DEATH BY CATTLE: EPRINOMECTIN ENDECTOCIDE EFFICACY IN MANAGEMENT OF MALARIA MOSQUITOES (*ANOPHELES QUADRIMACULATUS*)

by

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(Under the Direction of Nancy C. Hinkle)

ABSTRACT

In-lab bovine blood and cattle field efficacy testing were conducted to determine effects of LongRange® 5% eprinomectin extended-release injectable parasiticide on survival and fertility of *Anopheles* spp. mosquitoes. Mosquitoes were fed spiked blood in-lab and on cattle post-injection in the field. In-lab rates between 0.001 and 0.8 µl LongRange/ml of bovine blood were fed to mosquitoes, and cattle in the field were injected with single and double the label rate (1 ml LongRange per 50 kg). Mosquitoes that fed on single and double dose-treated cattle did not show mortality differing significantly following the majority of feedings on control cattle. Plasma analysis at the time of each feeding did not reveal detectable eprinomectin levels following day 7 post-treatment of cattle. LongRange is not a promising addition to the insecticidal zooprophylactic approach to mosquito management for vectors with high tolerance to macrocyclic lactones.

INDEX WORDS: zooprophylaxis, malaria, eprinomectin, cattle, mosquitoes

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. Malaria

For many pathogens, infection of a new host is entirely dependent on arthropods. Viruses, helminths, protozoa, and other microbes are capable of transfer from victim to victim by bites of blood-feeding mosquito species. The human malaria *Plasmodium* parasites accomplish their life cycle only in association with a single genus of mosquitoes, *Anopheles*. There are five human-infective species of *Plasmodium,* including *Plasmodium falciparum* Laveran*, Plasmodium vivax* Grassi & Feletti*, Plasmodium ovale* Stephens*, Plasmodium malariae* Grassi & Feletti*,* and *Plasmodium knowlesi* Sinton & Mulligan (Su 2010). The life cycles of *Plasmodium* spp. are very similar, beginning with the injection of sporozoites from an infective mosquito into a human host (Yang and Boddey 2017). Emerging from the African continent, malaria most likely originated in primates, spreading through the Nile valley and the Mediterranean region as well as Southeast Asia and Europe (Neghina et al. 2010).

Throughout history, malaria swept through civilizations, devastating communities long before identification as a vector-borne parasite (Kofoid 1934), and has likely been the most detrimental disease to humanity (Neghina et al. 2010). While the number of clinical cases on the African continent has dropped almost forty percent since 2000 (Khamis et al. 2018), and mortality has dropped by nearly fifty percent (Sande et al. 2017), malaria retains high transmission rates in many parts of the world despite eradication efforts, and disease suppression remains fragile and at risk of resurgence (Khamis et al. 2018). The disease also affects India and

much of Southeast Asia, but the majority of worldwide cases are found in sub-Saharan Africa (WHO 2015). Most of the world is also still at risk of endemic malaria due to travel, immigration, and the presence of vector-competent *Anopheles* mosquitoes globally, excluding the Antarctic. In 2015 the World Health Organization estimated just over 200 million malaria cases worldwide, with 90% of these occurring in African countries (Yaya et al. 2017). The majority of fatalities attributed to malaria are children, and infection is correlated with maternal and infant death (Lufele et al. 2017, Stanisic et al. 2015).

While malaria is both preventable and treatable, communities most impacted by this disease are often located in developing countries and rural agricultural regions lacking access to adequate medical resources. These agrarian communities are often irrigated, and livestock leave water-filled tracks, providing stagnant water sites suitable for mosquito reproduction. These conditions result in large malaria mosquito populations and challenging breeding site control (Mutero et al. 2004, Mutuku et al. 2006). Citizens may not have physical protection from the resulting large mosquito populations in the form of exclusion from homes and effective repellants. While recovery from the disease is common, malaria can be reoccurring in those infected and may cause permanent debilitation to individuals, impacting the futures of patients and their families (Collins and Jeffery 2007, Lobel et al. 2001). Poor health due to malaria infection has a substantial impact on developing countries' economic growth and rural communities via loss of production and product, causing a disease-poverty cycle (Benelli and Beier 2017, Yang and Boddey 2017).

1.2. *Anopheles* Mosquitoes

The mosquito has been accused of being the most deadly animal on earth due to many species' ability to transmit disease agents to animals and humans. In hematophagous mosquito

genera, the female blood feeds in order to procure protein for egg production. The *Anopheles* mosquito feeds by inserting the proboscis (made up of the mandibles, maxillae, labrum, labium, and hypopharynx) into mammalian skin. As a solenophage, it slides the flexible fascicles directly into a blood vessel, and uses a cibarial-pharyngeal pump system to draw a blood meal into the body (Kim et al. 2011, Kong and Wu 2011). This pump system is crucial to the injection of malaria parasites into the human host, as the injection of saliva by the mosquito includes the invading *Plasmodium* (Pingen et al. 2016). *Anopheles gambiae* Giles and *Anopheles arabiensis* Patton are two of the most common mosquitoes found in "hot spots" of rural agrarian communities where malaria is prevalent. In North America, especially in the Southeast, the most common malaria-competent mosquito is *Anopheles quadrimaculatus* Say. However, due to the lack of endemic malaria parasite, malaria is considered eradicated from the continent.

Anopheles gambiae is arguably the most problematic malaria vector for human hosts in sub-Saharan Africa. This mosquito bites most commonly indoors and rests inside homes, causing a high risk of exposure to humans. These mosquitoes have been targeted with insecticide-treated bed nets, breeding site control, and resting area pesticide treatment. *Anopheles arabiensis* is not so easily controlled by these measures, as it is more zoophilic and prone to outdoor biting (Gone et al. 2014, Massebo et al. 2015). Beyond behavioral differences, different species of anopheline mosquitoes may also differ in their susceptibility to pesticides, which alters necessary control measures depending on the region and most prevalent species (Dreyer et al. 2018).

1.3. Disease Management

Malaria control initiatives are most successful when they take into consideration both the *Plasmodium* parasite and *Anopheles* vectors. Control measures should focus on integrated control of both, as this has been shown to be the most affordable and efficacious approach

(Khamis et al. 2018). It is important to consider, too, that control of malaria does not refer to complete elimination, but rather transmission as low as one malaria case per thousand human hosts. This threshold is preferred over a goal of 100% elimination due to imported and dormant malaria cases, and realistic standards of public health (Sande et al. 2017). When focusing on treatment of the malaria parasite itself, *Plasmodium* spp. have been documented as being treated with botanical pharmaceuticals for centuries, and with synthetic pharmaceuticals more recently.

Ancient civilizations utilized wormwood (*Artemisia*) as a remedy for malaise caused by malaria, and extensive research has shown compounds from *Artemisia* spp. have insect repelling, larvicidal, and anti-plasmodial effects. Artemisinin, the component of these plants that acts as an anti-malarial, is a sesquiterpene lactone that is currently the leading treatment for uncomplicated *P. falciparum* and *P. vivax* cases (Weathers et al. 2014). The drug attacks malaria parasites in the schizont stage in host blood by inhibiting parasite hemoglobin digestion and malaria-specific metabolism pathways (Meshnik 2002). While combination with other anti-plasmodials such as mefloquine (novel synthetic blood schizonticide) in artemisinin-based combination therapies (ACTs) slow the spread of resistance to the drug by malaria parasites, resistance to artemisinin is on the rise and other anti-plasmodials such as quinine and chloroquine combined with antibiotics and synthetic drugs are also effective, depending on region and severity of disease (WHO 2017). Artemisinin is most effective against *P. falciparum*, the most detrimental malaria species (Benelli and Beier 2017). Both chloroquine and artemisinin are utilized as prophylactics, though there have been reported cases of *Plasmodium* resistance to chloroquine (Verschuere et al. 2017). The drug regimen and combination vary based on severity of the disease, the species of *Plasmodium*, parasite resistance, and patient pregnancy. Drawbacks to utilizing many of these

drugs include negative side effects and toxic effects in large doses or prolonged exposure (WHO 2017).

Barriers to successful vaccination lie in the biology of the *Plasmodium* parasites. They have multiple life stages, can lie dormant in the host, and have the five different human-infecting species; therefore vaccines have not proven to be very successful. The only malaria vaccine that has been fully developed is a subunit vaccine that targets the sporozoite stage of *Plasmodium* spp. in blood, but it does not have prolonged efficacy (Vaughan and Kappe 2017).

Due to the inability of *Plasmodium* to complete its life cycle in the absence of competent *Anopheles* mosquitoes, eradication initiatives focus on mosquito population management. The two pillars for control are indoor residual spraying (IRS) and insecticide-treated bed nets (ITNs) (Hewitt and Rowland 1999, Ye et al. 2017), which have proven extremely successful. Success of these methods depends on regional mosquito vectors and endophilic/exophilic habits, as they vary from one mosquito species to another.

