts for over 90% of malaria infections in children under five years old, who account for 78% of malaria related deaths (World Malaria Report, 2014). The impact of malaria goes well beyond the public health sector and has been a large impediment on the economic growth of African nations. Malaria prevention

and treatment costs African nations an estimated $12 billion USD annually and is responsible for an estimated loss of 1.3% gross domestic product per year (Gallup and Sachs, 2001; Sachs, 2002).

There is large variation in the reduction of malaria when analyzed by individual countries (Giardina et al., 2014). Only 55 of the 106 countries with ongoing malaria transmission in 2000 are on track to meet the Roll Back Malaria and World Health Assembly targets of reducing malaria case incidence rates by 75% by 2015. Of the twenty seven countries in sub-Saharan Africa with endemic malaria transmission, only three (Cabo Verde, Algeria and Sao Tome and Principe) are on pace to reduce malaria case incidence by 75% (WHO Malaria Report, 2014). Between 2005 and 2013, there was an increase in confirmed malaria cases and deaths in all age groups in 83 hospitals in Ghana and 186 hospitals in Nigeria (WHO, unpublished results). Meanwhile, there has been sub-country regional increases in malaria incidence and parasitaemia in Angola, Liberia, Equatorial Guinea, Mozambique, Senegal, Rwanda, and Tanzania since 2005 (Rehman et al., 2013; Giardina et al., 2014; Trape et al., 2014). Though significant progress had been made in malaria control over the past decade, it is clear that new interventions and treatments are needed to meet current malaria reduction goals and to foster region-wide elimination efforts. These will all be necessary to even consider the ultimate goal of malaria eradication.

1.2: Vector control to reduce malaria parasite transmission

The largest gains in malaria control have occurred through targeting the *Anopheles* mosquito vectors that transmit *Plasmodium* parasites. WHO estimates that current vector control interventions prevent 50 million malaria cases annually (WHO Global Plan for Insecticide Resistance Management, 2012). Current vector control strategies attempt to take advantage of

the endophagic (indoor feeding), endophilic (indoor resting), anthropophagic (feeding preference for humans) and night time feeding behaviors of adult female *Anopheles* mosquitoes (Takken and Knols, 1999; Takken and Verhulst, 2013). These behaviors are seen in major *Anopheles* vectors of malaria parasites around the world, including *An. gambiae* sensu stricto (s.s.), *An. coluzzi*, *An. arabiensis*, and *An. funestus* in Africa; *An. stephensi*, *An. punctulatus*, *An. minimus* and *An. dirus* in Asia and *An. darlingi* and *An. albimanus* in Latin America (Sinka et al., 2010b; Sinka et al., 2010a; Sinka et al., 2011; Basseri et al., 2012; Huho et al., 2013; Killeen, 2014). To take advantage of night time, indoor, human directed *Anopheles* host seeking, vector control strategies have focused on protecting individuals inside their homes at night through the use of long-lasting insecticide-treated bed nets and indoor residual spraying of insecticides on the walls and ceilings of households (Nahlen et al., 2003; Pluess et al., 2010; Raghavendra et al., 2011). Though each of these vector control tools have been shown to reduce disease transmission in the field, they each come with their own shortcomings and deficits, highlighting the need for the development of new vector control tools and integrated approaches at controlling vector populations.

A) Long lasting insecticide-treated bed nets

To protect against endophagic *Anopheles* vectors that primarily host seek at night, long-lasting insecticide treated bed nets (LLINs) were developed to protect individuals while sleeping. LLINs act as a physical barrier to mosquito bites with additional insecticidal properties to further reduce mosquito survival. LLINs were first used by American, German and Russian soldiers in World War II to protect troops from mosquitoes and other disease vectors at night (Lindsay and Gibson, 1988). LLINs treated with permethrin were first shown to reduce *P. falciparum* prevalence and incidence in children under five compared to children sleeping under non-treated

bed nets in Papua New Guinea in 1985 (Graves et al., 1987). Since then numerous studies have shown LLINs to be effective at reducing indoor resting densities of *Anopheles* vectors, malaria transmission and malaria-related illnesses and death in numerous malaria-endemic regions (Alonso et al., 1993; Gimnig et al., 2003; Lengeler, 2004b, a; Lindblade et al., 2004; Fegan et al., 2007; Noor et al., 2008; Lindblade et al., 2015).

Several international and domestic programs distribute LLINs free of charge or at a subsidized price. An estimated 44% of the population at risk for malaria infection slept under LLINs in 2013, up from an estimated 2% in 2004 (WHO Malaria Report, 2014). In 2013 alone, 142 million LLINs were delivered to sub-Saharan African nations and 214 million LLINs are scheduled to be delivered in 2014 (WHO Malaria Report, 2014). However, residual malaria transmission largely persists after full LLINs coverage due to improper net usage, compromised nets and mosquito bites which occur outdoors and during crepuscular hours when people are not protected by their bed nets (Killeen et al., 2006; Seyoum et al., 2012; Huho et al., 2013; Killeen, 2013, 2014).

B) Indoor residual spraying of insecticides

After LLINs, the second most employed vector control strategy is indoor residual spraying (IRS) of insecticides. IRS is simply the spraying of long-lasting, stable insecticide formulations on the interior walls and ceiling of households. IRS has been shown to cause an increase in mosquito mortality with the added benefit of mosquito repellency (Pluess et al., 2010). IRS was first used in the 1930s using pyrethrum extracts but was quickly replaced in the 1940s with dichloro-diphenyl-trichloroethane (DDT) (Najera et al., 2011). The Global Malaria Eradication Program (GMEP) of 1955-1968 was largely based on IRS of DDT. This campaign successfully eliminated malaria from North America, Europe, the Caribbean and parts of South and Central

America and Asia. However, GMEP was unsuccessful at eliminating malaria from Africa and many regions of South America and Asia where malaria persisted and even rebounded following the GMEP (Carter and Mendis, 2002; Najera et al., 2011).

Since the GMEP, IRS has been used on a much smaller scale to control vector populations rather than eliminate malaria from large geographical regions completely (Pluess et al., 2010). IRS spraying with DDT and other insecticides have proven to be effective at reducing *Anopheles* populations and malaria transmission in field settings across Africa and Asia including Benin, Mozambique, South Africa, India, Equatorial Guinea and in parts of Swaziland, Eritrea, Ethiopia, and Madagascar (Mabaso et al., 2004; Gunasekaran et al., 2005; Sharma et al., 2005; Sharp et al., 2007b; Sharp et al., 2007a; Overgaard et al., 2012; Rehman et al., 2013; Akogbeto et al., 2015). However, there are many variables which affect the effectiveness of IRS including the insecticide utilized, insecticide resistance, and the level of malaria transmission occurring (Pluess et al., 2010; Kim et al., 2012).

Of the 63 countries reporting IRS for vector control, 53 countries sprayed pyrethroids, 12 countries sprayed carbamates and 13 sprayed organophosphate-based insecticides. Globally, 124 million people were protected from malaria transmission through the use of IRS representing 4% of the global population at risk (WHO Malaria Report, 2014). There has been a decline in IRS usage from 11% of the global population at risk in 2010 due to financial restraints (WHO Malaria Report, 2014).

C) Larval Control

Larval control encompasses a broad range of strategies to eliminate mosquito larval habitats as well as biological and chemical interventions targeting mosquito larva directly. Historically, some of the most successful vector control campaigns have relied heavily on targeting mosquito

larva, including Fred Soper's eradication campaign to remove *Anopheles gambiae* from Brazil in the 1930s and early 1940s (Killeen et al., 2002b; Killeen et al., 2002a; Walker and Lynch, 2007). This was largely done through environmental modifications to eliminate larval breeding sites and use of the larvacide Paris Green (copper acetoarsenite) to directly kill mosquito larva (Killeen et al., 2002b; Killeen et al., 2002a; Walker and Lynch, 2007). Larval control lost popularity with the success of the DDT IRS in the GMEP campaign from 1955-1968 and the disease modeling work of Ronald Ross and George MacDonald which showed that reducing adult mosquito survivorship was much more effective at reducing malaria transmission (Macdonald, 1956; Walker and Lynch, 2007). Though DDT IRS campaign ultimately failed in Africa, the use of larval control has never returned as a major strategy for vector control.

However, several studies have shown that larval control can significantly reduce malaria transmission and provide additional benefits when combined with LLINs or IRS. The most widely used larvacide is the bacterial species *B. thuringiensis israelensis* (Bti) and *B. sphaericus* (Bs) (Walker and Lynch, 2007). These bacteria produce cryo-toxins which create pores in the mosquito larval midgut causing mortality (Bravo et al., 2007; Gomez et al., 2007). Bti and Bs have been shown to reduce *Anopheles* adult populations in Kenya, Zaire, Madagascar and India (Karch et al., 1991; Romi et al., 1993; Kumar et al., 1995; Fillinger et al., 2003). The larvacide pyriproxyfen, an insect juvenile hormone mimetic, has also been shown to significantly reduce adult populations of *An. minimus* and *An. maculatus* in Thailand as well as *An. culicifacies* and *An. subpictus* in Sri Lanka (Kanda et al., 1995; Yapabandara et al., 2001).

Larval control programs were only utilized in 38 countries in 2013 (WHO Malaria Report, 2014). Low usage is related to the difficulty in finding larval breeding sites which range from small, temporary pools without vegetation for *Anopheles gambiae* s.l. to large bodies of water

with emergent vegetation for *Anopheles funestus* (Gimnig et al., 2001; Mutuku et al., 2006; Tusting et al., 2013).

1.3: Insecticides used for malaria control

Chemical insecticides targeting the adult mosquito are the primary tool used to control malaria transmission. Currently, the only classes of insecticides used to target adult mosquitoes for malaria control are the pyrethroids, organochlorines (specifically DDT), organophosphates and methyl carbamates. These insecticide target components of the mosquito nervous system involved in chemical or electrical signaling where small functional disruptions result in mosquito mortality (Casida and Durkin, 2013a).

A) Pyrethroids

The insecticidal properties of pyrethrin extracts from the *Chrysanthemum cinerariaefulium* flower have been recognized and utilized since the 19th century (Elliott et al., 1973a; Soderlund and Bloomquist, 1989). These naturally-produced pyrethrins were the structural basis for the pyrethroid class of insecticides which were developed to control agricultural and public health insect pests. The first generation of pyrethroids were developed with enhanced insecticidal properties when compared to pyrethrins in the 1960s (Soderlund and Bloomquist, 1989). This includes bioallethrin, tetramethrin, resmethrin and bioresmethrin. The second generation of pyrethroids, which include permethrin, allethrin, cypermethrin and deltamethrin, were developed in the 1970s and had additional long-lasting properties (Elliott et al., 1973b).

Pyrethroids have grown to be the primary insecticide for controlling *Anopheles* vectors of malaria. They are the only approved insecticide for use in LLINs and are utilized in an estimated 75% of IRS campaigns (WHO Global Plan for Insecticide Resistance Management 2012; WHO Malaria Report, 2014). Pyrethroids are contact-dependent and can be absorbed through the insect

cuticle to bind its target within the mosquito hemocoel (Nkya et al., 2013). Pyrethroids cause loss of coordinated movement, periods of convulsive activity and ultimately paralysis and death in *Anopheles* mosquitoes (Beeman, 1982; Haynes, 1988; Kunkel et al., 2001; Cohnstaedt and Allan, 2011). *Anopheles* mosquitoes are exceptionally sensitive to pyrethroid exposure. Lethal concentrations of pyrethroids range from 0.05% for deltamethrin to 0.75% for permethrin in the WHO Bottle Assay against a variety of *Anopheles* mosquitoes (WHO Test procedures for insecticide resistance monitoring in malaria vector mosquitoes, 2013).

Pyrethroids act on the voltage-gated sodium channel (VGSC) causing channel dysfunction and aberrant action potential firing (Barnes and Verschoyle, 1974; Bloomquist and Soderlund, 1988; Soderlund and Bloomquist, 1989). VGSCs are located on neuronal axons and are responsible for the electrical currents which make up the action potential (Davies et al., 2007b). Pyrethroids have two different modes of action on VGSC, which depend on whether the compound includes or lacks an α-cyano group (Barnes and Verschoyle, 1974; Casida et al., 1983). Pyrethroids lacking an α-cyano group bind to the resting or inactivated state of the VGSC causing a shift in the voltage dependence of activation to more negative membrane potentials (Vais et al., 2001; Davies et al., 2007a; Casida and Durkin, 2013a). This is defined as the Type I pyrethroid mode of action. Type I pyrethroids include permethrin and allethrin. Type II action is caused by pyrethroids containing an α-cyano group which includes deltamethrin. Type II action is characterized by pyrethroid binding to the VGSC activated state, prolonging channel opening causing use-dependent depolarization, inactivation and blockage of action potential firing (Salgado et al., 1989; Salgado and Narahashi, 1993; Davies et al., 2007a; Casida and Durkin, 2013a).

B) Organochlorines

The organochlorine, DDT, was discovered in 1939 by the U.S. Department of Agriculture as a potent insecticide. It was first used during WWII as an insecticide and was the primary insecticide used in the large GMEP IRS campaigns (Sadasivaiah et al., 2007). Heavy usage of DDT declined after reports of resistance and reports on the potential long-term effects of DDT bioaccumulation on the ecosystem made famous by Rachel Carson in her book *Silent Spring* which led to the banning of DDT for agricultural spraying in 1972 by the Environmental Protection Agency (Carter and Mendis, 2002; Sadasivaiah et al., 2007; Raghavendra et al., 2011). However, the DDT ban allows for the use of DDT IRS for public health emergencies, such as malaria outbreaks (Raghavendra et al., 2011). Currently, ten countries utilize DDT in IRS campaigns, both because of its strong mosquitocidal properties and because it is the most cost-effective insecticide with an estimate IRS cost of $1.60 per house (Sadasivaiah et al., 2007). These DDT IRS have been shown to be highly successful in a low malaria transmission region of Madagascar as well as cause minor reductions in malaria transmission in high transmission regions of Uganda (Kigozi et al., 2012; Ratovonjato et al., 2014).

DDT targets the voltage-gated sodium channel and has a similar mode of action as type I pyrethroids by binding to the resting or inactivated state of the VGSC causing a shift in the voltage dependence of activation to more negative membrane potentials (Soderlund and Bloomquist, 1989; Davies et al., 2007a; Casida and Durkin, 2013a). DDT toxicity, using the standard WHO Bottle Assay, is 4% against a variety of *Anopheles* mosquitoes (WHO Test procedures for insecticide resistance monitoring in malaria vector mosquitoes, 2013). In addition to its toxic effects, DDT has strong non-contact repellency activity which enhances its ability to

reduce malaria transmission, though the molecular mechanism through which DDT causes repellency is not well understood. (Roberts et al., 2000; Grieco et al., 2007).

C) Organophosphates and Methyl Carbamates

Organophophate (OP) insecticides were developed during World War II as derivatives from sarin gas in the 1940 (Minton and Murray, 1988). OPs became popular in the 1970s because, unlike organochlorines such as DDT, they do no persist in the environment or bioaccumulate in the ecosystem (Minton and Murray, 1988; Carlock et al., 1999; Casida and Durkin, 2013a). Though OPs are relatively unstable in the environment, they are highly toxic to mammals due to their broad toxicity. The OPs recommended for vector control are fenitrothion, malathion and pirimiphos-methyl. OPs are distributed by IRS, though they are only employed in thirteen countries (WHO World Malaria Report, 2014). Low OP usage is due to high mammalian toxicity and relatively high costs compared to pyrethroids (WHO Global Plan for Insecticide Resistance Management 2012). Lethal concentrations of OPs range from 0.25% for pirimiphos methyl to 5% for malathion in the WHO Bottle Assay against a variety of *Anopheles* mosquitoes (WHO Test procedures for insecticide resistance monitoring in malaria vector mosquitoes, 2013).

The biological activities of methyl carbamates originated from Calabar beans which were used as a poison in West African witchcraft trials (Casida, 1963). The toxic constituent of the Calabar bean, physostigmine, was identified in 1925 as a methyl carbamate (MC) ester which is the only known naturally occurring carbamate ester (Casida, 1963). MC-based insecticides were developed from physostigmine in the 1960s in response to the high toxicity of OPs (Casida and Durkin, 2013a). Current MC-based insecticides used for malaria control are propoxur, fenitrothion and bendiocarb, all of which are applied only through IRS, but only utilized in twelve countries due to high costs compared to pyrethroids and DDT (WHO World Malaria

Report, 2014). Lethal concentrations of MCs range from 0.1% for propoxur and bendiocarb to 0.4% for carbosulfan in the WHO Bottle Assay against a variety of *Anopheles* mosquitoes (WHO Test procedures for insecticide resistance monitoring in malaria vector mosquitoes, 2013).

OPs and MCs both act as an acetylcholinesterase (AChE) inhibitor (Fukuto, 1990). AChE inactivates acetylcholine released into the neuronal synapse by hydrolyzing acetylcholine into choline and acetate (Casida and Durkin, 2013b, a). Acetylcholine is the primary excitatory neurotransmitter in invertebrates (Sattelle, 1977; Gepner et al., 1978). Inhibition of synaptic acetylcholine degradation causes dysfunctional excitatory electrochemical signaling resulting in paralysis and death in *Anopheles* mosquitoes (Minton and Murray, 1988). OPs phosphorylate the serine moiety in the AChE active site causing long-lasting inactivation of synaptic AChE which can last for over 24 hours (Fukuto, 1990; Casida and Durkin, 2013b, a). MCs also react with the same serine moiety in the AChE active site through a methylcarbamylation reaction. This reaction is not nearly as stable as the phosphorylation reaction with OPs and lasts around 30 minutes (Fukuto, 1990).

D) Current state of insecticide resistance

The heavy reliance on a limited number of chemical insecticides for malaria control has driven *Anopheles* populations to develop insecticide resistance worldwide. The mechanisms for resistance vary from target-site resistance, metabolic resistance, cuticular resistance and various forms of behavioral resistance (Ranson et al., 2011; Killeen et al., 2013; Killeen, 2014).

Target-site insecticide resistance is caused by point mutations in the target protein that reduces insecticide binding and efficacy. The number of insecticide classes used for malaria control are limited at four classes, but the number of insecticide targets is even more limited as

these four insecticide classes only target two proteins in the mosquito, the voltage-gated sodium channel and acetylcholinesterase (Fukuto, 1990; Davies et al., 2007a).

Point mutations in VGSC that confer resistance to pyrethroids and DDT are referred to as knockdown resistance (kdr) in reference to their phenotype to withstand exposure to these insecticides without being knocked down (Martinez-Torres et al., 1998; Ranson et al., 2000; Reimer et al., 2008; Ranson et al., 2011). The kdr mutation occurs at position 1014 with a mutation of a leucine residue. There are two kdr mutations that have been characterized in the field, and are known as kdr west which was first discovered in the Ivory Coast and kdr east which was first discovered in Kenya (Martinez-Torres et al., 1998; Ranson et al., 2000). The kdr west mutation is an adenine to thymine mutation which results in a leucine to phenylalanine mutation in the VGSC amino acid sequence (Martinez-Torres et al., 1998). The kdr east mutation is a thymine to cytosine mutation which results in a leucine to serine mutation in the VGSC amino acid sequence (Ranson et al., 2000). Both of these mutations are now found throughout the African continent (Ranson et al., 2011). Surprisingly, this residue does not directly interact with insecticide binding but alters channel kinetics to reduce insecticide efficacy on channel activity (Davies et al., 2007a).

Target-site resistance also occurs for OP and MC insecticides with a mutation in the AChE enzyme (Weill et al., 2003; Weill et al., 2004). A guanine to adenine point mutation in the Ace-1 gene which encodes for AChE, results in a glycine to serine mutation in the active site of AChE (Weill et al., 2004; Alout and Weill, 2008). It's hypothesized that this mutation blocks insecticide binding to its target in the AChE active site (Alout and Weill, 2008). This mutation has been reported in wild *An. gambiae*, *An. arabiensis* and *An. coluzzi* in multiple African

nations including Burkina Faso, Ivory Coast, Benin and Ghana (Djogbenou et al., 2008; Dabire et al., 2009; Essandoh et al., 2013; Dabire et al., 2014; Edi et al., 2014; Weetman et al., 2015).

The heavy reliance on chemical insecticides has also driven the development of a variety of metabolic insecticide resistance mechanisms in *Anopheles* populations (Hemingway et al., 2004; Li et al., 2007; Ranson et al., 2011). Metabolic resistance occurs through elevated activity or expression of xenobiotic detoxification enzymes which modulate and detoxify insecticides before they reach target sites within the mosquito. The three major classes of metabolic enzymes that confer insecticide resistance are cytochrome P450 monooxygenases (P450s), carboxyesterases and glutathione-S-transferases (GSTs) (Li et al., 2007). P450s act by oxidizing target substrates, making them more hydrophilic and easier to excrete (David et al., 2013). Cytochrome P450 mediated insecticide resistance has been reported in *Anopheles* mosquitoes in Ghana, Kenya, Tanzania, Cameroon and Mozambique (David et al., 2005; Vontas et al., 2005; Djouaka et al., 2008; Muller et al., 2008a; Muller et al., 2008b). A second class of detoxification enzyme that causes insecticides resistance is the carboxyesterases (Li et al., 2007). These enzymes break down xenobiotics by hydrolyzing ester moieties into an alcohol and an acid (Sogorb and Vilanova, 2002). Esterase-based resistance has been detected in wild *Anopheles* populations in multiple African nations including Burkina Faso and Benin (Aikpon et al., 2014; Toe et al., 2015). The third class of detoxification enzymes associated with insecticide resistance is the GSTs (Li et al., 2007). GSTs work by conjugating reduced glutathione to electrophilic residues on xenobiotic insecticides (Enayati et al., 2005; Ranson and Hemingway, 2005). There are multiple classes of GSTs expressed in mosquitoes. Of those classes, the delta and epsilon classes are most highly associated with insecticide resistance (Ranson et al., 1997b; Ranson et al., 1997a; Ranson et al., 2001; Ranson and Hemingway, 2005; Li et al., 2007). GST-based

insecticide resistance has also been reported in *Anopheles* populations across the African continent including the Ivory Coast, Benin and Cameroon (Nwane et al., 2013; Abdalla et al., 2014; Mulamba et al., 2014; Riveron et al., 2014). These three families of detoxification enzymes have broad substrate specificity and work on multiple classes of insecticides used for malaria control which creates the problem of cross resistance (Nauen, 2007; David et al., 2013). For example, the cytochrome P450 enzyme CYP6M2 has been experimentally shown to detoxify both pyrethroids and DDT (Mitchell et al., 2012; David et al., 2013).

Another relatively small form of insecticide resistance which has been reported in just three *Anopheles* populations is cuticular resistance (Vontas et al., 2007; Awolola et al., 2009). This form of resistance is caused by an upregulation of the cuticular proteins cplcg3 and cplcg4, which cause a thickening of the insect cuticle. Since most insecticides used for malaria control, such as LLINs and IRS, are exposed to *Anopheles* mosquitoes through contact-dependent diffusion through the cuticle, thickening the cuticle barrier reduces insecticide bioavailability in the hemocoel (Ranson et al., 2011).

Malaria control strategies attempt to take advantage of stereotypical mosquito behaviors associated with host-seeking and blood feeding. These behaviors include endophagy, endophily and host-seeking during nighttime hours (Killeen, 2014). Changes in these mosquito behaviors to avoid malaria control strategies are generally termed 'behavior resistance'. Different forms of behavioral resistance include exophagy and exophily, which allows the mosquito to avoid exposure to indoor insecticide applications (Chareonviriyaphap et al., 2013; Gatton et al., 2013; Killeen, 2014). Several *Anopheles* populations have also started host-seeking at earlier, crepuscular hours when more people are still awake and active and not protected under bed nets. This has been observed in *Anopheles* populations in Tanzania, Ethiopia, Bioko Island, Benin and

Senegal (Govella et al., 2010; Trape et al., 2011; Moiroux et al., 2012; Overgaard et al., 2012; Yohannes and Boelee, 2012; Sougoufara et al., 2014).

These forms of behavioral resistance allow malaria transmission to persist, even when full coverage of LLINs and/or IRS is implemented (Killeen, 2014). It is clear that current malaria control tools and strategies are not sufficient. Development of new vector control tools with novel modes of action and universal protection are required to further reduce and eliminate malaria transmission.

1.4: Ivermectin

The endectocide ivermectin (IVM) has arisen as a novel insecticide with strong potential for malaria control. IVM has several unique characteristics compared to other insecticides currently used for malaria control including a unique mode of action and a unique insecticide application through treating the populations of a malaria endemic region compared to insecticide application to households and bed nets. Mass drug administration (MDA) of IVM has been shown to reduce *Anopheles* survivorship, reduce *Plasmodium* sporozoite rates in *Anopheles* and shift the *Anopheles* population structure to younger mosquitoes which are less likely to transmit *Plasmodium* parasites (Chaccour et al., 2013).

A) Anthelminthic and insecticidal properties of ivermectin

Ivermectin is a semi-synthetic derivative of the compound avermectin, which is a natural fermentation product of the soil bacterium *Streptomyces avermectinius* (Burg et al., 1979; Campbell, 2012). The avermectin family of endectocides are 16-membered macrocyclic lactones each of which is comprised of a dissacharide, benzofuran and spiroketal moieties. The family also includes eprinomectin, abamectin, selamectin, doramectin and enamectin, though ivermectin has been shown to have the strongest nematocidal and insecticidal properties (Pitterna et al.,

2009; Butters et al., 2012b; Campbell, 2012). Ivermectin affects a broad range of parasitic nematodes that affect human, livestock and companion animals including *Onchocerca volvulus* (river blindness), *Brugia malayi*, *Wuchereria bancrofti* (lymphatic filariasis), *Loa loa*, *Dirofilaria immitis* (heartworm), *Ascaris* spp. and *Strongyloides* spp. intestinal roundworms, *Hyostrongylus* spp., *Ostertagia* spp. stomach worms, *Haemonchus* spp. pole worms, *Metastrongylus* spp., *Dictyocaulus viviparous* lungworms and *Trichuris* spp. whipworms (Lyons et al., 1981; Berry, 1991; Whitworth et al., 1991; Campbell, 1993; Datry et al., 1994; Moncayo et al., 2008; Hu et al., 2013; Lopes et al., 2014). Generally, IVM is not effective against *Anclyostoma* and *Necator* hookworms (Blair et al., 1983; Campbell, 1993; Tritten et al., 2012; Hu et al., 2013). IVM not only affects a wide range of parasitic nematodes, but is also highly potent at very low concentrations. Dogs infected with *Dirofilaria immitis* heartworms can be treated with IVM dosages as low as 0.001mg/kg of body weight (Campbell et al., 1983).

Interest in the effects of avermectins against hematophagous insects began when several avermectins were shown to be systemically active when applied to mice either orally and dermally against *Cuterebra* larvae in a high throughput screen (Drummond and Gingrich, 1972). This caught the interest research groups studying systemic insecticides to control tick infestations of cattle. Cattle were given IVM as an oral dose or subcutaneous injection and six tick species, both nymphs and adults, were fed on the treated cattle. Feeding on IVM-treated (50µg/kg) cattle prevented full engorgement and reproduction of *Amblyomma americanum* (L.), *A. cajennense* (F.), *A. maculatum* Koch, *Dermacentor andersoni* Stiles, *D. variabilis* (Say), and *Rhipicephalus sanguineus* (Latreille) ticks (Drummond et al., 1981). This proof of concept drove research into the effects of IVM-treated hosts on other hematophagous insects of public health interest. A single blood meal from an IVM-treated (2mg/kg) guinea pig or goat was shown to significantly

reduced the survivorship of *Glossina palpalis palpalis* tsetse flies, vectors of *Trypanosome* spp. parasites (Distelmans et al., 1983).

The first study on the effects of IVM on mosquito disease vectors proved that IVM reduced the survivorship of *An. stephensi*, *A. aegypti*, *C. pipiens* and *C. quinquefasciatus* (Pampiglione et al., 1985). As a larvacide, *C. pipiens* was the most susceptible to IVM, followed by *An. stephensi* and finally *A. aegypti*. When IVM was fed to adult mosquitoes, whether it was through cotton-wool treated with IVM or directly on an IVM-treated mouse, *An. stephensi* was the most susceptible where *A. aegypti* and *C. quinquefasciatus* were only moderately affected (Pampiglione et al., 1985). Since *An. stephensi* was the most susceptible mosquito species to IVM, further research was done on a broad range of *Anopheles* species to determine if these effects translated to other members of the *Anopheles* genus. Since the initial studies by Pampiglione, IVM-containing blood meals have been shown to reduce the survivorship of *An. gambaie*, *An. arabiensis*, *An. stephensi*, *An. quadimaculatus*, *An. punctulatus*, *An. koliensis* and *An. farauti* (Jones et al., 1992; Gardner et al., 1993; Bockarie et al., 1999; Foley et al., 2000; Fritz et al., 2009; Chaccour et al., 2010; Kobylinski et al., 2010). Importantly, the concentration of IVM in the blood that's necessary to reduce *Anopheles* survivorship can be achieved by taking a standard oral dose of IVM (150μg/kg), which is administered to humans as treatment for various parasitic worm infections (Kobylinski et al., 2010; Chaccour et al., 2013; Alout et al., 2014). The mosquito phenotype of IVM toxicity is flaccid paralysis which leads to death. Sub-lethal concentrations of IVM causes delayed re-blood feeding frequency, defecation rates, as well as motor deficiencies which has been shown through increased knockdown and inhibited recovery in blood fed females (Kobylinski et al., 2010; Butters et al., 2012a). However, human IVM plasma levels remain at mosquito-toxic levels for six days following a standard dose which

limits its potential to reduce malaria transmission over the course of a transmission season (Alout et al., 2014).

B) Ivermectin mass drug administration for onchocerciasis and lymphatic filariasis control

WHO and the World Bank established the Onchocerciasis Control Programme (OCP) in eleven West African nations in 1974 to combat high rates of onchocerciasis (river blindness). Originally this program funded insecticide spraying of fast-flowing rivers to kill larvae of the vector *Simulium* flies which transmit *Onchocerca volvulus* (Omura, 2008). With the discovery of the potent anthelmintic properties of IVM in the 1980s, OCP screened IVM as a potential tool for mass drug administration (MDA) to treat humans and control *O. volvulus* transmission (Omura, 2008). IVM proved to be effective in treating the clinical symptoms of river blindness with little to no side effects. IVM kills the microfilariae worms which causes the clinical symptoms of onchocerciasis but has minimal effects on the mature, female worms which produce these microfilariae. Therefore an infected person needs to be annually treated with IVM for the lifespan of the adult worms, which can be up to fifteen years (Thylefors, 2004). Onchocerciasis is most prevalent in tropical third world countries where many people would be unable to pay for IVM (brand name Mectizan®) treatments. In 1987, Merck began the Mectizan Donation Program under which they would donate IVM free of charge for the treatment of onchocerciasis for as long as it is required in Africa and six countries in South America (Omura, 2008). Since its inception, there have been over one billion IVM treatments freely distributed through this program (http:///www.mectizan.org).

