INTERACTION BETWEEN A SYSTEMIC ACARICIDE AND IMMUNOLOGICAL CONTROL OF *RHIPICEPHALUS* (*BOOPHILUS) MICROPLUS* (CANESTRINI) (ACARI: IXODIDAE)

A Thesis

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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May 2019

Major Subject: Entomology

ABSTRACT

Among the global agricultural sectors, livestock production is one of the most developed and dynamic. Breeding and domestication of livestock for food production is an enterprise that promotes economic stability at a global level. However, the southern cattle fever tick (SCFT) a.k.a the tropical cattle tick, *Rhipicephalus* (*Boophilus*) *microplus* (Canestrini), causes large economic losses in cattle production, particularly in tropical and subtropical parts of the world. In the United States losses were estimated to be ~$130.5 million in the late 1800's before the eradication program began. Ectoparasites develop easily and abundantly in tropics and sub-tropics and are responsible for large economic losses in the dairy and meat industry through weight loss, hide damage, and death from anemia.

The southern cattle tick *R. microplus* is a one-host tick species considered the most important ectoparasite of livestock in the world because of its association with high financial loss due to direct feeding (tick burden) and in the transmission of the hemoparasites *Babesia bovis, B. bigemina,* and *Anaplasma marginale,* the causative agents of babesiosis and anaplasmosis, respectively.

Rhipicephalus microplus has a high potential for population growth due to its relatively short life cycle and preference for cattle, reared in large numbers throughout the tropics and sub-tropics. Unfortunately, ticks in many parts of the world have evolved resistance to all pesticides available on the market, driving the development of new technologies to control this species.

Vaccination against ticks using the gut protein Bm86 has been shown to be effective against acaricide-resistant ticks. This technique has been successfully implemented in Puerto

Rico for the control of *R. microplus* on dairy and beef cattle. Observations from Puerto Rico indicate a potential interaction between anti-tick vaccination in conjunction with systemic acaricide use. Controlled animal studies were completed directly comparing efficacy of vaccination with and without systemic acaricide. Additionally, *in vitro* feeding of ticks with immunoglobulin-G (IgG) from vaccinated animals with several combinations of acaridides was used to screen antigen/acaricide combinations and to confirm results of field tests using animals. The results show that the vaccine had a synergistic interaction with the acaricide. Better and longer control was achieved with the combination than when either treatment was applied alone.

DEDICATION

I dedicate this dissertation to my mother Luz A. Rosario Laguna. Who was the most authentic, strong and tenacious women I have ever known. Who taught me to be the empowered woman that I am today. You taught me that the world was not built in one day and that no matter how difficult the road is, I will have the courage to finish it. To you mom, I will never fail you. Also to my father José Arocho who always gave me courage, solutions, and advice for passing the vicissitudes during the masters.

I learned early that when you emigrate you lose the crutches that have been the support until then, you have to start from scratch, because the past is erased in a stroke and nobody cares where you come from or what you have done before. We have to face the obstacles as they arise, do not waste energy fearing what may be in the future.

-Julia de Burgos-

ACKNOWLEDGEMENTS

First of all, I would like to thank you for your committee chair, Dr Teel for giving me the opportunity of being part of your last group of graduate students, special thanks for your patience to handle this explosive personality, thanks for mentoring, support and sweet advise to maintain my mental health during this adventure. Infinite thanks for developing my skills that I did not think I had, I am completely grateful that I am touched by a mentor as knowledgeable and with a great heart as you. Your kindness helped make this process more bearable and fun. Your knowledge is necessary for the world of acarology to be kept alive, so think seriously about writing a book because you are too young for a complete retirement. Thanks to all my committee members, Dr Robert J. Miller, Dr Craig Coats and Dr William E. Grant for the guidance during my adventure research.

Special thanks to Dr Miller (The big boss) who saved me from a vet school loan, and completely introduces me to the world of entomology. I will be infinite and eternally grateful for always guiding me and being there for me. Listen to each idea, and in one way or another, advise me. Thanks to the USDA CFTRL and Alberto Perez de Leon for the economic support to complete this dream, without you this would not be possible. Huge thanks to Dr Don Thomas, Mike Moses, Dave Krska, Brenda Leal, Cesar Agado, James Hellums, Homer Vazquez, Ruby Martinez, Summer De Luna, Joni Ortiz, and Ruben Ramirez for the time and effort invested with me in this project. You guys are one of the most important pieces of the project, you are the action behind the scene, that deserves recognition. The help during the data collection, blood taken, vaccination, cattle management, cleaning stalls, and support every time, are precious to

me. Each one of you leaves a ton of knowledge and story in my live so thank you, guys. Respect to who deserves it.

Guilherme Klafke and Jason Tidwell the two brightest minds, thank you guys for developing my skills inside the laboratory, that cost me a lot to develop. OBRIGADA, and THANKS, because you guys are one of the pillars in the construction process of this project. Thanks for devote me your time, for your sweetness, patience and calm to work with me, I will always be grateful for the knowledge they overflowed in me and I will keep them always in my heart. Dr Thomas, thank you for your advice and guidance, the world should be full of people like you. Thanks for always been there for me. I want be to like you when I grow up.

Special thanks to Deanna Bodine and all the Felix Guerrero Laboratory in Kerrville, for all the time and patience dedicated to my training with ELISA. Last but not least, a million thanks to the USDA APHIS VS team from Puerto Rico, because without them, it would not be here. Thanks to Dr Fred Soltero for polishing me in my field skills and trusting me. The most sincere thanks to "Josi" L. Rojas, "Pupi" Rojas, Arnaldo Ramos, Israel J. Gonzalez, Rebecca Jimenez, Adrian Ortiz, Rebekah Domenech, Eric Madera, Jorge Caballero, Jorge Cancel and all the rest of the team. Especially thanks to them because they were with me hand to hand, to collect a part of the data for this investigation, those who woke up at dawn, those who left their time and health in an arduous day of work. Thank you for tolerating me, understanding me, helping me on this journey, thanks to those who drive me while I slept, and to all those who became friends. From each and every one of you, I learned something. From you guys, I learned what it really is to work as a team and that work can also be fun. In the field, you learn much more than in a classroom and you were the best teachers. They have my greatest respect, and my eternal affection.

Thanks to my superhero Jose Arocho, who has always taught me that what matters is to believe in yourself and never quit. Him together at Fabio Tarazona, Alejandro Zamot, and Elisa Benitez have made the distance of being away from home not consume my self. Thanks to you I remain here standing and ready to continue for more, your support and all the hours that you have lost listening to me and comforting me gave me strength. Los amo.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a thesis committee consisting of Dr. Pete D. Teel (advisor), Dr. Robert J. Miller (co-advisor), Dr. Craig Coates from the Department of entomology and Dr. William E. Grant from the Department of Wildlife and Fisheries Sciences.

The data analyzed for Chapter 2 and 3 was provided by Kathleen Yeater the ARS USDA statistician.

All other work conducted for the thesis was completed by the student independently.

Funding Sources

The graduate study was supported by a USDA Cooperative Agreement number 59-3094-6-003 entitled *Investigating the Potential Interaction between Anti-tick Vaccination and Systemic Acaricide Treatments*.

NOMENCLATURE

AIT	Adult Immersion Test
ANOVA	One-Way Analysis Of Variance
ARS	Agricultural Research Service
APHIS VS	Animal & Health Inspection Service, Veterinary Service
BD	Becton Dickinson
BM86	Bovine Vaccine from Boophilus microplus in the year 1986
C-4	Carbon Fixation Pathway 4
CDC	Center for Disease Control and Prevention
CFSPH	Center For Food Security And Public Health
CFT	Cattle Fever Tick a.k.a Tropical Cattle Tick
CFTEP	Cattle Fever Tick Eradication Program
CFTRL	Cattle Fever Tick Research Laboratory
CY	Cydectin
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA viruses
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
ERS	Economic Research Service
FAO	Food and Agriculture Organization of the United Nations
GABA-A	γ-Aminobutiric Acid
Glu-Cl	Glutamate-Gated Chloride Channels

HLPE	High Level Panel Of Experts
HRP	Horseradish Peroxidase
IACUC	Institutional Animal Care and Use Committee
IgG	Immunoglobulin G
IF	Index of Fecundity
IFAT	Indirect Fluorescent Antibody Test
KBUSLIRL	Knipling-Bushland U.S. Livestock Insects Research Laboratory
LTT	Larval Tarsal Test
LIT	Larval Immersion Test
ML's	Microcyclic Lactones
MRM	Multiple Reaction Monitoring
N/A	Non applicable
NASS	National Agricultural Statistics Service
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PR	Puerto Rico
PTQZ	Permanent Tick Quarantine Zone
RH	Relative Humidity
RNA	Ribonucleic Acid
RNAi	RNA Interference
rRNA	Ribosomal ribonucleic acid
RPM	Revolutions Per Minutes
SCFT	Southern Cattle Fever Tick, *Rhipicephalus Boophilus microplus*

Spp.	Species
SST	Somatostatin
TBS	Tris-buffered saline
TG	Tick Guard
TMB	3,3 ', 5,5' -tetramethylbenzidine
TIPM	Tick Integrate Pest Management
USA	United States of America
US	United States
USDA	United State Department of Agriculture
USP	United States Pharmacopeia Unit
XG	Times Gravity

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1. INTRODUCTION

1.1 Livestock industry affected by *R. microplus*

Livestock production is one of the most developed, dynamic, and expanding sectors of the global agricultural economy (HLPE, 2016). Breeding and domestication of livestock for the production of food promotes economic stability at a global level (HLPE, 2016). Livestock production uses the most land and land resourses in the world due to the use of grass, cropland for feed production, and pasture. Presently almost 80% of all global agricultural land is used for livestock production (FAO, 2018). Livestock production contributes 40% of the global agricultural output, the food security of ~1.3 billion people is supported by this sector (FAO, 2018). Within the livestock industries, the milk industry is the most developed, followed by the meat industry. The United States is the largest importer and consumer of dairy products (FAO, 2016, Haley M. et al,. 2016). Additionally, the U.S. imported 1.81 million head of beef cattle in 2017 with a 6% increase forcast for 2018 (USDA ERS, 2018).

The livestock industry around the world and specifically in tropical and subtropical countries is threatened by various arthropod pests that reduce production through direct feeding, disease transmission, and irritation. In tropical climates such as Puerto Rico, where average temperatures are around 79°F throughout the year and the humidity is about 75%, economic losses due to various factors are common. Ectoparasites develop easily and abundantly in this climate and are responsible for large economic losses in the dairy and meat industry directly due to weight loss, reduction in milk production, hide damage, and death from anemia. Ectoparasites also cause

economic losses indirectly through the transmission of pathogens by tick hosts (Perez de Leon et al. 2012).

1.2 Life cycle and Development of *R. microplus*

The southern cattle tick *Rhipicehalus microplus* (Order Parasitiformes, Suborder Ixodida, Family Ixodidae, Subfamily Rhipicephalinae) is a hard tick that prefers to feed on bovines. However, it has been found to feed and reproduce on horses, donkies, deer, sheep, goats, and other wildlife to a much lower degree. In the 2000's, *Boophilus* was suggested to be a subgenus of *Rhipicehalus* based on the internal transcribe spacer 2, 18S rRNA, and the nucleotide sequence from 12S rRNA, also based in some morphological characters (Uilenberg et al. 2004). While this has generally been accepted by the scientific community there are morphological, molecular, and behavioral data contradicting this conclusion (Jonsson et al. unpublished data). *Rhipicephalus microplus* is an endemic species of areas between 35^{o}N and 35^{o}S latitude worldwide including Asia and India (Harwood et al, 1979). It has spread to all subtropical and tropical zones in the world including Australia, Madagascar, Africa, Mexico and other countries in Central and South America (Jonsson et al. 2001; Madder et al. 2011; Perez de Leon et al. 2012). It is sensitive to climatic extremes and, will not establish itself in places where rainfall is less than 500 mm for 60 days per year, or greater than 150 days of frost per year (Gothe, 1967: Yeomann et al. 1967). Recently, there have been reports of *R. microplus* spreading into areas of sub-saharian Africa historically infested with *Boophilus decoloratus*. As it spreads into these areas it is displacing *B. decoloratus* (Tønnesen 2004).

Rhipicephalus microplus is a one-host tick. All post-embryonic stages (larvae, nymph and adult) remain on a single animal. The engorged female is the only life stage that leaves the host, to lay eggs (Radunz, 1997; Murrell et al. 2003). This tick spends approximately 72-93% of is life

cycle in a non-parasitic stage (Murrell et al. 2003). Replete females drop on the ground (pre-oviposition period) and oviposit eggs (oviposition period-incubation period) under rocks, at the base of grass, or in grooves in the soil. After completion of the incubation period, the larvae eclose from the eggs. Larval development continues for approximately one week before questing for an animal host. Depending on the climate and time of year, larvae can live 9 months without feeding (cold months) (Hooker et al. 1912, Hitchcock 1955). In months where the conditions are sub-opimed this can be shorter.

The larvae have a Haller's organ on their front legs that can detect many chemicals emitted from potential host animals, including carbon dioxide (Sonenshine & Roe 2013). The Haller's organ can also detect heat. Changes in light also help larvae identify the presence of a host. To increase their chances to find a host, larvae climb to the highest part of grasses and exhibit questing behavior. Questing is identifyable when larvae are observed with front legs extended outward orienting toward the source of host. Larvae in a cluster link legs in order to travel together if a few individuals in a cluster come in contact with a host (Leal et al., 2017). In this way larvae work together to infest potential hosts, facilitating tick survival, as many individuals will now be able to attach to the animal, increasing the chances of offspring for the next generation. With attachment to the host, the non-parasitic phase ends (Sonenshine & Roe 2013). Larval attachment occurs mostly along the soft parts of the host skin such as the internal part of the thighs, back legs, and flanks (CFSPH 2007). The parasitic phase represents only 11-28% of the *R. microplus* life cycle, lasting ~21 days (USDA #485). After attachment, the six-legged larvae feed ~ 7 days. The larvae molt on the host and re-attach to other parts of the host as eight legged nymphs. The nymphal stage lasts ~7 days and molt to male and female adults. Adult females feed on small amounts of blood, ovaries develop, and copulation occurs. After mating, females complete a rapid engorgement of

~400ul of blood. Replete females begin to drop off the animal ~19-23 after the first larval attachment (Davey et al. 1982). The engorged female oviposits all eggs during the first 14 days after dropping from the host when under laboratory conditions (82°F and 70% humidity), despite the fact that during the first day a female can lay 80% of its egg mass. A normal sized egg mass can weight >150mg and have an hatchability of >80% (Davey et al. 1982).