While these tactics have successfully reduced populations of *An. gambiae* and other indoor-feeding species of mosquitoes, species such as *An. arabiensis* that have a more outdoor-oriented behavior have not been targeted as effectively by these measures (Massebo et al. 2015). Additionally, as IRS and ITNs have been in use for many years, behavioral changes due to long-term use cause mosquitoes previously managed by these tools to adopt a shift toward outdoor feeding (Bugoro et al. 2011, Sougoufara et al. 2016). In the last two decades, there has been a shift toward management tactics for outdoor feeding mosquitoes as changes in mosquito management tactics strive to accommodate this change in behavioral preference (Benelli and Beier 2017, Yakob et al. 2017). Susceptibility to the limited number of pesticides available for current management tactics is also a concern, and assessment of regional susceptibility to these

pesticides requires monitoring (Sande et al. 2017). This limits options for mosquito control even further, and larval habitat control is not a recommended tactic as it is in more developed regions where larval habitats are more manageable (Benelli and Beier 2017). The WHO has approved organochlorines, organophosphates, carbamates, and pyrethroids for IRS, but resistance and cross-resistance have been reported for these pesticides (Khamis et al. 2018). Sustainability in control is also important but is difficult to maintain in long-term mosquito management. Ideally, once management techniques have been deployed for an extended period of time, the mosquito population will be permanently reduced and transmission rates will remain moderated. Unfortunately, this is often not the case (Yukich and Chitnis 2017).

A study in Uganda showed that discontinuing residual spraying allowed pathogen transmission rates to increase to pre-program numbers (Raouf et al. 2017). This suggests that if spraying and other counter-measures do not continue until the parasite is eliminated, transmission rates increase again. As these mosquitoes become resistant to insecticide classes to which they are exposed, transmission rates may resurge to levels seen before spraying began. Integrated pest management, the practice of combining control tactics, is desirable compared to a more single-tactic management approach and is crucial to reducing resistance and developing a sustainable program (Benelli and Beier 2017). The use of a single-tactic system such as utilization of bed nets has shown failure in malaria control, encouraging a multi-faceted program of management (Zamawe et al. 2016, Zgambo et al. 2017). Transgenic mosquitoes or sterile insect technique for these vectors could be a longer-term solution to sustainability concerns and provide another integrated tactic, but current lack of cost-effectiveness and minimal field success have prevented these tactics from being implemented broad-scale for malaria management (Khamis et al. 2018).

1.4. Zooprophylaxis

In agriculture, trap crops divert pest insects from valuable cash crops. By surrounding target plants with these alternatives, insects and other arthropod pests are less likely to damage target crops. Trap crops are protective by appealing to arthropod instinctual preferences. This is done by planting trap crops that are preferable food sources to insects or that simply increase the number of landing points to decrease the probability of the pests locating target crops (Cheruiyot et al. 2018). The alternative crops are useful for diversion or for drawing pests to a centralized location so that they can be killed more efficiently (Hokkanen 1991). In human disease and vector control, humans are the target crop, and animals can be used as trap crops for blood-feeding arthropod vectors.

Preference in some malaria mosquito species for bovine or other livestock hosts over humans inspired the study of zooprophylaxis, which is the "general diversion of disease-carrying insects from humans to animals" (Saul 2003). Zooprophylaxis incorporates the use of dead-end hosts incapable of perpetuating the disease cycle (Kawaguchi et al. 2004). When mosquitoes bite cattle instead of humans, risk of human exposure to malaria and chances of mosquitoes acquiring infection are reduced, providing an additional prophylactic effect (Iwashita et al. 2014). The WHO has recognized zooprophylaxis as a potential measure of control since 1982 (Bogh et al. 2002). Domestic animals may already be present due to agricultural practice and be located around the home, drawing mosquitoes away from humans and diluting the host pool. This use of animals present in a community is termed "passive zooprophylaxis", while intentional contribution of livestock is "active zooprophylaxis" (Bogh et al. 2001). Animals used as trap crops can also be treated with a pesticide to kill mosquitoes and other biting vectors, or provide sub-lethal effects post-feeding, termed "insecticidal zooprophylaxis".

The success of passive zooprophylaxis is dependent upon the ratio of humans to animals, the distance from animals to humans, the ease of access to the alternative hosts, and the utilization of other integrated control measures when zooprophylaxis is used to reduce bites to humans (Sota and Mogi 1989). The species of mosquito, feeding preference (host, time, location), the type of housing, and geographical region also play a part in how the presence of cattle impacts the human transmission rate when untreated animals are used to dilute the host pool. Types of home, physical barriers to mosquitoes, and wealth have also been correlated with bite rates (Donnelly et al. 2016). Cattle distance from where the human hosts sleep is yet another contributing factor (Donnelly et al. 2015, Kawaguchi et al. 2004). Models have produced predictions and considered these variables when drawing conclusions about impacts of zooprophylaxis and its value in malaria control. System dynamics models attempt to keep track of the many different factors that impact transmission and the relationships involved. Epidemiological mathematical models have also been used to take into consideration factors such as human bite rates (Kaabi and Ahmen 2013, Kawaguchi et al. 2004, Nah et al. 2010, Sota and Mogi 1989).

Communities considering utilization of passive zooprophylaxis should assess these factors and determine whether passive or active zooprophylaxis measures are beneficial, harmful, or make no change. *Anopheles gambiae* has a distinct preference for humans, and so it was not surprising when numbers found inside houses did not change when cattle were placed outside the home, or when cattle odor bait traps were used for collecting this species (Mahande et al. 2007, Mayagaya et al. 2015). While some *An. gambiae* mosquitoes were receptive and pursued the cattle odor, *An. arabiensis* collections were more plentiful (Mahande et al. 2007). Mosquitoes may also take multiple blood meals from multiple hosts within a night and be willing

to bite livestock should they be available, even if their preference is for human odors (Ndenga et al. 2016). This works in reverse as well, however, as the more zoophilic *An. arabiensis* mosquito, which is a threat in much of sub-Saharan Africa, is also willing to take meals from multiple hosts, including humans, and may perpetuate malaria in regions where management tactics largely focus on more anthropophilic mosquitoes. Despite this affinity for cattle blood, *An. arabiensis* remains one of the most effective vectors of human malaria in Afro-tropical regions (Habetwold et al. 2004). When cattle are kept near the home, higher numbers of this species are exophilic feeders than endophilic, with more numerous collections in cattle sheds or outside than in the human shelters (Mahande et al. 2007, Mayagaya et al. 2015).

In contrast, without cattle around human homes, *An. arabiensis* do pursue humans inside homes more avidly, indicating that cattle are capable of luring away this species (Mayagaya et al. 2015). It should be noted that if not managed correctly, untreated cattle might increase blood-feeding resources and breeding sites, boosting the mosquito population (Kaabi and Ahmed 2013). Zooprophylaxis may produce a greater risk of mosquito exposure when used alone or with animals kept too close to humans, but livestock kept farther away from the home cease to be a threat and become protective with a pulling effect (Iwashita et al. 2014). Cattle kept inside compounds passively increase the likelihood of human bites and increase ambient mosquito numbers, causing increased parasitemia in humans, especially where strongly anthropophilic mosquito species are the primary vectors (Bogh et al. 2001, Bouma and Rowland 1995). Individuals who work around cattle either have higher numbers of bites or no difference from those whose professions do not place them in close proximity to livestock (Tirados et al. 2011). It has also been determined that cattle in the immediate proximity of humans may reduce landing counts and potentially bites to humans by *An. gambiae* (Maia et al. 2012), but in other cases the

mere presence of animals has been shown to increase the number of bites to humans, as in Chagas disease vectors (Gürtler et al. 2014). Still others have proven that cattle contribute to a reduction in cases of disease, such as onchocerciasis (Seidenfaden et al. 2001).

Many mosquito bites occur at night, which has been the motivation for insecticide-treated and non-insecticide-treated bed nets becoming a pillar of the malaria-control initiative (Bogh et al. 2002, Ranson et al. 2011). When bed nets were used in addition to cattle, there was an effective reduction in bites to humans as long as there were fewer than five cattle, but with cattle above this threshold, the residents were not protected due to a rise in mosquito numbers drawn to the area and providing additional blood meals (Kaburi et al. 2009). Integration of these strategies can provide a beneficial synergistic effect, reducing the mosquito population and lowering the number of bites to humans sleeping under nets (Bogh et al. 2002). This is especially useful when considering more anthropophilic vectors that may first pursue humans, but upon finding an impeding net may instead feed on cattle and other animals (Donnelly et al. 2016, Hassanali et al. 2008). This push-pull strategy improves passive zooprophylaxis efficacy (Donnelly et al. 2015).

An additional benefit to zooprophylaxis is the potential for decreased dependence on residual sprays and insecticide treated bed nets. Pyrethroids are the most commonly used pesticide class in IRS and ITNs and resistance to this class is becoming common (Ranson et al. 2011). With the addition of zooprophylaxis, spraying around bovine host sites could reduce the need for widespread spraying and instead target sites where mosquitoes are most likely to land (Kawaguchi et al. 2004). When assessing what livestock host to use for zooprophylaxis, cattle attract bites and can reduce vector presence near humans, while goats and sheep, when used passively, may draw vectors to the area and still allow for high transmission rates to humans

(Iwashita et al. 2014). Malaria mosquitoes have also reportedly blood-fed on horses, donkeys, chickens, and pigs (Bogh et al. 2001, Yamamoto et al. 2009).

1.5. Insecticidal Zooprophylaxis

While passive zooprophylaxis is dependent on many variables, some of those concerns no longer apply when considering implementation of insecticidal zooprophylaxis, which has been successful in control of mosquitoes and other vectors such as ticks and tsetse flies (Njoroge et al. 2017, Torr et al. 2007). Because many *Anopheles* species feed on multiple hosts, addition of insecticide-treated cattle converts zooprophylaxis into a tool for killing feeding mosquitoes (Laurent et al. 2017, Ndenga et al. 2016). Thus, cattle attracting more mosquitoes into the area may not have negative side effects if those mosquitoes die shortly after a blood meal. This addition of an insecticide may be sufficient to kill the additional vectors they attract and still reduce the overall population (Chaccour and Killeen 2016).