In 1995 the African Programme for Onchocerciasis Control (APOC) was established to support and broaden onchocerciasis control in Africa. Where the OCP was focused on insecticide spraying to control *Simulium* flies, APOC focused on the mass distribution of IVM. Since IVM

is extremely safe (Class IV toxicity), it can be administered by non-medical personnel with the dosage being determined by an individual's height. APOC took advantage of this classification to distribute IVM by community-directed distribution where afflicted communities become responsible for dispensing IVM (Omura, 2008). Community-directed mass drug administration of IVM has been repeatedly shown to reduce and in some cases eliminate parasitic worm infections including *Onchocerca volvulus* and *Wuchereria bancrofti* (Hoerauf et al., 2011; Tekle et al., 2012; Traore et al., 2012; Coulibaly et al., 2015).

C) Ivermectin mass drug administration for malaria control

It had previously been shown in the laboratory that IVM-containing blood can kills *Anopheles* vectors of malaria at IVM levels found in human blood after a standard dose of IVM (Kobylinski et al., 2010; Chaccour et al., 2013). Given that there is a high co-endemicity of malaria with onchocerciasis and lymphatic filariasis, it's possible that IVM MDAs for filarial worm treatment could also have a transient effect on local *Anopheles* vectors and malaria transmission. The first study on the effects of IVM MDA on wild *Anopheles* vectors was in 1994 in Papua New Guinea (Bockarie et al., 1999). They reported that *Anopheles punctulatus* caught in bedrooms of IVM-treated villages had a reduced survival rate of 63.2% over 48 hours (Bockarie et al., 1999). Survival rates were significantly reduced for *An. punctulatus* captured up to four days post IVM MDA, though there was no reduction in human biting rates suggesting that the reduced mosquito survivorship due to IVM MDA had no effect on human biting rates (Bockarie et al., 1999).

At first glance, it may appear that the effects of IVM MDAs on mosquito survivorship are not robust enough to reduce malaria transmission. However, modelling shows that transient reductions in mosquito survivorship can have a large impact on vectorial capacity and parasite

transmission (Wilson, 1993). The canonical Ross MacDonald equation of vectorial capacity defines the basic reproductive rate of transmission (R_0) as the expected number of secondary cases produced by a single infected individual (Macdonald, 1956). For example, an R_0 of two means that one infectious case generates on average over the course of its infectious period two additional infections in an otherwise uninfected population (Macdonald, 1956).

$$R_0 = \frac{ma^2p^nb}{-lnp}$$

Equation 1. Ross-Macdonald equation of vectorial capacity. m: vector density; a: daily probably of vector biting host; p: daily probability of vector survivorship; n: extrinsic incubation period; b: vector competence.

The variable with the largest impact on R_0 is p, the daily probability of vector survivorship which is exponentially linked to the extrinsic incubation period (EIP) of parasite development. EIP is defined as the time from when the parasite is acquired by the vector to when the vector becomes infectious and can transmit the parasite to subsequent hosts. For malaria parasites, the EIP is between ten to fourteen days depending on ambient temperature (Beier, 1998). Since IVM has been shown to reduce daily vector survivorship (p) and the daily probably of the vector biting a host, modelling suggests that IVM MDAs may have a significant effect on malaria transmission (Kobylinski et al., 2010; Kobylinski et al., 2012; Slater et al., 2014).

After Bockarie's initial discovery on the effects of IVM MDAs on *Anopheles* vectors of malaria, there was no follow-up work in the field on its potential effects on malaria transmission for nearly twenty years. In 2008 and 2009, the impact of IVM MDAs (150µg/kg), conducted by APOC and the Senegalese Ministry of Health, were measured on local *Anopheles gambiae* s.s. and *Anopheles arabiensis*. Indoor-resting mosquitoes were collected by backpack aspirators in the early morning and maintained for five days during which mosquito survivorship was monitored. The IVM MDA reduced both *An. gambiae* s.s and *An. arabiensis* survivorship by

over 10% for six days following the MDA (Sylla et al., 2010). Modelling the potential effects on malaria transmission revealed that IVM MDAs administered once a week at 100% coverage could reduce R_0 by nearly 90% (Sylla et al., 2010). Following this work, the effect of IVM MDA on the proportion of *Plasmodium falciparum* infected *An. gambiae* s.s. was studied. Indeed, *Plasmodium falciparum* sporozoite analysis of the head and thorax of *An. gambiae* collected before and two weeks following an IVM MDA in this same region of Senegal showed a 79% reduction in sporozoite rates giving the first evidence that IVM MDAs affect malaria parasite transmission (Kobylinski et al., 2011).

To prove the effects of IVM MDAs on malaria parasite transmission were significant and could be applied to different African regions with low, moderate or high malaria transmission, IVM MDAs were followed in Senegal, Liberia and Burkina Faso and the effects on local *Anopheles* populations were measured in 2008, 2009, 2012 and 2013 (Alout et al., 2014). IVM MDAs reduced *Anopheles gambiae* s.s. survivorship across the three field sites by an average of 88.2% ±2.6 pre-MDA to 53.44% ±3.4 during the first week post-MDA (Alout et al., 2014). However, the effects on survivorship returned to pre-MDA levels after this first week post-MDA. Since IVM MDAs specifically target adult blood fed female mosquitoes, modelling suggested that beyond reducing mosquito survivorship, IVM MDAs would also cause a shift in the population age-structure to younger mosquitoes (Foy et al., 2011). This is important because of the relatively long extrinsic incubation period (EIP) of malaria parasite development in *Anopheles* mosquitoes. Causing a shift in the population structure to younger mosquitoes would result in a lower proportion of infectious mosquitoes which can transmit malaria parasites (Foy et al., 2011). As predicted, IVM MDAs shifted the age structure of the local *An. gambiae* populations to younger ages (Alout et al., 2014). While the effects of an IVM MDA on mosquito

survivorship only last for a single week, the population structure was shifted for three weeks, suggesting IVM MDAs may have a longer effect on malaria transmission relative to mosquito survivorship (Alout et al., 2014). As predicted, IVM MDAs reduced the proportion of *P. falciparum* sporozoite positive *An. gambiae* for two weeks. Sporozoite rates went from 5.31% ±1.2 pre-MDA to 2.03% ±0.8 in the first week post-MDA to 1.19% ±0.7 in the second week after MDA (Alout et al., 2014).

The latest study on the effect of IVM on malaria parasite transmission examined the effect of artemether lumefantrine (AL) to clear asexual and immature gametocytes from a *Plasmodium falciparum* human paired with one or two doses of IVM to prevent mature gametocyte transmission to the malaria vectors *An. gambiae* and *An. funestus* (Ouedraogo et al., 2015). Treatment groups received either AL alone (6 doses at 0hr, 8hr, 24hr, 36hr, 48hr and 60hr), AL and one dose of IVM coinciding with the first dose (0hr) of AL (AL-IVM1), and AL and two doses of IVM coinciding with the first and fifth dose (0hr, 48hr) of AL (AL-IVM2). Blood was collected from each treatment group on days 1, 3 and 7, fed through an artificial membrane feeder to *An. gambiae* and *An. funestus*, and survivorship was monitored for 10 days. In summary, both *An. gambiae* and *An. funestus* survivorship significantly decreased when fed on blood taken one or three days after an IVM treatment. By seven days post IVM treatment, mosquito survivorship returned to levels no different than mosquitoes that fed on blood from AL-only treated patients (Ouedraogo et al., 2015). Thus, the blood from the AL-IVM1 treatment group reduced *Anopheles* survivorship for three days and blood from the AL-IVM2 treatment group reduced *Anopheles* survivorship for seven days compared to blood from AL only treated patients. Though IVM had an effect on *Anopheles* survivorship, there was no effect on *P. falciparum* development in the mosquito (Ouedraogo et al., 2015). Using this data as a model,

it's estimated that individuals in the AL-IVM1 had a 27.2% reduction in their contribution to malaria parasite transmission in the first week after treatment and that the AL-IVM2 group had a 35.4% reduction when compared to the AL treatment group (Ouedraogo et al., 2015).

In summary, IVM treatments have been shown to impact malaria transmission by decreasing vectorial capacity through a reduction in daily mosquito survivorship. This has been shown in the field following IVM MDAs in Senegal, Liberia and Burkina Faso as well as in a small clinical trial where IVM treatments were paired with AL. These achievements have prompted the formation of the Ivermectin Research for Malaria Elimination Network (IVERMEN) which has outlined a research agenda for studying the potential of IVM for malaria control (Chaccour et al., 2015). This includes (1) studying the effects of repeated IVM MDAs or single dose long-lasting formulations during the rainy season on clinical incidence of malaria; (2) studying the effects of a targeted population strategy where at-risk individuals or a subset of the population which represents a major reservoir of infection are treated with repeated or a single long-lasting dose of IVM; (3) studying the effects and safety of IVM MDA with artemisinin-combination therapy which would target both the parasite and vector (Chaccour et al., 2015).

1.5: Alternative vector control strategy – Mosquitocidal vaccine

IVM MDAs have shown that making host blood toxic to mosquitoes can have a major effect on critical variables associated with vectorial capacity and malaria transmission (Foy et al., 2011). However, a major disadvantage to IVM MDAs is the relatively short window of time where IVM levels are high enough in the host blood to affect blood feeding mosquitoes (Alout et al., 2014; Ouedraogo et al., 2015). A potential alternative to IVM MDA is developing an anti-mosquito vaccine. The concept is to immunize mosquito hosts, which would include humans and cattle, with a critical mosquito antigen. The host would have an immune response and create

antibodies against the mosquito antigen. Subsequently, if a mosquito were to take a blood meal from an immunized host, they would imbibe anti-mosquitoes antibodies which would then bind to their target within the mosquito to affect mosquito survivorship and fitness.

The concept of using vaccines to kill blood feeding arthropods gained validity with the success of TickGARD^{PLUS}, an anti-tick vaccine targeting the midgut antigen Bm86 of *Boophilus microplus* (Willadsen et al., 1989; Penichet et al., 1994; Rodriguez et al., 1995; Willadsen et al., 1995; Jonsson et al., 2000). Since then, anti-tick vaccine research has progressed quickly (Almazan et al., 2005; de la Fuente et al., 2007b; de la Fuente et al., 2007a). However, anti-mosquito vaccine research has not had the same successes.

The potential of a mosquitocidal vaccine was first studied in 1972 (Alger and Cabrera, 1972). In this report, rabbits were immunized with (1) supernatant of whole mosquito homogenate; (2) homogenate pellet; or (3) midgut homogenates from *An. stephensi*. *An. stephensi* mosquitoes that fed on five out of seven midgut homogenate immunized rabbits had increased mortality rates compared to adjuvant only controls (Alger and Cabrera, 1972). The increase in mortality rates ranged from an increase of 10-20% over the course of 21 days post feeding (Alger and Cabrera, 1972).

Since this initial discovery, a number of papers have been published proving that *Anopheles* mosquitoes which take a blood meal from an animal immunized against a mosquito tissue can sometimes have reduced survivorship (Noden et al., 1995; Almeida and Billingsley, 1998; Lal et al., 2001; Almeida and Billingsley, 2002; Foy et al., 2002; Foy et al., 2003). However, there are several limitations to these studies which have limited the development of a mosquitocidal vaccine. Most previous work on mosquitocidal vaccines have been conducted with some form of mosquito tissue homogenate as the antigen. This approach could never be developed into an

actual vaccine, which requires immunization with a specific protein antigen (Jacobs-Lorena and Lemos, 1995; Billingsley et al., 2008). It has resulted in slight and/or highly variable reductions in survivorship and fecundity, which has differed between mosquito species, immunized hosts and trials.

Currently, there have only been two reports on a mosquitocidal vaccine targeting a single, specific antigen (Foy et al., 2003; da Costa et al., 2014). The first study immunized mice with cDNA for the *An. gambiae* mucin (AgMuc1) or *An. gambiae* peritrophic matrix 1 (AgPM1) genes (Foy et al., 2003). AgMuc-1 is a GPI-linked protein expressed in the gut lumen while AgPM1 is a critical protein in the peritrophic matrix which is secreted around the blood meal in the midgut. While direct blood feeding of *An. gambiae* on AgPM1 immunized mice had no effect on mosquito survivorship, mosquitoes fed on AgMuc1 immunized mice had a significant decrease in survivorship of nearly 40% (Foy et al., 2003). Interestingly, when mosquitoes were artificially fed serum from AgMuc1-immunized mice mixed with human erythrocytes there was no effect on mosquito mortality suggesting that host immune effector cells were required for the mosquitocidal effects (Foy et al., 2003).

Recently, a study was published on the mosquitocidal effects of blood meals from mice immunized against the protein akirin (AKR). AKR is a transcription factor that interacts with NF-kB to control innate immune response gene expression (Goto et al., 2008). Blood feeding on akirin-immunized mice had no effect on *An. gambiae* survivorship (da Costa et al., 2014). However, mice were immunized with a recombinant AKR from *Aedes albopictus* rather than from *An. gambiae*. Also, AKR is an intracellular protein that is never exposed to factors in the extracellular space. It is likely that anti-AKR antibodies imbibed through a blood meal would not

have access to natively expressed intracellular akirin and therefore could not bind and affect mosquito fitness.

1.6: Glutamate-gated chloride channel

The primary target of IVM is the glutamate-gated chloride channel (GluCl) (Cully et al., 1994). GluCl is a member of the Cys-loop family of ligand-gated ion channels (CysLGIC) along with the nicotinic acetylcholine receptor, GABA-gated, glycine-gated, histamine gated and pH-sensitive chloride channels (Wolstenholme, 2012). GluCl is only expressed in invertebrates, with their closest vertebrate relative being the γ-aminobutyric acid-gated chloride channel (GABA-Cl) (Lynagh and Lynch, 2012b; Wolstenholme, 2012). GluCls were first discovered in the extrajunctional synapses of locust muscle in 1976 (Cull-Candy, 1976). The exact identity of the glutamate-gated receptors were not initially determined but were termed "H-receptors" because they gated a hyperpolarizing current (Cull-Candy, 1976). This glutamate-gated hyperpolarizing current was later found in the neuronal ganglia and muscle fibers of *Homarus americanus* lobsters and other crustaceans where they also discovered the inhibitory current was mediated by chloride ions (Marder and Paupardin-Tritsch, 1978; Lingle and Marder, 1981). Soon, glutamate-gated chloride currents were being identified in a wide range of invertebrates including insects, arachnids, crustaceans, mollusks, flatworms and roundworms (Cleland, 1996).

The first GluCl was cloned from *Caenorhabditis elegans* in 1994 through a fractionated cDNA library approach utilizing *Xenopus laevis* oocytes as a heterologous expression system and two-electrode voltage clamp to measure glutamate and IVM-induced currents in oocytes injected with fractionated pools of *C. elegans* mRNA (Cully et al., 1994). They discovered two GluCl genes, termed GluClα and GluClβ, with only 45% amino acid identity and disparate pharmacologies. Oocytes injected with GluClα were sensitive to IVM, but not glutamate while

oocytes injected with GluClβ were sensitive to glutamate, but not IVM. Oocytes injected with both GluClα and GluClβ were sensitive to glutamate and IVM (Cully et al., 1994). IVM activation of GluClα or GluClα and GluClβ caused nearly permanent channel opening which persisted after IVM was washed out of the extracellular solution. IVM also potentiated glutamate responses in oocytes injected with GluClα and GluClβ showing that both ligands could bind to GluCl channel simultaneously, suggesting that IVM bound to a unique site from the glutamate binding pocket. Further work has since shown that there are a total of six GluCl genes in *C. elegans*: *avr*-14, *avr*-15, *glc*-1, *glc*-2, *glc*-3 and *glc*-4 (*glc*-1 and -2 are the updated names for GluClα and GluClβ respectively) (Dent et al., 1997; Vassilatis et al., 1997; Horoszok et al., 2001; Wolstenholme, 2012).

The first insect GluCl was cloned from *Drosophila melanogaster* (Cully et al., 1996). Unlike *C. elegans*, there was only a single GluCl gene, which had low amino acid sequence similarity to the two *C. elegans* GluCl gene products. *X. laevis* oocytes expressing *Drosophila melanogaster* GluCl (DmGluCl) homomers were sensitive to both micromolar concentrations of glutamate and nanomolar concentrations of IVM (Cully et al., 1996). Again, IVM caused near permanent GluCl activation. While IVM still potentiated glutamate responses in DmGluCl, it was to a minor degree compared to CeGluCl. In insects, GluCl activity has been associated with motor and sensory systems as well as circadian rhythms (Wolstenholme, 2012). The initial discovery of GluCl was on extrasynaptic regions of locust muscles. Since then, GluCl has also been recorded in the dorsal unpaired median (DUM) neurons of the locust and cockroach (Washio, 2002; Janssen et al., 2007; Janssen et al., 2010). The DUM neurons are motor neurons which innervate the flight and leg muscles in both of these organisms. In *D. melanogaster*, GluCl activity has also been observed in larval neurons (Delgado et al., 1989). In the adult fly, GluCl

activity is involved in circadian rhythms and olfactory response. GluCl expression and activity has been shown on the large ventrolateral neurons in the supraesophageal ganglion. These neurons control circadian rhythms associated with rest and wake and are directly innervated by the dorsal clock neurons (McCarthy et al., 2011; Collins et al., 2012). GluCl is also expressed in the antennal lobe which contains neurons directly innervated by sensory neurons in the antenna. GluCl activity was shown to be critical to olfactory signaling as GluCl KO flies resulted in a loss of olfactory response in the antennal lobe (Liu and Wilson, 2013). GluCl signaling within the antennal lobe occurs between glomeruli in the antennal lobe where GABA-gated inhibitory signals occurs within glomeruli (Liu and Wilson, 2013).

A) Structure and Function of GluCl

GluCl channels are comprised of five subunits arranged around an ion pore. They contain a large N-terminal extracellular domain, which contains two characteristic disulfide bonds and the glutamate-binding pocket and four transmembrane domains with a large intracellular loop between transmembrane domains three and four (Lynagh and Lynch, 2012b, a). Subunit stoichiometry of the five subunits is currently unknown. In *C. elegans*, there is evidence that AVR-15 and GLC-2 co-assemble in the pharynx muscle in an unknown ratio. In *H. contortus*, there is evidence for GLC-2 and GLC-5 to co-assemble on the commisures of inhibitory motor neurons and in *D. melanogaster*, co-immunoprecipitation studies revealed that GluCl may co-assemble with a GABA-gated chloride channel subunit, RDL (Pemberton et al., 2001; Ludmerer et al., 2002; Portillo et al., 2003). However, these are the only reports of GluCl subunit stoichiometry and there is much left to understand on how GluCl channels are assembled *in vivo*.

The crystal structure of a modified version of the *C. elegans* GluClα (GluClcryst) was solved in 2011 and was a major breakthrough in our understanding GluCl structure function

relationships (Hibbs and Gouaux, 2011). The modified GluCl had 41 amino acid residues and 6 amino acid residues removed from the amino- and carboxy- terminus respectively and the 57 amino acid intracellular loop between transmembrane domains three and four were replaced with an Ala-Gly-Thr tripeptide. Additionally, a Fab region bound to the extracellular domains of the pentameric GluClcryst was needed to stabilize the structure for X-ray analysis. The GluClcryst crystal structure was originally solved as (1) GluClcryst with IVM bound and (2) IVM and glutamate bound (Hibbs and Gouaux, 2011). A second report was later published on the crystal structure of GluClcryst in the closed state which was stabilized without the Fab by the addition of phospholipid 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, and which provided evidence on the gating mechanisms of IVM-induced GluCl activation (Althoff et al., 2014). Comparison of the GluCl closed state compared to the GluCl + IVM bound state shows an increase in the distance of the intracellular face of the channel pore from 9.8Å to 10.3Å, an increase in the distance of the extracellular face of the channel pore from 12.6Å to 18.9Å and a decrease in the distance between the N-terminus in the extracellular domain from 43.0Å to 36.3Å (Althoff et al., 2014).

The crystal structure of GluClcryst bound to IVM and glutamate clearly shows the loops and residues involved in glutamate binding. Glutamate binds at the subunit interface in the extracellular domain. The glutamate-binding pocket is made of seven loops containing nine amino acid residues which interact with the glutamate molecule (Hibbs and Gouaux, 2011). These residues are highly conserved across GluCls from different species (Lynagh and Lynch, 2012b). Since the crystal structure for GluCl bound to glutamate has not been solved without IVM, the mechanism of glutamate-activation of GluCl has not been determined because a crystal structure for GluCl + glutamate alone has not been solved (Althoff et al., 2014).

The IVM-binding pocket was confirmed to be near the extracellular face of the transmembrane domain at the subunit interface (Hibbs and Gouaux, 2011). IVM is predicted to interact with transmembrane domains two and three of the (+) subunit and transmembrane domain one of the (-) subunit. Interactions with transmembrane domain two, which line the GluCl ion pore, are predicted to cause channel opening (Hibbs and Gouaux, 2011). Though the crystal structure of GluCl bound with IVM is solved, the exact residues that interact with IVM are still disputed (Lynagh and Lynch, 2012b). However, there are two residues that are sterically required for IVM to access its binding pocket. On transmembrane domain three, there's a requisite glycine residue that faces the opening of the IVM-binding pocket. Point-mutation experiments in the glycine receptor and comparison of IVM-sensitive and IVM-insensitive channels have confirmed the importance of this glycine residue (Lynagh and Lynch, 2010; Lynagh et al., 2011; Lynagh and Lynch, 2012b). The second requisite amino acid residue for IVM binding is a proline on transmembrane domain one found at the opening of the IVM-binding pocket. This proline disrupts the helical structure of transmembrane domain one creating space for the benzofuran moiety of IVM (Lynagh and Lynch, 2012b, a). In addition, this highly conserved residue disrupts the helical structure, exposing a backbone carbonyl that is thought to form an H-bond with IVM (Hibbs and Gouaux, 2011). In the original crystal structure report of GluClcryst, Hibbs and Gouaux predicted that the serine in position 15' on transmembrane domain two (TM2-15) is critical in IVM-binding and activity. The side chain of TM2-15 faces away from the ion pore and into the IVM-binding pocket. However, a comparison of TM2-15 between highly IVM-sensitive receptors show that this residue is not required (Forrester et al., 1999; McCavera et al., 2009; Kwon et al., 2010). Instead, the working hypothesis is that the residues facing the IVM-binding pocket on TM2 require a generally polar character to bind to

IVM, but that no specific residue on this domain must have a polar side chain (Lynagh and Lynch, 2012b). Comparison of the GluCl crystal structure in the apo vs IVM-bound state provides evidence on how IVM activates GluCl. Superimposition of the two structures shows the transmembrane domains undergo a "screw-axes-like movement". This rotation tips the pore-lining transmembrane domains by 8^{o} away from the ion pore which relieves the occlusion of the pore by leucine 254 (Althoff et al., 2014).

1.7: Summary

Reducing the burden of malaria is a stated priority of the WHO, UN and public health organizations worldwide. Current malaria control strategies are limited and their efficacy is in decline as *Anopheles* populations have developed a wide range of resistance mechanisms to current malaria control tools. Recent research has clearly shown the potential of IVM treatments as a new tool for malaria control (Sylla et al., 2010; Foy et al., 2011; Kobylinski et al., 2011; Alout et al., 2014; Ouedraogo et al., 2015). There is an obvious need to study the target of IVM, GluCl, in *Anopheles* mosquitoes to understand its physiological role and uncover potential mechanisms of IVM resistance. In addition, the effectiveness of IVM MDAs on important entomological factors associated with vectorial capacity and malaria transmission lends support to the concept of developing tools to make host blood meals toxic to mosquitoes. While IVM remains at mosquito-toxic levels for a relatively short period of time, a mosquitocidal vaccine which targets critical mosquito antigens to reduce mosquito survivorship and fitness could remain mosquito-toxic for a longer time period. Another potential advantage over endectocides like IVM is that a vaccine is unlikely to be toxic to the vaccinated hosts (humans or livestock) or to the humans that eat vaccinated livestock and drink their milk, or to off-target species in the

environment (Halley et al., 1993; Sommer et al., 1993; Hernandez et al., 1994; Strong et al., 1996; Errouissi et al., 2001; Salas et al., 2003). Therefore, developing a mosquitocidal vaccine targeting a specific and highly sensitive target in *Anopheles* vectors of malaria could have a major impact on malaria transmission.

Chapter 2: Characterization of the target of ivermectin, the glutamate-gated chloride channel, in *Anopheles gambiae* s.s.

2.1: Introduction

The latest WHO World Malaria Report estimates that in 2013 there were 198 million cases worldwide causing 584,000 malaria-related deaths. Nearly 90% of these deaths occurred in Africa where a primary vector of malaria is *Anopheles gambiae sensu stricto* (s.s.) (WHO World Malaria Report, 2014). Current malaria control programs primarily target malaria vectors through the use of long lasting insecticide treated bed nets and indoor residual spraying of pyrethroid-based insecticides. However, pyrethroid resistance is becoming widespread in many *An. gambiae* populations across Africa (Ranson et al., 2011; Trape et al., 2011). Out of the recent efforts to find new vector-targeting interventions with novel modes of action, the endectocide ivermectin (IVM) has arisen as a new candidate to control malaria transmission. IVM, when imbibed by vectors from host-treated blood meals, has proven to efficiently kill or disable *An. gambiae s.s.* both in the lab and the field (Kobylinski et al., 2010; Sylla et al., 2010). More recently, IVM mass drug administrations in multiple locations across west Africa have been shown to temporarily reduce the proportion of *P. falciparum*-infected *An. gambiae* in IVM-treated villages (Kobylinski et al., 2011; Alout et al., 2014). Sub-lethal doses of IVM have also been shown to inhibit the sporogony of *P. falciparum* in *An. gambiae* s.s and impair coordinated flight patterns (Butters et al., 2012a; Kobylinski et al., 2012). In clinical trials, IVM, in combination with artemether-lumefantrine, has been shown to reduce the likelihood of malaria transmission through reduction of mosquito survivorship (Ouedraogo et al., 2014). These studies demonstrate that IVM has promise as a novel malaria control tool.

The primary target of IVM is the invertebrate glutamate gated chloride channel (GluCl) (Cully et al., 1994; Cully et al., 1996; Janssen et al., 2007; McCavera et al., 2009; Janssen et al., 2010; Moreno et al., 2010), though it also has efficacy against other members of the invertebrate Cys-loop family of neurotransmitter receptors including the γ-aminobutyric acid- (Brown et al., 2012), histamine- (Zheng et al., 2002), and pH-sensitive chloride channels (Schnizler et al., 2005). Because IVM is used to control and treat parasitic nematode diseases (Omura, 2008), the majority of research on IVM targets has occurred in nematodes or model organisms, but the function of GluCl in mosquito disease vectors is unknown.

The purpose of this project was to characterize GluCl from *An. gambiae* in order to understand the physiological role of GluCl and how IVM may be affecting mosquito physiology. Cloning of the *An. gambiae* GluCl (AgGluCl) revealed unique splicing sites and products not previously predicted. We expressed AgGluCl clones in *Xenopus laevis* oocytes to measure its electrophysiological activity in response to glutamate and IVM. We also examined AgGluCl isoform-specific transcript levels across different tissues, ages, blood feeding status and gender and GluCl tissue expression in adult *An. gambiae*.

2.2: Materials and Methods

Mosquitoes. *Anopheles gambiae* s.s. G3 strain (origin The Gambia) were raised at 28-31^{o}C, 80% relative humidity on a 14:10 light dark cycle. Larvae were fed ground Tetramin® fish food daily. Adults were provided with water and 10% sucrose solution *ad libitum*. Colony mosquitoes were blood fed every 3 to 4 days on defibrinated calf blood. Defibrinated calf blood was prepared by the Colorado Serum Company (Denver, CO) where blood collection protocols are annually reviewed and approved by their IACUC.

AgGluCl Cloning. RNA was isolated from 10 female *An. gambiae* mosquitoes using TRIzol® (Invitrogen, Waltham, MA). Contaminating DNA was removed using an RNase-Free DNase Kit (Qiagen, Valencia, CA). cDNA library was constructed by reverse transcription of isolated RNA with the SuperScript® III Reverse Transcriptase Kit (Invitrogen, Waltham, MA) using poly dT and random hexamers. AgGluCl specific primers were designed for the putative AgGluCl gene from the VectorBase Community Annotation Database (*An. gambiae* PEST strain genomic sequence AGAP001434; Assembly: AgamP4). The following AgGluCl specific primers (5' to 3'): Fwd1: ATGGCCTCGGGCCATTTCTT; Rev1: TTAGTCCTCCTCCTCTTCGCG; Fwd2: ACGCGTCCCGTTCAGCGGAT; Rev2: ATCCGCTGAACGGGACGCG were used to probe the cDNA library. Positive fragments were cloned into pCR4 TOPO TA plasmid (Invitrogen, Waltham, MA) and sequenced at the Proteomic and Metabolomics Core Facility at Colorado State University.

Gene Structure and Domain Prediction. Signal peptide, transmembrane domains and phosphorylation sites were predicted using SignalP, TMHMM and NetPhos 2.0 on the Center for Biological Sequence Analysis website from the Technical University of Denmark (http://www.cbs.dtu.dk/). The AgGluCl genetic structure was created using Gene Structure Display Server Version 2.0 (http://gsds.cbi.pku.edu.cn/index.php). Amino acid sequence alignments were performed using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Homology Modeling. A homology model for the AgGluCl-a1 isoform was created using MODELLER 9.11 (Eswar et al., 2007). Individual chains of the *C. elegans* crystal structure (3RIF.pdb) were used as comparative models for creating AgGluCl-a1 backbone structures. The AgGluCl-a1 model was truncated to remove disordered termini and loop insertion regions (residues 1-35, 334-415, 448-451). Bound glutamate and ivermectin molecules were added to the

homology model through superposition with 3RIF.pdb coordinates. The SHARPEN modeling platform (Loksha et al., 2009) was used to perform combinatorial side chain optimization based on rotamers from the Dunbrack rotamer library (Dunbrack and Karplus, 1993).