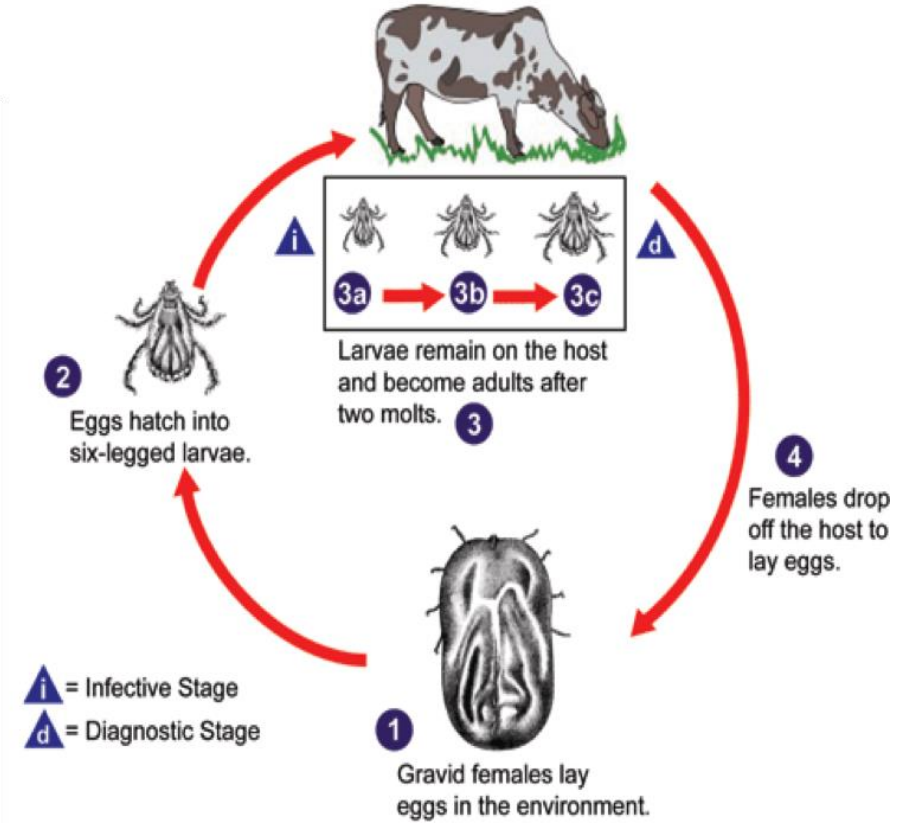

Figure 1. *Rhipicephalus microplus* life cycle "Reprinted from (CDC 2017)"

The length and survival rate of the non-parasitic phase varies depending on the temperature, type, and amount of vegetation, relative humidity, and seasonality (Utech et al. 1983; Wilson & Sutherst 1990). Therefore, developmental times can vary greatly between ecological

zones. Even local changes in topography or the presence of lakes, streams, or rivers can create micro-habitats that can affect tick development in localized areas (Legg, 1930; Londt et al. 1975, Davey et al. 1994). *Rhipicephalus microplus* has a high potential for population growth due to its relatively short life cycle and absence of specific host resistance (Sutherst 1987). In some areas of the world, five generations per year are possible. However, 3-4 generations per year are likely to be more common (USDA #485).

In sub-tropical climates, *R. microplus* can live the summer whithout feeding for 3 to 4 months. In tropical areas, *R. microplus* can survive without feeding for 8 to 9 months. (Hitchcock 1955). Therefore, tick management programs should take this into consideration when attempting to reduce tick numbers in order to decrease the risk of monetary losses due to tick feeding and tick-borne pathogens.

1.3 Tick-borne diseases transmited by *R. microplus*

This tick is a vector of potentially fatal tick-borne pathogens in tropical and subtropical zones. *Babesia bovis*, *B. bigemina*, and *Anaplasma marginale* are causal agents of bovine babesiosis and anaplasmosis, respectively (Jonsson et al. 2008). These diseases are collectively known as "tick fever" in some parts of the world (CFSPH 2008). Bovine babesiosis is a world-wide cattle hemoparasitic disease common in tropical and subtropical zones; caused by *Babesia* (Phylum Apicomplexa, Order Piroplasmida, Family Babesiidae) protozoan organism (Sonenshine & Roe 2013). *Babesiosis* was discovered in 1888 in Romania, but it was not until 1893 when Theobald Smith and Frederick Kilborne associated the presence of *Rhipicephalus* (*Boophilus*) *annulatus* (Say) with the infected cattle (Smith et al. 1893; Mosqueda et al. 2012; Schultz et al. 2008). This was the first observation of an arthropod vector transmitting a pathogen from infected

to non-infected hosts, a discovery that created the field of vector biology (Smith et al. 1893; Mosqueda et al. 2012; Schultz et al. 2008).

The most economically important pathogens of cattle are *B. bovis* and *B. bigemina* (De Waal, D.T et al. 2006), because they are the most prevalent in tropical and sub-tropical zones. These two species are important in Australia, South and Central America, Asia, Africa, and parts of Southern Europe (CFSPH 2008). *Babesia bigemina* is the most common form in Africa (CFSPH 2008). *Babesia bovis* and *B. bigemina* have been reported in Puerto Rico in the towns of Naguabo and Lajas confirmed by PCR tests (unpublished data R. Miller, 2016) and are endemic in the Mexican states bordering Texas.

The main host of *B. bigemina* and *B. bovis* is cattle. The first sign of Babesiosis is fever presenting 2 to 3 weeks after infection through tick feeding (Mosqueda et al. 2012). Fever is followed by anorexia, anaemia, and dark urine all caused by hemolysis of red blood cells. Animals appear weak, loose appetite, become depressed, and lethargic. Mortality in adult animals can reach 90% (CFSPH 2008). However, most animals can survive infection for week past the onset of fever. Therefore, it is recommended producers observe and monitor their animals closely for fever and other symptoms of *Babesia*. Most calves younger than 9 months will remain asymptomatic after infection with *Babesia* and retain this immunity through adulthood (CFSPH 2008).

Clinical signs of *Babesia* can vary depending on the diet and health of the herd, also on the age and species of the animals. Animals infected by *B. bovis* usually present severe symptoms like hemoglobinuria, hemoglobinemia, and neurologic disorders (Mosqueda et al. 2012). Hemoglobinuria is the presence of red cells in the urine whilst, hemoglobinemia is the excess of red cells in the blood plasma. Both are common symptoms of this disease.

Neurological symptoms are uncommon in infections with *B. bigemina*. Very rarely is there intrauterine infection of *Babesia*. However, then this does occur, a weak, anaemic, icteric, and dehydrated calf is produced.

In tick endemic areas, the diagnosis of Babesiosis is the most important tool to prevent the spread of disease to other animals in the herd (Mosqueda et al. 2012). Diagnosis can be made by the observation of symptoms in sick animals and confirmed by morphology in blood smears, polymerase chain reaction (PCR), transmission experiment, or serology (Mosqueda et al. 2012). In carriers, the PCR assay can be differentiated by species of *Babesia*. With the PCR assay, it is necessary to pay attention to the procedure as it is carried out as false positives can appear in the results. Polymerase chain reaction assay can detect exposure to the pathogen (Mosqueda et al., 2012). Serology, where the antibodies of *Babesia* are detected, is used for surveillance and export certification. These antibodies are detected by an indirect fluorescent antibody test (IFAT), or an ELISA (Mosqueda et al., 2012). For routine diagnosis, animal transmission techniques are used. In tissues, smears can identify species of *Babesia* with a thin film under oil immersion (Mosqueda et al., 2012). The film is stained with the animal blood and the x100 objective lens is used to help identify species (Mosqueda et al., 2012). Microscopic analysis is used at the acute stage of the disease (Mosqueda et al., 2012). The number of parasites inside the erythrocytes increases and this technique can detect them (Mosqueda et al., 2012). To achive improved results the cattle producer needs to be attentive to clinical symptoms that can be observed in the herd such as fever, anaemia, jaundice, and hemoglobinuria.

The animals are infected by the transmission of *Babesia* by tick species, mostly *R. microplus.* The tick is infested by ingesting parasites in the blood of cattle. Transmission is transovarial, with the meaning of transmiting disease from parents to offspring. *Babesia* zygotes

invade tick organs and multiply. Eggs are then infected as they are produced by the female. *Babesia bovis* can be transmitted by larvae 2-3 days after attachment while, *B. bigemina* can be transmitted only by nymphs and adults. *B. bigemina* matures in approximately 9 days after larval tick attachment (Mosqueda et al. 2012).

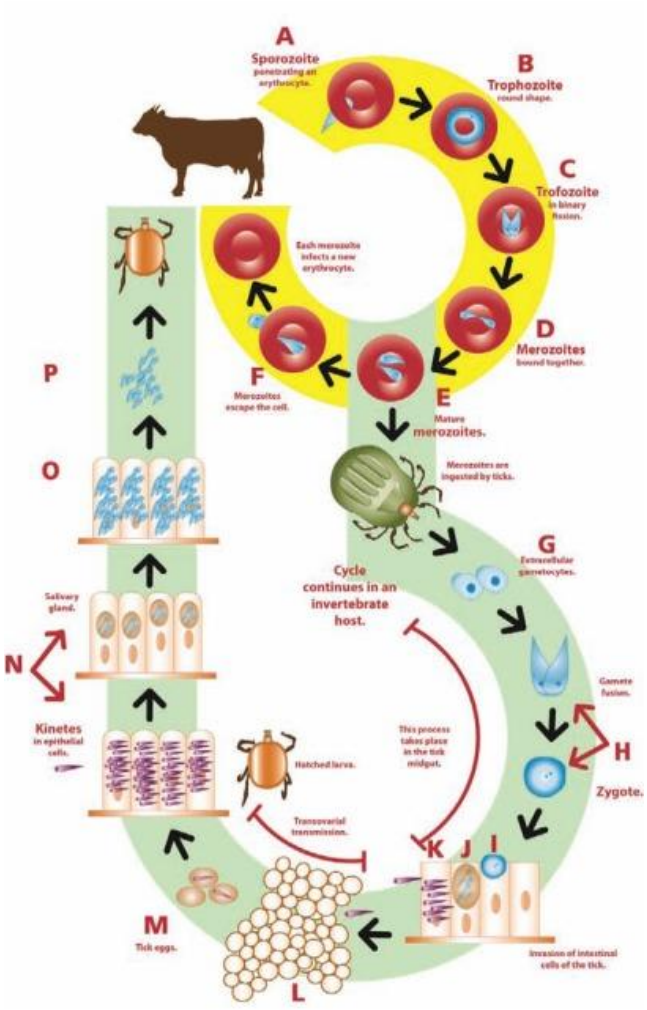

Figure 2. *Babesia bovis* life cycle "Reprinted from (Mosqueda et al. 2012)"

At the beginning the eradication program implement cultural methods because acaricides were not in the market. Pasture vacation was one of the most used technique, all the infested host were removed from the pastures or premises for a continued period of time. This ensure that the larvae would not survive when the host is absent. The eradication of the vector began by treating

all cattle with acaricides, this was the objective when the first eradication program was implemented. Cattle were treated in the erradication program every 2 to 3 weeks, with strong acaricides like arsenic compounds. Coumaphos is a broad spectrum organophosphate pesticide for use against ectoparasites including ticks, lice, and other species of insects. Attacking the vector reduces the incidence of disease, however the repeated use of pesticides to combat ticks can lead to the evolution of resistance.

The evolution of resistance in the cattle fever tick is a concern around the world. Therefore, researchers are using other methods such as anti-tick vaccines, to aid in their control. In Australia, Cuba, Mexico, Venezuela, Puerto Rico, and the Texas border region vaccines are used to combat *R. microplus*. Since 1943, bovine babesiosis was eradicated in USA, the disease is under control but *R. microplus* is developing resistance to acaricides in other parts of the world. However, now researchers are looking for other methods to kill ticks (APHIS, 2013). In tick endemic parts of the world, if an animal is diagnosed in time with babesia, treatment must be applied quickly. Treatments such as imidocarb or diminazene aceturate can protect animals and boost immunity (Mosqueda et al., 2012). Imidocarb, the most used babesiacide treatment, is a carbanilide derivative with antiprotozoal activity (Kuttler, K.L 1980). This drug can clear the parasites from the host with a subcutaneous or intramuscular administration (Suarez et al. 2011; Kuttler, K.L 1980). Diminazene aceturate is the most used anti-trypanosomal agent, and is found in the market in combination with antipyrine as a stabilizer, because this chemical has a low stability in water (Jensch, H. 1958; Fairclough, R. 1962). It is derived from surfen and is an aromatic diamidine (Mosqueda et al., 2012). Neither of these drugs are permitted for use in the USA. Currently, research is being performed to develop alternative diagnosis and treatment methods, aided by the complete sequencing of the *B. bovis* and *B. bigemina* genomes.