Several insecticides have been tested successfully for use in insecticidal zooprophylaxis. A single fipronil treatment in cattle killed all host-fed adult sand flies in India in the three weeks post-treatment, showing promise as a management tactic to reduce visceral leishmaniasis (Poché et al. 2013). Deltamethrin, a pyrethroid, reduces *An. arabiensis* populations when animals receive regular topical treatments and are kept near homes, although the resistance seen in other management tactics is predominantly in response to pyrethroids (Mahande et al. 2007). These mosquitoes were also killed successfully by oral dosing of Zebu cattle with ivermectin, eprinomectin, and fipronil (Poché et al. 2015). *Anopheles gambiae* fed on ivermectin-injected cattle were killed and fertility was lowered in survivors post-feeding (Fritz et al. 2009). *Anopheles culicifacies* Giles and *Anopheles stephensi* Liston also exhibited significant mortality when fed on ivermectin treated cattle, however, these treatments need to be reapplied every few

weeks in order to continue killing vectors throughout a season (Naz et al. 2013). Additional modes of action in pesticides used in insecticidal zooprophylaxis could provide additional rotation options for management of mosquitoes and other vectors, especially due to the ongoing reliance on pyrethroids.

1.6. Macrocyclic Lactones

Avermectins are macrocyclic lactones produced from fermentation of *Streptomyces* spp. bacteria, resulting in several potent endectocides. Included in this family of pesticides are ivermectin, doramectin, and eprinomectin, which have all been developed largely for endoparasite control (Shang et al. 2015). This class is valued for its broad-spectrum efficacy as well as low application rates compared to other pesticides and low accumulation in the environment (Naz et al. 2013, Vercruysse and Rew 2002). These drugs have been utilized for treatment of tropical diseases such as onchocerciasis, lymphatic filariasis, and mites (Chaccour et al. 2015). Ivermectin, a closely related macrocyclic lactone to eprinomectin, has been utilized in mosquito control longer than eprinomectin and has had success in malaria management initiatives. Ivermectin, moxidectin, doramectin, and eprinomectin have all been shown to negatively impact survival and fertility in anopheline mosquitoes, with eprinomectin and ivermectin having higher efficacy than the other macrocyclic lactones (Butters et al. 2012, Fritz et al. 2012).

Ivermectin injectables for livestock have resulted in promising insecticidal efficacy against zoophilic malaria vectors for up to one month (Naz et al. 2013). This is beneficial because livestock injectables provide a more prolonged efficacy than the approved oral endectocide treatments for humans (Chaccour and Killeen 2016). Mass drug administration of ivermectin to humans has shown evidence of reduced *An. gambiae* populations for up to a week,

significantly reducing survival and fertility of the mosquitoes and malaria parasite rates in residents of the community (Alout et al. 2014, Derua et al. 2015). Slow release injectable silicone implants provided long-term (up to 12 weeks) effective mortality in biting vectors when tested on rabbits, showing promise for macrocyclic lactones as a longer-term solution for insecticidal zooprophylaxis as a sustainable integrated management tactic (Chaccour et al. 2015).

While ivermectin has proven beneficial, eprinomectin was the first drug approved for domestic cattle use due to lower residue levels and broad-spectrum capabilities (Shang et al. 2015). Merck developed eprinomectin in 1996, derived from avermectin B1 (abamectin) (Shoop et al. 2001). As a macrocyclic lactone it is lipophilic, allowing persistence in the body for longer periods and providing the capability to concentrate in adipose tissue, potentially allowing the drug to congregate near the dermis where mosquitoes blood feed (Chaccour et al. 2015, Lanusse et al. 1997, Scott and McKellar 1992). Additionally, it is less lipophilic than other members of this class, resulting in lower concentrations in milk than other macrocyclic lactones such as moxidectin (Dupuy et al. 2008). Its mode of action is as a glutamate-gated chlorine channel binding agent, keeping the chloride channel open and paralyzing parasites by over-stimulation of the nervous system resulting in pest death (Fritz et al. 2012, Wolstenholme and Rogers 2005) as well as impairing invertebrate-specific neurotransmitters (Meyers et al. 2015). It is effective against internal cattle parasites as well as cattle mites and horn flies (*Haematobia* spp.) with no negative effects to animal health resulting from treatment at label rates (Hunter et al. 2012, Shoop et al. 1996). There has also been some evidence this class may act to reduce blood digestion in the stomachs of dipteran ectoparasites (Lyimo et al. 2017).

Eprinomectin was first developed as a pour-on due to its hydrophobic nature, but this use contributes more readily to resistance and overuse, and so attention has turned to formulation as

an oral dose or injectable (Shang et al. 2015). When compared, eprinomectin subcutaneous injection yielded a higher concentration in plasma than found in oral or pour-on treatments (Aksit et al. 2016, Baoliang et al. 2006, Wen et al. 2010). Following oral and subcutaneous delivery, macrocyclic lactones have remained in animals for several days post-administration, as demonstrated by research on ivermectin in pigs and cattle (Jones et al. 1992, Scott and McKellar 1992). More recently, studies have shown that eprinomectin, whether poured on, orally dosed, or injected, is also capable of remaining in animal tissues for up to a month and results in mortality to mosquitoes after they blood-feed on treated mammalian hosts (Askit et al. 2016, Lozano-Fuentes et al. 2016). While injected eprinomectin (and other macrocyclic lactones) persist in the animals longer than when externally applied, the use of eprinomectin in rural communities demands a long-term formulation that requires fewer applications. Repetitive dosing of animals can prove problematic due to needs for distribution, storage of the drug, and financial burden. Additionally, it may cause stress and increased labor to animal owners. LongRange, an eprinomectin injectable, may be the solution to these concerns.

1.7. LongRange

LongRange was developed by Merial (Boehringer Ingelheim) to provide a long-lasting endoparasite treatment with the goal of breaking the pasture cycle of cattle endoparasites (Soll et al. 2013). The 5% eprinomectin injectable is approved for beef cattle in North America, and treats for intestinal worms, lungworms, grubs, and cattle mites (Visser et al. 2013). The novelty of this drug is in its formulation, with eprinomectin in solution combined with polylactide-co-glycolic-acid (PLGA) 75:25, a polymer that retains a portion of the initial dose at the injection site. A gel matrix forms and gradually breaks down over time, distributing the second dose after the initial peak of the drug has waned. Antioxidant butylated hydroxytoluene and co-solvents N-

methyl-2-pyrrolidone and triacetin are included in this formulation. Administration is approved for dose volume of 1 ml per 50 kg of body weight in cattle. While this formulation is efficacious against internal parasites and mange in livestock (DeDonder et al. 2015, Pollock et al. 2016), relatively little is known about how it affects ectoparasites such as biting flies, including mosquitoes. The following manuscript details the study performed to determine whether LongRange might be an effective addition to the insecticidal zooprophylaxis management tactic.

CHAPTER 2

DEATH BY CATTLE: EPRINOMECTIN ENDECTOCIDE EFFICACY IN MANAGEMENT OF MALARIA MOSQUITOES (*ANOPHELES QUADRIMACULATUS*)[1]

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2.1. Abstract

In-lab bovine blood and cattle field efficacy testing were conducted to determine effects of LongRange® 5% eprinomectin extended-release injectable parasiticide on survival and fertility of *Anopheles* spp. mosquitoes. Mosquitoes were fed spiked blood in-lab and on cattle post-injection in the field. In-lab rates between 0.001 and 0.8 µl LongRange/ml of bovine blood were fed to mosquitoes, and cattle in the field were injected with single and double the label rate (1 ml LongRange per 50 kg). Mosquitoes that fed on single and double dose-treated cattle did not show mortality differing significantly following the majority of feedings on control cattle. Plasma analysis at the time of each feeding did not reveal detectable eprinomectin levels following day 7 post-treatment of cattle. LongRange is not a promising addition to the insecticidal zooprophylactic approach to mosquito management for vectors with high tolerance to macrocyclic lactones.

Keywords: zooprophylaxis, malaria, eprinomectin, cattle, mosquitoes

2.2. Introduction

The World Health Organization estimated over 200 million cases of malaria worldwide in 2015, with 90% occurring on the African continent alone (Yaya et al. 2017). While malaria is both preventable and treatable, communities most heavily impacted are in developing countries and rural agrarian regions lacking access to adequate medical resources. Additionally, with irrigation and large populations of livestock present, water-filled tracks and stagnant water sources provide plentiful habitat for larval development (Mutero et al. 2004, Mutuku et al. 2006). Thus, additional mosquito management tools are in high demand in agricultural communities of sub-Saharan Africa.

Anopheles gambiae Giles, an anthropophilic malaria vector prone to biting and resting inside homes, as well as other species with similar preferences, have been targeted with integrated tactics including insecticide treated bed nets (ITNs) and indoor residual sprays (IRS) (Tuno et al. 2010). Unfortunately, these species are exhibiting growing insecticide resistance and an adapted avoidance to these control measures (Bugoro et al. 2011, Sougoufara et al. 2016). *Anopheles arabiensis* Patton, another successful malaria vector, is difficult to target with these management tools, as it is more zoophilic and prone to outdoor biting (Gone et al. 2014, Massebo et al. 2015). Facing these challenges, it is important to pursue management tactics that utilize resources available to rural agrarian communities.