Quantitative Reverse Transcription PCR (qRT-PCR) of Mosquito Tissues. Mosquitoes were aged to 2, 6, and 14 days post emergence (DPE) and fed either a normal blood meal or a blood meal containing 11.75ng/mL IVM. Two DPE mosquitoes were fed a normal blood meal on day 2, while 7 DPE mosquitoes were fed on days 2 and 6, and 14 DPE mosquitoes were fed on days 2, 6, 10 and 14 to mimic normal blood feeding patterns. At 24 hours post blood feeding, mosquitoes were knocked down at 4^{o}C and sorted into four groups: non-blood fed females, blood fed females, IVM blood fed females and males. Mosquitoes were dissected into head, thorax and abdomen and pooled into three groups of ten (three biological replicates). RNA was extracted using TRIzol® (Invitrogen, Waltham, MA), normalized to 25ng/µL and stored at -80^{o}C. All blood feeds and dissections occurred between 2:00pm-3:00pm to control for circadian rhythms that might affect transcript levels.

qRT-PCR was performed with the SensiMix™ II Probe No-ROX Kit (Bioline, Taunton, MA) to determine AgGluCl isoform-specific transcript levels. AgGluCl primers were designed to pair with sequences on exons 2 and 4 and to amplify the alternatively spliced exons 3a. 3b and 3c (Fwd: GCATCAACGGAACCGACG; Rev: TCGGAACGTAAGCTGTACAC; amplicon length: 110bp or 113bp). Taqman probes were designed against sequences in exons 3a, 3b and 3c to delineate AgGluCl isoforms a1+a2, b and c (A: TCGTTCGAAGCATTATGACCATCAGTGAC; Probe B: TGTAAGAAGTATATCTAAAATCGATGACG; Probe C: GCCACACAAGTGTTCGTCAACATGTProbe). AgGluCl primers and probe specificity and

efficiency were validated on serially diluted AgGluCl splice isoforms cloned in the pCR-4 plasmid. All probes utilized FAM fluorophores and TAMRA quenchers. AgGluCl transcript levels were measured relative to ribosomal protein S7 (RPS7 Fwd: AGCAGCTACAGCACTTGATTATTGG; Rev: AGCAGCTACAGCACTTGATTATTGG; Probe: CCCGATTTCTCCGATCTTTCACATTCCA) (Blandin et al., 2004). All reactions were performed in technical duplicate with each well containing 25ng of RNA. All plates contained a no template control and positive controls of each AgGluCl splice isoform. AgGluCl splice isoform transcript levels were compared using 2-way ANOVA with Tukey's post-hoc analysis.

***In vitro* Transcription and Oocyte Expression.** AgGluCl cDNA was cloned into the pGEM-HE plasmid. The plasmid was linearized using the BspQ1 restriction enzyme. Capped mRNA was synthesized using the mMessage mMachine® T7 Ultra Kit (Ambion, Grand Island, NY). Stage 4-5 *Xenopus laevis* oocytes (EcoCyte Bioscience, Austin, TX) were maintained at 18^{o}C in Barth's solution (87.6mM NaCl, 2.4mM $NaHCO_3$, 1.1mM KCl, 0.32mM $Ba(NO_3)_2$, 0.4mM $BaCl_2$, 0.8mM $MgCl_2$, 15mM HEPES, pH 7.6). Oocytes were injected with 46nL of mRNA (500 ng/μL) using a Drummond Nanoject and micropipettes with a tip <10μm in diameter. Oocytes were incubated in Barth's solution for 72 – 120 hours at 18^{o}C before recording.

Oocyte Electrophysiology. Oocytes were recorded using two-electrode voltage clamp (GeneClamp 500B) held at -80mV. Oocytes were continuously perfused at a rate of 4mL/min via a peristaltic pump. The extracellular solution contained Barth's Solution plus glutamate or IVM (Sigma Aldrich, St. Louis, MO; mixture of two submoieties B1a and B1b, primarily containing B1a) as specified. IVM solutions were made from a 10mg/mL IVM stock dissolved in DMSO. Control solutions contained equal volumes of DMSO which never exceeded 1% and had no effect on the oocytes. Solution exchange was controlled via the BPS-8 Valve Control System

(ALA Scientific, Farmingdale, NY) and electronic valves. Electrodes of 1-5MΩ were filled with 1M $CsCl_2$ and 5mM EGTA pH 7.5 to prevent activation of endogenous calcium-activated chloride channels. As a negative control, we performed two-electrode patch clamp on non-injected and water-injected oocytes which never resulted in glutamate- or IVM-induced currents. As a positive control, we injected oocytes with a mammalian glutamate-gated cation channel (GluA1) (Partin, 2001) and successfully measured glutamate-induced currents. Current responses were acquired using an HP ProBook 6460b with an Instrutech ITC-16 interface under the control of AxoGraph X acquisition software. A dose-response curve was fitted to the data using the Hill equation:

$$I_{max} = \frac{1}{1 + \left(\frac{EC_{50}}{[L]} \right)^{H}}$$

where I_{max} is the maximal response, [L] is the ligand concentration, EC_{50} is the ligand concentration for the half maximal response and H is the Hill coefficient. The Hill coefficient is the number of ligands required to activate the channel. Five biological replicates were tested for each measurement.

Immunohistochemistry (IHC). Samples were prepared from female blood fed *An. gambiae* aged two to four days post-emergence. Twenty four hours after blood feeding, twenty four engorged females were knocked down at 4°C and injected intrathoracically with 46nL of 4% paraformaldehyde (Drummond Nanoject, Broomall, PA). Specimens were briefly washed in 70% ethanol before overnight fixation in 4% paraformaldehyde at 4°C. After fixation, specimens were paraffin embedded, cut into 5µm thick slices and mounted onto slides (Colorado HistoPrep, Fort Collins, CO). Slides were heated at 65°C for 10 minutes and treated with xylene to remove the paraffin layer and then re-hydrated with graded washes in ethanol and PBS. Specimens were

treated with graded washes of methanol and PBS to reduce autofluorescence. Slides were blocked for 2 hours with 10% non-fat dry milk and 0.1% Triton-X in PBS. Primary antibody staining consisted of 1:500 rabbit anti-AgGluCl IgG (prepared by GenScript, Piscataway, NJ) and 1:500 goat anti-HRP antibodies to stain neuronal tissue (Jan and Jan, 1982) (Jackson ImmunoResearch Laboratories, West Grove, PA) incubated overnight at 4^{o}C. Rabbit derived polyclonal anti-AgGluCl IgG was created against the N-terminal extracellular domain of AgGluCl-b by Genscript. Antibody specificity was verified through ELISA, western blot and immunostaining of C6/36 cells transfected with AgGluCl-a1 and AgGluCl-b (Meyers et al., 2015a). Specimens were washed with 0.05% Tween-20 Tris-based Buffer Solution (TTBS) and then incubated with 1:1000 donkey anti-rabbit Alexa 555 (Invitrogen, Waltham, MA) and 1:1000 donkey anti-goat Alexa 488 (Invitrogen, Waltham, MA) for 3 hours at room temperature. Slides were mounted with VectaShield® containing DAPI (Vector Lab, Burlingame, CA). Negative controls underwent the same procedure, but substituted histidine affinity-purified polyclonal rabbit IgG from non-immunized rabbits as the primary antibody.

2.3: Results

Cloning of AgGluCl Splice Isoforms and Gene Structure

We identified the putative *An. gambiae* GluCl (AgGluCl) gene using the basic local alignment search tool (BLASTp) algorithm on the VectorBase Community Annotation Database (https://www.vectorbase.org/) (Megy et al., 2012). We BLASTed the *An. gambiae* predicted amino acid sequence database (Assembly AgamP4) for similar amino acid sequences to the GluCl coding sequences from *C. elegans* (CeGluClα, GLC-1) (Cully et al., 1994) and D. *melanogaster* (DmGluClα) (Fig. 2.2) (Cully et al., 1996). Sequence analysis revealed one significant hit in the *An. gambiae* PEST strain genomic sequence (AGAP001434). This gene was

cloned out of a cDNA library created from mRNA isolated from adult blood fed female *An. gambiae*.

Vectorbase predicts AgGluCl (AGAP001434) contains 10 exons with exon 3 being alternatively spliced to produce three splice isoforms (https://www.vectorbase.org/) (Megy et al., 2012). However, our cloning resulted in the discovery of four splice isoforms containing DNA coding sequences not predicted by Vectorbase (Fig. 2.1A). The newly discovered splice isoform

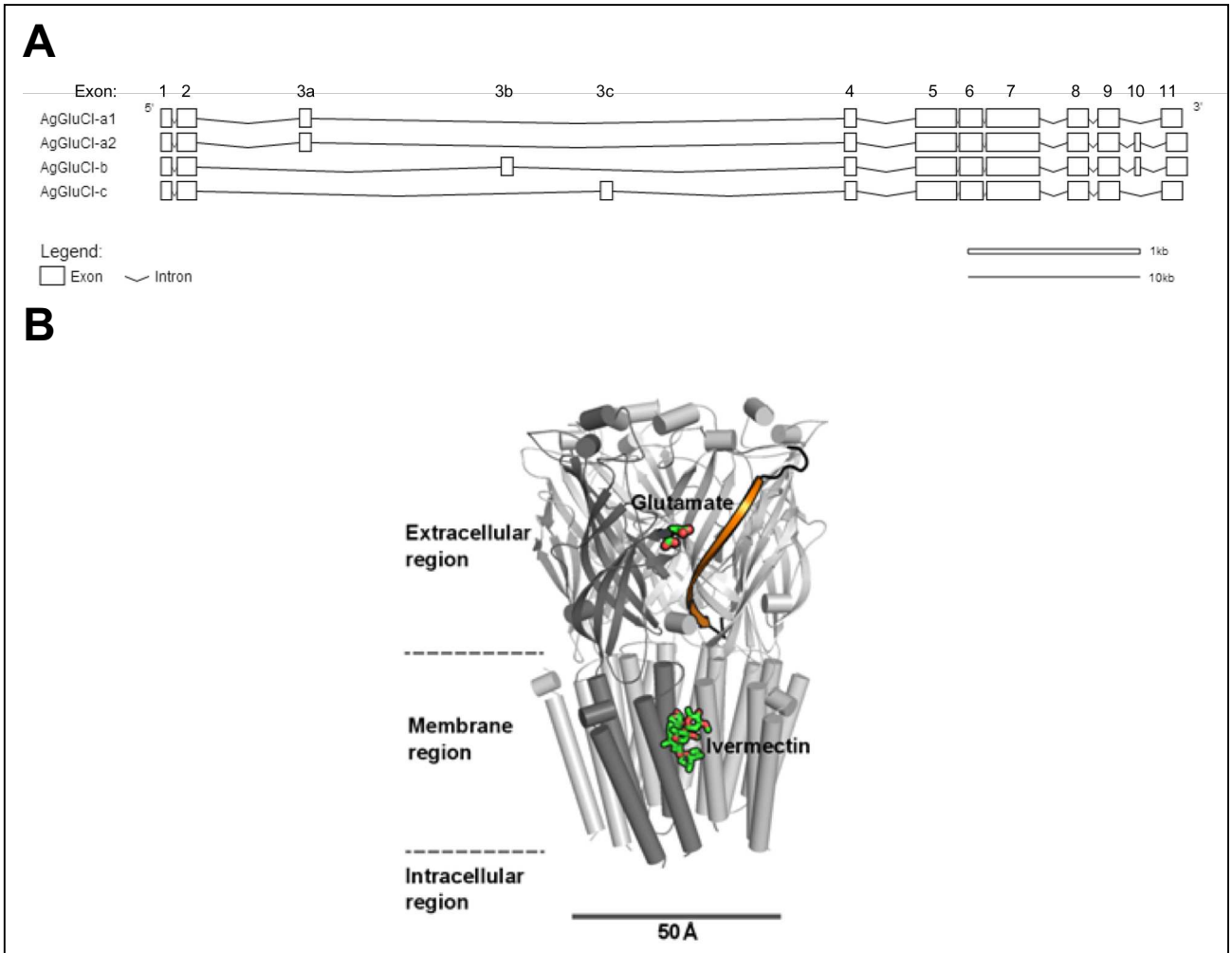


Figure 2.1. AgGluCl gene structure, predicted amino acid alignment and homology model. (A) Genetic structure of AgGluCl splice isoforms. (B) AgGluCl-a1 homology model with glutamate and IVM bound. The alternatively-spliced portion of the N-terminal extracellular domain (exon 3) is highlighted in orange. Experiments performed by Jacob Meyers, Lucas Johnson and Christopher Snow.

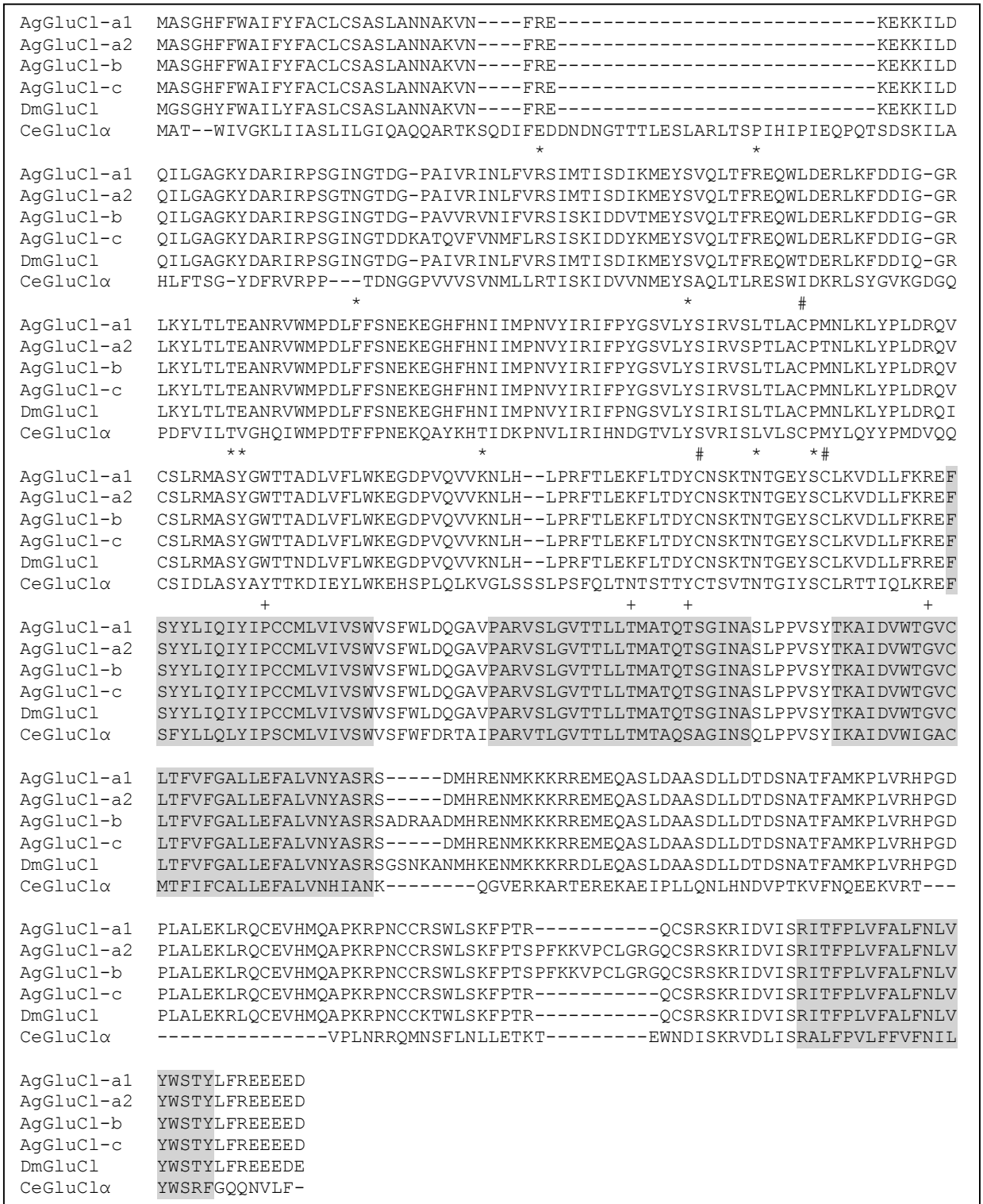


Figure 2.2. ClustalW alignment of AgGluCl amino acid sequences with *D. melanogaster* GluCl (DmGluCl) and *C. elegans* GluCl (CeGluCl). Grey highlights denote transmembrane domains. (*) glutamate binding residues; (+) IVM binding residues; (#) cysteine involved in disulfide bonds. Experiments performed by Jacob Meyers.

contains the same splicing pattern as the 'AgGluCl-a' named currently in Vectorbase (inclusion of exon 3a and splicing out of exons 3b and 3c), but incorporates additional nucleotide sequences. We propose to rename 'AgGluCl-a' to AgGluCl-a1 and to name the new splice isoform AgGluCl-a2.

We discovered two additional DNA sequences that are incorporated into the AgGluCl coding sequence that were not previously predicted. The first of these sequences is a small 15 nt sequence found only in AgGluCl-b (nt position 1007-1021). When we searched the Vectorbase genomic data for this sequence, we discovered it at the intron/exon junction of intron 7 and exon 8. Our data show that this sequence is spliced out of AgGluCl-a1, -a2 and –c, but included in splice isoform b. The second sequence is a 33 nt insertion found in the novel splice isoform, AgGluCl-a2 and AgGluCl-b. Positional analysis of this insertion showed that it occurs in predicted intron 9, between exons 9 and 10 (position 901-933). This sequence, previously predicted to be part of intron 9, seems to be a novel exon. These findings redefine the structure of the AgGluCl gene to incorporate an addition exon (exon 10) and change the overall number of AgGluCl exons to 11 (Fig. 2.1A).

AgGluCl Splice Isoform Protein Structure

Analysis of the putative AgGluCl gene products revealed a protein corresponding with chloride-permeable members of the Cys-loop family of neurotransmitter receptors. Members of this family contain a signal peptide, a large N-terminal extracellular domain containing 2 conserved disulfide bonds, four transmembrane domains, an anion permeable pore and a large intracellular loop between transmembrane regions 3 and 4 (Fig. 2.1B, Fig 2.2). ClustalW alignment of the gene product with the recently crystallized GluClα from *C. elegans* (CeGluCl)

and *D. melanogaster* GluCl (DmGluCl) revealed a protein with conserved residues associated with the glutamate and IVM binding pockets (Fig. 2.2) (Hibbs and Gouaux, 2011).

AgGluCl splice isoforms contain 2 major regions of heterogeneity in the protein structure. The first results from the alternative splicing of exons 3a, 3b and 3c. This encodes for a 22 amino acid region found on the N-terminal extracellular domain of the channel. This region contains loop G of the glutamate binding pocket (Fig. 2.1B, Fig 2.2) (Hibbs and Gouaux, 2011). The second region of heterogeneity is the result of alternatively spliced exon 8 (5 amino acids) and exon 10 (11 amino acids). The products of these alternative splicing events occur within the large intracellular loop between transmembrane domains 3 and 4 (Fig. 2.2). The splicing product of exon 8 is found immediately after transmembrane domain 3 and the splicing product of exon 10 is found prior to transmembrane domain 4. All GluCl crystal structures required the removal of this intracellular loop for crystallization, which is why it was not included in our AgGluCl homology model (Fig. 2.1B).

Functional Expression and Activity of AgGluCl

Heterologous expression of *An. gambiae* receptors and electrophysiological analysis pose technical challenges, but is important to understanding the physiological responses of a newly cloned channel. Previous attempts to heterologously express and functionally characterize other members of the Cys-loop family from *An. gambiae* have been unsuccessful (Jones et al., 2009). *X. laevis* oocytes were injected with AgGluCl mRNA and incubated for 72-120 hours for protein expression. Sufficient AgGluCl expression to permit measurable currents was successful on less than 5% of oocytes injected with AgGluCl mRNA. In oocytes expressing functional AgGluCl-b, glutamate evoked a rapidly activating inward current. AgGluCl-b responses were analyzed from glutamate concentrations ranging from 10μM to 300μM (Fig. 2.3A). A dose-response curve was

fitted to the data using the Hill equation. The EC_{50} for glutamate was 30.22µM ± 2.75 and the Hill coefficient was 1.93 ± 0.32, suggesting that more than one glutamate molecule is necessary

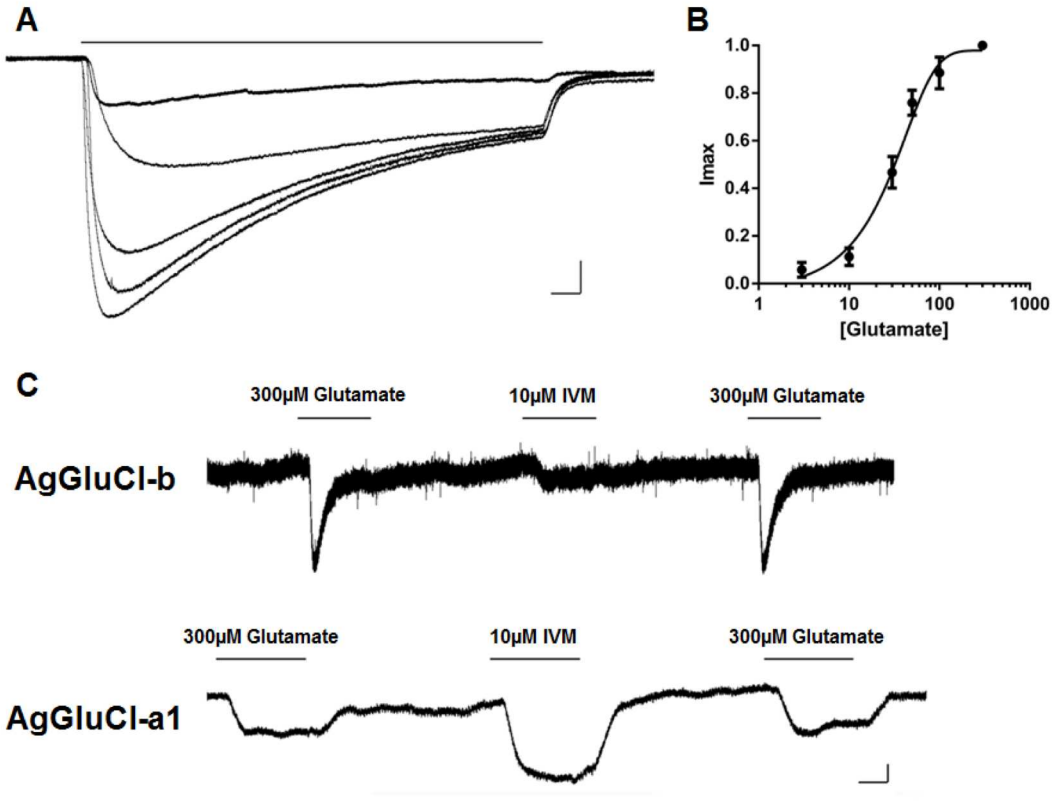


Figure 2.3. AgGluCl is activated by glutamate and IVM. (A) Representative currents of AgGluCl-b in response to 10µM, 30µM, 50µM, 100µM and 300µM glutamate. Scale: 10nA, 5sec. (B) Glutamate dose-response curve of AgGluCl-b. Data presented as mean and standard deviation. (C) Representative currents of AgGluCl-a1 and AgGluCl-b in response to glutamate and IVM. Scale: 2nA, 5sec. Experiments performed by Jacob Meyers.

to activate the channel (Fig. 2.3B). AgGluCl-b was insensitive to IVM (Fig. 2.3C).

IVM induced a slowly activating current in oocytes expressing AgGluCl-a1 (Fig. 2.3C). The peak current activated by 10µM IVM was 208.1% ± 60.8 (mean ± S.D.; n=5) greater than the peak current activated from the highest tested glutamate concentration (300µM). IVM did not potentiate subsequent glutamate-evoked currents in AgGluCl-a1 when compared to controls

(114.3% ± 16.1; mean ± S.D.; n=5). We were not successful at expressing AgGluCl-a2 or AgGluCl-c.

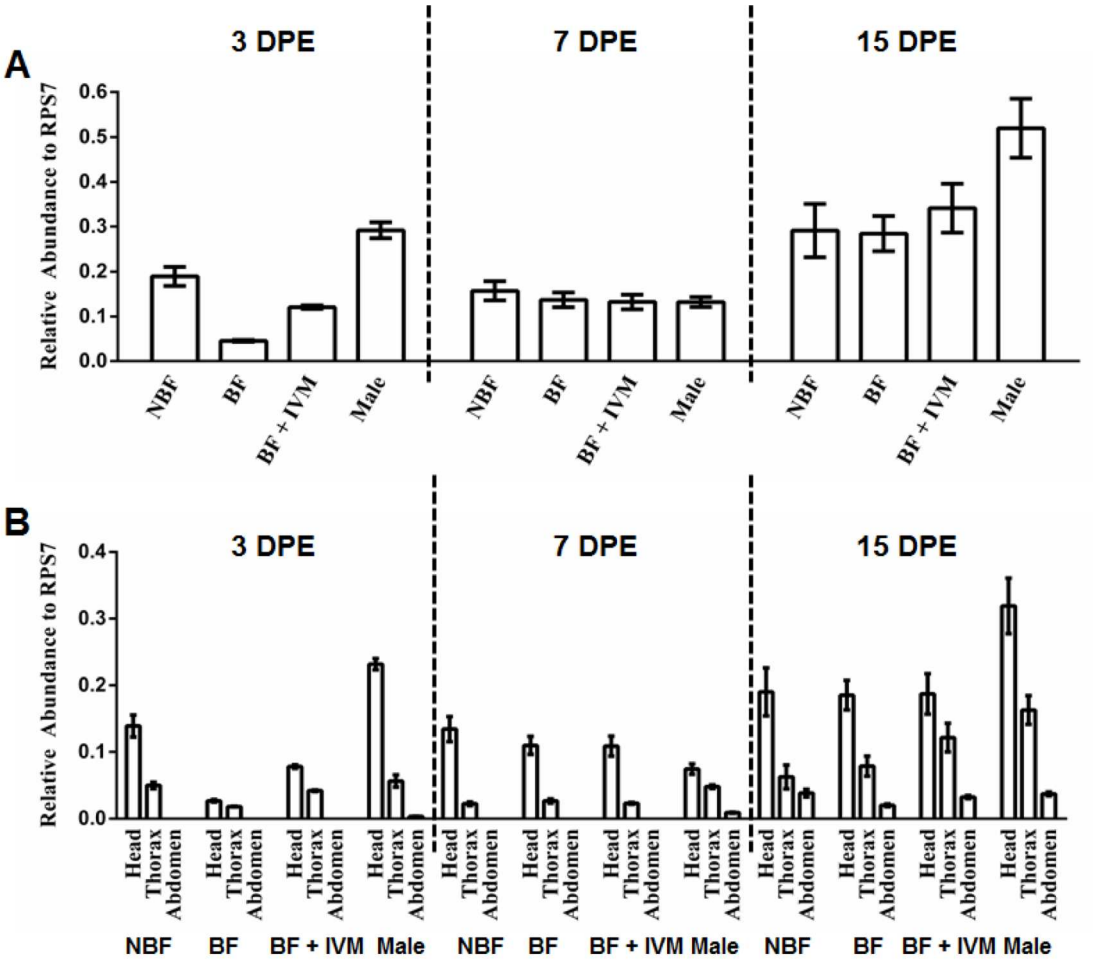
Transcriptional Profile of AgGluCl splice isoforms in adult *An. gambiae*

We measured AgGluCl isoform-specific transcript levels relative to the housekeeping gene ribosomal protein S7 (RPS7) across tissues, ages, blood feeding status and gender (Fig. 2.4). When total AgGluCl transcripts (all isoforms together) were analyzed by blood feeding status and age, the act of blood feeding significantly, but modestly, reduced total transcript levels in 3 day post-emergence (DPE) adult females ($p<0.0001$; n=3), but not those at 7 DPE ($p=0.6005$; n=3) or 15 DPE ($p=0.9742$; n=3). The addition of IVM to a blood meal significantly increased AgGluCl transcript levels in 3 DPE females ($p<0.0001$; n=3), but not 7 DPE ($p=0.9919$; n=3) or 15 DPE ($p=0.7133$; n=3) (Fig 2.4A). Adult female *An. gambiae* had significantly lower AgGluCl transcript levels when compared to males at 3 DPE ($p<0.0001$; n=3) and 15 DPE ($p<0.0001$; n=3) and relatively equal AgGluCl transcript levels at 7 DPE ($p=0.4158$; n=3) (Fig. 2.4A).

Analysis of AgGluCl tissue-specific expression revealed transcripts primarily in the head and thorax, with the highest transcript levels found in the head. This tissue-specific expression pattern was consistent across all ages, blood feeding status and gender except one ($p<0.05$ for all head vs thorax comparisons except 3 DPE BF head vs thorax: $p=0.6519$; n=3) (Fig. 2.4B). We detected no AgGluCl transcripts in the female abdomen until 15 DPE, the oldest age tested, while AgGluCl transcripts were always present in low levels in the male abdomen. The abdominal AgGluCl transcripts at 15 DPE were of relatively low abundance and predominately the AgGluCl-c isoform (Fig. 2.4B, 2.4C).

Analysis of isoform-specific expression patterns showed that the predominant AgGluCl isoforms are AgGluCl-a (our assay could not distinguish these related isoforms, the transcripts

could be either AgGluCl-a1 or –a2) and AgGluCl-b (Fig. 2.4C). Transcripts of these isoforms are found in relatively equal amounts across tissues, ages and blood feed status. AgGluCl-c transcripts were found at very low levels or completely absent in most tissues until 15 DPE when AgGluCl-c transcript levels were relatively high (Fig. 2.4C).