1.4 *Rhipicephalus microplus* eradication program in the USA

The Cooper Curtice hypothesis is simple. “Eliminate the vector and the diseases will be eliminated”. This hypothesis was strengthened by the observations of Smith and Kilborne in 1893 (Mosqueda et al. 2012). Babesiosis was eradicated between 1906 and 1943 from the United States, by eliminating *R. microplus* and *R. annulatus*, its vectors (FAO 1998). Before its eradication, babesiosis cost the U.S. an estimated $130.5 million in direct and indirect annual losses (FAO 1998). *Rhipicephalus microplus* and *R. annulatus* are sometimes present within areas near the border with Mexico, where these ticks are still endemic. For this reason, the USDA Animal and Plant Health Inspection Service Veterinary Services (APHIS-VS) maintains a permanent quarantine zone along the Texas-Mexico border. The USDA APHIS VS monitors cattle and wildlife that enter the United States by crossing the Rio Grande. The Cattle Fever Tick Eradication Program (CFTEP) treats every cow or horse that crosses the border with coumaphos dips (Graham & Hourrigan 1977). These officials are USDA-APHIS cowboys trying to maintain the front line on the Rio Grande free of ticks (Mullens et al. 2018). They are constantly looking for wildlife and infested cattle crossing in the shallow spots of the river (Mullens et al. 2018). Presently, there are several substantial outbreaks along the Rio Grande and Gulf Coast of Texas, (Mullens et al. 2018). These outbreaks will require substantial time and investment eradicate. It is imperative to develop better techniques to combat these vectors, avoid outbreaks, and control tick populations in the immediate and long term. Some factors that make this outbreak particularly difficult to control are the presence of wildlife (nilgai antelope and white-tailed deer) that serve as alternate hosts, large wildlife refuges within the quarantine area, and the lack of personel to inspect the large area that is currently infested. Other risks to the integrity of the overall eradication zone in Texas, are macro

weather patterns based on the solar cycle that can bring years of environmental conditions are appropriate to tick survival and physical factors such as the dam water level at several of the reservors along the quarantine zone allowing easy access of cattle and wildlife into Texas from Mexico (Perez de Leon et al. 2012; Mullens et al. 2018). There is a major concern that white tailed deer and nilgai antelope are effective alternate hosts for the cattle fever tick (Cardenas-Canales et al. 2011; Pound et al., 2010; Anderson et al. 2010) .

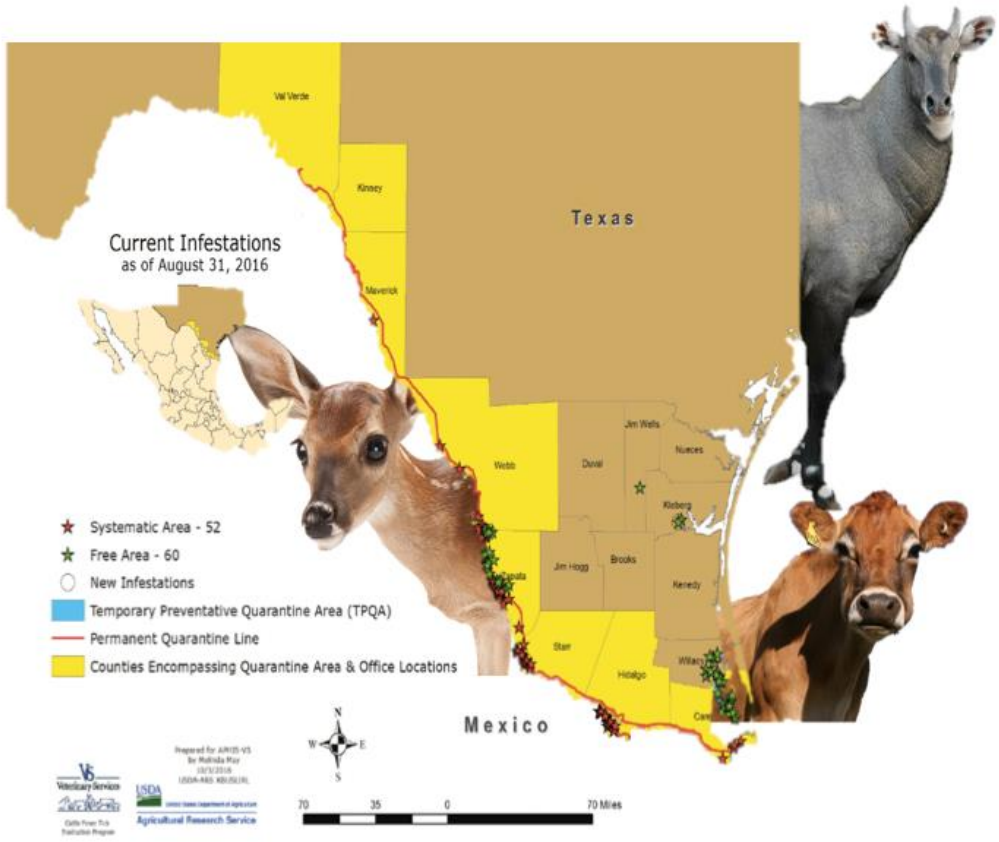

Figure 3. Actual eradication zone in USA (Figure Courtesy K. Lohmeyer 2018)

The Permanent Tick Quarantine Zone is approximately 580-miles-long, extended from Del Rio to Brownsville, Texas. The width of this zone is ~125 yards to ~8 miles. This territory includes

a tick free area, and a permanent tick quarantine zone through eight South Texas counties: Cameron, Hidalgo, Starr, Maverick, Kinney, Val Verde, Webb and Zapata.

Cattle Fever Ticks were introduced to the Americas in the early 1500's by European explorers carrying infested livestock (Anderson et al. 2010). In the early 1900's *R. annulatus* was established in states like North Carolina, South Carolina, Oklahoma, Alabama, Louisiana, Mississippi, and Arkansas (Ellenberger & Chapin, 1940). Babesia caused cattle mortality in the northern and the southern states, disrupting cattle movements and reducing economic growth. Therefore, the U.S. congress initiated a tick eradication program.

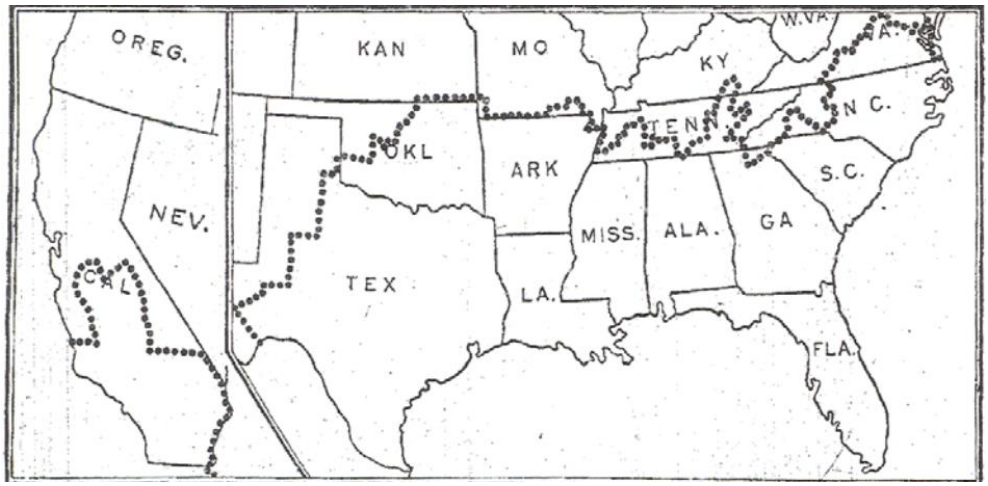

Figure 4. Cattle Fever Tick and Sothern Cattle Tick in 1900's "Reprinted from (Ellenberger and Chapin, 1940)"

The Cattle Fever Tick Eradication Program (CFTEP) was established in 1906, and was for the first example of a cooperative State and Federal effort (APHIS, 2013). The two main techniques used in this eradication program were: 1) the treatment of all cattle, horses, and mules systematically with arsenical dips every 2 weeks; and 2) the removal of all livestock from infested

pastures for 6-9 months, to ensure that no tick larvae remained (Molher, 1942).

The main techniques were not sufficient to eradicate ticks in some locations. In 1938 Florida was very difficult to eradicate because of the abundant white-tailed deer in the area in combination with a newly discovered tick species, *R. microplus*, found to be infesting these deer in favorable climatic conditions. Therefore, in 1939 the population of deer in affected parts of the state (Everglades) were reduced in order to erradicate the southern cattle fever tick infestation (APHIS, 2013). Louisiana was other state that had problems were the deer population. Around 1890, the deer population in 14 of 15 CFT-affected states was nearly extinct (Pound et al, 2010). The hunting laws during those years not established as they are currently, and for these reasons the tick populations were relatively easy to eradicate (Pound et al, 2010). As a result of these efforts in 1943, *R. annulatus* and *R. microplus* were eradicated from the US (APHIS, 2013). The PTQZ served as a buffer zone for the two tick species were not eradicated from this area (APHIS, 2013). In1961, Florida accomplished the eradication of the southern cattle tick, if this tick; had not been eradicated the losses today would be ~$3 billion per year across the USA (APHIS, 2013). Currently, the PTQZ has been greatly affected by native and exotic deer species crossing the quarantine zone (Pound et al. 2010). The climate within the Permanet Tick Quarantine Zone is conducive to the survival of fever ticks, *R. microplus* south east of Laredo, TX and *R. annulatus* north west of Laredo, TX. Relative humidity levels of 75% and lush areas of brush provide a favorable habitat for this tick (APHIS, 2013). These areas are commonly found in South Texas and areas adjacent within Mexico (Bram et al. 2001). If these ticks find their way into the US again, cattle production, cattle health, and the food supply industry will be at risk. A one-year outbreak in Texas incurs a minimum cost of $1.2 billion using historic figures, not including the surveillance and the infrastructure improvements that are needed to be implemented for inspection

(Anderson et al., 2010).

1.5 *Rhipicephalus microplus* eradication program in PR

Eradication was attempted in Puerto Rico using techniques that were successful in the Southeastern United States. Factors that affect the United States such as the introduction of wildlife along the border with Mexico and the broad amount of land are not shared with the island of Puerto Rico. The eradication program began in 1936, after the enactment of act #106, this act describes the spread of the cattle fever tick and the necessity of establishing a tick elimination program and preventive measures (Crom 1992). This eradication program involved the obligation to implement the systematic treatment of cattle with acaricides using dipping vats filled with arsenical acaricide every 14 days, for a period of 18 months following the instructions of the Secretary of Agriculture (Tate, 1941). After a pause due to World War II, *R. microplus* was eradicated by 1954 (Graham et al. 1977). The eradication in PR lasted for 24 years. Unfortunately, ticks were discovered in 1977 at a slaughterhouse in Mayagüez (Crom, 1992). The infested cattle were traced back to a farm in the town of Utuado (Crom, 1992). Another attempt at eradication began in 1979, but it was not successful due to insufficient funds available to create a robust eradication program (Crom, 1992). Therefore, only surveillance activities were carried until 1981.

In a third attempt at eradication in Puerto Rico the pesticide application method was changed to spray. This decision was made because the facilities that were built for dipping in the past eradication campaings were now in poor condition and there were insufficient funds to rehabilitate them (Crom 1992). The amount of *R. microplus* infested cattle increased from 40% to 90% by 1981, and the first case of *Babesia bovis* was recorded in April 1985. In spite of these setbacks, Puerto Rico is an island which gives it some advantages of isolation in comparison with the eradication program along the Texas/Mexico border. Additionally, there are no alternate

wildlife hosts in Puerto Rico as there are in Texas.

Currently, Puerto Rico has 257,285 head of cattle, of which 99,892 are from the dairy industry, and 96,026 are used for the meat industry (NASS USDA, 2012). At the moment, PR has 3 hard ticks species, which are the principal vectors of *A. marginale, B. bigemina* and *B. bovis.* One of the vectors is *R. microplus* (Crom, 1992). The estimated economic loss due to the presence of anaplasmosis and babesiosis which are vectored by *R. microplus,* carries a cost of US $ 20 million in Puerto Rico in 1989 (Crom, 1992). In 2007, cattle operations in PR were facing a yearly deficit of ~14,373,315 L (32,274,840 lbs) of milk and ~3,602,873 kg (7,926,321 lbs) of meat (Soto-Alberti, 1999, unpublished data). Despite this, Puerto Rico does not have an eradication program, and the dairy industry is still the most important agriculture sector for the national economy of the island. With a population of ~4 million inhabitants, the demand for milk has been decreasing. However, there still is a high comsumerism in a large percent age of the population. Fresh milk is not imported onto the island and all the milk that the local farms produce is used for national consumption. It is important to find a way to improve the control of the organisms that reduce production and cattle health.

1.6 Control Methods

Globally, more than 80% of cattle are infested by ticks (FAO 1987) . The methods used to combat at least two of the most important species, *R. microplus* and *R. annulatus,* are pasture rotation, environmental modification, and chemicals like microcyclic lactones (MLs) and conventional acaricides (Rodriguez, M. et al. 2004; Rodriguez, R.I. et al. 2018). The repeated use of acaricides leads to the development of resistance (Lovis et al. 2013). Resistance evolves relatively quickly in the *Boophilus* subgenus because of its rapid life cycle, improper treatment methods, and over-use or missuse by producers. “Resistance” is interpreted differently by many;

in this report, resistance is defined as "the development of a specific heritable trait(s) in a population of ticks, selected as a result of the population's contact with an acaricide" (Rodriguez, R.I. et al. 2018). This genetic selection results in an increase in the population survival percentage after exposure to a given concentration of that chemical (Rodriguez, R.I. et al. 2018). In addition to the resistance problem, over use of acaricides results in residues that can be found in products such as milk, meat, and the environment (Rodriguez, M. et al. 2004, Mulenga, A. et al. 2000, Zintl et al. 2003). Unfortunately, ticks in many parts of the world are evolving resistance to all pesticides available on the market. This is driving research to find new techniques to control *R. microplus*.

Resistance can result from the excessive use of a product, or if the applied dose is too low to achieve a lethal dose for heterozygotes in a population (Miller et al. 2005). Chemicals like amitraz, coumaphos, and diazinon are commonly used to combat *R. microplus*. However since 1980, *R. microplus* has been known to have resistance to these drugs in southern and eastern Mexico. Additionally, 8 strains of *R. microplus* were suspected to be resistant to organosphosphate in areas of Northern Mexico (Miller et al. 2005). The chemicals mentioned above were excessively used in the years, 1975 to 1985, in Mexico for the control of *R. microplus* during Mexico's tick eradication campaign (Li et al.2003). Resistance to these acaricides is a challenge to the eradication methods of the PTQZ in Texas. All cattle imported to the USA from Mexico must go through a tick inspection process, followed by a coumaphos treatment in a dip vat. If the cattle coming from Mexico are infested with coumaphos-resistant ticks, then unwanted ectoparasites may enter US territory.