Malaria mosquito preference for livestock hosts over humans in species such as *An. arabiensis* has inspired the study of zooprophylaxis, which is the "general diversion of disease carrying insects from humans to animals" (Saul 2003) and incorporates the use of dead-end hosts incapable of perpetuating disease cycles (Kawaguchi et al. 2004). *Anopheles arabiensis* in agricultural communities primarily feed on cattle, and for territories where mosquito populations are high, zooprophylaxis may hold potential as a management tool (Massebo et al. 2015). While the presence of livestock may reduce number of bites to humans by vectors (Bulterys et al. 2009), especially when combined with other control tactics (Iwashita et al. 2014), an insecticide applied to these alternative hosts should result in the additional death of feeding mosquitoes and overall reduction in mosquito populations. Several insecticide formulations have been tested successfully for use with this method of zooprophylaxis. Deltamethrin, a pyrethroid, has reduced *An. arabiensis, Anopheles stephensi* Liston, and *Anopheles culicifacies* Giles populations when livestock receive regular topical treatments and are kept near homes (Mahande et al. 2007, Rowland et al. 2001), and villages with treated cattle have reduced malaria cases transmitted by

malaria mosquito species in comparison to those without treated animals present (Rowland et al. 2001). Despite this effectiveness, mosquito resistance to this class of pesticide is a growing concern and additional modes of action are needed for effective management. Macrocyclic lactones, with broad-spectrum efficacy as well as low accumulation in the environment (Naz et al. 2013), have been assessed for use treating livestock with the goal of managing disease vectors. Topical treatments in livestock are effective against parasites for up to four weeks, and macrocyclic lactone injectables have shown prolonged efficacy compared to other classes. *Anopheles gambiae* and *Anopheles arabiensis* fed on ivermectin-injected cattle were killed rapidly following exposure and survivors exhibited lowered fecundity post-feeding (Fritz et al. 2009). *Anopheles quadrimaculatus* Say, the mosquitoes utilized in this study, were also killed effectively by ivermectin when fed in the lab and on treated mammal hosts (Jones et al. 1992). Eprinomectin, very similar in composition to ivermectin, was the first macrocyclic lactone approved for cattle due to low residue levels and broad-spectrum control (Shang et al. 2015). However, animals treated with topical or injected eprinomectin would need to be treated repeatedly within a season in order to maintain blood titers suitable for vector control due to previously tested formulations having limited residual activity. Oral, injectable, and topical treatments have not lasted longer than a month in the cattle at levels that produced significant mortality (Jiang et al. 2005, Lozano-Fuentes et al. 2016, Poche et al. 2015).

The extended-release 5% eprinomectin injectable LongRange® (Boehringer Ingelheim, Duluth, GA) is approved for beef cattle for control and prevention of intestinal worms, lungworms, cattle grubs, and sarcoptic mites (Soll et al. 2013, Visser et al. 2013), and may be a solution to the shorter efficacy of previously tested formulations. The eprinomectin is combined with poly-lactide-co-glycolic-acid 75:25 (PLGA), a polymer that allows a portion of the initial

dose to remain bound to a PLGA sphere-matrix at the injection site. This gel matrix breaks down gradually, and the eprinomectin continues to distribute for up to 150 days (Merial).

This study investigates the lethal concentrations of LongRange and technical grade eprinomectin for *An. quadrimaculatus* mosquitoes, with implications for mosquito species such as *An. gambiae* and *An. arabiensis*. Additionally, the concentration of the drug in the blood post-treatment was measured. Concentration of eprinomectin in the cattle was also bioassayed by blood feeding mosquitoes on treated bovine hosts, and mortality, fertility, and fecundity of mosquitoes following feeding were observed. Results contribute to the discussion of LongRange's value in rural agrarian communities where livestock are in close proximity to humans and where malaria mosquito populations and transmission rates are high.

2.3. Materials and Methods

Mosquito Rearing

The following reagent was obtained through BEI Resources, NIAID, NIH: *Anopheles quadrimaculatus*, Strain ORLANDO, Eggs, MRA-139, contributed by Mark Q. Benedict. Lab-reared *An. quadrimaculatus* eggs were received on wet filter paper and 2000 were washed into a metal pan (32x23x4 cm) of 600 ml 20ºC distilled water. Eggs hatched within 24-48 h and were transferred after 48 h of development via 3 ml propylene pipettes. Two hundred larvae per pan were separated and reared to adulthood at 24-27ºC using space heaters. These larvae and subsequent life stages were maintained on a 12 h photoperiod, with light-timer-activated sunrise and sunset (30 min) facilitated adult mating behavior. Mosquito larval diets consisted of ground tropical fish flakes (Tetramin, Blacksburg, VA) added at increasing increments as larval size progressed. Adults were fed ten percent sucrose in distilled water-saturated cotton and housed in 30x30x30 cm cages (BugDorm, MegaView Science Co., Taiwan).

Bovine Blood Collection

Blood was collected for colony rearing, in-lab testing, and HPLC testing from Angus cattle (*Bos taurus)* that were untreated with insecticide for one year leading up to the study. The University of Georgia cattle were secured in a headgate and haltered. Blood samples were collected from the jugular vein using 35 ml syringes and 16.5 gauge, 3.8 cm long needles (Monoject, St. Louis, MO) (Fig. 1). Blood was transferred into heparinized Vacutainer® tubes (PPT Plastic, 8 ml capacity, 16 mm x 100 mm Size, Green/Gray; BD Medical Systems, Franklin Lakes, NJ) immediately following collection and taken to the lab for refrigeration at 1.6°C.

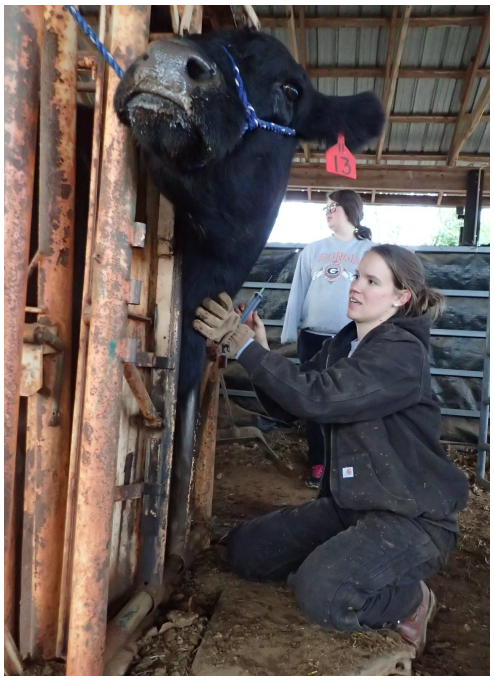

Fig. 1. Headgate and haltered cow during blood collection with syringe from jugular vein.

Blood collected from control animals was used for efficacy testing and colony rearing, while samples taken from treated animals during the study were processed for HPLC testing and bioassays. Animals were handled following IACUC #A3437-01 guidelines and safety protocols were followed, including utilization of a squeeze chute and headgate to ensure worker and animal

safety. The nine animals in this study were grazed on the same pasture and had unlimited access to water, with silage supplementation during winter months.

Mosquito Feeding

Rutledge feeders were used to feed mosquitoes heated blood (Lillie Glassblowers, Smyrna, GA; Rutledge et al. 1964). Parafilm® (Bemis Company, Inc., Oshkosh, WI) was stretched over the base of the Rutledge feeder and tubing was attached to hot water flow from a sink faucet to fill the outer pocket of the feeders. The Parafilm-covered bases were placed atop mesh of mosquito feeding cages to allow mosquitoes to penetrate Parafilm and feed. Each Rutledge feeder was held in place during feeding by a chemistry support stand and three-prong utility clamp (Fig. 2).

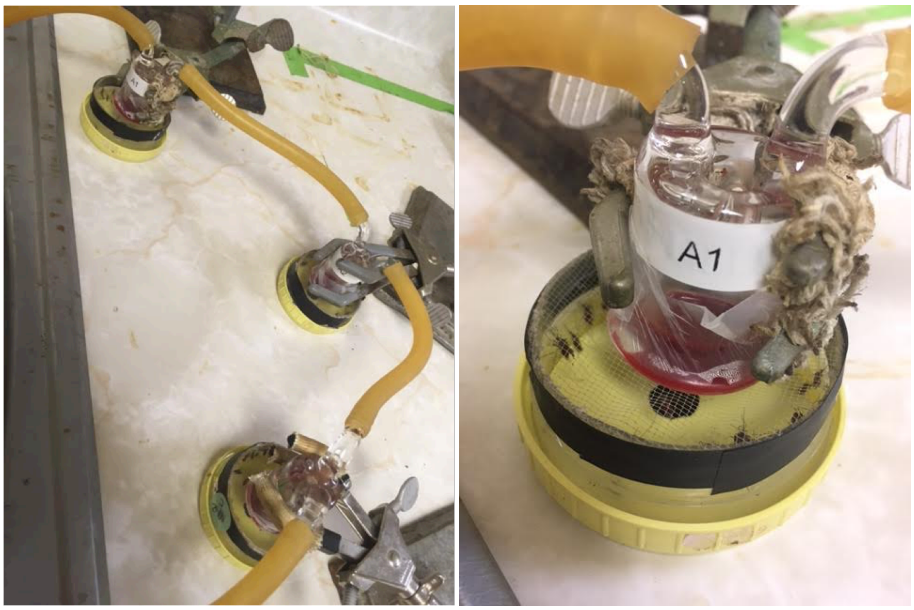

Fig. 2. Rutledge feeders and tubing held by utility clamps.