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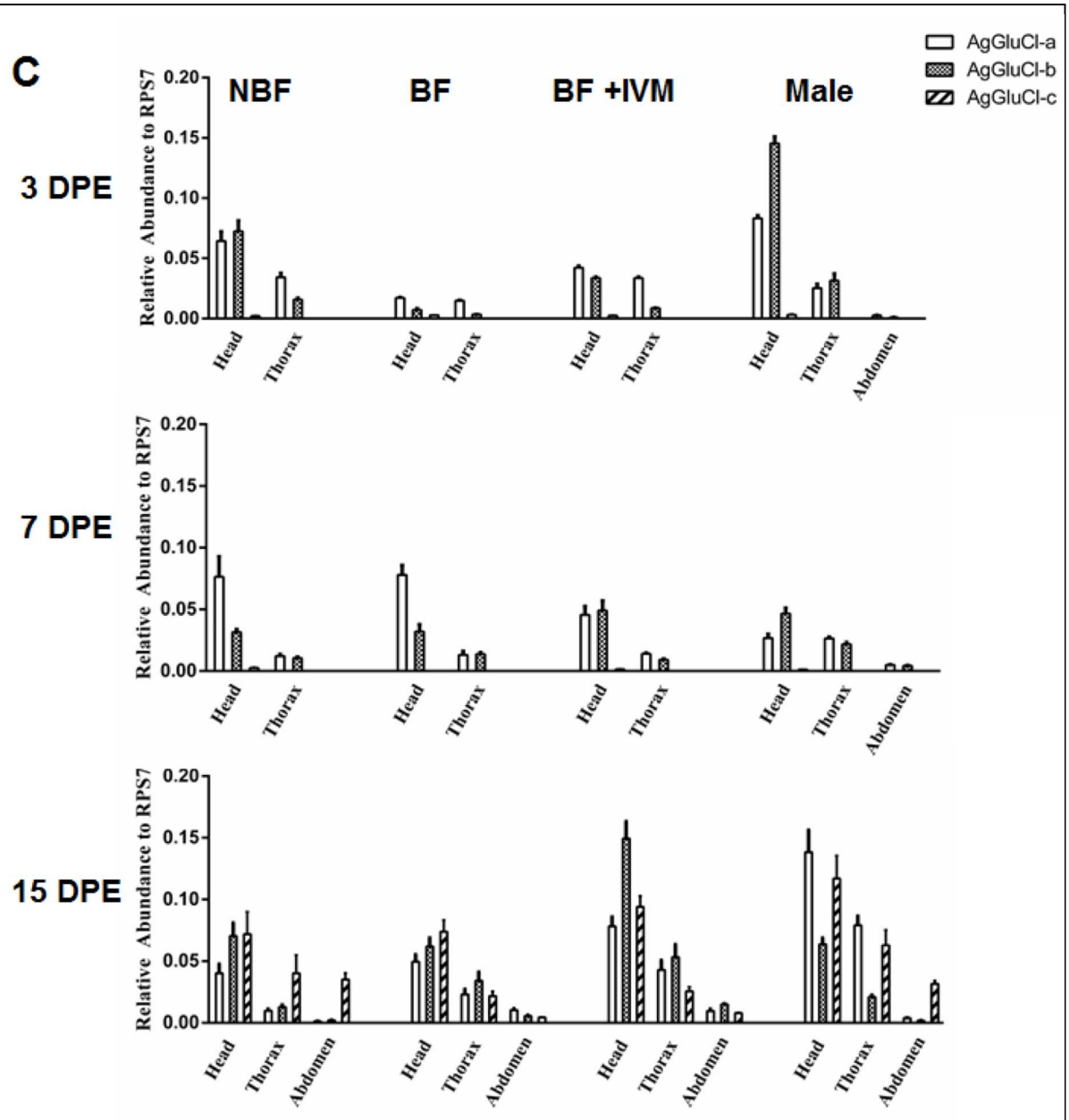

Figure 2.4. AgGluCl transcript levels across tissues, age, blood feeding status and gender. *An. gambiae* total AgGluCl transcript levels presented by age, blood feeding status and gender (A) and additionally presented by tissue (B). AgGluCl splice isoform-specific transcript levels presented by tissue, age, blood feeding status and gender (C). Note that the assay could not distinguish between isoforms AgGluCl-a1 and AgGluCl-a2. All data presented as mean and standard deviation. Experiments performed by Jacob Meyers and Wojtek Kuklinski.

GluCl expression in adult *An. gambiae*

Given that AgGluCl transcripts are abundant in the *An. gambiae* head and thorax, we wanted to examine which specific tissues express AgGluCl to begin to understand the physiological role of AgGluCl. We stained sagittal sections of whole adult female (2-4 DPE) mosquitoes for AgGluCl and the neuronal marker, anti-HRP IgG, (Jan and Jan, 1982) to distinguish neuronal tissue. The anti-AgGluCl IgG was created against the N-terminal extracellular domain of AgGluCl-b and has previously been shown to bind to multiple AgGluCl isoforms (Meyers et al., 2015a). Immunolabeling showed AgGluCl expression in the antennal

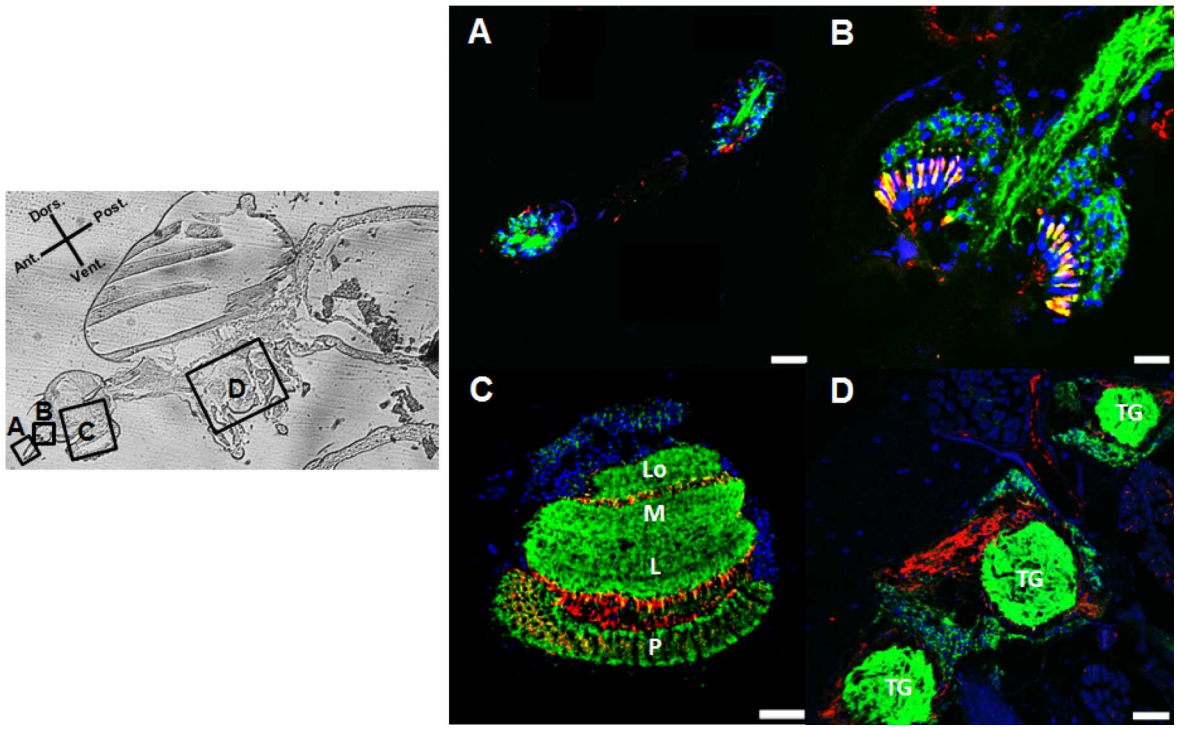

Figure 2.5. Representative images of adult, female *An. gambiae* immunoreactive tissues to AgGluCl IgG. Left: Differential interference contrast image of a whole, female *An. gambiae* sagittal section outlining the location and orientation of immunostained images (A) Two antennal segments. Scale: 10µM. (B) Johnston's Organ. Scale: 10µM. (C) Head: P: photoreceptors. L: lamina, M: medulla, Lo: lobula; Scale: 20µM. (D) Thoracic ganglia. TG: thoracic ganglia. Scale: 50µM. Red: AgGluCl; Green: Neuronal Marker; Blue: DAPI. Experiments performed by Jacob Meyers.

segments, Johnston's Organ, optic lobe, supraesophageal ganglion and thoracic ganglia (Fig. 2.5). There were small puncta of AgGluCl staining on antennal segments that could be associated with antennal sensilla (Fig. 2.5A). The Johnston's Organ, a mechanosensory organ in the pedicel associated with audition and flight coordination, has specific AgGluCl staining in the scolopidea (Fig. 2.5B). AgGluCl is present in the photoreceptors and each layer of the optic lobe which includes the lamina, medulla and lobula (Fig. 2.5C). AgGluCl staining was present on neuronal processes as well as cell bodies throughout the supraesophageal ganglion, though it is difficult to distinguish precisely which neuropil contain AgGluCl expression. Lastly, there was AgGluCl staining in all three thoracic ganglia which contain the motor neurons that control the flight and leg muscles (Fig. 2.5D).

2.4: Discussion

Community-directed mass drug administration of IVM is effective at killing *An. gambiae* mosquitoes (Sylla et al., 2010; Alout et al., 2014), inhibiting *P. falciparum* sporogony in *An. gambiae* (Kobylinski et al., 2012), and disrupting malaria parasite transmission in endemic villages (Kobylinski et al., 2011; Alout et al., 2014). Thus, IVM MDA has strong potential to be developed as a novel tool for malaria parasite transmission control. This study is the first to examine the primary target of IVM, GluCl, in any mosquito species.

The cloning of the AgGluCl gene from female *An. gambiae* mRNA revealed four splice isoforms where only three isoforms had previously been predicted (https://www.vectorbase.org) (Megy et al., 2012). These isoforms differ in their splicing of exons 3, 8 and 10. The splicing pattern of exon 3 is similar to GluCl splicing patterns recently discovered in other Dipteran species (Furutani et al., 2014; Kita et al., 2014). This exon encodes for the same number of amino acids (22-23 amino acids) and is found in the N-terminal extracellular domain containing

loop G of the glutamate binding pocket across *Musca domestica* and *Bombyx mori* (Furutani et al., 2014; Kita et al., 2014). Though this alternatively spliced exon codes for residues involved in glutamate binding, these isoforms have previously been shown to have equal sensitivities to glutamate and IVM in *Musca domestica* and *Bombyx mori* (Furutani et al., 2014; Kita et al., 2014). Interestingly, alternative splicing of exon 3 has also been shown to impact heterologous expression of BmGluCl (from *Bombyx mori*) in *X. laevis* oocytes (Furutani et al., 2014). BmGluCl-c had the strongest expression and cell surface density compared to isoforms a and b. Efficient BmGluCl expression required specific amino acid residues at the inter-subunit interface (Thr77, Thr78, Ser80 and Ile82) which are only found on BmGluCl-b and c but not isoform a (Furutani et al., 2014). These same residues are conserved in AgGluCl-a1 and a2, but not found in AgGluCl-b or c. This may explain why we had very little success in heterologous expression of AgGluCl-a1 and b in *X. laevis* oocytes and why the currents were relatively small. We also attempted to express functional AgGluCl isoforms in C6/36 cells (derived from *Aedes albopictus*), S2 cells (derived from *Drosophila melanogaster*), Sf9 cells (derived from *Spodoptera frugiperda*) and HEK293 cells (derived from *Homo sapiens*), but only were able to achieve non-functional expression in insect cells (see Fig 3.1) (Meyers et al., 2015a). This is also highlighted by previous unsuccessful reports attempting to heterologously express functional nicotinic acetylcholine receptors from *An. gambiae* (Jones et al., 2009).

The newly cloned AgGluCl isoforms also contained splicing sites not previously predicted for the gene. These splicing sites occur in the large intracellular loop between transmembrane domains 3 and 4. The amino acid insertions into the intracellular loop do not affect any potential phosphorylation sites as predicted by NetPhos 2.0 (Blom et al., 1999). The function of this region has been shown to impact channel kinetics and trafficking in other members of the Cys-

loop family and provides potential sites for post-translational modifications (Tsetlin et al., 2011; McKinnon et al., 2012). However, it is still unclear how the regions of heterogeneity between isoforms may affect channel activity and expression.

AgGluCl-b encodes a rapidly activating glutamate-gated chloride channel sensitive to micromolar concentrations of glutamate and requires cooperative glutamate binding for activity. These characteristics are similar to expressed and native GluCl from *Drosophila melanogaster* and *Locusta migratoria* (Cully et al., 1996; Janssen et al., 2007). We did not measure a full dose-response of glutamate on AgGluCl-a1 or the other two isoforms. However, preliminary data suggests that AgGluCl-a1 has a non-desensitizing response to glutamate while AgGluCl-b is desensitizing. Our data show that AgGluCl-b is insensitive to 10μM IVM which has been shown to activate all IVM-sensitive channels (Lynagh and Lynch, 2012b). Though AgGluCl-b is insensitive to IVM, it is currently unclear if its expression is linked to an IVM resistance phenotype. However, IVM agonizes AgGluCl-a1 and gates a slowly activating current that exceeds the maximum glutamate response. All AgGluCl isoforms contain the requisite amino acid residues in the IVM binding pocket for IVM-induced activation including the TM1-Pro and TM3-Gly (Fig. 2.2) (Lynagh and Lynch, 2012a, b). AgGluCl isoforms also contain polar residues hypothesized to coordinate IVM binding on TM domain 2 including Thr-M2-15'and Arg-M2-19' (Lynagh and Lynch, 2012b). These two isoforms differ in their splicing of exons 3, 8 and 10, which encode for regions in the N-terminal extracellular domain and large intracellular loop between transmembrane domains 3 and 4. It is unclear what residues encoded by these alternatively spliced exons are responsible for IVM sensitivity.

The magnitude of the IVM-induced peak current relative to the glutamate-induced peak current for AgGluCl-a1 is similar to *D. melanogaster* and *C. elegans* (Cully et al., 1994; Cully et

al., 1996). In our analysis, the IVM response of AgGluCl-a1 was reversible, which has not been observed in GluCls from other organisms. The only other member of the Cys-loop family that is reversibly activated by IVM is the histamine-gated chloride channel from *D. melanogaster* (Zheng et al., 2002). Though IVM does not have persistent activity on AgGluCl-a1 and does not activate AgGluCl-b, it is still highly effective at killing *An. gambiae* (Kobylinski et al., 2010). IVM-induced activity on AgGluCl-a2 and c splice isoforms is still unknown, as well as potential AgGluCl isoform heteromultimers and AgGluCl-expressing neurons, which may exhibit the more commonly observed persistent IVM-induced activity. Further research comparing AgGluCl isoforms will be necessary to fully understand its physiological role in *An. gambiae* as well as its potential as an insecticide target in regards to insecticide sensitivity and resistance development.

In response to a blood meal, AgGluCl transcript levels decrease modestly at 3 DPE, but are unaffected at 7 DPE and 15 DPE. We performed IFA staining of both NBF and BF 3 DPE female *An. gambiae* but did not observe a difference in staining intensity between the two physiological states. In this experimental design, the 3 DPE mosquitoes only received one blood meal, where 7 DPE and 15 DPE received additional blood meals prior to their final blood meal. This confounding variable could be an alternative explanation for the age-related AgGluCl transcriptional response to a blood meal. Previous microarray data showed that AgGluCl transcripts decrease in response to a blood meal matching our 3 DPE results, though the researchers do not report the age or number of blood meals received by the mosquitoes (Marinotti et al., 2006). AgGluCl isoforms are predominately found in the head and thorax. Interestingly, there are no AgGluCl transcripts found in the female abdomen until 15 DPE. AgGluCl staining was not observed in the female abdomen, but these experiments were only conducted on 2-4 DPE mosquitoes. It remains unclear what abdominal tissues could be the

source of AgGluCl transcripts and what role AgGluCl could play in the mosquito abdomen at this older age.

AgGluCl isoform-specific analysis shows that AgGluCl-a (isoforms a1 and a2 combined) and b are predominately expressed compared to AgGluCl-c. It is unclear from our analysis what the individual contributions are from AgGluCl-a1 and AgGluCl-a2 transcripts in the overall AgGluCl transcript measurements. We have shown that AgGluCl-b homomers are insensitive to IVM while AgGluCl-a1 homomers are IVM-sensitive. Our data suggests that if IVM resistance were to arise in *Anopheles*, it might occur through altered regulation of AgGluCl splicing favoring IVM-insensitive AgGluCl-b transcripts over IVM-sensitive AgGluCl transcripts. When we measured AgGluCl isoform-specific transcription in response to a single IVM-containing blood meal across mosquito tissues and ages, we did not detect a change in the proportion of AgGluCl-b isoforms compared to controls. However this does not preclude AgGluCl alternative splicing as a potential mechanism for IVM resistance in the field.

To begin to understand what physiological role GluCl has in *An. gambiae*, we stained sagittal slices of whole, adult female mosquitoes for AgGluCl and found expression in several vital organs involved in motor and sensory systems. There was significant AgGluCl expression in the thoracic ganglia, which contain the motor neurons that control the fight and leg muscles. GluCl is also expressed in the motor neurons of other invertebrates including *M. domestica* (Kita et al., 2013), *P. americana* (Zhao et al., 2004), *L. migratoria* (Janssen et al., 2007) and *D. melanogaster* larva (Rohrbough and Broadie, 2002). The observed phenotype of mosquitoes that take a blood meal containing IVM is paralysis (Kobylinski et al., 2010). Our data suggest that this paralysis may be due to disruption of GluCl on the motor neurons controlling the leg and flight muscles found in the thoracic ganglia.

We also observed GluCl staining in tissue associated with various sensory systems including the antennal segments, Johnston's Organ, optic lobe, and the supraesophageal ganglion. Chemosensory and thermosensory sensilla are found throughout the antennal segments and play an essential role in host-seeking behavior (Bowen, 1991). GluCl staining was also present in the scolopidia within the Johnston's Organ. These hair-like cells are connected to the basal plate of the Johnston's Organ, and transduce vibrations in the antenna for audition and flight balance (Gopfert and Robert, 2002; Ignell et al., 2005) Importantly, we have previously reported that sub-lethal doses of IVM impair coordinated flight movement, including increased knockdown and reduced recovery, which could be the result of impairing this sensory organ (Butters et al., 2012a). GluCl was present in all three neuropils of the optic lobes, which process visual signals from the compound eye and send projections to the supraesophageal ganglion (Fortini and Rubin, 1991). This is similar to GluCl expression in the optic lobe of *Musca domestica* (Kita et al., 2014). Lastly, GluCl expression was present throughout the supraesophageal ganglion though our broad immunohistochemical analysis did not allow for precise localization of which neuropil within the supraesophageal ganglion express AgGluCl. This ganglion is responsible for integrating sensory signals from the antennae and eyes (Ignell et al., 2005; Ignell and Hansson, 2005). GluCl is also expressed in the supraesophageal ganglion of *D. melanogaster* and has been shown to play a key role in circadian rhythms and olfactory processing (McCarthy et al., 2011; Liu and Wilson, 2013) Importantly, hemolymph has been shown to circulate to all of these structures, highlighting the fact that IVM in the hemolymph could affect physiological processes associated with these tissues found relatively far away from the midgut (Boppana and Hillyer, 2014).

As more research is conducted on the potential of IVM MDA for malaria control, it has become essential to understand its target, GluCl, in malaria vectors. This is the first report characterizing GluCl in any mosquito disease vector. We have described the genetic structure, splice-isoform specific transcription, activity and tissue expression of GluCl isoforms in *An. gambiae*. These findings give insight into how IVM affects *Anopheles* physiology and provides a potential mechanism for IVM resistance.

Chapter 3: Mosquitocidal properties of IgG targeting the glutamate-gated chloride channel in *Anopheles gambiae* s.s.

3.1: Introduction

Current insecticides for malaria control target critical proteins involved in neuronal signaling. This includes voltage-gated sodium channels (targeted by pyrethroids and organochlorines) (Zlotkin, 1999), aceytlcholinesterases (organophosphates and carbamates) (Casida, 1963; Fukuto, 1990), and glutamate-gated chloride channels (avermectins) (Campbell et al., 1983; Cully et al., 1996; Kobylinski et al., 2010; Sylla et al., 2010; Kobylinski et al., 2011). The glutamate-gated chloride channel of *Anopheles gambiae* (AgGluCl) and other Anopheline mosquitoes have proven to be an exceptionally sensitive target for the insecticidal drug ivermectin (IVM), when introduced through a blood meal (Jones et al., 1992; Gardner et al., 1993; Foley et al., 2000; Fritz et al., 2009; Chaccour et al., 2010; Kobylinski et al., 2010; Sylla et al., 2010). GluCl is a member of the cys-loop family of ligand gated ion channels. This channel is only expressed in invertebrates, where it gates an inhibitory chloride current on the post-synaptic membranes of neurons and muscle fibers (Cull-Candy, 1976; Fritz et al., 1979; Janssen et al., 2007; Janssen et al., 2010).

Given that GluCl can be targeted by drugs found in a blood meal and that GluCl is not expressed in mammals, we wanted to test the efficacy of AgGluCl as a candidate mosquitocidal vaccine antigen. The concept of using vaccines to kill blood feeding arthropods gained validity with the success of TickGARD^{PLUS}, an anti-tick vaccine targeting the midgut antigen Bm86 (Jonsson et al., 2000), and has continued with the more recent development of the subolesin/akirin antigens in tick and also recently mosquitoes (de la Fuente et al., 2011; de la Fuente et al., 2013; da Costa et al., 2014). Research into mosquitocidal vaccines has been

conducted since the 1940s, but with much less success, with most strategies focusing on targeting midgut antigens (Dubin et al., 1948; Hatfield, 1988a; Lal et al., 2001; Foy et al., 2003). In most experiments, animals were immunized against heterogeneous mosquito tissue homogenates, which led to variable reductions in survival and fecundity in multiple mosquito species, including animals immunized against head tissue homogenates where we would expect AgGluCl expression to occur (Almeida and Billingsley, 1998; Foy et al., 2002). Consequently, an efficacious anti-mosquito vaccine has never been developed despite decades of intermittent research (Jacobs-Lorena and Lemos, 1995; Willadsen and Billingsley, 1996; Billingsley et al., 2008).

We administered a polyclonal anti-AgGluCl immunoglobulin G (anti-AgGluCl IgG) to *An. gambiae* mosquitoes through a blood meal or directly into the hemocoel by intrathoracic injections to determine and quantify its broad mosquitocidal activity in *An. gambiae*. We also examined the effects on survivorship of co-administering anti-AgGluCl IgG with a known GluCl agonist, IVM, to study the mechanism of action of anti-AgGluCl IgG.

3.2: Materials and methods

Mosquitoes. *Anopheles gambiae* s.s. G3 strain (origin The Gambia) were raised at 28-31^{o}C, 80% relative humidity on a 14:10 light dark cycle. Larvae were fed ground Tetramin® fish food daily. Adults were provided with water and 10% sucrose solution *ad libitum*. Colony mosquitoes were blood fed every 3 to 4 days on defibrinated calf blood.

Production and Verification of anti-AgGluCl IgG Specificity. Polyclonal anti-AgGluCl IgG was prepared by GeneScript USA, Inc (Piscataway, NJ). Briefly, two rabbits were immunized against five milligrams of the recombinant N-terminal extracellular domain of AgGluCl produced in *Escherichia coli* (244 amino acids in length). Immunizations were boosted twice at

two week periods. Rabbit serum was collected and affinity-purified to isolate polyclonal rabbit IgG. Antibody specificity to AgGluCl was verified via ELISA, western blot and immunostaining of C6/36 cells (derived from *Aedes albopictus*) (Singh and Paul, 1969) transfected with AgGluCl cloned into the pIB/V5-His TOPO® plasmid (Life Technologies™ Grand Island, NY) (Figure 3.1B, 3.1C).

Blood Feed with Artificial Membrane Feeder. Two to four day post-emergence mosquitoes were fed defibrinated calf blood spiked with purified rabbit anti-AgGluCl IgG (Genscript, Piscataway, NJ), purified rabbit control IgG (Genscript, Piscataway, NJ), IVM, or vehicle via glass bell feeders (Lillie Glass Feeders, Symrna, GA) sealed with pig sausage casing and heated to 37^{o}C. Mosquitoes were given 30-45 minutes to feed after which they were knocked down briefly at 4^{o}C for sorting and removal of unfed and partially fed mosquitoes. Fully-engorged mosquitoes were monitored for survivorship for 4 days following the blood meal.

Intrathoracic Injection of anti-AgGluCl IgG. Adult female mosquitoes were briefly knocked down at 4^{o}C for intrathoracic injection. Glass capillaries (Drummond Scientific, Broomall, PA, 3-00-203-G/XL) were pulled using a Flaming/Brown Micropipette Puller Model P-87 (Sutter Instruments, Novato, CA) to create microcapillary injection needles. Mosquitoes were injected with 69 nL of either 958 ng/mL anti-AgGluCl IgG or control IgG using a Drummond Nanoject II Automatic Injector (Drummond Scientific, Broomall, PA, 3-000-204) and glass microcapillary injection needles. Following injection, mosquitoes were maintained for four days and survivorship was monitored.

Survivorship Analysis and anti-AgGluCl IgG LC_{50} Determination. Replicates were pooled and analyzed by Logrank Test (Mantel-Haenzel method; proportioned hazards model) with 95% confidence intervals using Prism software (GraphPad, La Jolla, CA). LC_{50}

determination followed previously described analysis (Kobylinski et al., 2010). Briefly, survivorship replicates were pooled into a non-linear mixed model with probit analysis, which accounted for background mortality in the control group and assessed replicate effects, to calculate the lethal concentration which killed 50% of mosquitoes (LC_{50}). Wing lengths from 20 mosquitoes from each group were measured and compared across groups to ensure mosquito size did not affect survivorship (Lounibos et al., 1995).

Serial anti-AgGluCl IgG Blood Feeds. Mosquitoes were serially fed blood meals containing 282μg/mL of anti-AgGluCl IgG every 4 days for 20 days. Dead mosquitoes and the number of eggs deposited were monitored daily. Mosquitoes that did not re-feed were discarded and censored in the analysis. One hundred seventy eggs per group per day were collected and monitored for 2 days after which emerged larvae were counted and discarded. Replicates were pooled and analyzed by Logrank Test (Mantel-Haenzel method; proportioned hazards model) with 95% confidence intervals. Analysis was done using Prism software (GraphPad Software).

Mice AgGluCl Immunizations and Serial Feeding. Ten female CD1 mice were immunized against the bacterially-expressed N-terminal extracellular domain of AgGluCl (Genscript). The antigen was mixed with TiterMax® Gold adjuvant (Sigma-Aldrich) to a final concentration of 300μg/mL. Twenty five microliters was injected intramuscularly into each leg of the mice. Negative controls were injected with bovine serum albumin mixed with the adjuvant. Mice were immunized three times over 6 weeks. Blood was collected (200μL) from the submandibular vein before immunizations and two weeks following the final immunization to verify anti-AgGluCl antibody production. Fifty mosquitoes were serially fed every four days and their survivorship monitored. Mosquitoes that did not re-feed were discarded and censored in the analysis. There was no significant difference between the groups of mosquitoes that fed on each mouse;

subsequently the data was grouped together for analysis. All animal procedures were approved by the Colorado State University IACUC board.

3.3: Results

Production and verification of anti-AgGluCl IgG specificity

Polyclonal anti-AgGluCl IgG was generated in rabbits against the bacterially-expressed N-terminal extracellular domain of AgGluCl. Antibody titer and specificity against the recombinant immunized protein was verified by ELISA (512,000), western blot against the recombinant immunized AgGluCl extracellular antigen, and immunolabeling of C6/36 cells (derived from *Aedes albopictus*) expressing native, full-length AgGluCl (Figure 3.1A, 3.1B).

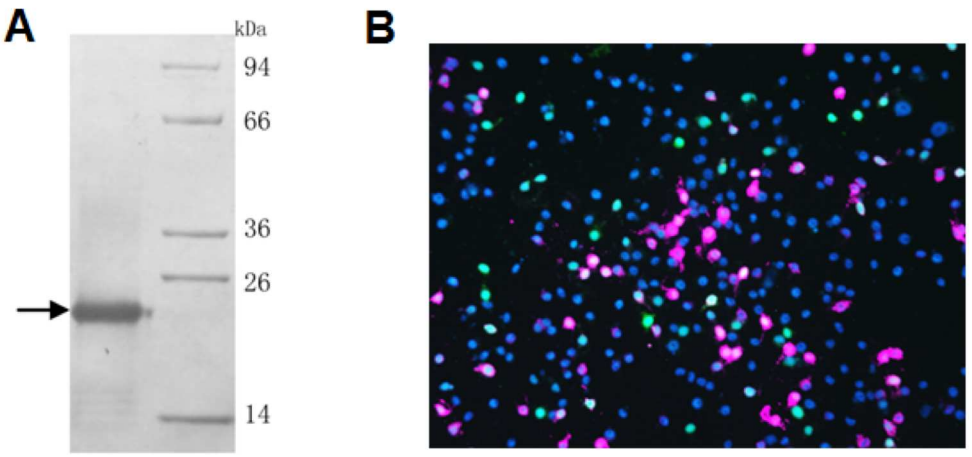

Figure 3.1. Anti-AgGluCl IgG specificity. (A) Western blot of anti-AgGluCl IgG against the bacterially-expressed AgGluCl extracellular domain. (B) C6/36 cells transfected with AgGluCl and eGFP (green). Cells are immunostained for anti-AgGluCl IgG (pink) and DAPI (blue). Experiments performed by Jacob Meyers and Genscript.

Anti-AgGluCl IgG kills *An. gambiae* when administered through a blood meal or intrathoracic injection.

To measure the effects of anti-AgGluCl IgG on *An. gambiae* survivorship, we fed mosquitoes on blood meals containing anti-AgGluCl IgG ranging from 1.0 - 3.0mg/mL. Blood feeding of five different anti-AgGluCl IgG concentrations showed that anti-AgGluCl IgG induces a strong, dose-dependent mosquitocidal effect (Figure 3.2A). Non-specific polyclonal (control) rabbit IgG, when blood fed at the highest concentration tested, did not affect mosquito mortality. These results were consistent across three replicates. We calculated the LC_{50} of anti-AgGluCl IgG to be 2.82mg/mL [2.68, 2.96] (n=1,499) (Figure 3.2A).