Figure 5. Acaricide resistance strains of *Rhipicephalus* ticks around the world "Reprinted from (Rodriguez, R.I. et al. 2017)"

Organophosphates and MLs are the main products used to control ectoparasites, including many ticks. But as with the rest of products available on the market, several strains of ticks have developed resistance to these compounds (Rodriguez-Vivas et al. 2006 ; Perez-Cogollo et al. 2010). For this reason, researchers are now using Tick Integrated Pest Management (TIPM) techniques; where they integrate the application of two or more technologies to control tick populations (Rodriguez, R.I. et al., 2014).

The combination of chemical, non-chemical, cultural and mechanical methods are now popular to attack these vectors. The formulations of chemicals available for tick control treatments are flowable products, emulsifiable concentrates, wettable powders, hand operated spray, spray box, injection, pour-on, dipping vat, ear tag, food supplements, intra-ruminal bolus, air needle-free injection and pheromones-acaricide-impregnated devices among others (George et al., 2004). Farmers are combining mixtures of acaricides which inturn has driven the evolution of resistance to the mixtures tolevels higher than the single product alone. The benefit was short

lived it is gone (Rodriguez, R.I. et al., 2017).

Researchers found by the end of the 19th century, that animals that had undergone natural *Babesia* infections, had long lasting immunity and that blood from recovered animals did not produce such severe form of the disease in recipient cattle (Connoway &Francines, 1899). This was exploited by immunizing cattle against babesiosis in many countries (Callow, 1984, Gray et al., 1989, De Vos & Potgieter, 1994, Benavides et al., 2000, Pipano, 1995). There were many attempts around the world to create vaccines with infected blood collected from infected animals, but it was not until 1996 that the countries of South Africa, Australia, and Ireland created frozen vaccines for *B. bigemina* and *B. bovis*. They were produced by the Onderstepoort Veterinary Institute (De Waal, et al., 2006). Following vaccination, the protective immunity develops for 3-4 weeks, however there is no experimental data on the long-term persistence of *Babesia* in the animal (De Vos, 1979 ; Pipano et al., 2002) For *B. bovis* the protective immunity after a single vaccination lasts several years, in the case of *B. bigemina* the duration is shorter (De Waal, et al. 2006). Unfortunately, this vaccine is not commercialized in the United States or Puerto Rico.

Because resistance has developed to every acaricide on the market in many tick-endemic areas of the world, and there are no effective vaccines against *Babesia* available in the US or Puerto Rico, the development of new approaches to tick control are needed. One such technology is vaccination to induce an immunological response against tick infestation (Mulenga et al., 2000, Johnston et al., 1986; Willadsen & Jongejan 1999). The most successful commercial vaccines so far against *R. microplus* consists of 2 products based on the same technology. They were created in Cuba and Australia. The first vaccine used a recombinant antigen, Bm86, identified from the gut of semi-engorged adult female ticks (Willadsen & Kemp 1988; Willadsen et al., 1989;), and has the name of Bm86 (found in *Boophilus microplus* in the year 1986). It was produced by

recombinant DNA techniques from which the two products, as previously mentioned, were created (Rand et al., 1989, Turnbull et al., 1990, Rodriguez et al., 1994). One of the vaccines was created in Cuba in 1993; the Cuban Bm86-based vaccine is known as GavacTM. Gavac was marketed in 1993 and after its registration was used in Latin America in field trials (Rodriguez et al., 1995a, b). These field trials, located in Colombia, Mexico, and Cuba, indicated that the vaccine gave successful results against tick populations at those locations (Rodriguez et al. al., 1995a, b; De la Fuente et al., 1998, Redondo et al., 1999). The Cuban vaccine was based on published Australian research, however the Cuban's were able to market their vaccine quicker due to fewer industry regulations. The Australian vaccine was released on the market in 1994, under the name TickGARD^{TM} (Willadsen 1995). There it became the top selling tick control product purchased by the Australian dairy industry, until it was pulled from the market after a merger created a new company with competing products.

An experimental Bm86-based vaccine produced by Zoetis, (Parsippany-Troy Hills, New Jersey) was receintly developed in the USA. It is based on the Bm86 antigen, but contains a modern adjuvant. This vaccine was used on 6 farms in Puerto Rico in the villages of Sabana Grade, Aibonito, Isabela, Moca, Naguabo, San Sebastian (Miller, R. 20017, unpublished data). It is the first vaccine to be approved on the island against ticks in both dairy and beef cattle (Miller, R. 2017, unpublished data). In addition, to Puerto Rico, it was approved for use in the USDA Cattle Fever Tick Eradication Program in Texas (Miller, R. 20017, unpublished data). In Puerto Rico, vaccination with Bm86 reduced the need for acaracide treatment by 70%. This is similar to the results of field studies in Venezuela and Cuba (Valle et al., 2004, Vargas et al., 2010). Tick vaccines have been shown to decrease *R. microplus* populations over time and could affect the transmission of tick-borne pa thogens by decreasing the vector capacity of ticks. Additionally,

it may be that continued use of anti-tick vaccination creates an immune response in initially non-reactive inviduals, which increases its effectiveness in a herd over time. To date, little is known about then the interaction of vaccine and acaricide. It has been observed that the use of the vaccine drastically reduces the need for traditional acaricides. What is not known is if there is a true additive, or synergistic interaction, or if the two technologies are simply working independently.

1.7 Study Objectives

This project is an investigative study of *R. microplus* control consisting of two parts, an *in vivo* animal field study and a series of *in vitro* laboratory bioassays. The goal of this study is to provide a method to control ticks more sustainably than the exclusive use of chemical acaricide, reducing the selection for acaricide resistance, chemical residues in animal products and the environment. The objectives of this study were set to address the main question: Is there a synergistic interaction between anti-tick vaccination and systemic acaricide treatment?

The two study objectives were:

1) Determine if there are synergistic interactions between anti-tick vaccination and acaricide treatment using a an *in vitro* field trial model.
2) Compare the efficacy of Bm86 and systemic acaricides for suppression of *R. microplus* using an *in vitro* feeding technique.

2. ARTIFICIAL FEEDING TECHNIQUES

2.1 Introduction

Animal studies are costly because cattle must be purchased, fed, and kept in isolation with proper care. Animals can suffer from skin inflammation, joint problems, anemia, and stress by being confined in small spaces. These situations raise ethical situations that can generate negative public opinion to studies with live organisms. Additionally, *R. microplus*, has high host specificity for cattle (Evans et al., 2000). Therefore, the use of less expensive serogate hosts for chemical efficacy studies or antigen testing is not possible (Evans et al., 2000). What is left is to develop *in vitro* techniques to rear this tick and test combinations of treatments in a more cost-effective way as pre-screening or alternative to *in vitro* studies. In this study, laboratory bioassay techniques have been developed to avoid the use of on-animal studies.

Historically, *R. microplus* has been tested with numerous topical bioassay techniques such as the larval immersion test (LIT) (larval immersed mostly in macrocyclic lactones and amitraz) (Rodriguez-Vivas et al 2006a, Perez-Cogollo et al., 2010), the adult immersion test (AIT) (engorged females immersed in technical or commercial acaricides) (Guerrero et al., 2014), the larval tarsal test (LTT), and the larval packet test (Stone & Hadock, 1962). These tests have been used to evaluate new topical acaricides and combinations of compounds. They are also commonly used to test pathogenicity of entomopathogenic fungi (Klafke et al., 2006, FAO, 2003; Ribeiro et al., 2007, Frazzon et al. Al., 2000). These tests are also recommended by the FAO to study acaricide resistance in ticks (FAO 2004).

Other bioassay techniques involve the artificial feeding of ticks. Since a blood meal is provided to the tick it is a better assessment of systemic acaricides. This bioassay can help to

identify key elements of the host-tick relationship and facilitates the assessment of the test product, identify key elements in the biology and physiological aspects of blood feeding, protein actions, and chemical compound intake. *In vitro* feeding is useful not only for systemic acaricides but it has been found useful in anti-tick vaccine screening (Varella, A. et al., 2011).

The first artificial feeding bioassay technique using glass capillary tubes was developed in the 1950s, to artificially feed *Hyalomma excavatum, H. dromedarii, Dermacentor reticulatus,* and *R. sanguineus* ticks, but these were not fed until repletion (Chabaud, 1950; Gregson, 1957). The necessary conditions for *in vitro* feeding of *Rhipicephalus spp*. vary depending on the researcher. Conditions like the position of mouth parts, tube angle, tube type, tube aperture, anti-coagulant preferences, host blood species, blood temperature, tick stage, and serum versus whole blood all change between researchers (Lew-Tabor et al., 2014). Over time, the *in vitro* technique was improved. In 1960 Joyner and Purnell demonstrated that palps can be inserted into the capillary tube, and later demonstrated that ticks successfully fed whether the palps were inside or outside of the capillary tube (Joyner & Purnell, 1968, Purnell & Joyner, 1967). This was also tested with *R. appendiculatus* (Joyner &Purnell, 1968, Purnell & Joyner, 1967). Another important factor discovered in 1943 was that mated ticks fed and engorged more actively than when unmated (Gregson, 1943).

The testing of anticoagulants in artificial feeding experiments is one of the principal steps of the process. The most commonly used anticoagulants are EDTA, heparin, and citrate. When semi-engorged *R. microplus* females were artificially fed it was found they preformed better when fed heparinised blood but some researchers used another anticoagulant like EDTA (Willadsen et al., 1984). Egg production was maximized for *R. microplus*, fed bovine blood compared to rabbit, guinea pig or rats (Willadsen et al., 1984). *Rhipicephalus microplus* must be

reared until the semi-engorged female stage on cattle prior to *in vitro* feeding. This allows the tick to produce regulatory hormones naturally leading to a normal maturation (Varella et al., 2011). When the ticks are artificially de-attached from the host the feeding hormones are already present in the tick and they continued the feeding behaviour. To measure the feeding success, weight gain, and egg hatchability are used as determinants factors are used (Bennett, 1974).

Artifical feeding has been used successfully in the evaluation of systemic acaricides and vaccines (Lew-Tabor et al., 2013). This technique is well suited for vaccine evaluation because it simulates the normal route in which ticks are exposed to the antigen on animal. Only a few *in vitro* studies report the effect of feeding antibodies or antiserum for some tick species. *Rhipicephalus appendiculatus* fed with rabbit serum showed inhibition of artificial feeding as a result of physical gut disruption or altered feeding behavior (Losel et al. 1993). The explanation for this reponse was that ticks sensed and resisted the taste of serum following detection by the gustatory sensilla in the cheliceral denticles (Losel et al. 1992). Antibodies can have an effect on the gut or feeding behavior of the tick, for this reason artificial feeding can be a good option to screen for potential vaccine antigens (Lew-Tabor et al 2014). Studies with *R. microplus* have been conducted to test two different monoclonal antibody targets of potential vaccines, but the data from this study were not disclosed (Gonsioroskiet al., 2012).

If the results of an *in vitro* test are validated *in vivo*, the results are more reliable, and for this reason the present study was divided in two parts: 1) an *in vitro* testing of the combination of systemic acaricide with tick anti-serum and antibodies, and 2) *in vivo* field controlled study. The *in vitro* mitigates secondary variables that are impossible to eliminate in a field study and allows for flexibility in changing experimental conditions before they are tried in the field (Baldridge et al., 2007). The benefits of this technique make it essential for testing of purified antibodies

(IgG), to screen antigen/acaricide combinations, and to determine if vaccine-pesticide synergy interactions occur.

Other methods to test potential vaccine or drug targets are the RNA interference (RNAi) dsRNA knockdown techniques (Almazán et al., 2010; Barnard et al., 2012). The host antibody response to the target cannot be examined by this technique, which causes lack of association with immunity and the natural process of feeding do not occur (Lew-Tabor et al., 2014). But this technique can be used to complement screening of vaccine studies with *in vitro* feeding conditions. This provides another option for researchers to screen for potential antigens in vitro, prior to in vivo animal trials (Lew et al., 2014).

2.2 Methods

2.2.1 Overview

This project was carried out at the Cattle Fever Tick Research Laboratory (CFTRL), USDA, ARS, inside the Moorefield Airbase in Mission, Texas (IACUC #2017-01). For this, five Hereford (*Bos taurus*) beef calves were maintained in confined barns with screened sides for the study. One of these animals was used as the donor of fresh blood. The rest remaining three animals were used as hosts for *R. microplus* to realize the in *vitro* study. Each calf, received two scoops (Tolco, 11399) of 3 quarts of grain twice per day and water ad libitum while in stanchion. Their health was checked daily. No adverse affects or medical treatments were required thoughout the study.

2.2.2 Cattle infestation

Stanchioned calves had the hair on both sides shaved and a patch made from muslin cloth fixed to the calf with rubber contact cement (DAP, Non-Flammable, Baltimore MD). This patch was closed until the infestation occurred. At the time of the infestation the patch was opened in

the upper center part, with a horizontal aperture. A vial containing larvae from 250 mg of eggs (approximately 2,500 larvae) was placed inside the patch, and the patch was closed with staples and duct tape (Duck, Avon, OH). The *R. microplus* larvae used to infest the calves were from Deutch strain (reference strain susceptible to all acaricides). This strain was originally collected in Webb County, Texas and has been reared in a laboratory using standard procedures following the IACUC SOP # 017-01 protocol (Davey et al., 1980). The patch was under constant monitoring for approximately 16 to 18 days after infestation.

Figure 6. Patch protocol in confined cattle

2.2.3 Tick collection and preparation

After day 16 post infestation, semi-engorged females were carefully collected with bent tip forceps (FisherScientific, 16100110) placed on the bucal parts of each tick. These ticks were transported to the laboratory and used on the same day. Tick mouthparts were examined and cleaned microscopically using a dissecting microscope (OMANO). Cement cones or remaining dermal tissue were carefully removed with a 4-1/4in overall leng forceps size (Dumont, Roboz).

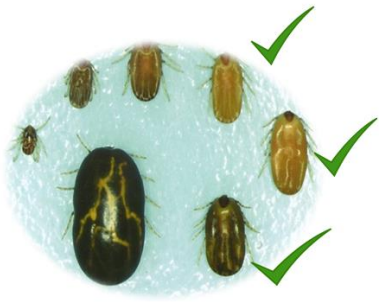

Figure 7. Selection of stage for in vitro, from the seven days of adult fully engorgement

Each semi-engorged female tick collected on days 4-6 of engorgement was randomly selected , weighed and stuck dorsally on a double-sided tape (Gorilla double-side mounting tape, touch & clear®, Inc (Cincinnati, Ohio) in a plastic petri dish. Micro-pippette tips with 2mL of capacity, (model RC-L2000 Rainin, Mettler Toledo) were placed over the mouth parts including palps and hypostome. The 2 mL tips were held at an approximately 45^{o} angle supported by a ball of wax onto the petri dish (Figure 8).