LongRange-Treated Blood

In order to establish dosage-mortality regression lines and LC_{50} for LongRange when fed to *An. quadrimaculatus*, untreated blood was collected from cattle to be used for selected dilutions in the lab. The label rate of LongRange for beef cattle is 1 ml LongRange per 50 kg of cattle body weight. Based on the average weight of our cattle (545 kg) and blood volume expected for each (40 liter),

peak drug concentration in the blood can be expected to reach up to 0.3 µl LongRange per ml of blood. Blood was treated at 0, 0.001, 0.01, 0.025, 0.05, 0.1, 0.125, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.5, and 0.8 µl LongRange/ml bovine blood, and 2 ml of blood was offered via Rutledge feeders to 20 mosquitoes per container, transferred by aspirator.

Dilutions were prepared using a micropipette measuring increments of 0.1 µl from 0.0 µl - 1.0 µl. Mosquitoes were fed for 45 min or until all fed to repletion. For each blood-feeding test, a cage of mosquitoes was fed an untreated blood sample as control in addition to treated mosquitoes. Each test day, ≥90% mosquitoes fed to repletion. Each treatment rate was replicated three times with containers of 20 mosquitoes and those that did not feed were removed.

Observations

Post-feeding, blood-fed mosquitoes were aspirated into labeled observation containers and provided sugar water. Dead mosquitoes were counted and survivors were subsequently observed at 24 h, 48 h, and 72 h post feeding for in-lab testing, and field trial mosquitoes were observed daily leading up to 100% mortality.

Technical Grade Eprinomectin Efficacy

Technical grade eprinomectin (PESTANAL®, analytical standard Sigma-Aldrich, St. Louis, MO) was diluted in dimethyl sulfoxide (DMSO) and further diluted in blood for mosquito feeding. A DMSO stock solution of 18.18 ppm was used to create dilutions of 909 ppb, 272 ppb, 227 ppb, 136 ppb, 91 ppb, 55 ppb, 45 ppb, 27 ppb, 9 ppb, and .91 ppb in blood and fed to mosquitoes. Three replicates for each rate and two controls (equivalent DMSO + blood, blood) were included for each test. Combining 1000 ml of DMSO and 0.02 g of technical grade eprinomectin produced the initial DMSO stock solution of 18.181 ppm eprinomectin. Then 0.5 ml of this solution was diluted into 9.5 ml of blood to produce 10 ml. The remaining rates were prepared via serial dilutions. Twenty

mosquitoes per feeding cage were aspirated from a colony of mosquitoes of equal age (4-7 day post-eclosion) and fed on Rutledge feeders for each rate. Mosquitoes were moved following 45 min feeding time into observation containers and observed at 1, 24, 48, and 72 h post-feeding. Mortality counts were taken for each time point.

Field Study

Sterile plastic cups (8 cm dia, 12 cm tall) were cut in half and tulle was attached over the top of the resulting cylindrical containers. LongRange eprinomectin (5%) formulation was injected into cattle in the field in order to bioassay treated cattle. Nine untreated seven-year-old black Angus cows of similar weight were divided into three treatment groups by a random number generator: three single LongRange doses, three double LongRange doses, and a control group of three cattle left untreated (Table 1). Four days prior to treatment, baseline blood samples were taken. A veterinarian delivered doses for the average weight of the herd, 544 kg, which was equivalent to one injection of 12 cc of the drug for animals given a single dose as a prescapular subcutaneous injection on the left side of the animal. Double-dose animals received 12 cc at both the left and right injection triangles (24 cc). At two days post-treatment, 20 mosquitoes were aspirated into each of 18 feeding containers and put into heated insulated boxes to be taken to the field and fed on cattle.

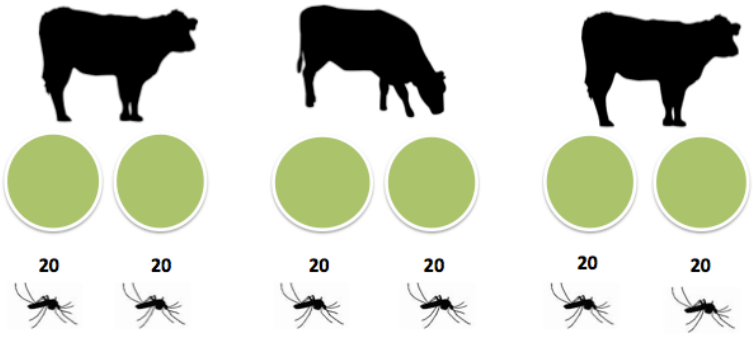

Fig. 3. Distribution of mosquitoes for each treatment group of three cattle.

Field trials took place December-February 2016-2017. Cattle were caught individually in the headgate and clippers were used to shave two patches on the back of the cow to allow mosquitoes direct access to the hide. Two containers of mosquitoes were labeled by cow tag number and attached to its back by prepared strips of duct tape (0.3 m) with small amounts of glue (Kamar Heatmount Detectors Adhesive, Steamboat Springs, CO) on the adhesive side of the tape. Two adhesive tape strips were used per container, placed in an x-shape, and pressed against the shaved hide of the animal (Fig. 3, Fig. 4). After each set of cages was attached, blood was collected from the jugular vein and animals were released to stand in a paddock for 45 min to allow mosquito feeding to repletion.

Fig. 4. Placement of mosquito feeding cages beneath adhesive tape in x-pattern on shaved patch of cow's back.

Cattle were moved once again through the chute and feeding cages were removed and placed into insulated boxes, which were returned to the lab and mosquitoes were transferred to observation containers. Number of blood-fed mosquitoes was counted and mortality was documented at 1 h, followed by counts each day until all mosquitoes were dead. This protocol was

repeated at eight subsequent time points for a total of nine feeding days post-treatment (d 2, 7, 10, 14, 28, 35, 49, 63, 77 post-treatment).

Table 1
Cattle-mosquito distribution in field study

Treatment*	Cow ID	Weight (kg)	Gender	Breed	Age (Yrs)	No. Mosquitoes/Cow
Control	45	666	F	Angus	6	40
Control	48	628	F	Angus	6	40
Control	65	570	F	Angus	6	40
Single	49	583	F	Angus	6	40
Single	51	566	F	Angus	6	40
Single	71	545	F	Angus	6	40
Double	13	607	F	Angus	6	40
Double	19	576	F	Angus	6	40
Double	52	561	F	Angus	6	40

*Label dose of LongRange

Oviposition and Hatch Rates

Eggs laid by mosquitoes fed on-host were collected by aspirating eight mosquitoes per cow per feeding date at 48 h post-feeding into vials (4x7 cm, one mosquito/vial) containing a circle of wet filter paper as oviposition substrate. Following oviposition, 3 ml water was added to facilitate hatching. Egg numbers were counted on the first and second day of oviposition, and hatch rates were recorded once hatching was complete.

Blood Processing

At the time of each mosquito feeding in the field, 12 ml of bovine blood was collected from each animal and was taken to the lab to be processed. Each blood sample was placed into tubes (BIPEE Graduated Plastic Centrifuge Tubes, 15 ml, Conical-Bottom) and centrifuged at 2500 x g for 12 min at room temperature (24-27ºC). Plasma was separated into 2 ml microcentrifuge tubes (two per sample, 2 ml plasma total) using a micropipette and stored at -80ºC.

Protein Precipitation

To determine eprinomectin concentrations in the blood at each feeding post-treatment, supernatant from each blood collection was analyzed by HPLC. These samples were processed by protein precipitation and extraction as described previously by Baoliang et al. (2008). Plasma samples were thawed in a warm water bath and were combined with 1 ml acetonitrile (Sigma-Aldrich, St. Louis, MO) in a micro-centrifuge tube (2 ml polypropylene snap cap tubes, Globe Scientific, Paramus, NJ) for the 90 samples in order to precipitate protein. This 1:1 solution in micro-centrifuge tubes was mixed on a vortex for 4 sec and placed on a rotating turntable to mix for 20 min. Tubes were subsequently centrifuged at 7500 x g for 5 min to pull protein and impurities to the bottom of the tube. The supernatant containing the eprinomectin was decanted and stored in micro-centrifuge tubes. The protein pellet was discarded.

Solid Phase Extraction

Supernatant samples were stored at -80ºC until extractions were performed. C_{18}-SD disc cartridges (Chrom Tech Inc., Apple Valley, MN) were used for extractions and were primed with 2 ml of acetone (Sigma-Aldrich, St. Louis, MO) eluted through the cartridges with 10 ml syringes into waste. Then, 2 ml of distilled water was eluted slowly to leave a thin layer of water above the cartridge disc (both acetone and water were measured with a 3 ml plastic pipette, VetMed USA, Naperville, IL). A 1.5 ml sample of supernatant from the plasma samples was added to the cartridge with a micropipette and was eluted slowly through the column into waste. To clean the sample, 2 ml of water was eluted through the column. To remove the sample containing eprinomectin, 1 ml of acetone was then permitted to elute by gravity into a 14 ml centrifuge tube. This was repeated for all 90 samples, then centrifuge tubes were placed uncapped in a nitrogen drier at 40°C for 60 min.

Tubes were removed, capped and stored at -80°C until derivatizations could be completed in preparation for HPLC analysis.

HPLC

When calibrating HPLC equipment, an initial standard was prepared by combining 20 ml of acetonitrile (Sigma-Aldrich, St. Louis, MO) and 20 ml of 1-methylimidazole (Sigma-Aldrich, St. Louis, MO) in a 50 ml tube. This solution (ACN/Imidazole) was used for all following derivatizations. For the initial calibration sample, 100 µl of ACN/Imidazole and 150 µl of trifluoroacetic anhydride (Sigma-Aldrich, St. Louis, MO) were combined in a clean 150 ml centrifuge tube using a micropipette and allowed to react for 30 sec, then placed on a vortex for 5 sec to ensure reaction completion. A three-drop sample was transferred to an HPLC tube and placed in automated HPLC machine (Agilent Technologies, Alpharetta, GA). Water and a 1:1 ratio of acetonitrile and tetrahydrofuran were loaded as polar and non-polar reagents, respectively. This sample was run to ensure no impurities were present.