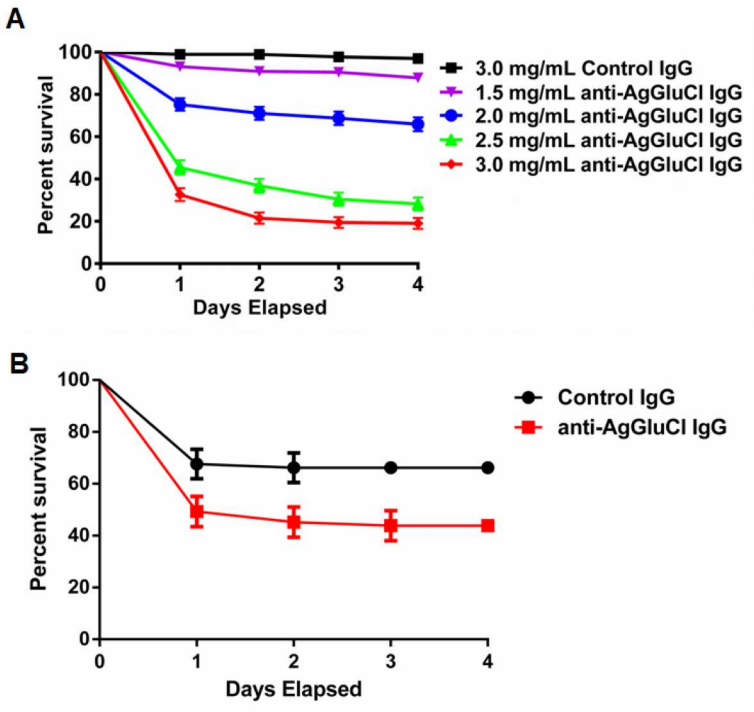

Figure 3.2. Anti-AgGluCl IgG kills *An. gambiae* when administered through a blood meal or directly into the hemocoel. (A) Survival curves of *An. gambiae* after feeding on a single blood meal containing anti-AgGluCl IgG. (B) Survival curves of *An. gambiae* after a single intrathoracic injection of anti-AgGluCl IgG. Data presented as mean and S.E. Experiments performed by Jacob Meyers and Meg Gray.

To verify that anti-AgGluCl IgG was affecting survivorship by translocating across the midgut and binding to AgGluCl that we previously found only expressed in the hemocoel (Meyers et al., 2015), we injected a physiologically relevant concentration (958 ng/mL) of anti-AgGluCl IgG (Vaughan et al., 1990)

or control rabbit IgG directly into the hemocoel by intrathoracic injection. A single injection of anti-AgGluCl IgG significantly reduced *An. gambiae* survivorship over four days (p=0.0083; hazard ratio: 2.322 [1.242, 4.30], n=141) compared to control (Figure 3.2B).

Though a single blood meal containing concentrations of anti-AgGluCl IgG that could be expected in immunized animals was ineffective at killing mosquitoes, we tested the possibility that repeated blood meals containing a physiologically-relevant concentration ($1/10^{th}$ the LC_{50}: 282µg/mL) of anti-AgGluCl IgG could have an effect on mosquito fitness over time. To test this hypothesis, we serially fed

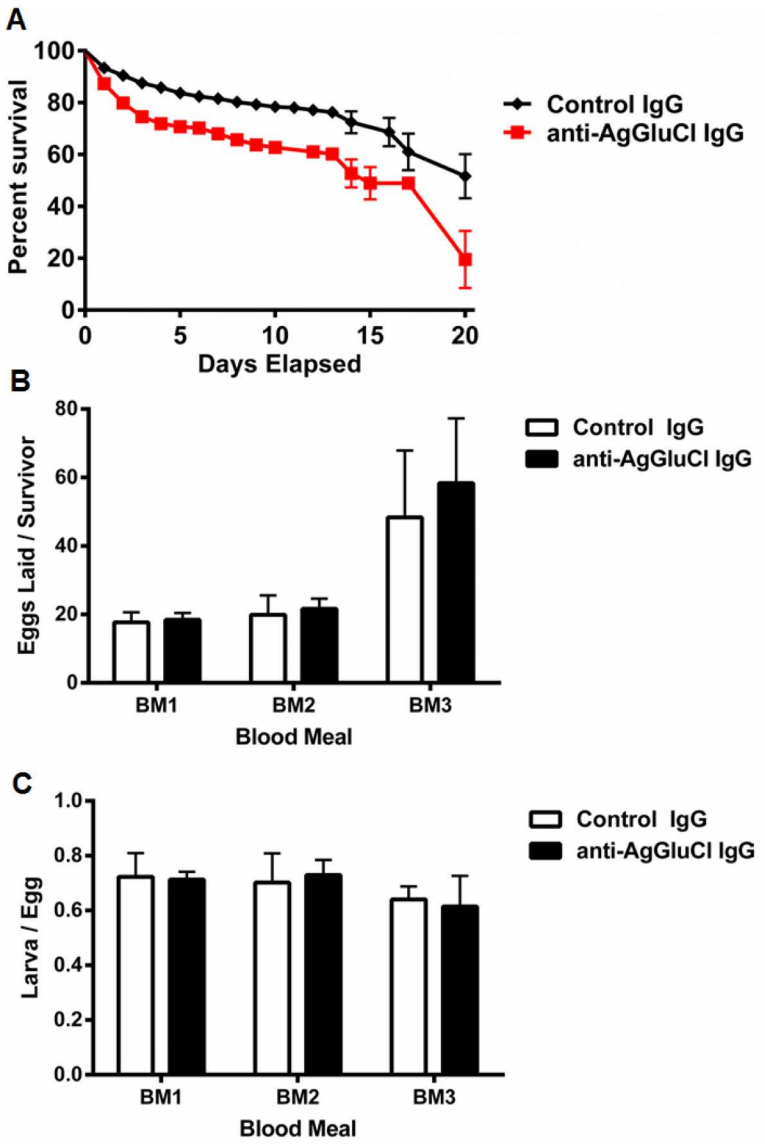

Figure 3.3. Serial feeding of a physiological concentration of anti-AgGluCl IgG kill *An. gambiae* mosquitoes. (A) Survival curves of *An. gambiae* that feed on multiple blood meals containing AgGluCl IgG. Blood feeds occurred on days 0, 4, 8, 12, 16 and 20. (B) Number of eggs laid per surviving female mosquito per number of blood meal consumed. (C) Number of larva developed per egg per number of blood meal consumed. All data presented as mean and standard error. Experiments performed by Jacob Meyers and Tim Burton.

mosquitoes blood meals containing anti-AgGluCl IgG every four days and measured survivorship, number of eggs laid/female, and egg-hatch success. Mosquitoes that consumed repeated anti-AgGluCl IgG in successive blood feeds exhibited significantly reduced survivorship compared to controls ($P<0.001$, Hazard ratio=1.898 [1.553-2.495], n=1,374) (Figure 3.3A). There was no effect on number of eggs laid/female or egg hatch success (Figure 3.3B, 3.3C).

Serial blood feeding directly on AgGluCl-immunized mice does not affect mosquito survivorship

To examine the mosquitocidal potential of targeting AgGluCl with antibodies ingested from a natural source, we immunized mice against the N-terminal extracellular domain of AgGluCl and serially fed mosquitoes directly on the immunized mice. Groups of 50 mosquitoes were blood fed on the mice every four days and survivorship was measured daily until all of the mosquitoes died or failed to re-blood feed at the 4 day interval. ELISA experiments demonstrated that immunized mice produced antibodies against the AgGluCl antigen ranging from 128,000 – 256,000. However, the mosquitoes were largely unwilling to take blood meals from restrained mice and only 112 of the original 387 mosquitoes took all three blood meals. The survivorship of the mosquitoes that serially fed on

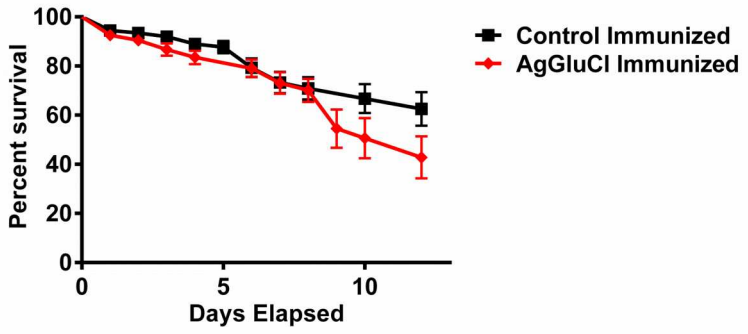

Figure 3.4. Survival curves of *An. gambiae* mosquitoes that had multiple blood feeds on immunized mice. Blood feeds occurred on days 0, 4, 8, and 12. Data presented as mean and standard error. Experiments performed by Jacob Meyers and Brian Foy.

the immunized mice was not significantly lower than the controls when the experiment ended due to mosquitoes refusal to re-blood feed at the last time point (P=0.1384, Hazard ratio=1.365 [0.899-2.163], n=112) (Figure 3.4).

Anti-AgGluCl IgG reverses the IVM-induced mortality

We hypothesized that anti-AgGluCl IgG would inhibit channel activity by binding to regions on the extracellular domain of AgGluCl that are critical for channel opening. To test this, we co-administered anti-AgGluCl IgG with ivermectin (IVM), a known GluCl agonist. We fed *An. gambiae* a single blood meal containing the LC_{75} of IVM (Kobylinski et al., 2010) and a physiologically-relevant concentration (282µg/mL) of anti-AgGluCl IgG (Stoute et al., 1997) and monitored survivorship over four days (Figure 3.5). IVM alone and IVM mixed with control non-specific rabbit IgG produced significant mosquitocidal effects relative to vehicle control groups (IVM vs. PBS: p<0.0001; hazard ratio: 8.930 [5.765, 11.99]; n=208) (IVM + Ctrl IgG vs. PBS: p<0.0001; hazard ratio: 8.641 [6.576, 14.31]; n=193). Control IgG had no effect on IVM toxicity (IVM vs. IVM + Ctrl IgG: p=0.9213; hazard ratio: 0.9890 [0.7497, 1.290]; n=225). A single blood meal containing a physiologically-relevant concentration of anti-AgGluCl IgG did not significantly reduce mosquito survivorship (anti-AgGluCl IgG vs. PBS: p=0.0621; hazard ratio: 1.837 [0.9894, 3.531]; n=183). However, the combination of IVM and anti-AgGluCl IgG attenuated

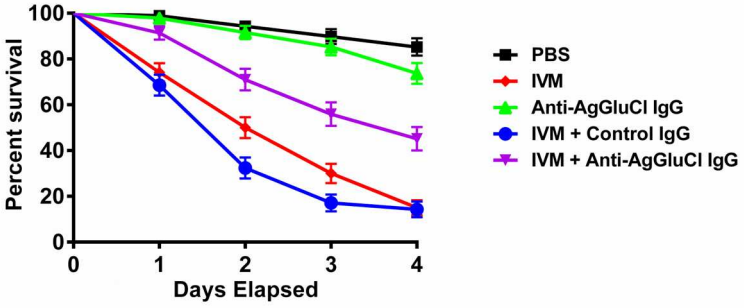

Figure 3.5. Survival curves of *An. gambiae* after feeding on a single blood meal containing PBS, IVM, anti-AgGluCl IgG, IVM + control IgG and IVM + anti-AgGluCl IgG. Data presented as mean and S.E. confidence interval. Experiments performed by Jacob Meyers.

the mosquitocidal effects of IVM alone (IVM vs. IVM + anti-AgGluCl IgG: P<0.0001; hazard ratio: 2.022 [1.842, 3.471]; n=117), suggesting anti-AgGluCl IgG partially blocks IVM-induced activation of AgGluCl.

3.4: Discussion

In the present study we have shown that IgG targeting the N-terminal extracellular domain of AgGluCl reduces *An. gambiae* survivorship when mixed with calf blood and administered through an artificial membrane feeder. To our knowledge, this is the first example of a single antigen that can be targeted by IgG in a mosquito blood meal to consistently and significantly reduce mosquito survivorship. Previous vaccine development strategies targeting mosquitoes have immunized animals with whole mosquito tissues which could never be employed as a vaccine antigen (Alger and Cabrera, 1972; Almeida and Billingsley, 1998) or against a single midgut antigen. These resulted in small and highly variable reductions in survivorship and fecundity and differed between mosquito species, immunized hosts and trials (Dubin et al., 1948; Alger and Cabrera, 1972; Hatfield, 1988a, b, c; Ramasamy et al., 1988; Vaughan and Azad, 1988; Srikrishnaraj et al., 1993; Noden et al., 1995; Almeida and Billingsley, 1998; Lal et al., 2001; Almeida and Billingsley, 2002; Foy et al., 2003; Billingsley et al., 2008; da Costa et al., 2014). In contrast, blood meals containing anti-AgGluCl IgG consistently reduced *An. gambiae* survivorship across all replicates in a dose-dependent manner.

The LC_{50} concentration of anti-AgGluCl IgG in a blood meal, and the range of anti-AgGluCl IgG concentrations that reduced *An. gambiae* survivorship, are greater than IgG concentrations found in an immunized human host (Shawler et al., 1985; Stoute et al., 1997). Such a vaccine would not benefit from natural boosting as the host is not naturally exposed to AgGluCl epitopes, so any vaccine would depend on relatively high antibodies titers. Our anti-

AgGluCl IgG was created against the large 244 amino acid extracellular domain. AgGluCl epitope optimization targeting highly antigenic regions of this domain or passive immunization of monoclonal antibodies against targeted extracellular AgGluCl epitopes may improve anti-AgGluCl IgG toxicity. Further, anti-AgGluCl IgG specificity should be determined against crude *An. gambiae* tissues as specificity has only been tested against the bacterially produced antigen and AgGluCl-expressing C6/36 cells.

Repeated blood meals containing a physiologically-relevant dose of AgGluCl produced a mosquitocidal effect over 20 days. The data suggest that repeated binding of anti-AgGluCl IgG to its target causes the mosquitocidal effect. Alternative explanations are 1) that *An. gambiae* is unable to remove anti-AgGluCl IgG efficiently allowing antibody concentrations to accumulate and persist in the hemolymph, potentiating its effect in the mosquito; 2) only older blood fed mosquitoes are sensitive to the lower titer of antibodies ingested; 3) anti-AgGluCl IgG binds to non-specific targets affecting *An. gambiae* survivorship. Regardless, these data are highly relevant because wild *An. gambiae* blood feed almost exclusively on humans approximately every 2-3 days (Beier et al., 1988; Beier, 1996). Furthermore, a malaria control strategy that preferentially targets aged mosquitoes that have taken multiple blood meals may act as a "late-life acting" insecticide. Since *Plasmodium spp* take 10-14 days to develop in the midgut and invade the salivary glands of *An. gambiae* (Beier, 1998), the mosquitocidal properties of ingested AgGluCl IgG would only effect older females that can transmit *Plasmodium spp* but have already laid the majority of their eggs, thus reducing the selective pressure that drives resistance development (Read et al., 2009; Billingsley, 2010). Mosquitoes that were fed on mice immunized with the AgGluCl extracellular domain did not affect survivorship before the experiments ended prematurely due to mosquitoes refusal to blood feed (P=0.1384, Hazard

ratio=1.365 [0.899-2.163]). During this experiment, a high percentage of mosquitoes were censored from analysis due to failure to re-feed (Percent censored: Controls: 72.8%, AgGluCl: 69.1%), affecting our ability to measure a significant mosquitocidal effect to the standard threshold of P=0.05. Nevertheless, these minor effects on mosquito survivorship could have major effects in the field because of the critical role mosquito daily survivorship on disease transmission. The concentration of anti-AgGluCl IgG used for intrathoracic injections is within the range of IgG concentrations found in *Anopheles stephensi* hemolymph three hours after feeding on rats immunized against *Plasmodium falciparum* circumsporozoite protein (Vaughan et al., 1990). This suggests that *An. gambiae* survivorship might be affected when serially fed directly on an AgGluCl-immunized host. The mosquitocidal effects of anti-AgGluCl IgG injected into the hemocoel also suggest that anti-AgGluCl IgG is binding to its target expressed outside of the midgut to affect *An. gambiae* survivorship.

When we co-administered anti-AgGluCl IgG with the potent GluCl agonist IVM, we show an attenuation of IVM-induced mortality. This suggests that anti-AgGluCl IgG reverses or blocks the effects of IVM. We predict that AgGluCl antagonism could disrupt inhibitory glutamatergic synaptic communication and potentially cause neuronal hyper excitation. It is still unclear how anti-AgGluCl IgG directly affects AgGluCl activity; it could directly inhibit channel opening, disrupt Cl^- ion flow through the pore. Antibody binding could also exclude glutamate binding or it could indirectly affect activity by causing AgGluCl to be internalized into intracellular vesicles. The likely explanation is that anti-AgGluCl IgG binding to multiple extracellular epitopes of AgGluCl antagonizes the channel and prevents IVM-induced opening of the channel. Though the conformation of AgGluCl would change with IVM binding, it seems unlikely that this would affect the binding of the polyclonal anti-AgGluCl IgG since the

antibodies likely bind to multiple epitopes on the extracellular domain of AgGluCl, while IVM binding occurs in between subunits on residues found on the AgGluCl transmembrane domain (Hibbs and Gouaux, 2011).

Further testing of anti-AgGluCl IgGs would be necessary to determine its mode of action with the optimal experiment being testing the effects of anti-AgGluCl IgG on AgGluCl-expressing *X. laevis* oocytes. Auto-immune antibodies against other channels have been shown to antagonize channel activity in mammals, which is likely the same mechanism of action of anti-AgGluCl IgG (Weber et al., 2000; Wang et al., 2007; Briani et al., 2008; Vernino et al., 2008). However, GluCl is not expressed in any mammalian species, making it unlikely that anti-AgGluCl IgG will have auto-immune effects in humans or livestock. The mammalian receptors most closely related to the insect GluCl are the GABA-gated chloride channel and glycine-gated chloride channel (Lynagh and Lynch, 2012b) but their amino acid similarity with AgGluCl is <35%. Nevertheless, anti-AgGluCl IgG reactivity to these channels and other neurotransmitter receptors should be tested in the design of any vaccine that would use AgGluCl epitopes as vaccine antigens.

A mosquitocidal vaccine targeting the highly conserved AgGluCl has major potential against multiple *Anopheles spp.* which have previously been shown to pass IgG through their midgut including *Anopheles arabiensis*, *Anopheles stephensi*, *Anopheles funestus* and *Anopheles albimanus* (Vaughan and Azad, 1988; Beier et al., 1989). Further research testing the effects of anti-AgGluCl IgG on mosquito survivorship and fitness of *An. gambiae* and other *Anopheles* spp. after feeding on animals immunized against extracellular AgGluCl epitopes will be required to discern the potential of AgGluCl as a target antigen of a pan-*Anopheles* vaccine and as an intervention to control malaria parasite transmission.

Chapter 4: AgRDL and AgVGSC as a target of a mosquitocidal vaccine and tissue expression in *Anopheles gambiae* s.s.

4.1: Introduction

Our results describing the mosquitocidal properties of anti-AgGluCl IgG suggest that other neuronal proteins could be used as candidate antigens for a mosquitocidal vaccine. Ideal candidates are the extracellular domains of other well-known insecticide targets which are dynamic and undergo conformational changes during activity. The *An. gambiae* GABA-gated chloride channel (resistance to dieldrin; AgRDL) is another member of the cys-loop ligand-gated ion channels with a similar structure and physiological function to AgGluCl. AgRDL is involved in inhibitory neurotransmission of the invertebrate central nervous system (Buckingham et al., 2005), and is the target of the avermectin, cyclodiene and phenyl pyrazole classes of insecticides (Hemingway and Ranson, 2005; Lees et al., 2014). The avermectins agonize AgRDL activity while the cyclodienes and phenyl pyrazoles antagonize AgRDL activity (Buckingham et al., 2005; Hemingway and Ranson, 2005). Since AgRDL has a similar structure and physiological role as AgGluCl, we hypothesize that targeting this channel with antibodies through a blood meal will similarly affect *An. gambiae* survivorship.

The *An. gambiae* voltage-gated sodium channel (AgVGSC) is the target of dichlorodiphenyltrichloroethane (DDT) and the pyrethroid class of insecticides (Soderlund and Bloomquist, 1989). VGSCs are expressed by neurons and trafficked to the axon hillock and along the axon to initiate and propagate action potentials (Davies et al., 2007a, b). DDT and pyrethroids reduce the rate of VGSC closing, prolonging the sodium current and causing aberrant, repetitive action potential firing (Vijverberg et al., 1982).

VGSCs are also the target of multiple classes of spider, scorpion and snail toxins, demonstrating that peptides binding to VGSC extracellular residues can affect channel function (Nicholson, 2007; King et al., 2008; Stevens et al., 2011; Klint et al., 2012). Additionally, several VGSC toxins are insect specific and do not affect vertebrate VGSCs. This includes hainantoxin-I, Magi 2, Tx4(6-1), scorpion α-insect toxins, scorpion β-toxins, μ-agatoxins, σ-palutoxins and curtatoxins (Figure 4.1) (King et al., 2008). Hainantoxin-I blocks VGSC ion conductance by binding to site 1 of the insect VGSC (Li et al., 2003). Magi 2, Tx4(6-1) and scorpion α-insect toxins bind to site 3 of the insect VGSC, inhibiting channel inactivation and causing a minor shift in voltage dependence to more hyperpolarizing membrane potentials (Cestele and Catterall, 2000; King et al., 2008). Scorpion β-toxins, μ-agatoxins, σ-palutoxins and curtatoxins bind to site 4 of the insect VGSC causing a shift in voltage dependence to more hyperpolarizing membrane potentials (King et al., 2008).

Previous work in other organisms has shown that antibodies against RDL and VGSC antagonize channel activity, suggesting that blood meals containing these antibodies will affect *Anopheline* physiology and survivorship (Vassilev et al., 1989; Weber et al., 2000; Hoftberger et al., 2013; Jarius et al., 2013; DeFelipe-Mimbrera et al., 2014). We hypothesize that targeting the insect VGSC toxin sites 1, 3 and 4 on the AgVGSC will affect mosquito survivorship. In addition, we performed immunofluorescence staining of adult, female *An. gambiae* to uncover immunoreactive tissues to each anti-*Anopheles* IgG.

4.2: Materials and Methods

Mosquitoes. *Anopheles gambiae* s.s. G3 strain (origin The Gambia) were raised at 28-31^{o}C, 80% relative humidity on a 14:10 light dark cycle. Larvae were fed ground Tetramin® fish food daily.

Adults were provided with water and 10% sucrose solution *ad libitum*. Colony mosquitoes were blood fed every 3 to 4 days on defibrinated calf blood.

Production and Verification of anti-*Anopheles* IgGs Specificity. Polyclonal anti-AgRDL IgG was prepared by GeneScript USA, Inc (Piscataway, NJ). The recombinant N-terminal extracellular domain of AgRDL (minus the signal peptide) was produced in *Escherichia coli* (230 amino acids in length). Three AgVGSC extracellular antigens were assembled *in vitro* and linked at the N-terminus to the immunogen keyhole limpet hemocyanin (AgVGSC-1 residues: 284-296; AgVGSC-2 residues: 861-870; AgVGSC-3 residues: 1,683-1,697). Two rabbits were immunized against five milligrams of each *Anopheles* antigen. Immunizations were boosted twice at two week periods. Rabbit serum was collected and affinity-purified to isolate polyclonal rabbit IgG. Antibody specificity was verified via ELISA and western blot.

Blood Feed with Artificial Membrane Feeder. Two to four day post-emergence mosquitoes were fed defibrinated calf blood spiked with purified rabbit anti-*Anopheles* IgG (Genscript, Piscataway, NJ) or purified rabbit control IgG (Jackson Immunoresearch Laboratories Inc., West Grove, PA) via glass bell feeders (Lillie Glass Feeders, Symrna, GA) sealed with pig sausage casing and heated to 37^{o}C. Mosquitoes were given 30-45 minutes to feed after which they were knocked down briefly at 4^{o}C for sorting and removal of unfed and partially fed mosquitoes. Fully-engorged mosquitoes were monitored for survivorship for 4 days following the blood meal.

Intrathoracic Injection of anti-*Anopheles* IgG. Adult female mosquitoes were briefly knocked down at 4^{o}C for intrathoracic injection. Glass capillaries (Drummond Scientific, Broomall, PA, 3-00-203-G/XL) were pulled using a Flaming/Brown Micropipette Puller Model P-87 (Sutter Instruments, Novato, CA) to create microcapillary injection needles. Mosquitoes

were injected with 69 nL of either 958 ng/mL anti-*Anopheles* IgG or control IgG using a Drummond Nanoject II Automatic Injector (Drummond Scientific, Broomall, PA, 3-000-204) and glass microcapillary injection needles. Following injection, mosquitoes were maintained for four days and survivorship was monitored.

4.3: Results

Production and verification of anti-*Anopheles* IgG specificity

Polyclonal anti-AgRDL IgG was generated in rabbits against the bacterially-expressed N-terminal extracellular domain of AgRDL (AGAP006028; Assembly: AgamP4) (Figure 4.1). Antibody titer and specificity against the recombinant immunized protein was verified by ELISA (1:256,000) and western blot.

MSLTIEVPHAKSPSLGVLILTLNLALFLPQTINRTPPYVLAGTGGGSMLGDVNISAILDSFSV
GYDKRVRPNYGGPPVEVGVTMYVLSISSLSEVKMDFTLDFYFRQFWTDPRLAYRKRPGVETLS
VGSEFIKNIWVPDTFFVNEKQSYFHIATTSNEFIRIHHSGSITRSIRLTITASCPMNLQYFPM
DRQLCHIEIESFGYTMRDIRYFWKDGLSSVGMSSEVELPQFRVLGHRQRATEINLTTGNYSRL
ACEIQFVRSMGYYLIQIYIPSGLIVIISWVSFWLNRNATPARVALGVTTVLTMTTLMSSTNAA
LPKISYVKSIDVYLGTCFVMVFASLLEYATVGYMAKRIQMRKQRFMAIQKIAEQKKQQQADAN
HPPPPPPVASDHSHGHGHGHSHTHQHTPKQQMGSRSGTMQNVPPNMGSRGCSIVGPLFQEVRF
KVHDPKAHSKGGTLENTINGGRGGGQPGGGGPPPGGPPGGGGGGPDEESAAPQHLIHPGKDIN
KLLGITPSDIDKYSRIVFPVCFVCFNLMYWIIYLHVSDVVADDLVLLGEEK

Figure 4.1. *An. gambiae* RDL predicted amino acid sequence. Gray highlight denote transmembrane domains. Anti-AgRDL IgG was designed against the 230 amino acid N-terminal extracellular domain (bold). Experiments performed by Jacob Meyers.

Designing antibodies targeting AgVGSC poses multiple challenges compared to AgGluCl, AgRDL and other members of the cys-loop family of LGIC. The AgVGSC gene encodes for all four subunits of the channel where AgGluCl and AgRDL genes encode for a single subunit of the channel which is later assembled in the endoplasmic reticulum. This makes the AgVGSC gene (6357bp) much larger and difficult to clone compared to the AgGluCl (1374bp) and AgRDL (1668bp) genes (http://www.vectorbase.org). While members of the cys-loop family of

LGICs have large N-terminal extracellular domains which make favorable targets for antibodies, AgVGSC only has small extracellular loops which are more difficult to target for antibody binding (Figure 4.2B).

Anti-AgVGSC-1 IgG is designed against a twelve amino acid antigen on the extracellular loop of subunit 1 connecting TM domains 5 and 6 of the AgVGSC (Figure 4.2A, B). This is the largest AgVGSC extracellular loop at 107 amino acids and includes the Site 1 toxin binding site for tetrodotoxin, saxitoxin and other peptide toxins (Figure 4.2A, B). Anti-AgVGSC-2 IgG is designed against a ten amino acid antigen on the extracellular loop of subunit 2 connecting TM

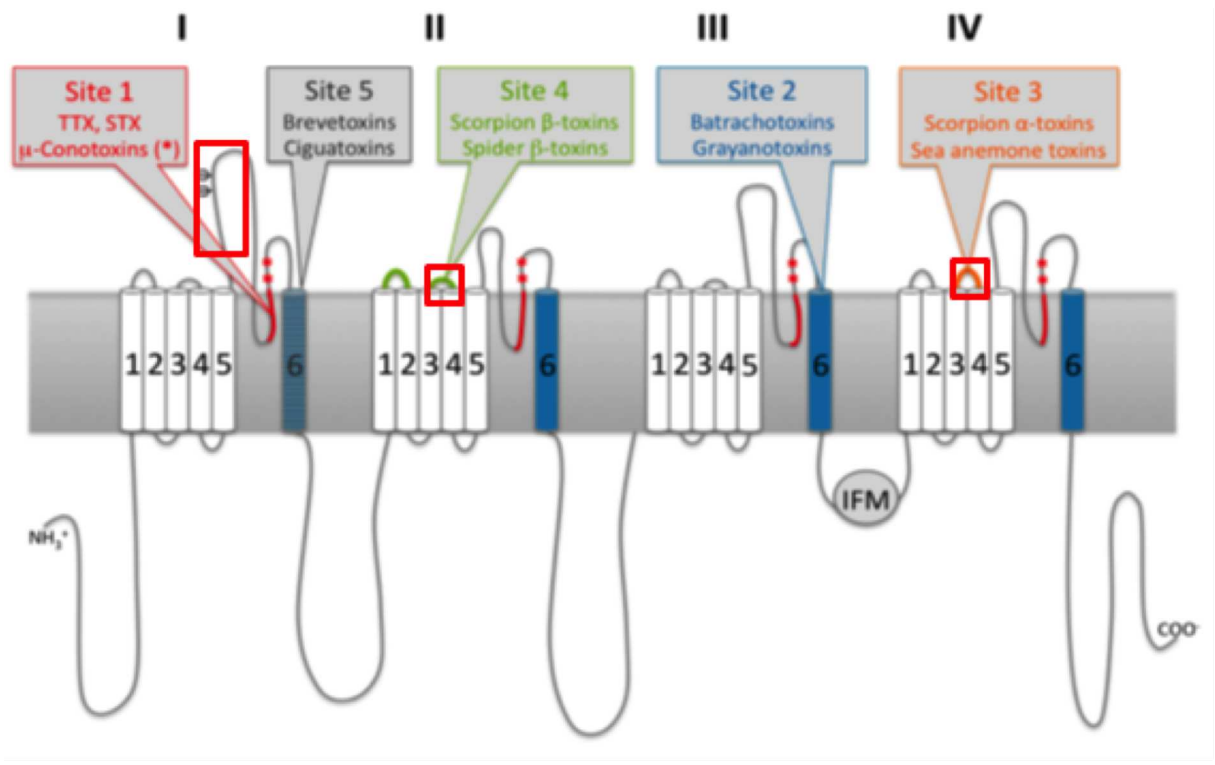

Figure 4.2. *An. gambiae* voltage-gated sodium channel. (A) Cartoon schematic of AgVGSC depicting channel topology and neurotoxin binding sites. Red boxes denote antigen location for anti-AgVGSC-1, -2 and -3 IgGs, respectively. Adapted from Stevens et al., 2011.