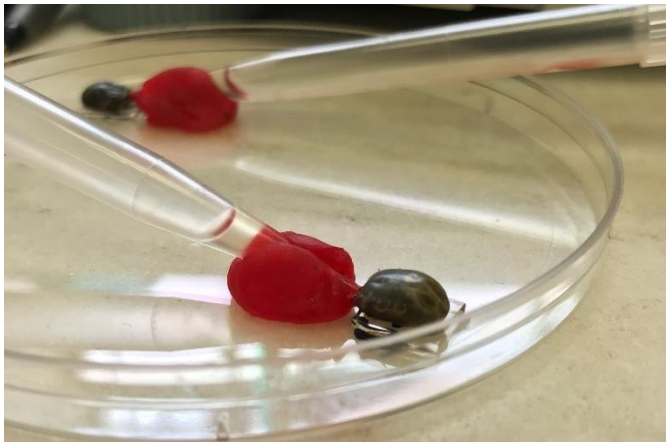

Figure 8. Micro-pippette tip held at an approximately 45^{o} angle

After ticks and tips were positioned, the tips were filled with 200µl of blood. The petri dishes were placed inside an aquarium and maintained at a temperature of 28ºC with a relative humidity of 87% that were monitored constantly with a thermo-hygrometer VWR (98090309, Control company) and the HOBO Pro VZ (U23-001, Onset Company) monitored the temperature and RH. After approximately one hour and thirty minutes of setting the ticks in the aquarium, ticks were checked for blood leaking or if feeding had not commenced. In these cases, tips were repositioned and spilled blood cleaned. Approximately 18 hours after initial feeding, tips and wax ball were removed. Ticks were collected, and fully engorged ticks were re-weighed. Ticks that did not feed or die, were discarded. The weight gain was used to determined which ticks fed. Each individual fully engorged female was placed in a labeled pre-weighed and sterile 2-dram shell vial (15X45 mm 03339030C, FisherScientific) with a cotton ball as a cap. These vials were placed inside the aquarium at 28ºC and 87% RH. After 14 days each tick was monitored for egg laying, vials were re-weighed, dead females removed and eggs examined. The vials with eggs were incubated again for daily monitoring of larval hatch. The larval hatch (percentage of emergence) was estimated by visual examination, 14 days after the first larvae hatch.

2.2.4 In vitro feeding assays

Three preliminary studies were carried out using the *in vitro* feeding technique: I) anti-coagulant preference; II) moxidectin bioassay, and III) fluazuron bioassay. These preliminary studies had two main objectives: 1) to optimize the *in vitro* feeding technique and 2) determine the best concentration of moxidectin to be used in the *in vitro* studies with Bm86 anti-sera and purified Bm86 IgG.

2.2.5 Anti-coagulant preference assay

In order to determine the best anti-coagulant to be used on the *in vitro* feeding assays, bovine blood was collected from tick-naïve cattle by venipuncture of the jugular vein using Vacutainer® tubes (Becton Dickinson) treated with two different anticoagulants: sodium heparin and Ethylenediaminetetraacetic acid (EDTA). For sodium heparin-treated blood, nine milliliters of blood were drawn into a 10 mL tube containing 158 USP units of sodium-heparin. For the EDTA-treated blood, 3 ml were drawn into a 4ml tube containing 7.2mg/ml final concentration of EDTA. The blood was collected from the donor animals within 3 hours of the tests being conducted.

One experimental control group was formed of ticks to evaluate any effects attributed to tick mortality due to adhesive properties of the tape used to restrain ticks during artificial feeding. All tick treatment groups were incubated for ~18h in 28ºC and 87% RH. Experimental groups 1 and 2 were fed with 200 µL and 400 µL of EDTA-treated blood, respectively. Experimental groups 3 and 4 were fed with 200 µL and 400 µL of sodium-heparin treated blood, respectively.

2.2.6 Fluazuron assay

Fluazuron is an acaricide growth regulator that inhibits the incorporation of chitin during the moulting between life-stages of ticks (Oliveira et al., 2012). It is a systemic acaricide and can kill ticks susceptible to this drug by disrupting its life-cycle. This product was used in the *in vitro* feeding assay to verify its feasibility to be used in combination with anti-tick vaccine in integrated tick control.

Initially, a diluent solution was prepared with 0.4 mL of Triton X-100 in 19.6 mL of acetone. This solution contained 2% Triton X-100 in 100% of acetone. The fluazuron technical

powder (Sigma-Aldrich) was diluted in 10 mL of the diluent solution to prepare a stock solution at a final concentration of 1%. The stock solution was pre-diluted 1:10 in the diluent. One milliliter of the pre-dilution was added to 9 mL of demineralized water to prepare the working solution (0.01% of fluazuron, 10% acetone and 0.2% of Triton X-100). The working solution was diluted 1:10 in heparin-treated fresh blood to give a final concentration of 0.001% of fluazuron (10 ppm), 1% of acetone and 0.02% of Triton-X. The 10 ppm fluazuron-treated blood was diluted 1:10 to give a final concentration of 1 ppm.

The control groups consisted in non-treated blood and blood mixed with the vehicle of fluazuron solution (water solution of 10% acetone and 0.02% Triton X-100). Treated groups were fed with blood treated with a final concentration of 10 ppm and 1 ppm of fluazuron. Each tested group consisted of 30 ticks.

2.2.7 Moxidectin assay

Moxidectin is a mylbemicin within the class of macrocyclic lactones used to control endo- and ecto-parasites (endectocide) (Nolan et al. 2012). Moxidectin was used in the *in vitro* feeding assay to verify its feasibility to be used in combination with anti-tick vaccine in integrate tick control.

Moxidectin technical powder (Sigma-Aldrich) was diluted in 10 mL of acetone to prepare a stock solution at 1%. A pre-dilution was made with 6 mL of the stock solution and 4 mL of acetone (final concentration 0.6%). One milliliter of the pre-dilution was used to prepare the use solution in combination with 99 mL of H_2O and 20 µL of Triton-X 100 (final concentration 0.006% moxidectin, 1% acetone and 0.02% Triton X-100). The use solution was used to prepare four dilutions that were used to treat the blood (600 ppm, 60 ppm, 6 ppm, and 0.6 ppm) were 1.8 mL of blood was added to each 25 mL falcon sterile tube. The dilution began with the addition

of 200 µL of the use solution to the 600 ppm tube. For the 60 ppm dilution 0.2 mL of the 600 ppm sterile tube was added in series until the tube of 0.6 ppm.

The control groups consisted in non-treated blood and blood mixed with the vehicle of moxidectin solution (solution of 1% acetone and 0.02% Triton X-100). Each tested group consisted of 30 ticks.

2.2.8 Antibody plus moxidectin assay

Ticks in the experimental group 1, were artificially fed a mixture of 90 µl of serum containing IgG from Bm86-vaccinated cattle and 110 µl of heparinize fresh blood. Experimental groups 2 and 3 were fed a mixture of 0.1 ppm of acaricide (Moxidectin), IgG and heparinized fresh blood. IgG used for this combination was extracted from the second part of this project described in Chapter 3, were blood collected from 10 pre- and post- vaccinated beef cattle (Angus and Hereford). The serum was tested for anti-body levels using ELISA. The IgG was purified from samples with highest measured concentrations of antibodies with the Nab ™ Spin kit. This purified IgG was offered in blends of fresh blood and acaricide by *in vitro* feeding to ticks in this group.

2.2.9 Enzyme-Linked ImmunoSorbent Assay (ELISA)

Whole blood was previously collected from the jugular vein of the 20 Angus and Hereford cattle vaccinated with Bm86 anti-tick vaccine described in Chapter 3, in 2 SST vacutainer (367985, Becton Dickinson) tubes per animal. Tubes were identified with the date and the number of the corresponding animal. Serum was harvested after, whole blood was permitted to stand for 30 minutes at room temperature. The vacutainer with clot was centrifuged at 3400XG RPM for 30 minutes and 200µl of the supernatant was transferred to vacutainers and kept at -20°C (Henry, 1979; Thavasu, et al 1992).

Selection of sample sera with the highest IgG concentration to the Bm86 vaccine was determined by evaluating pre- and post-vaccination sera for each animal using ELISA. This protocol was optimized for the bovine H+L antibody and vaccinated bovine serum. A protocol by Felix D. Guerrero (pers, comm) that is divided in two days was followed. On the first day Bm86 antigen was diluted in 10ug/ml of BupH Carbonate-Bicarbonate Buffer. One Hundred microliters of the diluted solution was added to an ELISA plate (0.1 ug/well) (Thermo Fisher, Waltham, MA, USA), this was used to coat 96 well sealed plates overnight at room temperature (40^{o}C). Sera was serially diluted to 1: 100-1:8000 in TBS (TBS / 0.05% Tween 20-10% Blocker BLOTTO dilution buffer (Thermo Fisher, Waltham, MA, USA) (Guerreo F.D., 2016, personal communication). Goat anti-Rabbit IgG (H + L) antibody, Horseradish Peroxidase (HRP) conjugate, prepared from antibodies that have been adsorbed against bovine IgG to minimize cross reactivity was introduced. The (H+L) were impregnated in the wells for 1 hour after the incubation of the plates with a 1x wash buffer and 20X TBS 20 buffer, and the secondary antibody (Peroxidase Labeled Rabbit anti-bovine IgG) (Thermo Fisher, Waltham, MA, USA). The TMB substrate solution (3,3 ', 5,5' -tetramethylbenzidine) (Thermo Fisher, Waltham, MA, USA) was added to the wells and incubated for 20 minutes in a dark room. The TMB is responsible for the color reaction, chromogen solution may be used as a substrate for HRP, Addition of sulfuric acid stop solution changes the color to yellow, enabling accurate measurement of the intensity at 450nm using a spectrophotometer (ELx800 Absorbance Reader, BioTek, Winooski, VT, USA) or plate reader. Which will help us to enabling the determination of antibody titers using an OD450 nm value at least twice as high as the negative control serum (Almazan, C. et al., 2005).

2.2.10 IgG Purification

Serum with high concentrations of antibodies was selected for IgG purification by means of Nab ™ Spin kit. In this procedure, serum is added to a microcentrifuge spin column to immobilized protein resin and enables quick purification. The column is equilibrated before the serum is added with 2mL of Binding Buffer. This binding buffer removes all the nonbound sample components for 10 minutes in a end-over-end shaker. After saving the flow through of the binding buffer step, the colum was washed 3 times with the binding buffer. One hundred microliters of neutralization buffer and 1ml elution buffer were added to the column after 1 minute in the centrifuge. This last step provided us the three fractions of the samples of purified proteins. The three portions of flow-through were stored, and the column was washed with 3ml of elution buffer, 3 ml of PBS and with 3 ml of storage solution stores at 4^{o}C for later use. The column can be used 10 times before losing binding capacity. The determination of which fraction has more antibodies is measured by the relative absorbance at 280nm and using neutralizarion buffer as a control solution.

2.2.11 Statistical Analysis

The following parameters were recorded for each individual tested in the bioassays and considered in the analysis if the results. i) initial female weight (g); ii) post-fed female weight (g); iii) egg mass weight (g); iv) estimates of percentage of larval hatch. The following indexes were calculated: a) engorgement ratio (post-fed females weight / initial females weight); b) conversion (egg mass weight / post-fed females weight), and c) estimated number of larvae / female [(egg mass weight x larval hatch) x 20,000)].

Statistical analysis for the *in vitro* feeding assays were calculated using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. *P* values < 0.05 were

considered statistically different. ANOVA and graphs preparation were performed using Sigma Plot 14 (Systat Software, Inc. 2017).

2.3 Results

2.3.1 Tick and equipment optimization

Measurements of weight gain after artificial feeding indicated that ticks at day 5 and 6 of the seven days of engorgement process were better candidates for the *in vitro* feeding than ticks on day 4. The engorgement process last from when the larvae molt to the adult until the adult fully engorged. Other factors that influence the feeding process are mouth part cleaning, ticks that had damaged mouthparts or derbis in the capitulum did not feed correctly.

2.3.2 Anti-coagulant preference

Ticks that were placed in the double-sided sticky tape without receiving any blood meal had an average weight loss of 2 mg. This experimental group was used to determine if the chemicals on the sticky tape may affect the results of the study. In this bioassay, two anticoagulants were tested: heparin and EDTA, in two volumes: 200 µL and 400 µL; in this test we observed that a heparin-treated blood meal resulted in higher larval hatching rates (76 and 70% for 200 and 400 µL of blood, respectively) in comparison with the EDTA-treated blood (1 and 13%, for 200 and 400 µL of blood, respectively). The tick survival with EDTA-treated blood was low in comparison with the heparin-treated blood fed ticks. The average egg mass weight was lower in both EDTA-treated groups with values of 6 and 8 mg each one in comparison with the free blood group with 100 mg of egg mass weight. This free blood group produce 95% of hatchability. Heparin did not compromise female engorgement (figure 9), egg production (figure 10) and hatching and showed to be the less toxic anticoagulant comparing to EDTA. There was no statistical difference between the two volumes of heparin-treated groups in any of the parameters analyzed (Table 1).

Table 1. Results of the artificial feeding assay with heparin- and EDTA-treated blood with semi-engorged females of *Rhipicephalus microplus*.