In order to create an eprinomectin reference curve, blood was spiked with three known rates of eprinomectin (0.125 µl/ml blood, 0.3 µl/ml blood, and 0.7 µl/ml blood) and analyzed alongside untreated control. Derivatizations were performed based on modifications to those used by Montigny et al. (1990). The HPLC Mobile phase was THF, acetonitrile, and water at a 45:45:30 v ratio. Flow rate was maintained at 2 ml/min and column specifications were as follows: Waters Spherisorb 5 micron, 4.6 x 250 mm, C6 with Xterra 5 micron, RP18 3.9 x 20 mm guard column, 30°C. Detection was made using FluorEssence (ex=365, em=475). The 90 dried plasma samples were derivatized using 100 µl ACN/Imidazole and 150 µl of trifluoroacetic anhydride and reacted for 30 sec, followed by mixture on the vortex for 5 sec. Samples were placed into a 2 ml microtube and centrifuged at 7500 x g for 5 min to ensure no impurities remained in the sample. Three drops

of each of these samples were then loaded into four individual HPLC tubes and loaded into the HPLC tube station. The HPLC was calibrated and samples analyzed according to Montigny et al. (1990). A linear curve was determined for the known presence of LongRange per ml of bovine blood. Eprinomectin appeared on chromatographs at approximately 2.9 min for this calibration. The 90 test samples were then loaded into HPLC machine. Chromatographs were integrated by hand and plotted on our reference curve to determine the amount of eprinomectin in each sample.

Statistical Analyses

The LC_{50} and LC_{95} for *An. quadrimaculatus* were determined for LongRange and technical grade eprinomectin using PoloPlus software and were visually represented by plotting log mortality against rate of eprinomectin. All other tests were completed in R statistical software package (R 3.2.1). Shapiro-Wilkes tests were run to determine normality for all data, and Kaplan-Meier survival curves and log-rank tests were completed to compare mortality of each treatment group over time. Chi squared tests were used to determine significance of percent of mosquitoes that oviposited and for the number of eggs that hatched for each treatment group. Lastly, a Poisson regression was utilized to compare numbers of eggs laid by mosquitoes, followed by a Tukey analysis to refine significance.

2.4. Results

Mosquito mortality observed for populations fed on treated and untreated bovine hosts are depicted in Table 2. For seven of nine trials there were no differences in survival of blood-fed mosquitoes ($p \geq 0.05$), with mosquitoes fed on animals treated with single and double doses showing similar survival as those fed on untreated control animals and to each other (Fig. 6). A Kaplan-Meier summary of survival for combined nine mosquito feeding days in the field and

resulting mortality is shown in Figure 5, for which treatment groups did not differ from the control or one another (p = 0.25).

Table 2

Kaplan-Meier log-rank tests for individual trials of host-fed mosquito survival post-feeding

D Post-Treatment	DF	p-Value[a]
-4	2	0.614
2	2	0.947
7	2	0.445
10	2	≤0.001*
14	2	0.308
28	2	≤0.001*
35	2	0.063
49	2	0.066
63	2	0.304
77	2	0.472

[a]Probability from Kaplan-Meier log-rank test
*Significant value

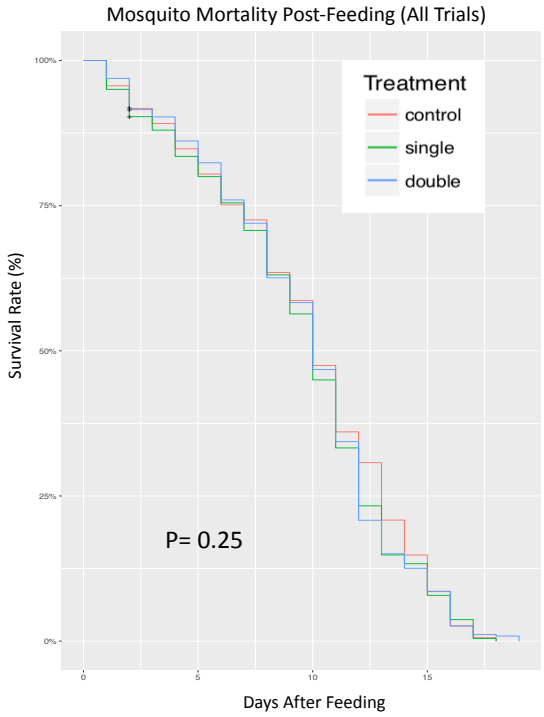

Fig. 5. Kaplan-Meier curve for mosquito survival for all trials comparing the three treatment groups with no differences in mosquito mortality (p = 0.25).

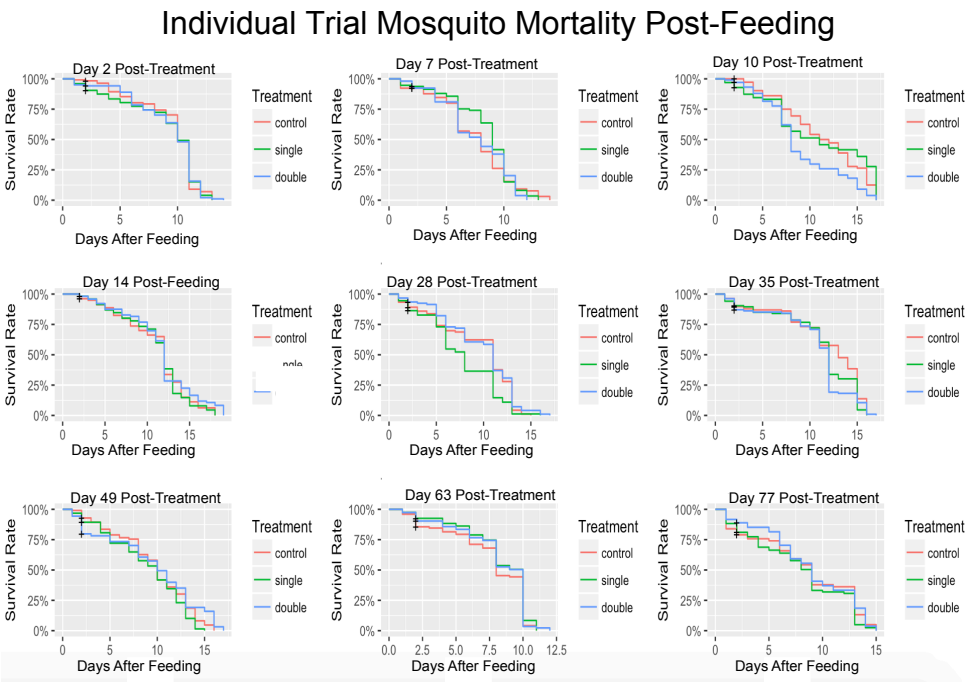

Fig. 6. Kaplan-Meier analyses for all trials post-treatment for cattle with significant differences shown following feeding on-hosts on days 10 and 28.

Mosquitoes fed on days 10 and 28 post-treatment experienced significantly different mortality between treatment groups (Table 2, Fig. 7). Mortality of mosquitoes fed on double-dosed cattle on day 10 was higher than those fed on single-dosed cattle ($p \leq 0.001$) and untreated cattle ($p = 0.002$), though control and single dose-fed mosquitoes did not differ ($p = 0.382$. Mosquitoes fed on single dosed cattle at day 28 exhibited higher mortality than control ($p \leq 0.002$) and double dosed cattle-fed mosquitoes ($p \leq 0.001$), but control and double dosed cattle-fed mosquitoes did not differ for day 28 fed mosquitoes (Table 3).

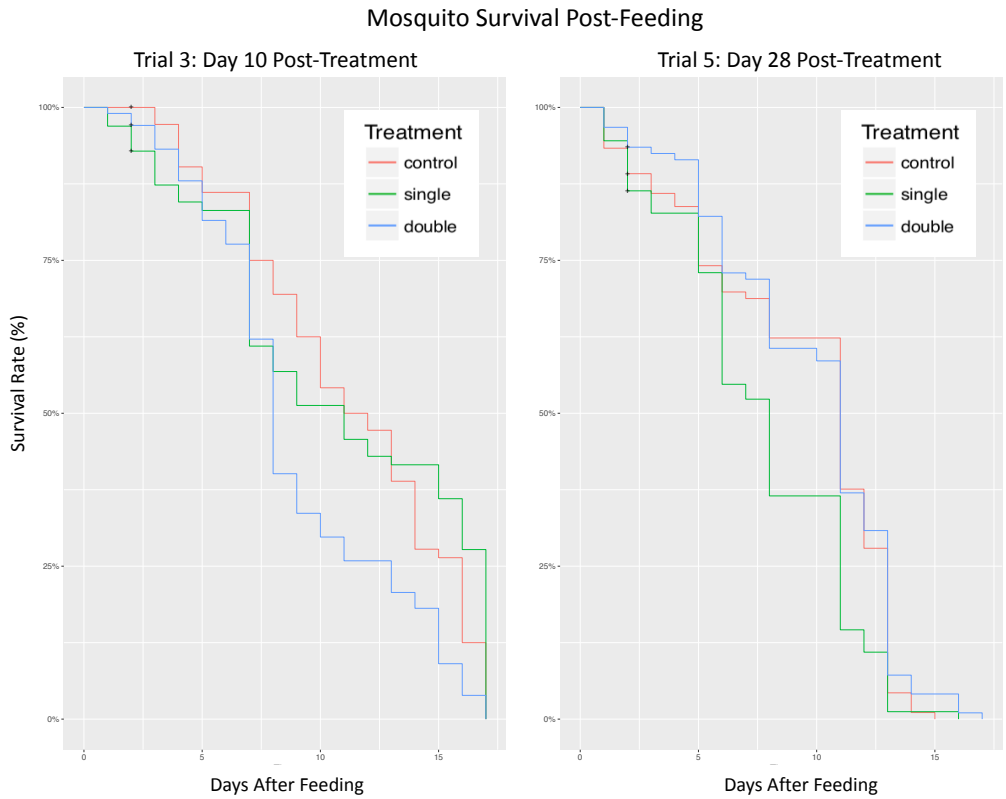

Fig. 7. Kaplan-Meier survival of mosquitoes post-feeding on day ten shows higher mortality over time for those fed on double-dosed cattle than when fed control ($p \leq 0.002$) or single dose ($p \leq 0.001$) blood. Day 28 mosquitoes fed on single-dosed cattle had higher mortality over time than either control ($p \leq 0.002$) or double-dosed cattle ($p \leq 0.001$).