B

MTEDSDSISEEERSLFRPFTRESLQAIEARIADEEAKQRELERKRAEGEDEDEGPQPDPTLEQ
GVPVPVRMQGSFPPELASTPLEDIDSFYSNQRTFVVISKGKDIFRFSATNALYVLDPFNPIRR
VAIYILVHPLFSLFIITTILVNCILMIMPTTPTVESTEVIFTGIYTFESAVKVMARGFILQPF
TYLRDAWNWLDFVVIALAYVTMGIDLGNLAALRTFRVLRALKTVAIVPGLKTIVGAVIESVKN
LRDVIILTMFSLSVFALMGLQIYMGVLTQKC**IKEFPLDGSWGN**LTDESWELFNSNDTNWFYSE
SGDIPLCGNSSGAGQCDEGYICLQGYGKNPNYGYTSFDTFGWAFLSAFRLMTQDYWENLYQLV
LRSAGPWHMLFFIVIIFLGSFYLVNLILAIVAMSYDELQKKAEEEEAAEEEALREAEEAAAAK
AAKLEAQQAAAAAAANPEIAKSPSDFSCHSYELFVGQEKGNDDNNKEKMSIRSEGLESVSEIT
RTTAPTATAAGTAKARKVSAASLSLPGSPFNLRRGSRGSHQFTIRNGRGRFVGVPGSDRKPLV
LSTYLDAQEHLPYADDSNAVTPMSEENGAIIVPVYYANLGSRHSSYTSHQSRISYTSHGDLLG
GMTKESRLRNRSARNTNHSIVPPPNANNLSYAETNHKGQRDFDLTQDCTDDAGKIKHNDNPFI
EPAQTQTVVDMKDVMVLNDIIEQAAGRHSRASDHGVSVYYFPTEDDDEDGPTFKDKALEFLMK
MIDIFCVWDCCWVWLKFQEWVAFIVFDPFVELFITLCIVVNTLFMALDHHDMDPDMEKALKSG
NYFFTATFAIEATMKLIAMSPKYYFQEGWNIFDFIIVALSL**LELGLEGVQG**LSVLRSFRLLRV
FKLAKSWPTLNLLISIMGRTVGALGNLTFVLCIIIFIFAVMGMQLFGKNYTDNVDRFPDHDLP
RWNFTDFMHSFMIVFRVLCGEWIESMWDCMLVGDVSCIPFFLATVVIGNLVVLNLFLALLLSN
FGSSSLSAPTADNETNKIAEAFNRISRFSNWIKMNLANALKFVKNKLTSQIASVQPTGKGVCP
CISSEHGENELELTPDDILADGLLKKGIKEHNQLEVAIGDGMEFTIHGDLKNKAKKNKQIMNN
SKVIGNSISNHQDNKLDHELNHRGVSLQDDDTASIKSYGSHKNRPFKDESHKGSAETMEGEEK
RDASKEDLGIDEELDDEGEGDEGPLDGELIIHAEEDEVIEDSPADCCPDNCYKKFPVLAGDDD
APFWQGWGNLRLKTFQLIENKYFETAVITMILLSSLALALEDVHLPQRPILQDILYYMDRIFT
VIFFLEMLIKWLALGFKVYFTNAWCWLDFIIVMVSLINFVASLCGAGGIQAFKTMRTLRALRP
LRAMSRMQGMRVVVNALVQAIPSIFNVLLVCLIFWLIFAIMGVQLFAGKYFKCVDKNKTTLPH
EIIPDVNACKAENYSWENSPMNFDHVGKAYLCLFQVATFKGWIQIMNDAIDSRDVSFVGKQPI
RETNIYMYLYFVFFIIFGSFFTLNLFIGVIIDNFNEQKKKAGGSLEMFMTEDQKKYYNAMKKM
GSKKPLKAIPRPRWRPQAIVFEIVTNKKFDMIIMLFIGFNMLTMTLDHYKQSETFSAVLDYLN
MIFICIFSSECLMKIFALRYHYFIEPWNLFDFVVVILSILGLVL**SDIIEKYFVSPTLLR**VVRV
AKVGRVLRLVKGAKGIRTLLFALAMSLPALFNICLLLFLVMFIFAIFGMSFFMHVKDKSGLDD
VYNFKTFGQSMILLFQMSTSAGWDGVLDGIINEEDCLPPDNDKGYPGNCGSSTIGITYLLAYL
VISFLIVINMYIAVILENYSQATEDVQEGLTDDDYDMYYEIWQQFDPDGTQYVRYDQLSDFLD
VLEPPLQIHKPNRYKIISMDIPICRGDMMFCVDILDALTKDFFARKGNPIEETAELGEVQQRP
DEVGYEPVSSTLWRQREEYCARLIQHAWKRYKQRHGGGTDASGDDLEIDACDNGCGGGNGNEN
DDSGDGATGSGDNGSQHGGGSISGGGGTPGGGKSKGIIGSTQANIGIVDSNISPKESPDSIGD
PQGRQTAVLVESDGFVTKNGHRVVIHSRSPSITSRTADV

Figure 4.2 cont. *An. gambiae* voltage-gated sodium channel. (B) Predicted amino acid sequence for AgVGSC (AGAP004707). Grey highlights denote transmembrane domains. Red, underlined residues are targets for anti-AgVGSC-1, -2 and -3 IgGs, respectively. Experiments performed by Jacob Meyers.

domains 3 and 4 (Figure 4.2A, B). This short extracellular loop is also the Site 4 toxin binding site for scorpion and spider β-toxins. Anti-AgVGSC-3 IgG is designed against a fifteen amino acid antigen on the extracellular loop of subunit 4 connecting TM domains 3 and 4 (Figure 4.2A,

B). This short extracellular loop is also the Site 3 toxin binding site for scorpion α-toxins, Magi 2 and Tx4(6-1). The peptide sequence for all three AgVGSC antigens were compared to the predicted amino acid sequence of all *An. gambiae* proteins using the BLAST search tool on VectorBase and no other *Anopheles* proteins were found to contain a similar antigen (http://www.vectorbase.org).

Blood meals containing anti-AgRDL IgG may reduce the survival of *An. gambiae*

To measure the effects of anti-AgRDL IgG on *An. gambiae* survivorship, we fed mosquitoes on blood meals containing anti-AgRDL IgG ranging from 1.5 - 3.0mg/mL. Our preliminary data shows that blood feeding of three different anti-AgRDL IgG induces a significant mosquitocidal effect (Figure 4.3). Non-specific polyclonal (control) rabbit IgG, when blood fed at the highest concentration tested, did not affect mosquito mortality. These results were consistent across three replicates. All three concentrations of anti-AgRDL IgG tested equally reduced mosquito

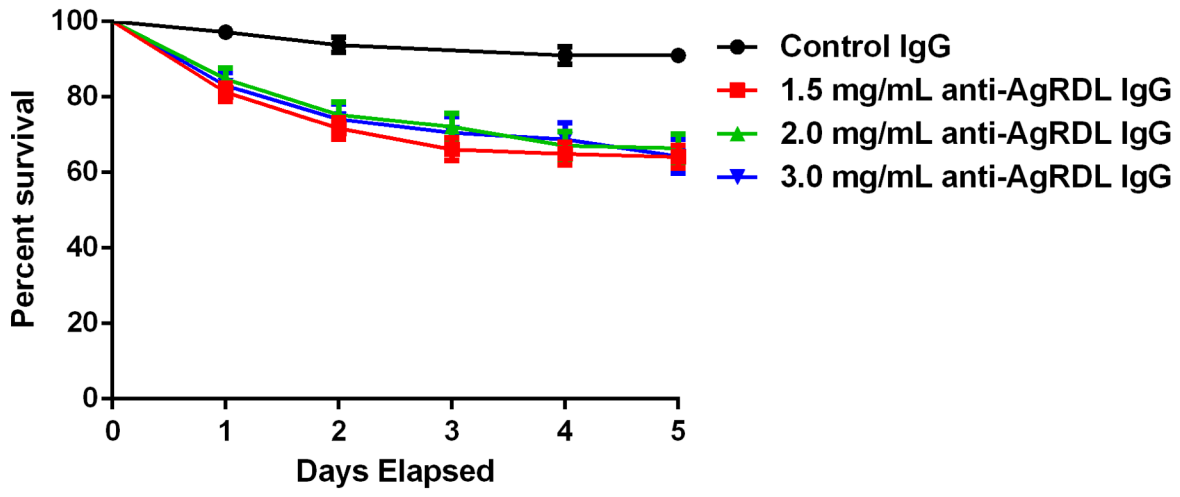

Figure 4.3. Blood meals containing anti-AgRDL IgG may reduce the survival of *An. gambiae* mosquitoes. Survival curves of *An. gambiae* after feeding on a single blood meal containing anti-AgRDL IgG. Data represent two replicate blood feeds and are presented as mean and S.E. Experiments performed by Jacob Meyers and Jasmine Donkoh.

survivorship (1.5mg/mL anti-AgRDL IgG: Hazard ratio = 3.456 [2.281, 5.235], n=251; 2.0mg/mL anti-AgRDL IgG: Hazard ratio = 3.785 [2.294, 6.244], n=158; 3.0mg/mL anti-AgRDL IgG: Hazard ratio = 4.668 [2.649, 8.223], n=112). Since none of the anti-AgRDL IgG concentrations tested reduced mosquito survivorship by over 50% we could not calculate the LD_{50} of blood meals containing anti-AgRDL IgG.

Intrathoracic injection of anti-AgVGSC-1 and -3 IgGs may reduce the survival of *An. gambiae*

A preliminary experiment of the effects of anti-AgVGSC IgGs on *An. gambiae* survivorship was conducted with anti-AgVGSC-1 and -3 IgGs. A physiologically relevant concentration (956ng/mL) of IgG (Vaughan et al., 1990) was injected into the hemocoel of adult, female *An. gambiae* mosquitoes and survivorship was monitored for four days (Figure 4.4). The one

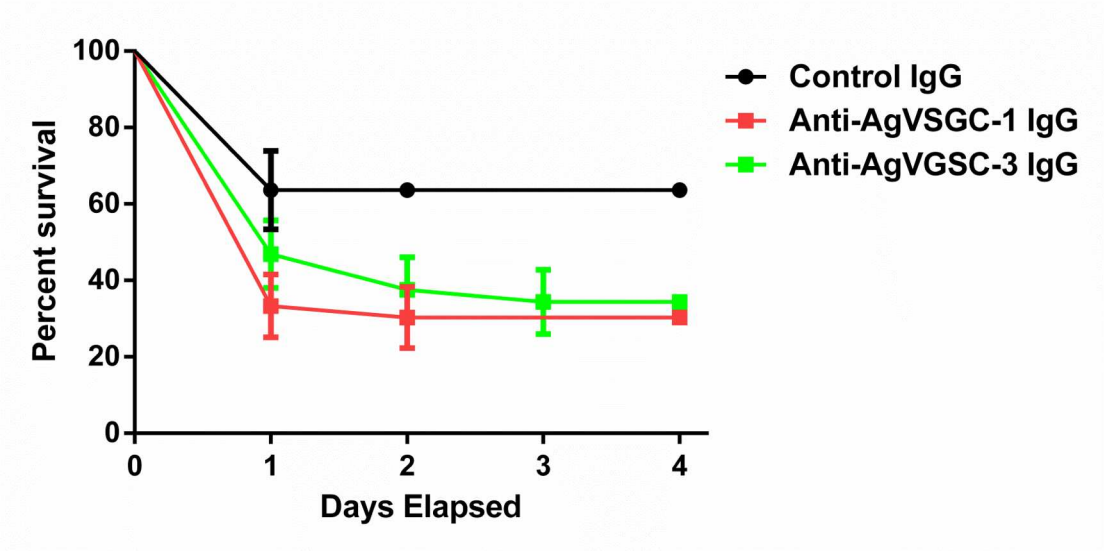

Figure 4.4. Intrathoracic injection of anti-AgVGSC IgGs may reduce the survival of *An. gambiae* mosquitoes. Survival curves of *An. gambiae* after a single intrathoracic injections containing anti-AgVGSC IgGs. Data represent one un-replicated experiment, and are presented as mean and S.E. Experiments performed by Jacob Meyers and Meg Gray.

experiment showed that both anti-AgVGSC-1 and -3 IgGs equally reduced mosquito survivorship relative to controls (Anti-AgVGSC-1 IgG: Hazard ratio = 1.958 [1.027-6.881], n=55; Anti-AgVGSC-3 IgG: Hazard Ratio = 1.894 [1.271-5.559], n=54). The effects of anti-AgVGSC-2 IgG on *An. gambiae* survivorship have not been tested.

AgRDL expression in adult, female *Anopheles gambiae*

Though blood meals containing anti-AgRDL IgG seem to reduce *An. gambiae* survivorship, immunofluorescent staining of sagittal slices of whole adult non-blood fed female *An. gambiae*

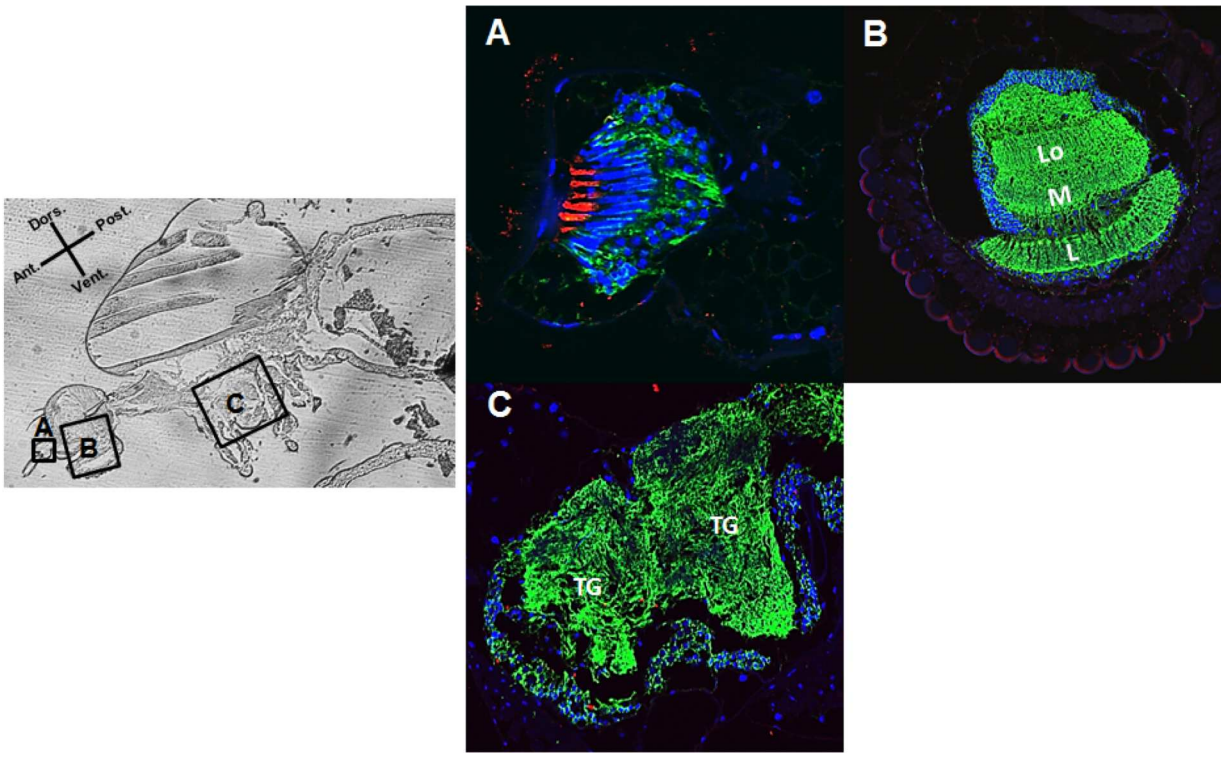

Figure 4.5. Adult, female *An. gambiae* immunoreactive tissues to anti-AgRDL IgG. (A) Johnston's Organ. Left: Anterior, Right: Posterior. (B) Head: L: lamina, M: medulla, Lo: lobula; (C) Thoracic ganglia. Top: Dorsal, Bottom: Ventral; TG - thoracic ganglia. Red: AgRDL; Green: Neuronal Marker; Blue: DAPI. Experiments performed by Jacob Meyers and Jasmine Donkoh.

showed very little tissue expression. While we expected to observe AgRDL staining in a broad range of neuronal tissue, there was only AgRDL staining in the Johnston's Organ (Figure 4.5A). There was no AgRDL staining in the supraesophageal ganglion, thoracic ganglia or any other neuronal or non-neuronal tissues (Figure 4.5B, C).

Immunostaining of three anti-AgVGSC IgGs in adult, female *Anopheles gambiae*

Though each anti-AgVGSC IgG antigen target was predicted to be specific to AgVGSC, which seems to be a single, unduplicated and non-heterogenous gene in the genome, the three anti-AgVGSC IgGs each had a unique staining pattern in adult, female *An. gambiae*. Anti-

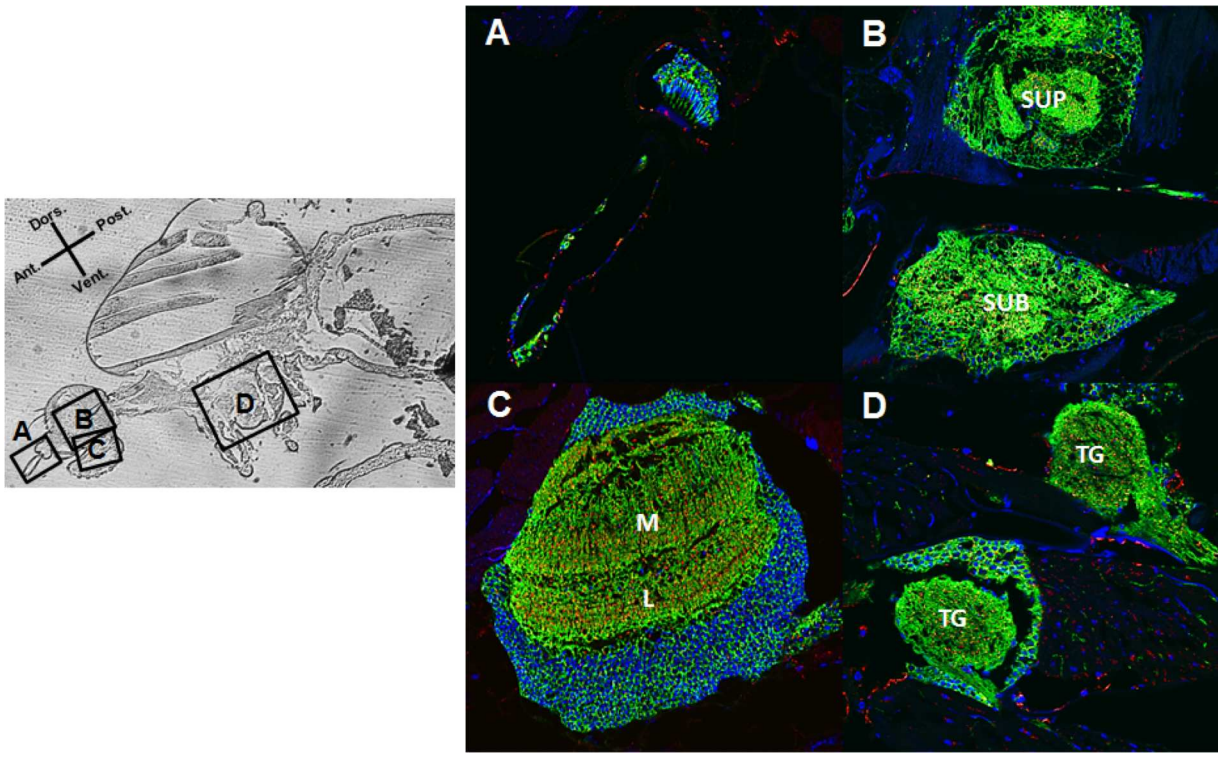

Figure 4.6. Adult, female *An. gambiae* immunoreactive tissues to anti-AgVGSC-1 IgG. (A) Antennal segment and Johnston's Organ. Bottom-left: Anterior, Top-right: Posterior. (B) Head: SUP: supraesophageal ganglion, SUB: subesophageal ganglion. (C) Optic Lobe: L: lamina, M: medulla. (D) Thoracic ganglia. Top: Dorsal, Bottom: Ventral; TG - thoracic ganglia. Red: AgVGSC-1; Green: Neuronal Marker; Blue: DAPI. Experiments performed by Jacob Meyers and Jasmine Donkoh.

AgVGSC-1 IgG stained the antennal segments, Johnston's organ, supraesophageal ganglion, subesophageal ganglion, optic lobe and thoracic ganglia (Figure 4.6). Staining was localized to the neuronal processes and was not found on neuronal cell bodies. Anti-AgVGSC-2 IgG stained the ommatidia, ventral nerve cord, muscle fibers and neuronal processes of the thoracic ganglia (Figure 4.7). Lastly, anti-AgVGSC-3 IgG stained puncta within the lens, muscle fibers, ventral nerve cord leading to the thoracic ganglia and the thoracic ganglia (Figure 4.8).

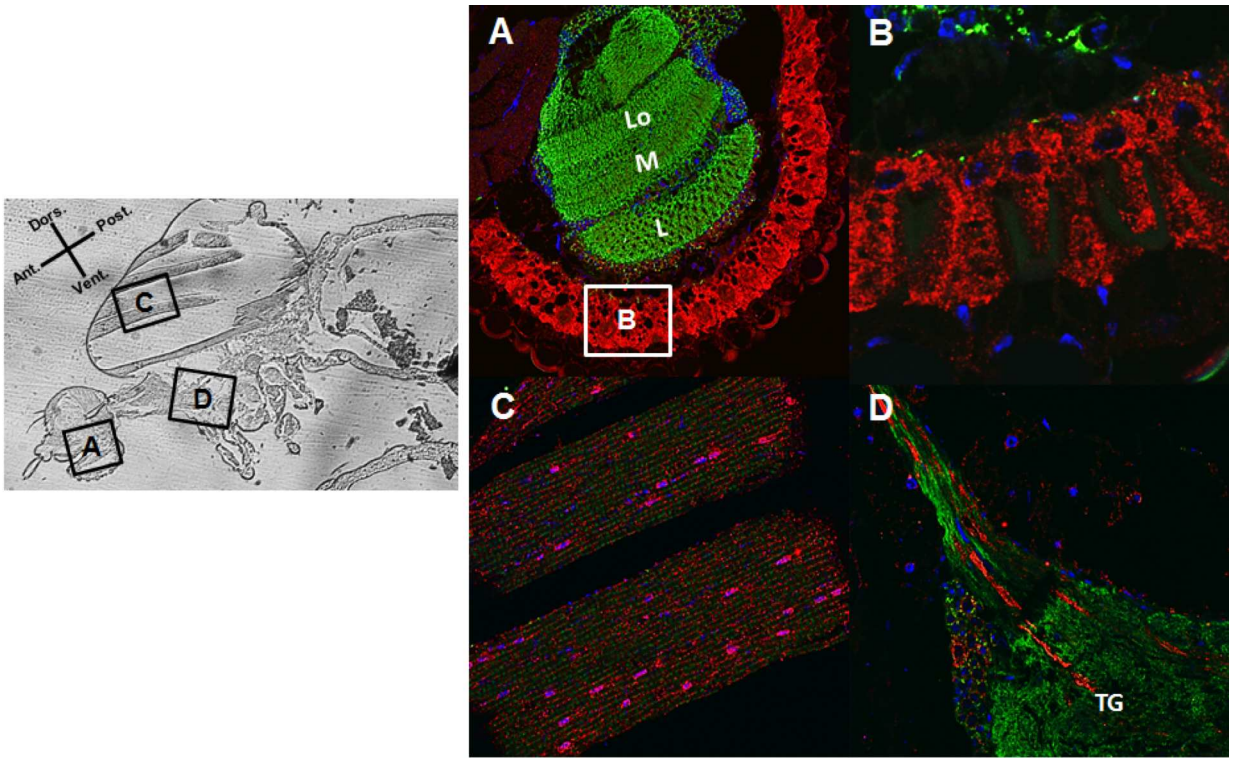

Figure 4.7. Adult, female *An. gambiae* immunoreactive tissues to anti-AgVGSC-2 IgG. (A) Head: L: lamina, M: medulla, Lo: lobula. (B) Photoreceptors. (C) Muscle fibers. (D) Thoracic ganglia. Left: Anterior, Right: Posterior; TG - thoracic ganglia. Red: AgVGSC-2; Green: Neuronal Marker; Blue: DAPI. Experiments performed by Jacob Meyers and Jasmine Donkoh.

4.4: Discussion

We discovered that a single blood meal containing anti-AgRDL IgG may reduce *An. gambiae* survivorship. The sensitivity and intensity of blood meals containing anti-AgRDL IgG on mosquito survivorship are unique from blood meals containing anti-AgGluCl IgG. While

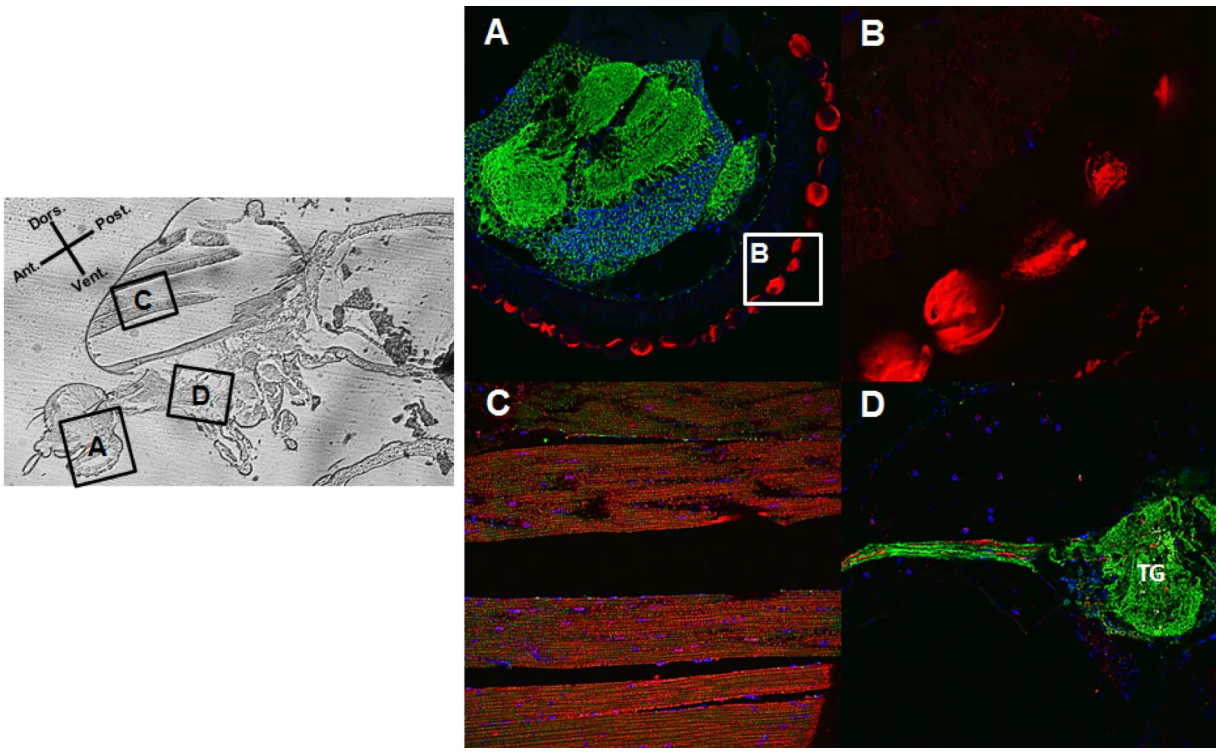

Figure 4.8. Adult, female *An. gambiae* immunoreactive tissues to anti-AgVGSC-3 IgG. (A) Head. (B) Photoreceptors. (C) Muscle fibers. (D) Thoracic ganglia. Left: Anterior, Right: Posterior; TG - thoracic ganglia. Red: AgVGSC-3; Green: Neuronal Marker; Blue: DAPI. Experiments performed by Jacob Meyers and Jasmine Donkoh.

blood meals containing 3.0mg/mL of anti-AgGluCl IgG killed nearly 80% of blood fed *An. gambiae* mosquito over four days and there was a clear dose-dependent killing effect, 3.0mg/mL of anti-AgRDL IgG killed only 27% of blood fed *An. gambiae* mosquitoes compared to controls and we observed no dose-dependent killing effect. It should be noted that anti-AgRDL IgG specificity was only confirmed through western blot and ELISA against the bacterially-produced antigen. Further testing of anti-AgRDL IgG specificity is necessary to determine if it binds naturally-expressed AgRDL and whether or not it binds other, non-specific proteins. Such data would then clarify whether the observed modest mortality effects of anti-AgRDL IgG are true or an artifact. However, the relatively minor mortality effects of anti-AgRDL IgG compared to anti-AgGluCl IgG may be explained by a differential role of AgGluCl and AgRDL in the adult,

female mosquito. While AgGluCl is expressed in the antenna, Johnston's Organ and the cell bodies and neuronal process of the supraesophageal ganglion and thoracic ganglia (Meyers et al., 2015b), AgRDL expression was only observed in the Johnston's Organ of adult, female *An. gambiae*. RDL staining in adult *D. melanogaster* shows staining in the antennal lobes, optics lobes and throughout the supraesophageal and subesophageal ganglia (Aronstein and Ffrench-Constant, 1995). There was no staining observed in the thoracic or abdominal ganglia (Aronstein and Ffrench-Constant, 1995). However, the fly age was not reported and flies do not take blood meals, which have a major effect on transcriptional patterns and protein expression in mosquitoes (Dana et al., 2005; Marinotti et al., 2005; Rinker et al., 2013). Both antibody blood feeds and immunostaining were performed on non-blood fed female, adult mosquitoes aged two to four days. We have previously shown that age, tissue and blood feeding status can affect AgGluCl transcriptional levels (Fig. 2.4). It is possible these same variables affect AgRDL expression. It is also possible that young, non-blood fed adult female *An. gambiae* do not utilize AgRDL for GABA-mediated inhibitory neurotransmission. A better understanding of AgRDL gene regulation may clarify why a maximal concentration of anti-AgRDL IgG has such marginal effects on mosquito survivorship. It may also point out different mosquito life stages on which anti-AgRDL IgG may be more effective at reducing *An. gambiae* survivorship.

AgGluCl is the only gene in the *An. gambiae* genome encoding for a glutamate-gated chloride channel while AgRDL is one of three genes encoding for a GABA-gated chloride channel along with ligand-gated chloride channel homologue 3 (LCCH3) and GABA and glycine-like receptor of *Drosophila* (GRD) (Buckingham et al., 2005). AgRDL has low amino acid homology to both AgLCCH3 and AgGRD (<30%) (http:///www.vectorbase.org) so it is unlikely that anti-AgRDL IgG would bind to AgLCCH3 or AgGRD. RDL has also been shown

to form heteromultimers with LCCH3 in *D. melanogaster* and *Apis mellifera* (Hosie et al., 1997; Dupuis et al., 2010). If this occurred in *An. gambiae*, it could reduce the ability for anti-AgRDL IgG to bind AgRDL subunits.