Group	N	Mean initial weight (mg)	Mean final weight (mg)	Mean engorgement ratio	Mean egg mass weight (mg)	Mean conversion	Mean larval hatch (%)	Mean lumber of larvae / female
Blood free	29	43.103 (1.992) a	41.141 (1.941) a	0.953 (0.005) a	8.563 (1.192) a	0.197 (0.021) a	60 (7.975) a	107.414 (20.422) a
EDTA-200 µL	30	46 (1.941) a	125.61 (8.032) b	2.806 (0.189) b	1.117 (0.584) a	0.025 (0.012) b	10.17 (5.021) b	17.448 (10.124) a
EDTA-400 µL	30	44.693 (2.008) a	120.61 (12.943) b	2.87 (0.336) b	1.363 (0.525) a	0.035 (0.013) b	9.6 (5.099) b	15.767 (8.566) a
Heparin-200 µL	30	41.867 (1.642) a	105.948 (5.804) b	2.639 (0.187) b	40.27 (4.337) b	0.35 (0.03) c	77.15 (6.445) c	733 (81.517) b
Heparin-400 µL	29	43.369 (1.959) a	112.981 (12.758) b	2.716 (0.325) b	47.137 (7.445) b	0.0358 (0.032) c	79.73 (5.035) c	713.769 (118.073) b

N: number of females; a, b, c: values indicated with different letters in the same column are considered statistically different at the Tukey's test with a P value < 0.05.

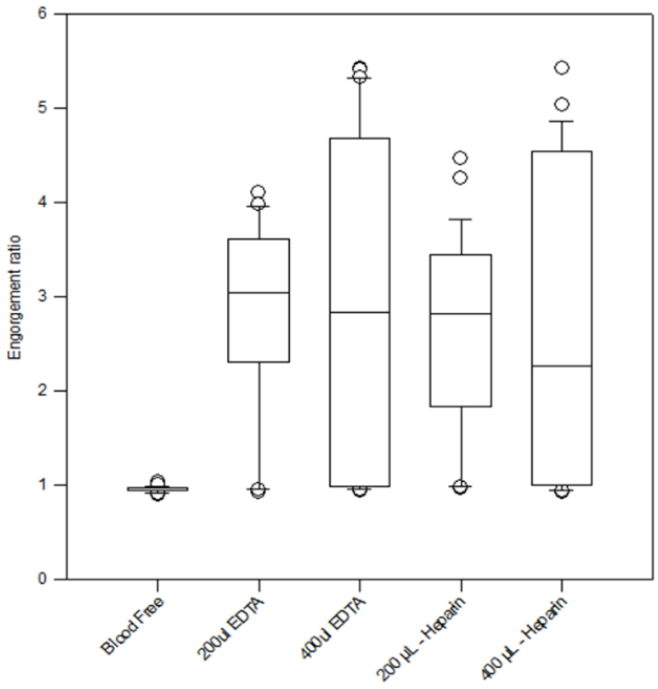

Figure 9. Engorgement ratio of anticoagulant testing group. In his anticoagulant testing groups there is no significant different between the means. The groups of 400 µl EDTA and Heparin had more variability in comparison to the groups fed with 200 µl.

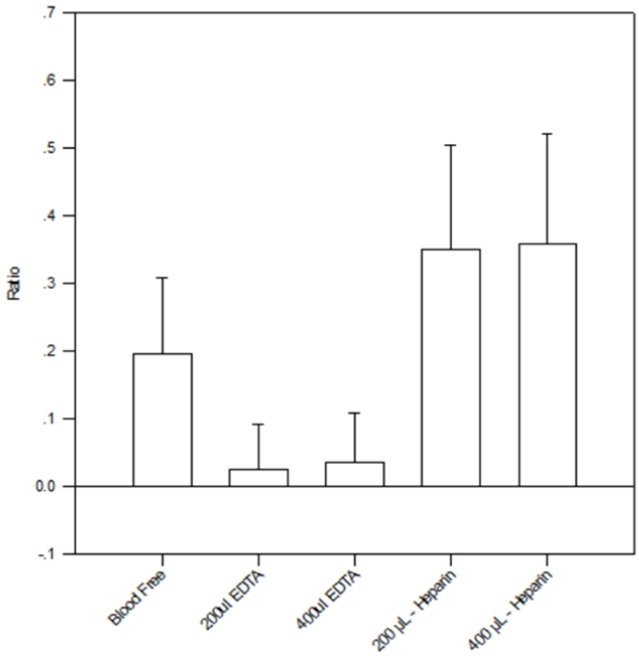

Figure 10. Conversion of egg mass/female weight of anticoagulant testing group. The blood free group had a ratio of conversion of egg mass/female weight of ~0.2. Both EDTA groups 200 and 400 µl had ratios near 0.05. Heparin groups had ratios of ~0.35 respectively.

2.3.3 Fluazuron

Fluazuron 10 ppm treatment group resulted in a complete blockage of larval hatching (figure 11). The group that was fed with 1 ppm had 36.6% mortality and only 11% larval hatch. Not only was the hatchability percent affected by the amount of fluazuron in the blood, but also the average egg mass weight in the 1 ppm (48 mg) group is much higher than the 10 ppm (10 mg); 30 mg of difference by adding 9 ppm in the concentration. No significance difference in means was observed in initial weight, final weight, egg mass and conversion between the treatment and control groups. A reduction and difference is observe in the mean larval hatch

percent and the mean number of larvae per female (Table 2). Were in both fluazuron 10 ppm present means of zero, and fluazuron 1 ppm present lower means in comparison with the control groups (Table 2). Is important to recognize that in the 10 ppm group tick imbibed more blood (75 mg) than in the 1 ppm (67 mg); this product works as a mite growth regulator and as this two arthropods share the same order it may be this reason why the group that fed more with a high amout of product had an high mortality.

Table 2. Results of the artificial feeding assay with fluazuron -treated blood with semi-engorged females of *Rhipicephalus microplus*.

Group	**N**	**Mean initial weight (mg)**	**Mean final weight (mg)**	**Mean engorgement ratio**	**Mean egg mass weigh t (mg)**	**Mean conversion**	**Mean larval hatch (%)**	**Mean number of larvae / female**
Control (Blood)	14	52.929 (4.678) a	103.588 (13.112)a	2.354 (0.248) a	73.225 (5.752) a	00.737 (0.04) a	78.75 (6.105) a	1164.85 (147.834) a
Control (Blood+Vehicle)	29	46.831 (2.85) a	116.986 (8.721) a	2.418 (0.153) a	73.41 (4.84) a	0.642 (0.0201) a	80 (5.617) a	1229.038 (124.745) a
Flu 1 ppm	30	44.387 (2.604) a	97.912 (9.394)a	2.117 (0.173) a	62.753 (6.15) a	0.636 (0.0462) a	7.647 (5.253) b	76.612 (58.451) b
Flu 10 ppm	30	53.953 (2.914) a	106.663 (8.285) a	2.004 (0.099)a	71.1 (6.24) a	0.665 (0.0427) a	0 (0) b	0 (0) b

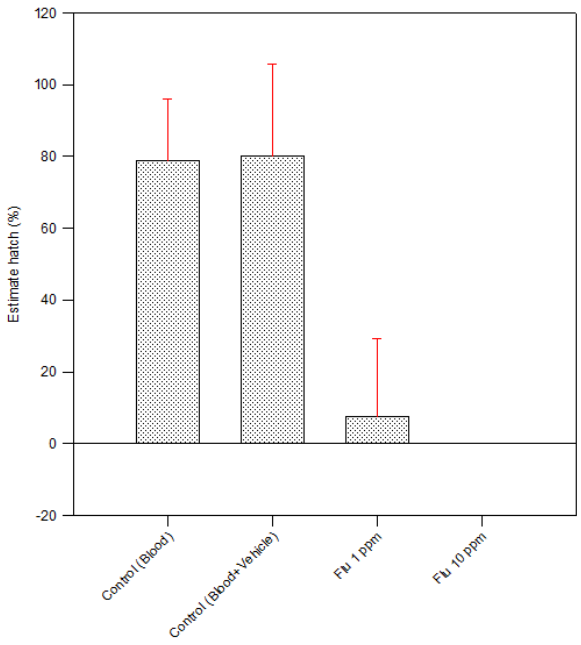

Figure 11. Fluazuron effect on larval hatch and control groups. There was no significant difference between the two control groups. An estimate hatching percent of ~78, ~80, ~12, and 0 of control, control (blood+acetone), Fluazuron 1 ppm, and Fluazuron 10 ppm were observed respectively.

2.3.4 Moxidectin

This broad-spectrum endectocide in 600 ppm does not express any hatching capacity, or number of larvae per female, although the average weight of the egg mass was 44 mg (Table 3). The group fed with 60 ppm had an average egg mass weight of 57 mg that produce only 3% of hatchability, this group only fed 26 mg of moxidectin. The average weight gain is increased as the concentration of moxidectin in the blood administered to the ticks reduced. The 6 ppm group had an average egg mass weight of 58 mg and a hatchability percent of 21% in an average weight gain of 36 mg. The last group tested was 0.6 ppm of moxidectin in the blood had an

average weight mass gain of 70 mg and a 60% of hatchability but the egg mas average weight it was only 11 mg, the lower egg mass average in all the group that moxidectin was tested. It is observed that by increasing the amount of moxidectin in the blood reduce the average of weight gain; there is an inversely proportional relationship.

Table 3. Results of the artificial feeding assay with moxidectin -treated blood with semi-engorged females of *Rhipicephalus microplus*.

Group	N	Mean initial weight (mg)	Mean final weight (mg)	Mean engorgement ratio	Mean egg mass weight (mg)	Mean conversion	Mean larval hatch (%)	Mean number of larvae / female
Control (Blood+Vehicle)	15	43.72 (2.294)a	77.6 (5.23)a	1.787 (0.112) a	42.071 (6.655)a	0.506 (0.0741) a	69.214 (12.149)a	721.214 (151.258)a
Mox 0.6 ppm	27	36.881 (1.862)a	112.242 (7.27) b	2.969 (0.16)b	57.626 (6.381)a	0.493 (0.0469) a	55.105 (9.799) a	789.895 (154.155)a
Mox 6 ppm	26	35.135 (2.076)a	62.327 (4.124)a,c	1.84 (0.0748) a	29.25 (3.126)b	0.491 (0.0513) a	12.273 (6.126) b	110.636 (57.457) b
Mox 60 ppm	31	40.623 (1.768)a	57.521 (3.91) a,c	1.444 (0.0517) c,d	1.389 (1.389)c	0.0205 (0.0205) b	0.526 (0.526) b	2.789 (2.789) b
Mox 600 ppm	30	40.27 (2.352)a	44.168 (2.717) c	1.199 (0.0311) d	0.947 (0.947)c	0.0389 (0.0389) b	0 (0)b	0 (0) b

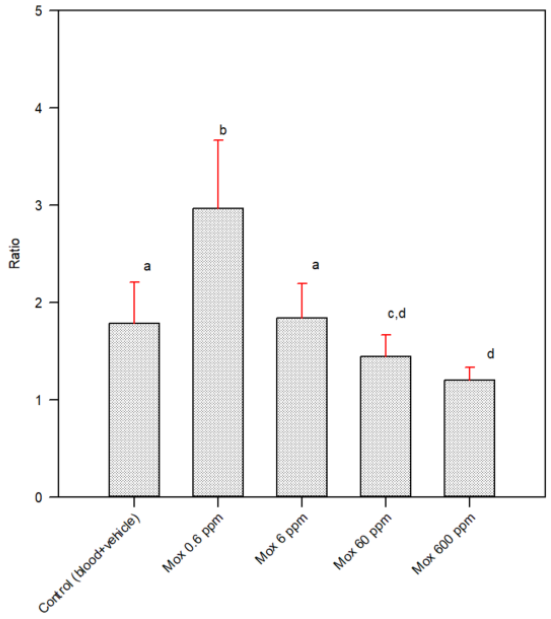

Figure 12. Engorgement ratio of moxidectine treated group. The engorgement ratio of females produced across the entire study was significantly different for group fed with 0.6 ppm of moxidectin, control group and moxidectin 6 ppm are similar in ratios as moxidectin 60 and 600 ppm. Estimated ratio of engorment was ~1.7, 3, ~1.8, ~1.6, ~1.5 for the control, Mox 0.6, Mox 6, Mox 60 and Mox 600 ppm respectively.

2.3.5 IgG assay

There is no differenece in means until the egg mass weight, were a difference between the treatment groups and the control groups is observed in the means. A trend of lower values from mean egg mass weight to mean number of larvae/female were observed in the IgG+Mox group. This trend value, support the field study part and the Puerto Rico project, but the high mortality of ticks in the control groups do not allow us the corroborate this results. For this reasons this test will be repeated in the future.

Table 4. Results of the artificial feeding assay with IgG with semi-engorged females of *Rhipicephalus microplus*.

Group	N	Mean initial weight (mg)	Mean final weight (mg)	Mean engorgement ratio	Mean egg mass weight (mg)	Mean conversion	Mean larval hatch (%)	Mean number of larvae / female
Control	19	54.81 (3.04)a	75.31 (6.07)a	1.35 (0.10) a	50.82 (3.91) a	0.68 (0.03) a	79.15 (8)a	721.214 (151.258) a
IgG	20	58.63 (22.72) a	61.90 (8.87) a	1.84 (0.20)a	18.46 (7.79) b	0.23 (0.09)a	27.08 (11.40) b	789.895 (154.155) a
IgG+Mox	19	38.75 (1.76)a	139.55 (73.40) a	2.96 (1.46)a	3.33 (3.33) b	0.07 (0.07)b	0 (0)b	110.636 (57.457)b

2.4 Discussion

2.4.1 Anti-coagulant preference

The group that did not receive any blood meal lost an average of 2 mg of weight is due to desiccation; water loss by environmental stress and the prolonged time without blood meal (Rosendale et al. 2017). This is a mechanism of how the tick reserve energy until they can find a blood meal. They are free of blood by in vitro but they were collected from the cow on day 16 after infestation, so the group already fed a portion of the blood meal and are partially engorge so they produce an egg mass average weight of 100mg. In the hatchability express a 95%, this defines that the chemicals compounds from the mounting tape (Gorilla Glue®, Inc, Cincinnati, Ohio) do not affect the health of the egg mass, the tick itself or the larvae.