Table 3
Pair-wise log-rank comparisons for feeding days 10 and 28 post-treatment

Day 10	p-value	Day 28	p-value
Control vs. Single	0.382	Control vs. Single	0.002
Single vs. Double	≤0.001	Single vs. Double	≤0.001
Control vs. Double	0.002	Control vs. Double	0.369

Numbers of eggs did not differ significantly between treatments following each feeding day on-host (Fig. 9). Percent mosquitoes that oviposited post-feeding ($p \geq 0.05$) and hatch rates ($p \geq 0.05$) did not differ between treatment groups (Fig. 8, Fig. 10).

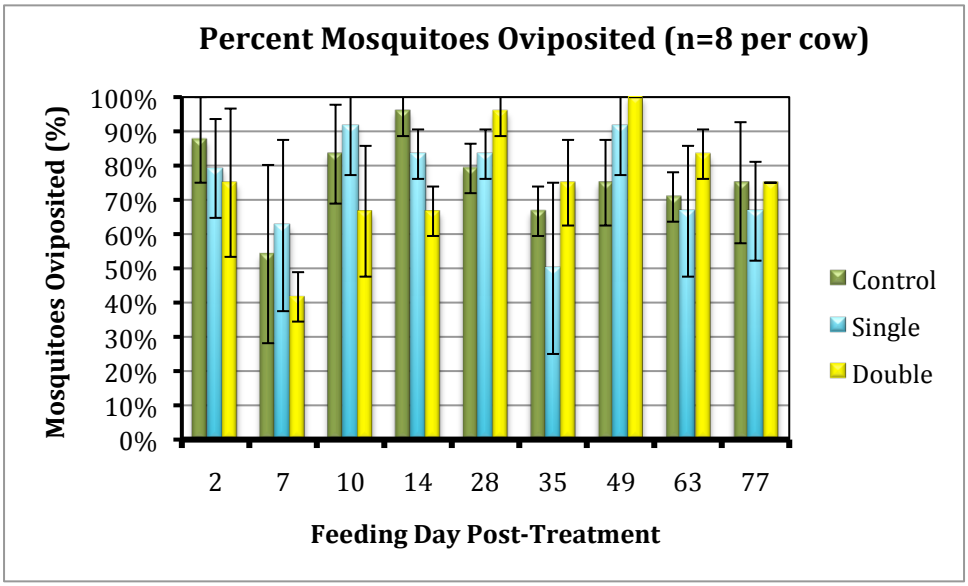

Fig. 8. Percentage of mosquitoes (n=8 mosquitoes per cow) oviposited post-feeding on treated hosts per treatment did not differ between those fed on control, single, and double dosed bovine hosts ($p \geq 0.05$). D 77 consisted of fewer replicates due to cows being unavailable.

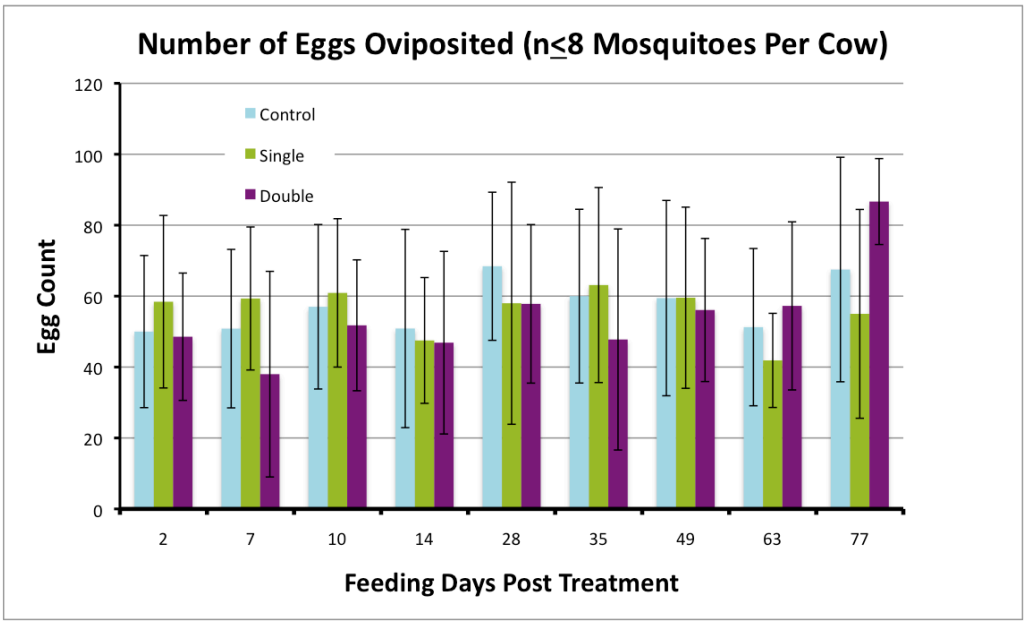

Fig. 9. Number of eggs oviposited by mosquitoes (n=8 mosquitoes per cow) blood-fed on bovine hosts did not differ between treatment groups for any of the feeding days following treatment ($p \geq 0.05$). D 77 consisted of fewer replicates due to cows being unavailable.

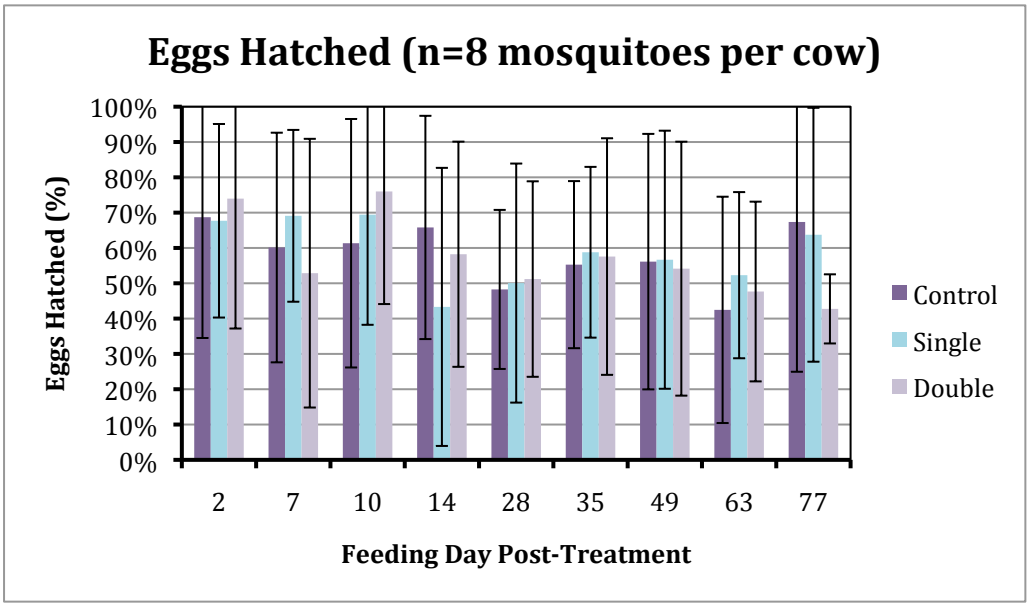

Fig. 10. Number of eggs hatched (n=8 mosquitoes per cow) did not differ between treatment groups for any of the feeding days following treatment ($p \geq 0.05$). D 77 consisted of fewer replicates due to cows being unavailable.

Efficacy tests for technical-grade eprinomectin produced an LC_{50} of 317 ng/ml of plasma (Table 4) and LC_{95} of 1,080 ng/ml plasma.

Table 4

LC_{50} for Eprinomectin-Fed *Anopheles* spp. mosquitoes

Anopheles Species	Oral LC_{50} ng/ml plasma (95% CI)	Citation
An. quadrimaculatus	317.1 (257.4, 381.6)	Present Study
An. arabiensis	8.5 (7.2, 10.0)	Fritz et al. 2012. J. Med. Entomol.
An. gambiae	23.6 (19.3, 26.7)	Butters et al. 2012. Acta Trop.