We previously showed that anti-AgGluCl IgG reverses the mosquitocidal effects of the AgGluCl agonist IVM (Meyers et al., 2015b). If anti-AgRDL IgG can be shown to truly bind native AgRDL, it may also reverse the potential mosquitocidal effects of an AgRDL agonist. As both IgGs would target inhibitory neurotransmitter receptors and likely have the same effects on their targets, we hypothesize they would synergize when applied together in a potential vaccine targeting both AgGluCl and AgRDL.

Since IgGs against CysLGIC insecticide targets are effective at reducing mosquito survivorship, we wanted to test the efficacy of IgGs against other neuronal insecticide targets. The most widely used class of insecticide is the pyrethroids which target the *Anopheles* voltage-gated sodium channel (WHO World Malaria Report, 2013). Very preliminary assays on the effect of anti-AgVGSC IgGs on *An. gambiae* show that a single, physiologically relevant concentration of both anti-AgVGSC-1 and -3 IgGs intrathoracically injected into the hemocoel of adult, female *An. gambiae* significantly reduce mosquito survivorship. Both anti-AgVGSC-1 and -3 IgGs equally reduced mosquito survivorship, which was also equivalent to the reduction in mosquito survivorship of anti-AgGluCl IgG intrathoracic injections. This suggests that imbibing blood meals containing AgVGSC-1 and -3 IgGs may also reduce mosquito survivorship.

Though both anti-AgVGSC-1 and -3 IgGs had a similar effect on *An. gambiae* survivorship, all three anti-AgVGSC IgGs had different staining patterns on sagittal slice of whole adult, female *An. gambiae*. Further testing is necessary to determine the specificity and action of these

three anti-AgVGSC IgGs against naturally expressed AgVSGC through western blot and perfusion onto AgGluCl-expressing *X. laevis* oocytes.

Anti-AgVGSC-1 IgG stained the expected tissues for AgVGSC which are the neuronal processes, but not cell bodies, of all neuronal tissues. This includes the antenna, Johnston's organ, supraesophageal ganglion, subesophageal ganglion, ventral nerve cord and thoracic ganglia. This mirrors the VGSC expression pattern of other insects including *Drosophila melanogaster* and *Musca domestica* (Tseng-Crank et al., 1991; Hong and Ganetzky, 1994; Castella et al., 1997). Since anti-AgVGSC-1 IgG stained nearly all neuronal tissues, we would expect ingestion of anti-AgVGSC-1 IgG to affect a broad range of physiological systems including sensory, motor as well as the reproductive, digestive and endocrine systems.

Surprisingly, anti-AgVGSC-2 and -3 IgGs did not have this same pattern of staining. Anti-AgVGSC-2 IgG stained the photoreceptors, muscle fibers, ventral nerve cord and thoracic ganglia while anti-AgVGSC-3 IgG stained puncta within the lens of the ommatidia, as well as the muscle fibers, ventral nerve cord and thoracic ganglia. Currently it is unclear why anti-AgVGSC-2 and -3 IgGs have unique staining patterns from the predicted AgVGSC staining pattern observed with anti-AgVGSC-1 IgG and observed in other insects (Tseng-Crank et al., 1991; Hong and Ganetzky, 1994; Castella et al., 1997). One explanation is that anti-AgVGSC-1 IgG targets the largest extracellular loop of AgVGSC and therefore might be the most likely to bind to AgVGSC while anti-AgVGSC-2 and -3 IgGs target small extracellular loops. By targeting these small extracellular loops, it is possible that anti-AgVGSC-2 and -3 IgGs are binding to a small proportion of expressed AgVGSC (ventral nerve cord and thoracic ganglia) and not to other neuronal tissues where we would expect AgVGSC expression. However, this does not explain why anti-AgVGSC-2 and -3 IgGs are staining the muscle fibers, photoreceptors

or puncta within the lens. This suggests that these IgGs are also binding other proteins, although a BLAST search of the amino acid sequences of predicted proteins from the *An. gambiae* genome come up with no hits. Antibody specificity has only been tested by ELISA and western blot against the antigen peptide used to immunize rabbits from which these IgGs were isolated. Further work is necessary to demonstrate the specificity of these anti-AgVGSC IgGs to functional expressed AgVGSC.

Our findings that anti-AgGluCl IgG reduces *An. gambiae* survivorship drove the hypothesis that IgGs against other critical neuronal insecticide targets may also be effective at affecting mosquito fitness. The preliminary data presented in this chapter suggesting that anti-AgRDL IgG as well as three anti-AgVGSC IgGs may affect mosquito survivorship suggest our initial hypothesis may be correct. Further *in vitro* work is necessary to prove the mosquitocidal effects of these antibodies as well as *in vivo* experiments immunizing animals against these mosquito antigens and testing the mosquitocidal effects of directly feeding *An. gambiae* on immunized animals.

Chapter 5: Anti-AgGluCl IgG: Mosquitocidal properties and antibody translocation in three diverse mosquito disease vectors (Diptera: Culicidae)

5.1: Introduction

Mosquito-borne diseases, such as malaria, dengue and West Nile virus, account for an estimated 1,434,000 deaths annually and 60,056,000 disability adjusted life years (World Health Report, 2012). These diseases are transmitted by Anopheline and Culicine mosquitoes. Historically, the most successful examples of vector-borne disease control have been through targeting mosquito vectors through the use of chemical insecticides. However, cross-cutting strategies that affect these diverse mosquito disease vectors are currently limited to broad spectrum insecticides, to which resistance has become widespread (Corbel et al., 2007; Saavedra-Rodriguez et al., 2007; Norris and Norris, 2011; Ranson et al., 2011). Development of novel control strategies is necessary to maintain control of, and further eliminate the transmission of mosquito-borne diseases.

We previously showed that blood meals containing anti-AgGluCl IgG significantly reduces *An. gambiae* survivorship (Meyers et al., 2015a). Since GluCl is found outside of the midgut, it is important to understand which mosquito species permit IgG to translocate from the blood meal into the hemolymph. Previous literature shows that antibody translocation is not uniform across mosquito species (Vaughan and Azad, 1988; Jeffers and Michael Roe, 2008). Antibodies have been shown to translocate across the midgut of *An. gambiae*, and other *Anopheles* spp. for up to 48 hours post ingestion (Vaughan and Azad, 1988; Beier et al., 1989). There are conflicting reports concerning antibody translocation across the midgut of *Aedes aegypti*, though most reports show that there are barriers to this process (Hatfield, 1988c, b; Ramasamy et al., 1988; Vaughan and Azad, 1988; Jacobs-Lorena and Lemos, 1995). *Culex tarsalis* has never been tested

for this process, but *Culex pipiens* have been shown to have little to no antibody translocation occuring directly following blood feeding (Vaughan and Azad, 1988).

We administered a polyclonal anti-AgGluCl IgG to *Aedes aegypti* and *Culex tarsalis* through a blood meal or directly into the hemocoel by intrathoracic injections to determine and quantify its broad mosquitocidal activity across these diverse mosquito species. In parallel, we compared GluCl tissue expression and antibody translocation into the hemolymph of *A. aegypti* and *C. tarsalis* compared to *An. gambiae*.

5.2: Materials and Methods

Mosquitoes. *Anopheles gambiae* s.s. G3 strain (origin The Gambia), *Aedes aegypti* (Vergel) and *Culex tarsalis* (California) were raised at 28-31^{o}C, 80% relative humidity on a 14:10 light dark cycle. Larvae were fed ground Tetramin® fish food daily. Adults were provided with water and 10% sucrose solution *ad libitum*. Colony mosquitoes were blood fed every 3 to 4 days on defibrinated calf blood.

Blood Feed with Artificial Membrane Feeder. Two to four day post-emergence mosquitoes were fed defibrinated calf blood spiked with purified rabbit anti-AgGluCl IgG (Genscript, Piscataway, NJ), purified rabbit control IgG (Genscript, Piscataway, NJ), IVM, or vehicle via glass bell feeders (Lillie Glass Feeders, Symrna, GA) sealed with pig sausage casing and heated to 37^{o}C. Mosquitoes were given 30-45 minutes to feed after which they were knocked down briefly at 4^{o}C for sorting and removal of unfed and partially fed mosquitoes. Fully-engorged mosquitoes were monitored for survivorship for 4 days following the blood meal.

Intrathoracic Injection of anti-AgGluCl IgG. Adult female mosquitoes were briefly knocked down at 4^{o}C for intrathoracic injection. Glass capillaries (Drummond Scientific, Broomall, PA, 3-00-203-G/XL) were pulled using a Flaming/Brown Micropipette Puller Model P-87 (Sutter

Instruments, Novato, CA) to create microcapillary injection needles. Mosquitoes were injected with 69 nL of either 958 ng/mL anti-AgGluCl IgG or control IgG using a Drummond Nanoject II Automatic Injector (Drummond Scientific, Broomall, PA, 3-000-204) and glass microcapillary injection needles. Following injection, mosquitoes were maintained for four days and survivorship was monitored.

Hemolymph Extraction and Dot Blot. Hemolymph samples were taken from *An. gambiae*, *A. aegypti* and *C. tarsalis* three hours following blood feeding on de-fibrinated calf blood (control) or de-fibrinated calf blood containing 3.0mg/mL rabbit-derived anti-AgGluCl IgG. Hemolymph was extracted by hemocoel perfusion as previously described (Vaughan et al., 1990). Briefly, a small perforation was made on the dorsal portion of the abdomen between the 7^{th} and 8^{th} segment. Mosquitoes were intrathoracically injected with PBS, using glass microcapillary injection needles, until a drop (~0.5µL) of hemolymph protruded from the previously made abdominal tear. This hemolymph was collected into a glass microcapillary, extruded into a microcentrifuge tube and stored at -80^{o}C. Each sample contained pools of ten mosquito hemolymph extractions.

Fifty microliters of anti-AgGluCl IgG standards (3ng/mL – 3µg/mL) and extracted mosquito hemolymph were blotted onto nitrocellulose paper using the Bio-Rad Bio-Dot SF Microfiltration Apparatus (Bio-Rad Laboratories, Hercules, CA) following the manufacturer protocol. Since mosquito hemolymph extractions yielded very little volume, we diluted 5µL of hemolymph with 45µL PBS for blotting onto the nitrocellulose paper. Blots were blocked with 3% BSA in 0.05% TTBS for one hour on a rocker. Blots were treated with a 1:50,000 dilution of goat anti-rabbit IgG (H+L) HRP conjugate (Thermo-Scientific, Waltham, MA) in 1% BSA TTBS solution for three hours on a shaker and then washed for 15min in TTBS. Antibodies were visualized using

SuperSignal West Femto Maximum Sensitivity Substrate (Thermo-Scientific, Waltham, MA) per manufacturer protocol. Fluorescence was measured using IVIS-200 Optical Imaging System (Xenogen, St. Hopkinton, MA) and quantitated using Living Image 3.0 software (Caliper Life Science, Waltham, MA). Anti-AgGluCl IgG standards were fit with a linear regression curve, which was used to estimate the concentration of anti-AgGluCl IgG in the mosquito hemolymph.

Immunohistochemistry (IHC). Whole mosquito specimens were prepared from female blood fed mosquitoes aged two to four days post-emergence. After blood feeding, engorged females were injected intrathoracically (Drummond Nanoject, Broomall, PA) with 46nL of 4% paraformaldehyde. Specimens were briefly washed in 70% ethanol before overnight fixation in 4% paraformaldehyde at 4^{o}C. After fixation, specimens were paraffin embedded, cut into 5µm thick slices and mounted onto slides (Colorado HistoPrep, Fort Collins, CO). Slides were heated at 65^{o}C for 10 minutes and treated with xylene to remove the paraffin layer and then re-hydrated with graded washes in ethanol and PBS. Specimens were treated with graded washes of methanol and PBS to reduce autofluorescence. Slides were blocked for 2 hours with 10% non-fat dry milk and 0.1% Triton-X in PBS. Primary antibody staining consisted of 1:500 anti-AgGluCl IgG (prepared by GeneScript, Piscataway, NJ) and 1:500 goat anti-HRP (Jackson ImmunoResearch Laboratories, West Grove, PA) incubated overnight at 4^{o}C. Specimens were washed with 0.05% Tween-20 Tris-based Buffer Solution (TTBS) and then incubated with 1:1000 donkey anti-rabbit Alexa 555 (Invitrogen, Waltham, MA) and 1:1000 donkey anti-goat Alexa 488 (Invitrogen, Waltham, MA) for 3 hours at room temperature. For midgut staining, midguts were dissected from two to four day old females and fixed in 4% paraformaldehyde overnight. Midguts were blocked with 3% bovine serum albumin (BSA) in TTBS for one hour and stained with 1:500 anti-AgGluCl IgG for three hours. Specimens were subsequently washed

in TTBS and stained with 1:1000 donkey anti-rabbit Alexa 555 (Invitrogen, Waltham, MA) and Acti-stain 488 phalloidin (Cytoskeleton Inc., Denver, CO) for one hour. Slides from either preparation were mounted with VectaShield® containing DAPI (Vector Lab, Burlingame, CA). Negative controls underwent the same procedure, but substituting histidine affinity-purified polyclonal rabbit IgG from non-immunized rabbits as the primary antibody.

5.3: Results

Anti-AgGluCl IgG kills *A. aegypti* and *C. tarsalis* when administered through intrathoracic injections but not through a blood meal.

Since GluCl is highly conserved across multiple mosquito species (percent homology *An. gambiae* vs *A, aegypti*: 92.47%) (Figure 5.1), we tested to see if anti-AgGluCl IgG had similar mosquitocidal properties in *A. aegypti* and *C. tarsalis* as we observed in *An. gambiae*. When introduced through a blood meal, anti-AgGluCl IgG had no effect on survivorship in *A. aegypti* up to a concentration of 4.0 mg/mL anti-AgGluCl IgG (p=0.6124; hazard ratio: 0.4526 [0.06213, 3.297], n=80) (Figure 5.2A). *C. tarsalis* blood fed on anti-AgGluCl IgG up to 3.5mg/mL also had no effect on survivorship (p=0.0867; hazard ratio: 2.131 [0.8966, 5.066]; n=161) (Figure 5.2C).

Figure 5.1. Mosquito GluCl alignment. ClustalW alignment of predicted amino acid sequences for AgGluCl-b (AGAP001434) from *A. gambiae* and AeGluCl (AAEL003003) from *Ae. aegypti*. Asterisks denote identical amino acid residues. Black bars denote transmembrane domains. Experiments performed by Jacob Meyers.

To test if anti-AgGluCl IgG could affect mosquito survivorship if it was artificially made to cross the midgut, we intrathoracically injected *A. aegypti* and *C. tarsalis* with anti-AgGluCl IgG. When a physiological relevant concentration of anti-AgGluCl IgG (958 ng/mL) (Vaughan et al., 1990) was administered through a single intrathoracic injection, it reduced survivorship in both *A. aegypti* (p=0.0221; hazard ratio: 2.969 [1.169, 7.537]; n=122) and *C. tarsalis* over four days (p=0.0237; hazard ratio: 2.536 [1.132, 5.682], n=124) (Figure 5.2B, 2D).

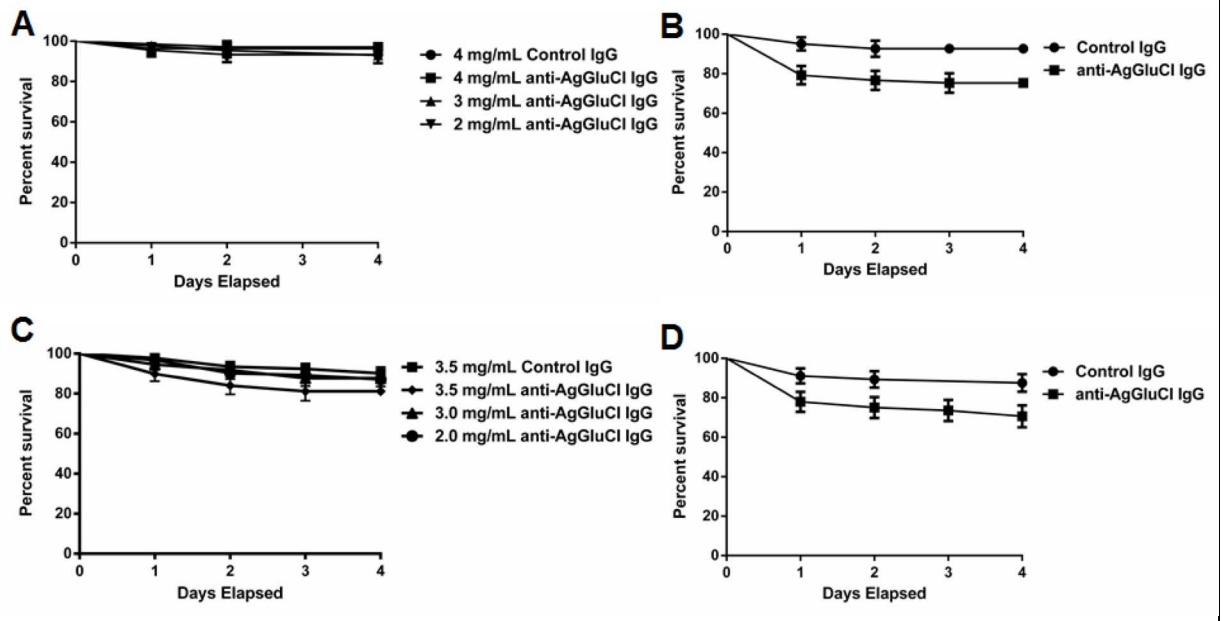

Figure 5.2. Anti-AgGluCl IgG kills *A. aegypti* and *C. tarsalis* when administered directly into the hemocoel, but not through a blood meal. Survival curves of *A. aegypti* (A) and *C. tarsalis* (C) after feeding on a single blood meal containing anti-AgGluCl IgG. Survival curves of *A. aegypti* (B) and *C. tarsalis* (D) after a single intrathoracic injection of anti-AgGluCl IgG. Data presented as mean and S.E. Experiments performed by Jacob Meyers and Meg Gray.

IgG is found in the hemolymph of *An. gambiae*, but not *A. aegypti* or *C. tarsalis*, following a blood meal containing anti-AgGluCl IgG.

To determine if IgG introduced through a blood meal is able to traverse the midgut into the hemocoel of *A. aegypti*, *C. tarsalis* and the anti-AgGluCl IgG blood meal susceptible *An. gambiae*, we fed mosquitoes a blood meal containing anti-AgGluCl IgG and extracted their hemolymph at three hours post blood feeding. Hemolymph, diluted in PBS from the extraction procedure, was blotted for the rabbit-derived anti-AgGluCl IgG. Anti-AgGluCl IgG was detected in the diluted hemolymph of *An. gambiae* that had fed on anti-AgGluCl IgG containing blood meals, but not *A. aegypti* or *C. tarsalis* (Figure 5.3A). A standard curve of anti-AgGluCl IgG was

created from 3ng/mL to 3µg/mL to estimate the amount of anti-AgGluCl IgG that had diffused into the *An. gambiae* hemolymph. From this standard, we calculated 12.92ng/mL anti-AgGluCl IgG in the hemolymph of *An. gambiae* following a blood meal containing anti-AgGluCl IgG (Figure 5.3B). This calculation compensated for the dilution of hemolymph in PBS.

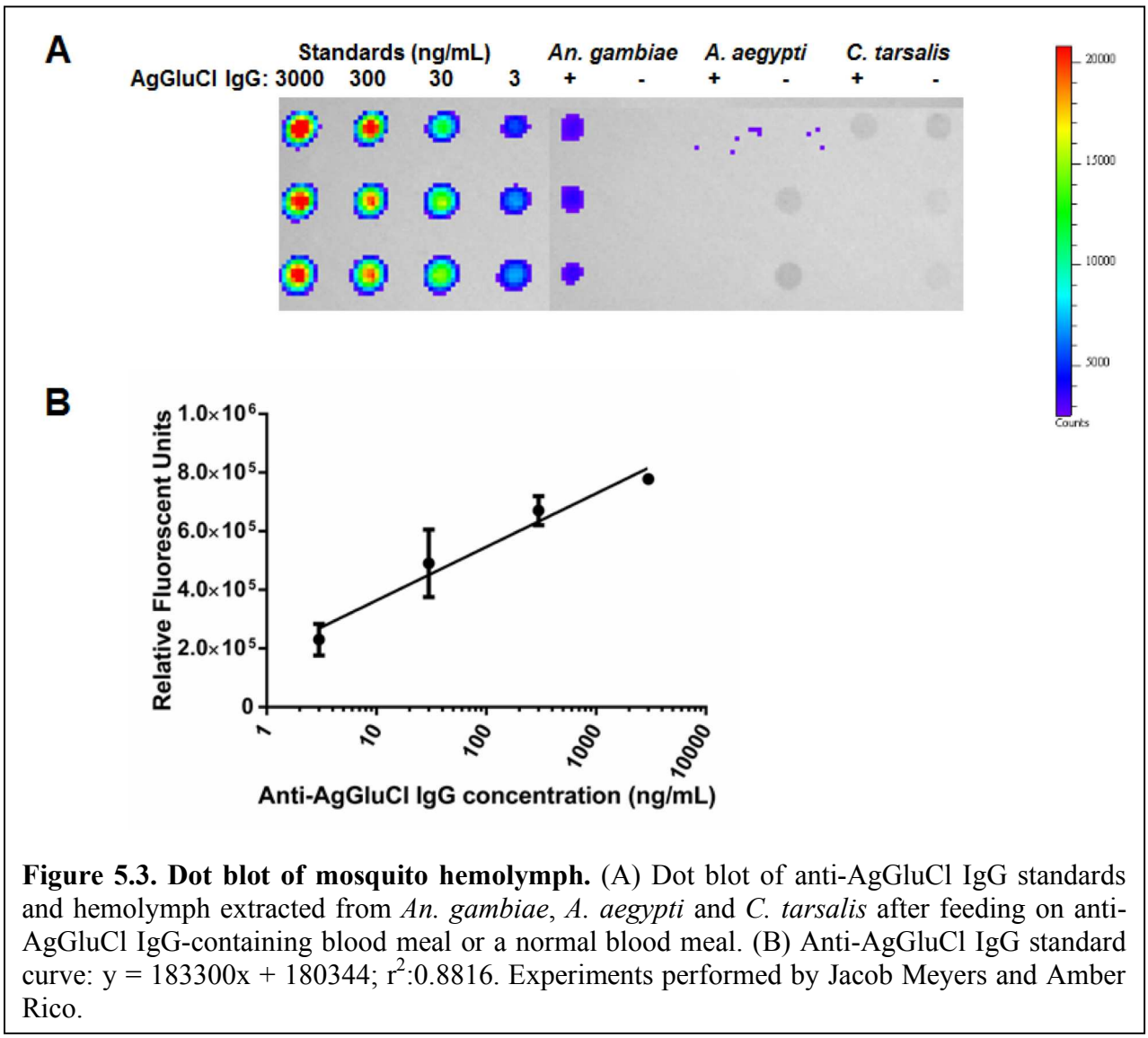

Figure 5.3. Dot blot of mosquito hemolymph. (A) Dot blot of anti-AgGluCl IgG standards and hemolymph extracted from *An. gambiae*, *A. aegypti* and *C. tarsalis* after feeding on anti-AgGluCl IgG-containing blood meal or a normal blood meal. (B) Anti-AgGluCl IgG standard curve: y = 183300x + 180344; r^2:0.8816. Experiments performed by Jacob Meyers and Amber Rico.

GluCl expression in *A. aegypti* and *C. tarsalis*

Given that anti-AgGluCl IgG reduces the survivorship of *An. gambiae*, *A. aegypti* and *C. tarsalis* when introduced into the hemocoel, we wanted to examine GluCl tissue expression to understand what physiological systems anti-AgGluCl IgG could be affecting. We have previously shown that AgGluCl is expressed in antenna segments, the Johnston's Organ, head and thoracic ganglia of adult, female *An. gambiae* (Table 5.1) (Meyers et al., 2015).

Table 5.1. GluCl expression in *An. gambiae*, *A. aegypti* and *C. tarsalis*. Experiments performed by Jacob Meyers.

	An. gambiae	*A. aegypti*	*C. tarsalis*
Antenna	+	+	+
Johnston's Organ	+	+	+
Head	+	+	+
Thoracic Ganglia	+	+	+
Anterior Midgut	-	+	+
Posterior Midgut	-	-	+

We stained sagittal sections of whole adult female mosquitoes for GluCl and a neuronal marker (Jan and Jan, 1982) to distinguish neuronal tissue. Immunolabeling of *A. aegypti* and *C. tarsalis* showed GluCl expression in the antennal segments, Johnston's Organ, optic lobe, supraesophageal ganglion and thoracic ganglia, just as previously seen in *An. gambiae* (Table 5.1) (Meyers et al., 2015). There were small puncta of GluCl staining on antennal segments

localized at the base of hair sensillae (Fig. 5.4A, 5.5A). The Johnston's Organ, a mechanosensory organ in the pedicel associated with audition and flight coordination, has

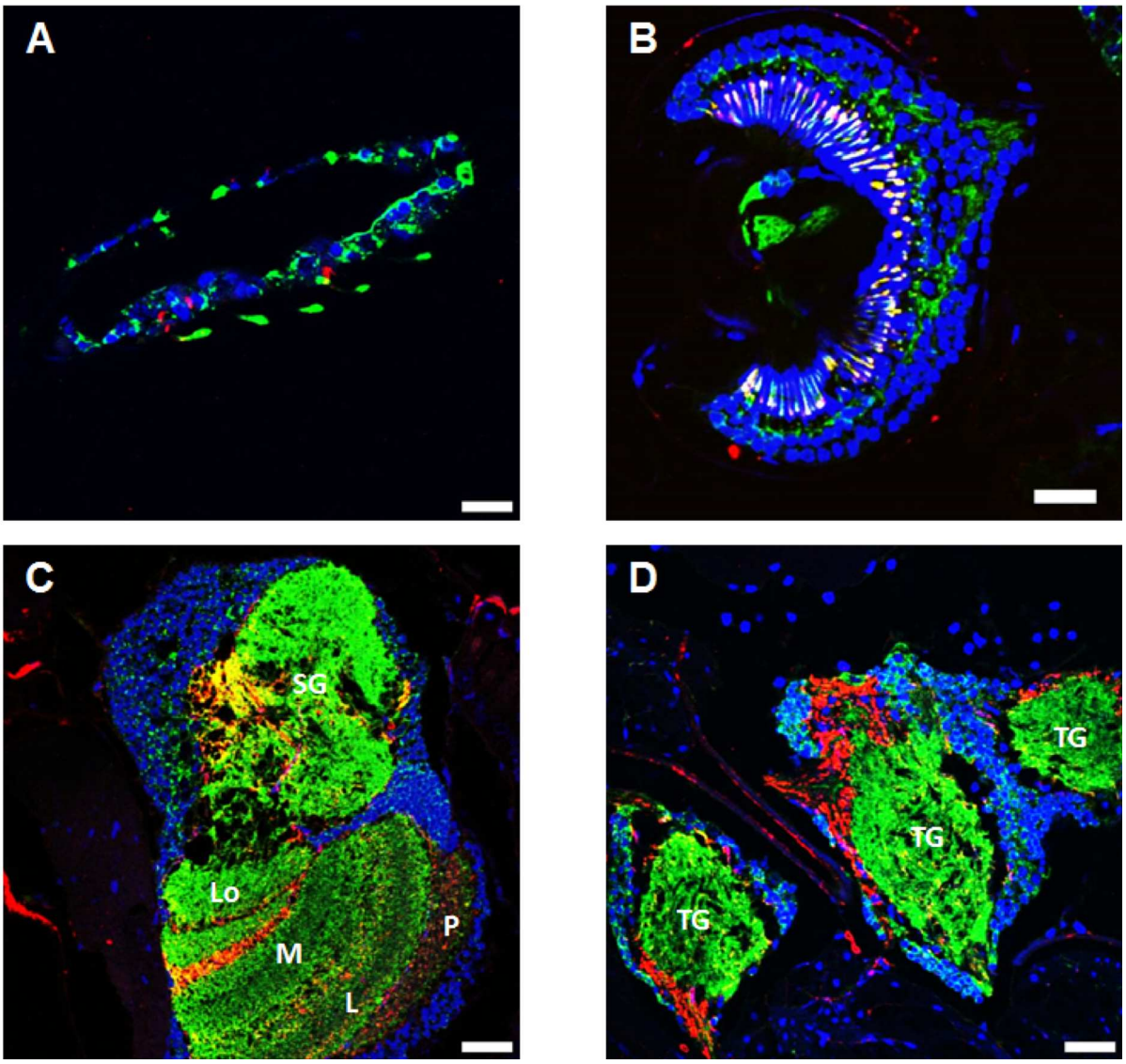

Figure 5.4. Adult, female *A. aegytpi* immunoreactive tissues to AgGluCl IgG. (A) One antennal segment. Left: Proximal towards head, Right: Distal from head. (B) Johnston's Organ. Left: Anterior, Right: Posterior. (C) Head: P- photoreceptors. L – lamina, M – medulla, Lo - lobula; SG: supraesophageal ganglion. (D) Thoracic ganglia. Top: Dorsal, Bottom: Ventral; TG - thoracic ganglia. Red: AgGluCl; Green: Neuronal Marker; Blue: DAPI. Scale: 50µM. Experiments performed by Jacob Meyers.