The 200 ul ethylenediaminetetraacetic acid (EDTA), had a higher average weight gain mass (124 mg) than the 400 ul EDTA fed group (105 mg) both are not a significant difference

but in the percent of hatchability, we can observe a direct effect in this extra consume of EDTA. The group that fed 124 mg of EDTA only had a 1% of hatchability in the other side the group that fed less present an increment in hatchability of 13%. The mode of action of EDTA as an anticoagulant in the blood is by removing calcium ions from the blood clotting system (Butenas & Mann 2002). The exact role of calcium ions in tick feeding is still an enigma, according to Saito and Pereira in stage III-IV of the oocytes, in the cytoplasm of salivary gland secretory cells of some tick species and semi-engorged *R. sanguineus* the presence of calcium sphered are identified (Saito et al 2005). In other insect genera like *Drosophila* the process which a mature egg initiate the embryo development needs a calcium wave for the egg activation (Kaneuchi et al. 2015). Also, *Rhodnius prolixus* a blood-sucking insect had the presence of a calcium-binding phosphoprotein in the oocytes and hemolymph (Silva-Neto et al. 1996). By analyzing this calcium presence in oocytes not only in ticks but also in insects, it may suggest that this calcium is necessary for the development of oocyte, ionic balance or embryo skeleton formation (Lew-Tabor et al. 2014). EDTA bind calcium ions strongly and this may be the reason why the group of ticks that fed higher amount of EDTA in the blood show a lower hatchability in comparison with the other group of EDTA 400ul and heparin blood groups.

Heparin treated blood fed ticks demonstrated a successful engorgement and hatchability. The heparin group that was fed with the double amount of blood only show an average egg mass weight of 48 mg. Thirty-six percent of egg mass average of the group that was fed with 200 ul. This may be due to increment exposure of the anticoagulant. Heparin is the chose anticoagulant due to a successful larval emergence, and weight gain. This was also demonstrated by Lew-Tabor in 2014, and by Waladde in 1993 (Waladde et al 1993).

In terms of comparison of the control groups, all the groups reflect an initial weight gain with no significant difference in means (Table 1), which determines that all the groups received a similar blood meal before the detachment of the animal. The means of final weight, engorgement ratio and egg mass weight (Table 1, figure 9) had no difference except for the blood free group because it did not receive any blood meal after being detached from the animal. Heparin group presents a significant difference in means in comparison with the other control groups in egg mass and in a number of larvae/female; because it was the most efficient anticoagulant and the one the ticks fed more and do not affect the reproductive efficacy of them (Table 1). In the conversion of egg mass/female weight, it is visible the means difference of the blood free, heparin and EDTA groups (Figure 9). The EDTA group had a lower mean than the blood free group, this is defined by the fact that although they received a blood meal after being detached that blood meal affects the reproductive outcome (Table 1). In the conversion is visible the preference of heparin as a most effective anticoagulant tested in this study, with a 3.5 ratio. Larval hatch percent show a significant different between the EDTA and the rest of the groups (Table 1), due to the action of EDTA with the calcium ions necessary for the larval hatching.

2.4.2 Fluazuron group

Acetone was used in one of the control group to monitor the effect of the medium that helps to solubilize acaricides. It was used to verify its toxicity in the ticks since it would be used as a moxidectin and fluazuron solvent. The average weight gain, egg mass, and hatchability are similar in the group with acetone and the fresh blood alone, which indicates that acetone is a good vehicle for acaricides without affecting the reproductive power and survival of the tick.

There is no significant difference between the initial weight of any of the group tested in the fluazuron set (Table 2), this as in the anticoagulants group expresses a similitude of the blood

meal intake after the tick was unattached from the animal. A higher mortality is expressed in the fluazuron treated groups in comparison with both control groups (Table 2), this explains the similitude in means during the final weight, engorgement ratio, egg mass weight and conversion (egg mass/ female final weight). The major difference is observed by the larval hatching and the estimated number of larvae by a female where the means of the 2 control groups and the 2 fluazuron groups are aligned in similitude (Table 2, Figure 11). A predominant reduction of hatching percent is observed by both fluazuron treated group but most predominant by the group that received 10ppm. This group reflects a 0% hatching (Table 2, Figure 11). The control groups show a hatchability above 70% and the fluazuron treated group only 11%. Fluazuron works as a mite and ticks growth regulator and inhibitor of the chitin synthesis. If the chitin synthesis is inhibited the formation of larvae and eggshell is affected, and this can be the reason why the groups fed with fluazuron express less hatchability.

2.4.3 Moxidectin group

As we saw in the rest of the testing groups there is no significant difference in the means of initial weight, the blood meal intake was similar for all the group. Ticks on the moxidectin group show a particular behavior called dose-response the more moxidectin parts per million in the blood the lower the average of weight gain by the ticks and lower hatchability percent (Table 3). With this, we can observe that the action of moxidectin is not only reflected in the larval hatch, but it can also be determined its effect at the moment of the weight after feeding. Moxidectin group fed with 60 and 600 ppm show mortality of less than half of the initial 30 tick tested after the in vitro feeding. The group fed with 0.6 ppm of moxidectin present a difference in terms of final weight, engorgement ratio and egg mass weight (Table 3, Figure 12). It even shows higher mean than the control group in this two characteristics. This may show that the

blood with moxidectin in low concentration may have a greater palatability than the blood for the ticks but did not have enough concentration to interfere with the blood intake as the group of 600 ppm. The larval hatching percent and the estimate of larvae by the female present to similitude in means of the 3 higher concentrations of moxidectin in the blood (6 ppm -600 ppm). The most adequate concentration in the blood to measure the effect of moxidectin is 0.6 ppm because it does not have high mortality after feeding and the results of egg mass and hatchability can be measured. The moxidectin selectively binds to the GABA-A and glutamate-gated chloride ion channels in invertebrates these receptors are related to locomotion, mediating sensory inputs into behavior and feeding (Wolstenholme 2012). This explains the behavior of the engorgement ratio and the initial weight reduction while increasing the concentration of moxidectin in the blood.

2.4.4 IgG group

Immunoglobulin G groups were tested but the mortality of control group (blood plus the carrier) was elevated that results can not be used to present relevant data. This test will be repeated for future studies.

2.4.5 Additional observations

The adequate weight to fed an *R. microplus* tick artificially is 40 mg to 80 mg the average weight gain is higher when ticks are in this range. Is almost impossible to determine a tick weight when they are attached to the animal, but by observational analysis, the ticks on day 5 and 6 of the engorgement phase are the best candidates. Ticks on the 3rd and 4th day of engorgement are more propense to do not feed any blood from the tip because do not produce cement or do not have the pick of feeding high to continue the blood intake. The ticks on this early stage had smaller mouthparts and are more propense to produce blood pools around before

the 18 hours of completer the feeding process. In conclusion, ticks that weight 40 mg in days 5th or 6th of the engorgement period are a better candidate for in vitro feeding.

Other interesting observations occur in the tick mouth part cleaning process. In the cleaning process, other organisms were observed walking around the outside surface of the exoskeleton of the ticks. *Bovicola bovis* a cattle biting louse that fed only by chewing and *Chorioptes bovis* a mite of the Sacoptiformes order that fed on dander and skin debris were observed in higher numbers during the cleaning process. The patch placed on the animal to collect the ticks is developing a micro-ecosystem where a variety of Diptera and all these other ectoparasites that also affect the animal health and behavior were observed.

3. FIELD STUDY: INTERACTION OF ANTI-TICK VACCINATION WITH BM86 AND MOXIDECTIN

3.1 Introduction

Biological, physical, and chemical methods are commonly employed for tick control. Vaccination is an alternative control method for ectoparasites (Rodríguez-Mallon 2016). This technique works well with the other common control methods and studies report long term protection against ticks (De la Fuente 2006). Since 1908 researchers have investigated immunological suppression as an alternative method to control ticks (Willadsen 1987; Willadsen et al. 1988). In nature immunological control can be realized through the adjudication of two approaches, direct immunization inducing an immune response with an isolated tick antigen or natural exposure to the parasite and by inducing an immune response through repeated tick feeding (Willadsen 1987).

Anti-tick vaccines are designed to induce significant immunity to tick infestation by the utilization of specific protein antigens (Willadsen 2004). The antigen is a foreign protein that the immune system of the animal can recognize as a potential threat. After the initial exposure, the immune system maintains a memory and keeps a record of it. Therefore, when the antigen enters to the organism again the immune system quickly recognizes it and attacks it readily.

Anti-tick vaccine technology is based on a protective concealed antigen from the gut protein Bm86 originally discovered from semi-engorged adult *R. microplus* female ticks (Willadson P., 2008; 1988). This Bm86 protein was obtained by splicing a foreign DNA fragment into a molecule that can replicate it and amplifying the fragment. This method is called DNA recombinant technology. Anti-tick vaccines containing the recombinant Bm86 antigen

have been commercially available outside the U.S. since the early 1990s (Willadsen P., 2008). One of the first countres to develop this vaccine was Australia, under the name of TickGARD and marketed by Hoechst Animal Health (Willadsen et al. 1989). Cuba also marketed a Bm86-based vaccine under the name of Gavac produced by Heber Biotec S.A in La Havana, other countries in Latin America like Venezuela used this vaccine in their markets from 1994 to 1997. Countries including Mexico, Cuba, Brazil, Argentina and Colombia conducted field trials and as result, they found a successful tick population control with this recombinant vaccine.

When the cattle are vaccinated an immunological reaction is induced in the tick gut and the uptake of the antibody cause damage to the parasite (Wiladsen et al. 1989). Tick survival, engorgement, and egg laying capacity are reduced in ticks that consume blood from vaccinated animals. The reaction occurs between the antibodies and the Bm86 bound to the surface of the gut epithelial cells in the tick. Bm86 antibody binding inhibits the endocytotic activity of these cells (Wiladsen et al. 1989). This inhibition interrupts bloodmeal ingestion.

An anti-tick vaccination with Bm86 has been shown to greatly reduce the number of southern cattle fever ticks in Australia, Cuba, Mexico, and Venezuela (Valle et al., 2004, Vargas et al., 2010). Tick vaccines have been shown to decrease tick populations over time and could affect the transmission of tick-borne pathogens by decreasing the vector capacity of ticks by interfering with the development of pathogens (Glen Scoles, personal communication). Anti-tick vaccination has been shown to decrease the number of pesticide applications needed. Therefore, it is possible that vaccine-pesticide interactions occur. Australian scientists demonstrated that the persistent activity of moxidectin, a macrocyclic lactone, was three times longer when used in Bm86-vaccinated rather than non-vaccinated cattle. However, they did not publish this data (Dr.

David Kemp, lead scientist on this project, passed away before enough supporting data could be collected).

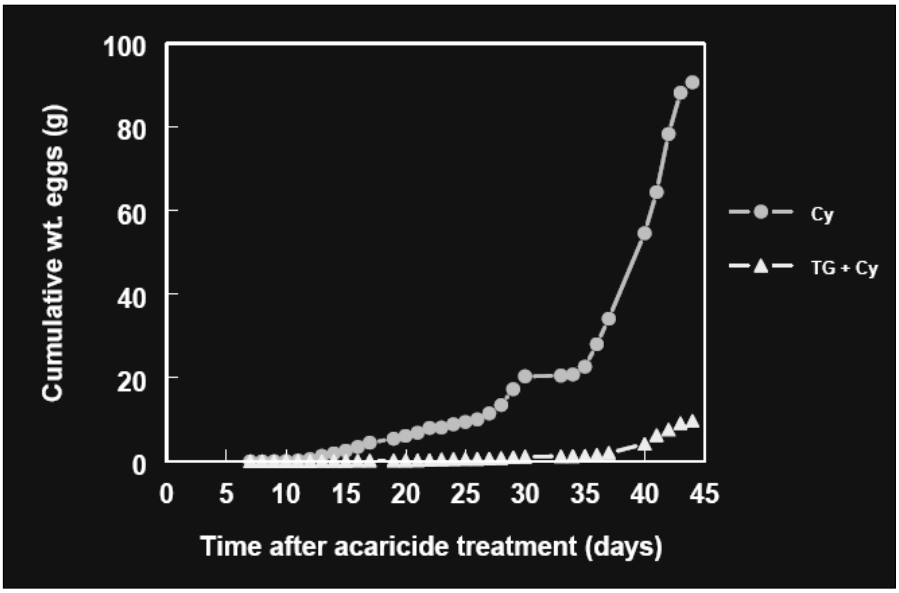

Figure 13. Egg production of unpublish Kemp study. Egg production was delayed in moxidectin (Cy, cydectin) + Bm86 (TG, TickGuard) vaccinated animals versus moxidectin only treated animals by 24 d (12 versus 38 d, respectively, Kemp et al. Unpublished Data).

Macrocyclic lactones (MLs) are important compounds for the control of tick populations. MLs are divided into two groups, milbemycins (moxidectin and milbemycine oxime) and avermectins (ivermectin, abamectin and doramectin) (Klafke et al. 2006). These compounds are used in different parts of the world for the control of internal gastrointestinal parasites such as nematodes and microfilariae. They are also used against external parasites e.g. ticks, mites and mange that can cause huge economic damage to livestock and pets field (Klafke et al. 2006). Macrocyclic Lactones are neurotoxins. These compounds have a high affinity to glutamate-gated chloride channels (Glu-Cl), which result in somatic musculature paralysis which directly causes

parasite death (Cully et al., 1994). The paralysis is developed by a slow and irreversible membrane conductance increase by the opening of these channels (Cully et al., 1994).

Resistance to macrocyclic lactones has evolved in many *R. microplus* populations thoughout the world. Intense use or under-dosing of these compounds are major factors that influence the evolution of tick resistance in ticks. Early detection of resistance is essential in order to avoid further selection of resistant ticks by using the same active ingredient and to delay the spread of resistance. The integration of two different methods of control that have a different mechanism on the tick system will help to delay the tick resistance. Anti-tick vaccination is well suited for this as it has a different mode of action and reduces the need for repeated acaricide treatments.

The objective of this study was to determine if an interaction exists between vaccination with Bm86 and treatment with macrocyclic lactone in order to better understand how Bm86 vaccination can be incorporated into an integrated tick management program.

3.2 Methods:

3.2.1 Overview

The protocol of this study was approved by the Institutional Animal Care and Use Committee of the USDA-ARS KBUSLIRL (IACUC #2017-01). It was conducted in accordance with accepted practices described in the "Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching, 3rd Ed.", as promulgated by the Federation of Animal Science Societies.