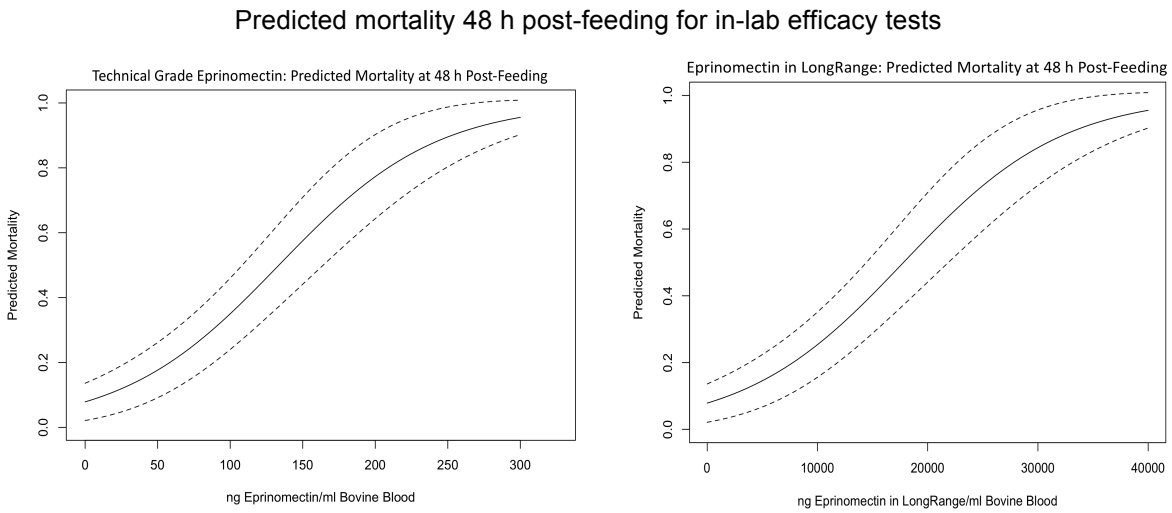

Fig. 11. Predicted mortality of *An. quadrimaculatus* mosquitoes fed varying concentrations of technical grade eprinomectin (a) and LongRange (b) in bovine blood in-lab via Rutledge feeders.

HPLC analysis of blood collected from animals on mosquito blood-feeding days detected eprinomectin levels in double-dosed host cow plasma on the first two feeding days post-treatment, day two and day seven. Day two exhibited eprinomectin concentrations in plasma that ranged from 7.5-25.42 ng/ml plasma, and day seven double-dosed cattle maintained 8.39-12.03 ng/ml plasma (2.5-8.33 ng eprinomectin/ml blood). For the remaining feeding dates day 10, 14, 28, 35, 49, 63, and 77, and for single dosed animals, eprinomectin rates were below detection ($\leq$ 7.5 ng eprinomectin/ml plasma) (Table 5).

Cattle responded well to LongRange treatments without changes in behavior or well-being. However, for the final feeding on day 77 post-treatment, three animals were removed from the study due to calving within 24 h previous to the time of mosquito feeding.

Table 5
Detectable levels of LongRange eprinomectin in plasma (HPLC)

Dose	DAT*	ng of Eprinomectin/ml plasma
Double	2	7.50
Double	2	25.42
Double	2	21.08
Double	7	12.03
Double	7	10.24
Double	7	8.39
All Other	All Other	< 7.50

*Days after treatment

2.5. Discussion

Despite eradication efforts since the 1940s, over 200 million cases of malaria are still recorded annually, with rural sub-Saharan Africa accounting for 90% of the remaining transmission (WHO 2017, Yaya et al. 2017). Risk of resurgence looms on the horizon as limited numbers of pesticides approved for malaria mosquitoes lose efficacy, with developing anopheline resistance and observed behavioral shifts from historically indoor to outdoor-feeding preferences (Bugoro et al. 2011, Khamis et al. 2018). Insecticidal zooprophylaxis has shown to be effective in reduction of malaria mosquito populations in rural communities where livestock are in close association with humans, but has exhibited limited duration of efficacy with current pesticide formulations.

For this project, testing the long-lasting LongRange 5% eprinomectin formulation and its effects on malaria mosquitoes, high survival rates in mosquitoes fed on treated hosts over 73 days post-treatment provide evidence that LongRange was unable to achieve desired mosquito management for *An. quadrimaculatus* vectors. Mortality in mosquitoes fed on cattle treated with a double-dose of LongRange did not occur until day ten post-treatment, while in-lab mosquitoes fed LongRange were killed successfully within three days following feeding. Should this significantly higher mortality have been caused by eprinomectin, this mortality was outside of

the window of time that mosquitoes would be able to reproduce and potentially transmit disease, which does not support LongRange use in *Anopheles* zooprophylaxis. Single dose-treated mosquitoes did exhibit higher mortality following feeding on day 28 post-treatment, but this rate of mortality was higher than that of double dose-fed mosquitoes and so mortality was not likely associated with eprinomectin treatment, which was shown in the lab to kill more mosquitoes with increasing concentration in the blood. These results were also observed outside the window of time in which malaria is capable of becoming transmissible by the mosquito, and would fail to limit the mosquito's ability to spread disease before death (Table 3, Fig. 8).

The LongRange formulation consists of excipient and organic compounds to improve solubility, as well as Theraphase^{TM} technology polymer beads (PLGA). These microscopic, eprinomectin-laden spheres bind together at the injection site and break down over time in order to achieve the long-term drug delivery LongRange provides. It has been reported that the highest concentration of eprinomectin in the cattle is at the initial injection, with a waning concentration over the first two months (Forbes 2012). Throughout the months following treatment, the polymer breaks down and provides up to 150 days of effective concentration of eprinomectin, killing internal parasites (Soll et al. 2013). However, due to a lack of mortality in this study during the first month when eprinomectin titers should be highest in the bovine blood, the decision was made to shorten the observation period for this study and the full 150 days of advertised efficacy period was not tested. Instead, the treated cattle were bioassayed until the approximate time that eprinomectin blood-levels were expected to once again increase, at approximately 70 days.

The series of tests performed in the lab confirm that *An. quadrimaculatus* has a higher tolerance for eprinomectin than is available in treated cattle, even at the highest titer in the blood

immediately following treatment. Mosquito LC_{50} determined in the lab (316 ppb eprinomectin) was much higher than the LC_{50} of 8.5 ppb for *An. arabiensis* determined by Fritz et al. (2012) (Table 5) and indicates that differing species have varying tolerances to eprinomectin, just as they do to ivermectin (Dreyer et al. 2018). *Anopheles quadrimaculatus* LC_{50} (316 ng/ml of plasma) was also greater than the amount of eprinomectin present in the cattle (7.5-25 ng eprinomectin/ml plasma) even at double the label rate, which should represent the highest volume of drug in the blood over the duration of the study (Table 6). This amount of eprinomectin in the blood of the cattle at these dates following injection is similar to the initial plasma profile determined by Soll et al. (2013) in the first two weeks following treatment (4-25 ng eprinomectin/ml plasma) and reported by Merial. To achieve control of this species of mosquito using this formulation, dose per cow would have to be increased $\geq$ 20-fold, and higher than double label rate dose to cattle can cause weight loss, limited weight gain, and abscess or irritation of the injection site requiring wound care (LongRange Technical Manual). Eprinomectin treatment at these rates had no effect on *An. quadrimaculatus* capability to oviposit and did not affect the number of eggs laid (Fig. 9). Dreyer et al. (2018) reported that several other anopheline mosquito species exposed to 32-1300 ng ivermectin/ml plasma were impacted significantly in comparison to controls in number of eggs laid. Fecundity was not impacted by even the highest dose throughout the present study, despite the fact that Dreyer et al. (2018) also reported significantly reduced hatch rates in mosquitoes fed as little as 8 ng ivermectin/ml plasma. With such low concentrations of eprinomectin in the bovine blood for the present study (7.5-25 ng/ml plasma), these results are not surprising.

Much higher levels of eprinomectin were required to kill mosquitoes when fed in LongRange formulation directly in blood in the lab, as would be expected, since much of the

eprinomectin remained bound in poly–lactide-co-glycolic acid (PLGA) spheres. These spheres contain pores saturated with eprinomectin, which expel only a portion of the drug on initial contact with bovine tissue (Shang et al. 2015). The remaining eprinomectin in the LongRange formulation would have remained unavailable and bound to spheres during uptake by the mosquito during in-lab efficacy determination.

In conclusion, eprinomectin injected as LongRange formulation is not an effective addition to insecticidal zooprophylaxis when considering use for *Anopheles* spp. mosquitoes with tolerances higher than 7.5-25 ng eprinomectin/ml bovine plasma, or high tolerances similar to *An. quadrimaculatus*. Populations of adult mosquitoes with these higher tolerances would be largely unaffected and only the highest rate of the LongRange formulation has any potential to impact the survival of future generations. Mosquitoes with a lower tolerance for eprinomectin and other macrocyclic lactones have potential to experience higher mortality, and other malaria vectors such as *An. arabiensis* mosquitoes have been reported to have lower tolerance to macrocyclic lactones and should be investigated for management by this tactic (Fritz et al. 2012, Dreyer et al. 2018). PLGA-bound pesticides are a promising addition to insecticidal zooprophylaxis for long-term efficacy and eprinomectin, macrocyclic lactones, or other pesticide classes should be investigated further for this use. The use of macrocyclic lactones for control of mosquitoes would not only be advantageous by utilizing resources already available in rural high-risk agrarian communities, but also benefit bovine owners by increasing health of the animals. Next steps to determine the impacts of this formulation and other macrocyclic lactones on mosquitoes with lower tolerances should be pursued.

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2.7. Competing Interests

The authors declare that they have no competing interests.

2.8. Ethics Approval

The use of vertebrate animals was approved by the University of Georgia Institutional Animal Care and Use Committee (A2015 03-026-Y2-A0).

2.9. References

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