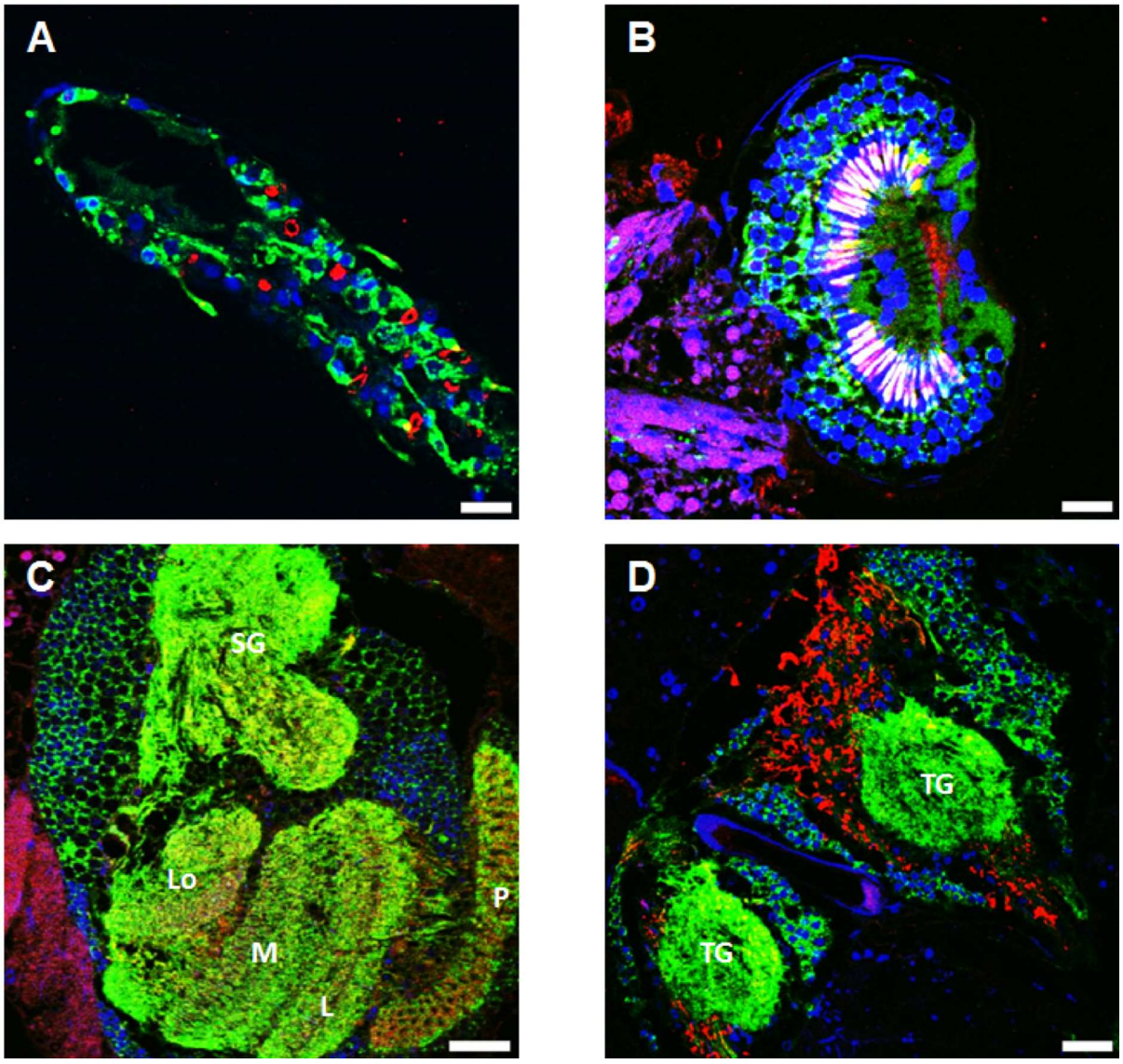

Figure 5.5. Adult, female *C. tarsalis* immunoreactive tissues to AgGluCl IgG. (A) Two antennal segments. Left: Distal from head, Right: Proximal towards head. (B) Johnston's Organ. Left: Posterior, Right: Anterior. (C) Head: P- photoreceptors. L – lamina, M – medulla, Lo - lobula; SG: supraesophageal ganglion. (D) Thoracic ganglia. Top: Dorsal, Bottom: Ventral; TG - thoracic ganglia. Red: AgGluCl; Green: Neuronal Marker; Blue: DAPI. Scale: 50μM. Experiments performed by Jacob Meyers.

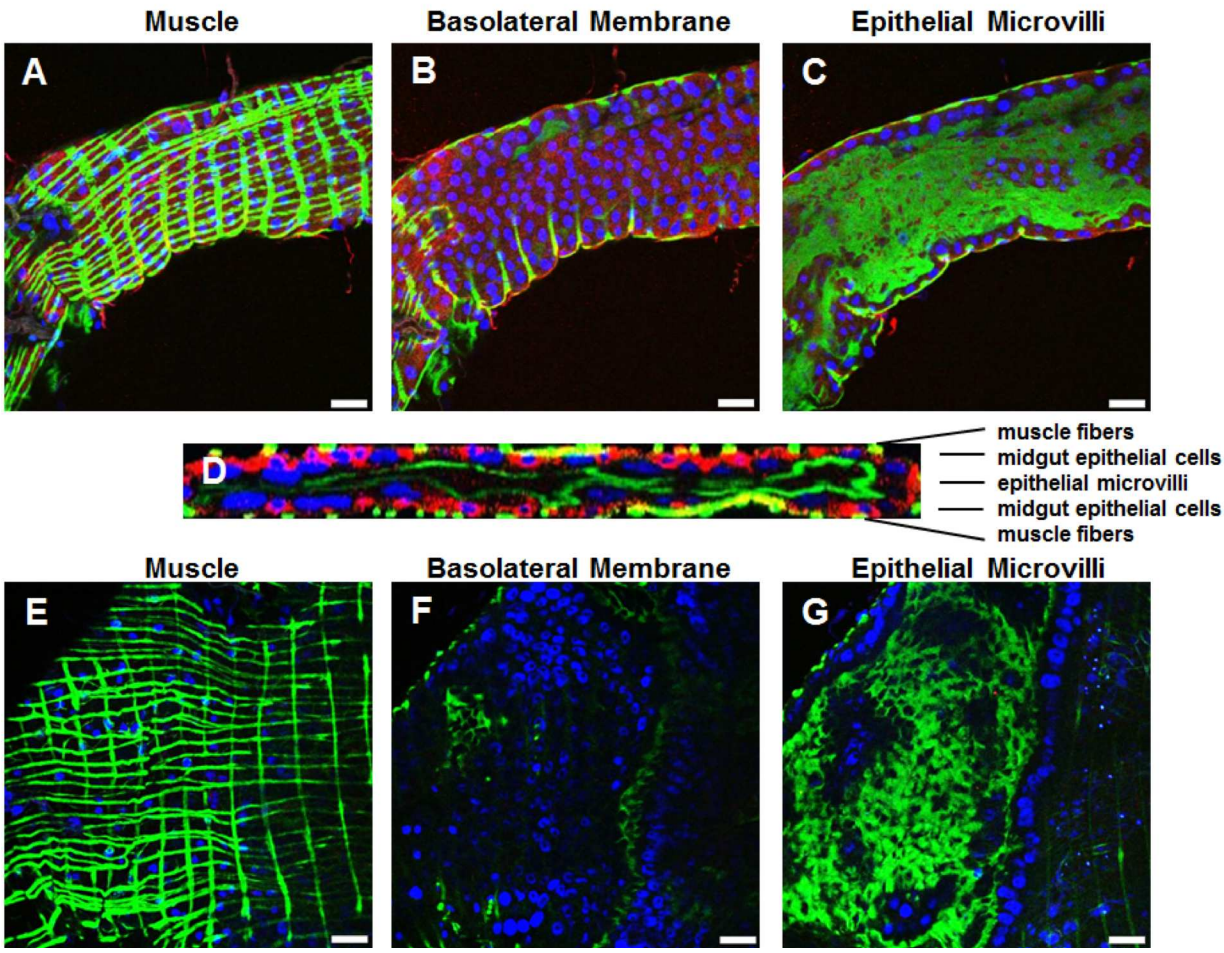

Figure 5.6. Immunostaining of dissected *A. aegypti* anterior and posterior midgut. Confocal slices of the muscle, basolateral membrane and epithelial microvilli of the anterior (A-C) and posterior (E-G) midgut. Confocal stack through the thickness of the anterior midgut (D). Red: GluCl; Green: actin; Blue: DAPI. Scale: 50μM. Experiments performed by Jacob Meyers.

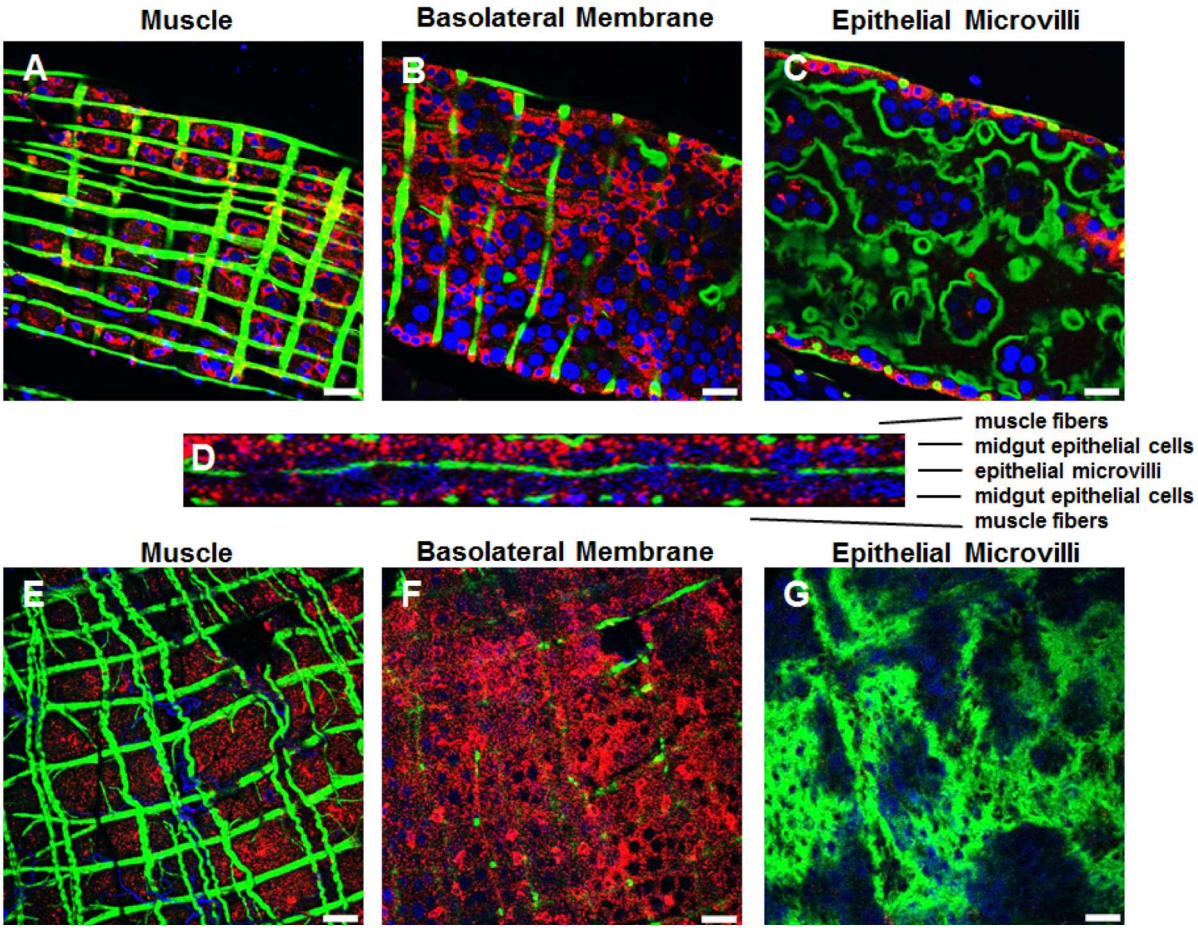

Figure 5.7. Immunostaining of dissected *C. tarsalis* anterior and posterior midgut. Confocal slices of the muscle, basolateral membrane and epithelial microvilli of the anterior (A-C) and posterior (E-G) midgut. (D) Confocal stack through the thickness of the posterior midgut (D). Red: GluCl; Green: actin; Blue: DAPI. Scale: 50µM. Experiments performed by Jacob Meyers.

specific GluCl staining in scolopidea (Fig. 5.4B, 5.5B). GluCl staining is present in neuronal tissues found in the head (Fig. 5.4C, 5.5C). Lastly, there was GluCl staining on neuronal cell bodies found in all three thoracic ganglia (Fig. 5.4D, 5.5D).

Surprisingly, *A. aegypti* and *C. tarsalis* exhibited unique GluCl staining on the midgut epithelium, which was not present in *An. gambiae*. Midguts were dissected and individually stained to visualize the precise location of GluCl in the midgut. In *A. aegypti*, GluCl expression was restricted to the anterior midgut and was not present in the posterior midgut (Fig. 5.6). In *C. tarsalis*, GluCl expression was found throughout the anterior and posterior midgut (Fig. 5.7). In both *A. aegypti* and *C. tarsalis* GluCl expression was confined to the basolateral side of the midgut epithelial cells but not on the epithelial microvilli that project into the midgut lumen (Fig. 5.6D, 5.7D).

5.4: Discussion

Since GluCl is highly conserved across multiple mosquito species, we tested to see if blood meals containing anti-AgGluCl IgG could affect the survivorship of two important Culicine disease vectors, *A. aegypti* and *C. tarsalis*. Anti-AgGluCl IgG did not affect *A. aegypti* or *C. tarsalis* survivorship when administered through a blood meal. When a physiologically-relevant concentration of anti-AgGluCl IgG was directly injected into the hemocoel, it reduced the survivorship in both *A. aegypti* and *C. tarsalis*. Surprisingly, all three mosquito species had the same reduction in survivorship to intrathoracic injections of anti-AgGluCl IgG. This suggests that anti-AgGluCl IgG has a similar affinity for the GluCl across these mosquito species and that GluCl plays a similar, critical physiological role in all three species.

Previous research has shown that whole IgG passes through the midgut of *An. gambiae* (Vaughan and Azad, 1988). There are conflicting reports on antibody translocation in *A. aegypti*, but most work has shown that this process does not occur (Vaughan and Azad, 1988; Jacobs-Lorena and Lemos, 1995). There have been no reports on antibody passage across the midgut of *C. tarsalis*, though other *Culex* species have been shown to have little to no antibody passage across the midgut (Vaughan and Azad, 1988). In agreement with previous work, we only detected anti-AgGluCl IgG in the diluted hemolymph of *An. gambiae* but not *A. aegypti*, nor did we find anti-AgGluCl IgG in the diluted hemolymph of *C. tarsalis* post blood feeding. These results provide an explanation for the sensitivity of these three mosquito species to blood meals containing anti-AgGluCl IgG. Using our standard curve, we detected 12.92ng/mL of anti-AgGluCl IgG in *An. gambiae* hemolymph. This is well below the concentration found in *An. stephensi* following blood feeding on an immunized rat (900-958ng/mL) (Vaughan et al., 1990). However, in our experiment we added rabbit-derived anti-AgGluCl IgG to calf blood that already contained calf IgG. We believe the low anti-AgGluCl IgG concentration we detected is because the rabbit-derived anti-AgGluCl IgG comprises only a small portion of the total IgG in the blood meal. It's also possible that a major portion of anti-AgGluCl IgG remained bound to target antigens throughout the hemocoel, sequestering it from our hemolymph extraction. Though the concentration of anti-AgGluCl IgG in the hemolymph may not reflect what would be present in the hemolymph following a blood meal from an AgGluCl-immunized host, it still verifies the presence of IgG in the hemolymph and confirms that IgG is able to translocate across the *An. gambiae* midgut. It is not clear how IgG in a blood meal translocates into the hemocoel of *An. gambiae*, nor is it known what the barriers are to this process in *A. aegypti* or *C. tarsalis*.

Further research is required to examine this key divergence in midgut physiology between *Anophelinae* and *Culicinae* mosquitoes.

To begin to understand what physiological role GluCl has in *A. aegypti* and *C. tarsalis*, we stained sagittal slices of whole, adult female mosquitoes for GluCl and found a similar neuronal GluCl expression pattern to what we previously discovered in *An. gambiae* (Meyers et al., 2015). There was only one tissue that had disparate GluCl expression between these three mosquito species. This was the midgut, which exhibited GluCl staining in *A. aegypti* and *C. tarsalis* but not *An. gambiae* (Meyers et al., 2015). GluCl expression in the midgut came as a surprise because *A. aegypti* and *C. tarsalis* were not sensitive to anti-AgGluCl IgG when administered through a blood meal. Closer examination of GluCl midgut expression through direct immunostaining of dissected midguts showed that GluCl expression only occurred on the basolateral surface of the midgut epithelial cells and was not present on the epithelial microvilli found in the midgut lumen of both *A. aegypti* and *C. tarsalis*. This means that GluCl antigens are not directly exposed to the blood meal and that IgG would need to translocate across midgut epithelial cells to bind to extracellular epitopes of GluCl found on the basolateral surface of the midgut epithelium. Our data show that IgG is unable to translocate into the hemocoel in *A. aegypti* and *C. tarsalis,* which is why GluCl on the basolateral surface of the midgut is not affected by anti-AgGluCl IgG-laden blood meals. At present, there is no evidence that anti-AgGluCl IgG insensitivity in *A. aegypti* and *C. tarsalis* is related to GluCl expression on the midgut. Though GluCl on the midgut could be acting as a "sink" and binding anti-AgGluCl IgG from a blood meal before it could reach neuronal expressed GluCl in the head and thorax, it has been previously shown that anti-*Rickettsia typhii* antibodies also do not translocate from the blood meal to the hemolymph in *A. aegypti* and *C. pipiens* giving further evidence that this

refractory phenotype to anti-AgGluCl IgG-laden blood meals is due to a barrier in antibody translocation (Vaughan and Azad, 1988). GluCl expression is confined to the anterior midgut of *A. aegypti* and not present in the posterior midgut, where GluCl expression was detected in both the anterior and posterior midgut of *C. tarsalis*. It is unclear what role GluCl has on the midgut epithelium or why it is differentially expressed across these three mosquito species, but its role in chloride ion transport suggests it might be involved in maintaining ionic balance during midgut alkanization and digestion (del Pilar Corena et al., 2005).

Targeting GluCl through a vaccine strategy will not be effective at killing Culicine mosquitoes because IgG is unable to translocate into the hemocoel following a blood meal. However, a mosquitocidal vaccine targeting a specific Culcine midgut antigen expressed in the midgut lumen could still be developed since this approach would not require antibody translocation across the midgut.

Chapter 6: Conclusions and remarks

6.1: Target of ivermectin in *Anopheles gambiae*: the glutamate-gated chloride channel

If IVM MDAs are to become a malaria control tool targeting *Anopheles* vectors of malaria, a thorough understanding of its molecular target is necessary to understand how IVM affects *Anopheles* physiology and to predict and potentially avoid potential mechanisms of resistance. We showed that AgGluCl is highly expressed in a broad range of neuronal tissues in the mosquito head and thorax, and likely is involved in a broad range of neurophysiological functions including processing of sensory signals and motor control. AgGluCl expression in these critical tissues suggests why IVM is toxic to *Anopheles* mosquitoes at very low concentrations. Transcriptional analysis of AgGluCl gene regulation showed that AgGluCl is highly transcribed in the head and thorax throughout the lifetime of the adult *An. gambiae*. AgGluCl transcripts generally were highest in the head, increased with age and decreased after a blood meal. In cloning AgGluCl, we discovered a novel, fourth splice isoform which was not previously predicted as well as a novel exon and splice site. We were able to heterogously express and functional analyze two of the four AgGluCl isoforms (a1, b) in *Xenopus laevis* oocytes using two-electrode voltage clamp. Though all four isoforms contain the predicted critical amino acid residues for IVM sensitivity, AgGluCl-b homomers were insensitive to IVM while AgGluCl-a1 homomers were highly sensitive to IVM.

While our initial characterization of AgGluCl answered many important questions concerning its role in *An. gambiae* physiology and as a target of IVM, it also created many questions. We still do not know the functional characteristics of AgGluCl isoforms –a2 and –c to glutamate or IVM as well as natively expressed AgGluCl. In discovering an IVM-insensitive AgGluCl splice isoform we have found a potential IVM resistance mechanism through

alternative splicing. A shift in transcriptional regulation toward IVM-insensitive AgGluCl splice isoforms could potentially occur to create IVM-resistant *An. gambiae*. This would be a novel insecticide resistance mechanism that has never occurred with any other insecticide target. A single blood meal containing IVM did not affect AgGluCl splice isoform transcriptional patterns towards IVM-insensitive AgGluCl-b transcription but this does not preclude a shift in alternative splicing after repeated sub-lethal IVM exposures across multiple generations. While our analysis of AgGluCl expression of 2DPE mosquitoes showed no expression in the abdomen, there were AgGluCl transcripts amplified from the abdomen of 15 DPE females. It is still unknown what abdominal tissues could be expressing AgGluCl in older, adult *An. gambiae* mosquitoes and how this could affect IVM sensitivity in young vs old *An. gambiae*.

6.2: Mosquitocidal vaccine

Research into a mosquitocidal vaccine has been conducted intermittently since the 1970s, though little to no research in this field has occurred in over a decade. Given the capability of IVM to create mosquito-toxic blood meal, we hypothesized that antibodies, targeting the same protein as IVM, in a blood meal could similarly reduce *An. gambiae* survivorship. Indeed, anti-AgGluCl IgG, when imbibed in a blood meal, reduced *An. gambiae* survivorship. This was the first example of antibodies in a blood meal, targeting a single protein outside of the midgut, reducing mosquito survivorship. Intrathoracic injection and serial feeding of physiologically relevant concentrations of anti-AgGluCl IgG reduced *An. gambiae* survivorship suggesting that anti-AgGluCl IgG toxicity might occur when taken directly from an AgGluCl-immunized animal. Initial testing of blood feeding *An. gambiae* directly on AgGluCl-immunized mice showed that a single blood meal does not affect survivorship, but that serial feeding may reduce mosquito survivorship over time. This potential breakthrough opens the door for testing other

proven chemical insecticide targets as immunological insecticide targets. We began by testing the mosquitocidal effects IgG targeting the GABA-gated chloride channel "resistance to dieldrin" because of its similarity to AgGluCl. Our preliminary data suggests that blood meals containing anti-AgRDL IgG may reduce *An. gambiae* survivorship.

Many chemical insecticides have been developed from biologically produced toxins. For example, the pyrethroid class of insecticides was developed from the pyrethrins from Chrysanthemum flowers and the avermectin class of endectocides were developed from the fermentation product of the soil bacterium *Streptomyces avermectinius*. In following with those successful strategies, we hypothesized that insecticidal peptide toxins produced from organisms such as spider, scorpions and pufferfish may direct potential antigen targets for developing a mosquitocidal vaccine. To test this hypothesis we developed three IgGs against peptide toxin sites on the *An. gambiae* voltage-gated sodium channel. Our very preliminary results suggest that these antibodies may also be toxic to *An. gambiae* mosquitoes when imbibed through a blood meal.

The initial success of blood meals containing IgG targeting AgGluCl and other *Anopheles* neuronal proteins drove us to test the broad potential of this strategy against Culicine mosquitoes. GluCl and other neuronal proteins are highly conserved and across Anopheline and Culcine mosquitoes so we hypothesized that blood meals containing anti-AgGluCl IgG would similarly reduce *A. aegytpi* and *C. tarsalis* survivorship. However, this was not the case. We later showed this was due to a difference in antibody translocation from the blood meal into the hemolymph. While we showed *An. gambiae* allowed antibodies to pass into the hemolymph following a blood meal, there was a barrier to this process in *A. aegypti* and *C. tarsalis*. We still do not understand what causes this barrier, but it prevents the development of a mosquitocidal

vaccine strategy to broadly target all mosquito disease vectors. However, a pan-*Anopheles* vaccine has major potential against multiple *Anopheles spp.* which have previously been shown to pass IgG through their midgut including *Anopheles arabiensis*, *Anopheles stephensi*, *Anopheles funestus* and *Anopheles albimanus* (Vaughan and Azad, 1988; Beier et al., 1989).

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Appendix I.

AI.1: Sub-lethal effects of IVM and anti-AgGluCl IgG on *An. gambiae* vision

Since the target of IVM and anti-AgGluCl IgG is expressed in the optic lobe of the *An. gambiae* supraesophageal ganglion, we wanted to test if sub-lethal concentrations of IVM and anti-AgGluCl IgG had an effect on visually-based *An. gambiae* behavior. We utilized a visual bioassay which measured the color-based landing preference of female *An. gambiae* previously described and validated on *A. aeygpti* (Muir et al., 1992). Briefly, the test cage (60cm x 60cm x 60cm) was made from Perspex Plexiglas. Painted black and white cardboard squares, each measuring 6in x 6in, were arranged in a Latin square design and attached to the center of one of the vertical walls of the test cage. At least fifty adult, female *An. gambiae* mosquitoes aged 2-6 DPE were released into a test cage for ten minutes and their final landing location was noted in three biological replicates. Experimental groups were graphed and statistically analyzed with paired t-tests on GraphPad Prism Software.

Non blood-fed female *An. gambiae* showed a strong landing preference for black squares (45.3% ± 2.9%) over white squares (4.0% ± 1.2%) (p-value=0.0018; n=150; t=23.43; df=3) (Fig A1.1a). To see if *An. gambiae* survivors of an IVM-containing blood meal had altered vision, we fed mosquitoes an LC_{25} concentration of IVM (11.75ng/mL) and tested their visually-based landing behavior at 24hr, 48hr and 72hr post blood meal (PBM) (Fig A1.1b). To confirm the toxic effects of IVM, we also monitored daily survivorship over four days (Fig A1.1c). At 24hr PBM, neither control nor IVM-fed mosquitoes landed on white or black squares at a high rate as the mosquitoes remained on the bottom of the test cage and were not active. We believe this was due to the post-blood meal effects which cause mosquitoes to become less active. We also noted that many mosquitoes had a dark abdomen suggesting the remnants of an undigested blood meal.

By 48hr PBM, the control fed *An. gambiae* became active and a significant proportion landed on the black squares compared to the white squares (Black: 16.8% ± 6.2%; White: 1.4% ±.8%; p-value=0.0067). By 48hr PBM, the IVM-fed *An. gambiae* also had a significant proportion land on the black squares compared to the white squares (Black: 14.5% ± 3.9%; White: 1.1% ± 0.7%; p-value=0.0253). At 48hr PBM, there was no difference in the proportion of mosquito which landed on black

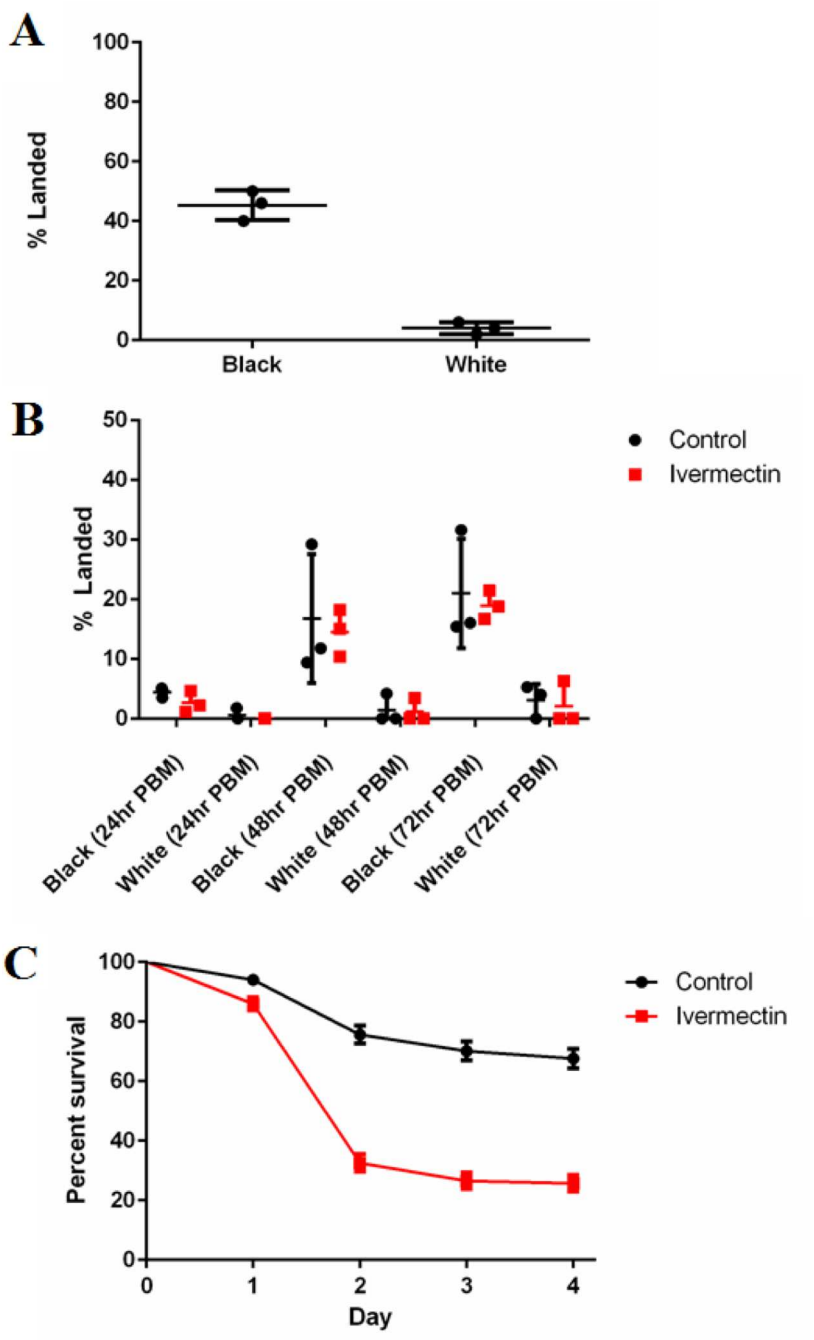

Figure A1.1. IVM does not affect *An. gambiae* visually-based landing behavior. (A) Percentage of non-blood fed *An. gambiae* mosquitoes landing on black and white colors. (B) Percentage of non-blood fed *An. gambiae* mosquitoes landing on black and white colors following a control or IVM-containing blood meal. (C) Mosquito survivorship following a control or IVM-containing blood meal. All data presented as mean and S.E. Experiments performed by Jacob Meyers.

or white squares between control and IVM-fed mosquitoes (Black: p-value=0.9922; White: p-value>0.9999) which did not change at 72hr PBM (Fig S1.1b).

These results suggest that *An. gambiae* survivors of an IVM-containing blood meal have no side effects on their vision by 48hr PBM. However, it is still unclear if IVM has an effect on vision at 24hr PBM since neither IVM-fed or control-fed mosquitoes were active, which was necessary to measure landing behavior. It is possible that IVM-fed mosquitoes had visual deficits at 24hr PBM that we could not measure given this confounding variable. Itis also possible that IVM from a blood meal does not reach the optic lobe located in the mosquito head. It may initially saturate the thoracic ganglia, which also expresses AgGluCl and are located in closer proximity to the midgut.

Appendix II.
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