3.2.2 Study site

All cattle used in this study were held at the USDA-ARS KBUSLIRL for minimum of 3 weeks due to pre-study medication requirements. After, the cattle were transported to USDA-ARS Cattle Fever Tick Research Laboratory (CFTRL) at Moore Air Field located near Edinburg, TX, 26.3871°N, 98.3376°W, elevation 66 m. The CFTRL facilities are fully accredited by the American Association of Laboratory Animal Care. Twenty-three cattle were brought to the CFTRL, 20 of the 23 were selected to be part of the study. The remaining three were not included in the study due to poor health, poor weight gain, or aggressive behavior. Twenty *Bos taurus* (Black Angus & Hereford) animals of approximately 136–227 kg (600-1,000 lbs) and ~12 months old were used in this study. Animals were held in Pasture 7. Pasture 7 is ~40 acres with 50% covered by common South Texas woody plants – mesquite (spp), blackbrush (spp), brasil (spp), etc. with the open areas covered with buffelgrass (*Pennisetum ciliare syn.*). Buffelgrass is a C-4 grass native to Africa, a perennial cespitose plant (Cox et al., 1988; Ibarra-Flores et al., 1995). This grass is perfect for the South Texas climate because it can tolerate desert habitats and respond with a vigorous growth when erratic rainfall events occur which is the typical climate of South Texas (Sanderson et al., 1999). Nutritional quality of this grass is influenced by

environmental and management factors, but levels of calcium, potassium, sodium and magnesium are adequate for cattle production throughout the year (Hanselka, 1988). Phosphorus are generally low and fluctuate in response to the rainfall (Hanselka, 1988). Other types of grass and succulents like prickly pear (*Opuntia sp.*) are part of the grassland of pasture 7.

Figure 14. CFTRL pastures map and location

3.2.3 Study groups

All animals were randomly assigned to treatment groups. One group was vaccinated (Bm86 Immunomodulator, Zoetis, Kalamazoo, MI) at 0 and 28 days according to the label instructions (2.0 ml per animal; 3/4", 18g needle; IM). A second group was given a single moxidectin (Cydectin, 1%, Bollinger Ingelheim) injection (1", 18g needle; SC) according to the label on day 61. A third group was vaccinated on days 0 and 28 and given a single moxidectin injection 1% SC on day 61 according to the label instructions. The fourth group served as an

untreated control. All personnel working with cattle and ticks were blinded to the treatment groups. Employees performing rectal temperatures, clinical observations, injection site reaction scoring, tick counts, and laboratory assays were unware of group allocation and the treatment each animal received.

Table 5. Treatment Groups

Treatment	n	Day of Treatment	Day of Bi-Weekly Infestation Start
BM86	5	0 and 28	42
Moxidectin	5	61	42
BM86/Moxidectin	5	0 and 28 (BM86), 61 Moxidectin	42
Control	5	N/A	42

3.2.4 Ticks and Tick infestation

All animals were artificially infested with Deutch (susceptible strain) *R. microplus* larvae. The Deutch strain of *R. microplus* was originally collected in 2008 from Dimmit County Texas and has been reared in the laboratory using standard procedures (Davey et al 1980). "Free-release" infestations were made by gluing a vial of tick larvae (125 mg, ~2250) to individual calves on the dorsal midline between the scapulae and removing a cotton plug covering the opening to the vial allowing the larvae to infest the animal freely without confinement. Infestations were made twice per week starting on day 42 through to the end of the study. Day 42

was two weeks after the boosting vaccination. Therefore, all life stages of the tick (larvae, nymphs, and adults) were on host animals prior to moxidectin treatment.

3.2.5 Tick Counts

Twice per week (on infestation days), counts of standard females (attached adult female ticks, 0.4 – 8.0 mm in length) were made on the entire left side of each animal in the study. Standard females are in the "rapid engorgement" phase of their development. Generally, these are mated female ticks ~6-14 days old. They will be replete and off the host within 24 hours to oviposit eggs in the environment. Up to 10 engorged females from each animal were collected and brought back to the laboratory, held at 28^{o}C, 98% RH, and a photoperiod of 12:12 L:D for fertility and fecundity estimation. The index of fecundity (IF) was calculated by multiplying the average weight of eggs produced by collected females, by the total number of females counted per animal, and multiplying the product by the percent hatch of the egg mass:

$$IF = (\text{average egg mass weight x females}) \times \% \text{ hatch}$$

The number of live larvae produced per female was calculated by multiplying the IF by 20,000 which is approximately the number of eggs in one gram.

3.2.6 Rectal temperatures

Rectal temperatures were measured before and after every vaccine injection (days 0-3, and 28-31). The measurements were obtained with a thermometer, (Safety 1st 3-in-1Nursery Thermometer) with battery-operated digital read out.

3.2.7 Blood collection and serum extraction

Ten milliliter BD vacutainer blood tubes were used for obtaining 10 ml blood samples. The plastic serum tubes have spray-coated silica and are used for serum determinations in chemistry, serology, and immunohematology. After the collection of the whole blood from the jugular vein from each calf, samples were centrifuged (1,000 rpm for 30 min). Serum was divided into 5 aliquots of 200 ul and immediately frozen for later processing.

3.2.8 Pharmacokinetics moxidectin

Preliminary liquid chromatography tandem mass spectrometry (LC/MS/MS) method parameters have been investigated for optimized moxidectin detection. It was necessary to implement multiple reaction monitoring (MRM) to have nanomolar sensitivity. A parent ion (*m/z*) corresponding to the analyte of interest was selected and fragmented into its daughter ions. The two most intense daughter ions were filtered to the detector. These two daughter ions were used to identify and quantify the amount of analyte present in the sample. By varying the amount of energy in the collision cell, the relative intensity of the daughter ions was controlled.

The TurboVap (Multi-Well Evaporation Systems, Glas-Col) system was used in the sample prep step for rapid solvent evaporation using a steady stream of ultra-high purity nitrogen gas, and a heating block with temperature control up to 100°C.

Sample preparation methods were based on previous ivermectin analysis completed at University of Texas at San Antonio but optimized for moxidectin. The extract was further purified, evaporated to dryness using a TurboVap, and reconstituted in methanol. The ivermectin extraction protocol was used for the extraction of the moxidectin. Two milliliter sample serum was aliquoted into a 15 mL centrifuge tube. Six milliliter acetonitrile was added to the tubes containing the sample. The sample was vortexed for 30 seconds and sonicated for 10 minutes,

followed by centifrugation at 4,000 RPM for 30 minutes and then the supernatant was poured into a new tube and evaporated to dryness. The sample was reconstituted in acetonitrile and aliquoted into LC/MS/MS autosampler vials for analysis.

Mass spectrometric parameters were optimized by direct infusion. Once the extraction efficiency and necessary sensitivity was achieved, samples were be prepped and analyzed. An internal standard of another avermectin (ivermectin, doramectin, ect) was be investigated for additional calibration validity.

3.2.9 ELISA

See Chapter 2 for the description of ELISA proceedures.

3.2.10 Statisticsal Analysis

The dependent measurements of the treated and control cattle groups over time were analyzed for statistical differences ($P < 0.05$) using the GLIMMIX Procedure in SAS (version 9.2). The daily (repeated) measures were modeled with the unstructured covariance structure to account for the correlated measures of the individual calves.

3.3 Results

Animal health was not impacted by any treatments or procedures during this experiment. There were no observed rectal temperature increases post vaccination and no injection site reactions were observed for either the vaccination or moxidectin treatments. Seasonal climate fluctuations did not alter animal health.

There was a significant difference (d.f.=3, F=8.9, P<0.001) in the cumulative number of adult ticks collected from the treatment groups when compared to the control group over the 87-day observation period (Fig. 14). The cumulative number of adult females (±SE) observed were 1,100, (±228), 1,156 (±228), 1,327(±228), and 2,539 (±228) for Bm86+moxidectin, moxidectin,

Bm86, and control groups, respectively. The cumulative control of standard females throughout was 57, 54, and 48% for the Bm86+moxidectin, Bm86, and moxidectin treatments, respectively (Fig. 15). The observation period represented 65-150 days after the initial Bm86 vaccination and 2-87 days post moxidectin injection.

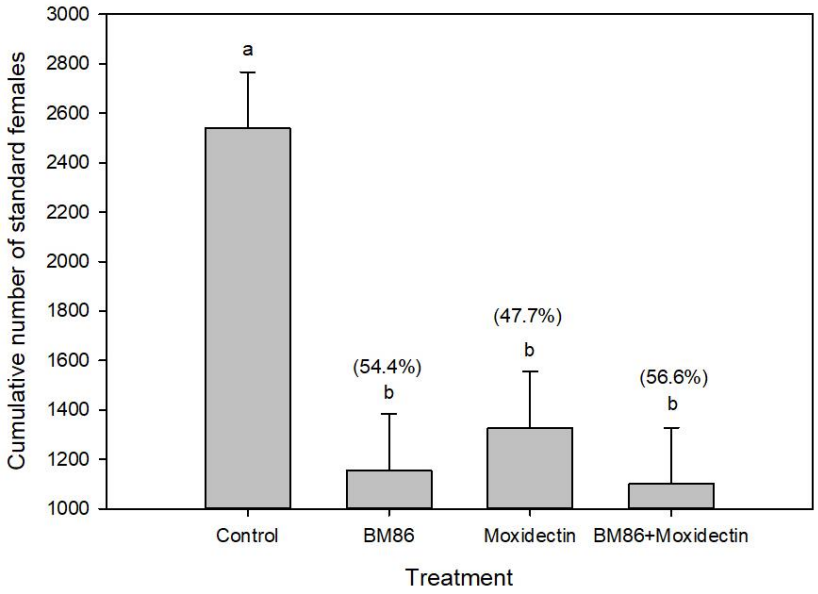

Figure 15. Cumulative standard female counts (% Control) by treatment group. All cattle were artificially infested twice weekly with ~5,250 larvae and allowed to graze under field conditions.

The cumulative estimate of live larvae produced across the entire study was significantly different (d.f.=3, F=5,94,876, P<0.001) for all treatment groups (Fig. 16). Estimated production of live larvae (±SE) was 682,158 (±370), 792,545 (±398), 1,043,679 (±456), and 3,654,265 (±854) for the Bm86+moxidectin, Bm86, moxidectin, and control groups, respectively. The cumulative percent control of larvae was 81.3, 78.3, and 71.4% for Bm86+moxidectin, moxidectin, and Bm86 treatments, respectively (Fig. 16).

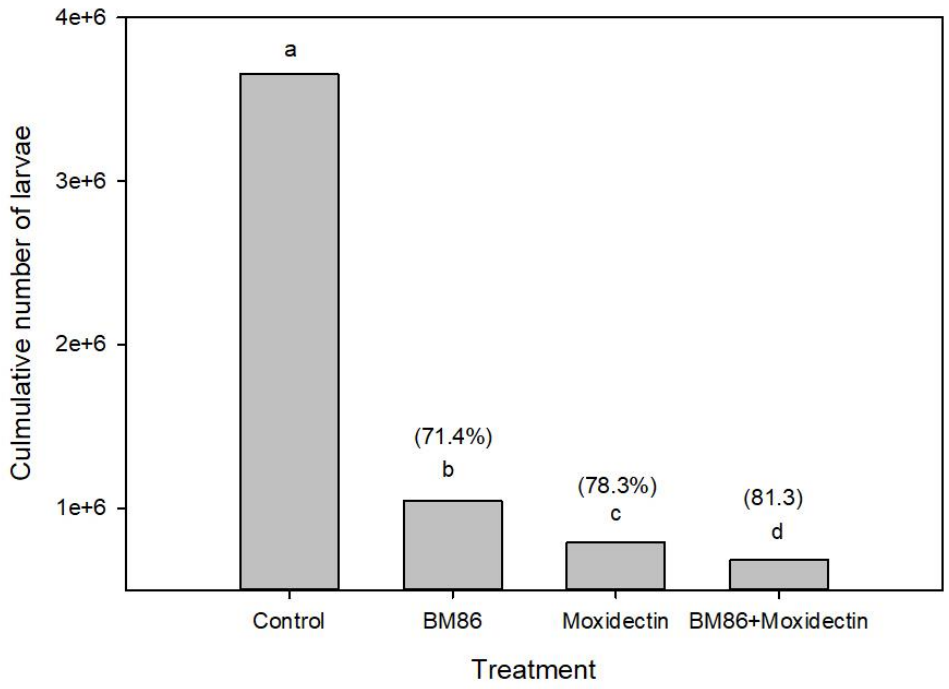

Figure 16. Cumulative number of live larvae (% Control) by treatment group. This calculation takes into account the total number of females collected, the average amount of eggs produced per female, and the observed percent hatch of the eggs.

There was a significant difference in the daily number of observed standard females (d.f.=3, F=9.68, P<0.0001), female weight (d.f.=3, F=2.62, P<0.05), egg mass weight per female (d.f.=3, F=,11.8, P<0.0001), and index of fecundity (d.f.=3, F=, P<0.0001) between the treated groups and the control group. However, there was no difference among the different treatment groups in these categories. For percent conversion of blood meal, there was only an observed significant difference (d.f.=3, F=23.14, P<0.0001) between the control and Bm86 treatment with

the moxidectin and moxidectin+Bm86 treatment (Table 6). There were no significant differences in egg hatch among all the treatment groups and the control group (d.f.=3, F=0.04, P<0.99, Table 6). For daily IF observations, there was a significant difference (d.f.=3, F=10.7, P<0.0001) between the control and all treatment groups. Among the treated groups IF was significantly lower for the Bm86+moxidectin and moxidectin-only treatment than the Bm86-only treated group (Table 6).

Table 6. Mean (±SE) of the number of females, average female weight, egg mass weight per female, percent conversion of blood meal, percent hatch and index of fecundity throughout the study.

Experimental Group	N	Mean(±SE)					
		Total Females	Ave Fem WT	Egg Mass Wt/Fem	% Conversion	% Hatch	IF
BM86+Cydectin	5	56.2(±10.02)^{b*}	0.164(±0.05)^{b}	0.066(±0.02)^{b}	41.1(±3.0)^{b}	32.1(±14.2)^{a}	0.12(±0.03)^{c}
Cydectin	5	66.1(±10.02)^{b}	0.197(±0.05)^{b}	0.077(±0.02)^{b}	36.0(±4.3)^{b}	29.2(±14.2)^{a}	0.12(±0.03)^{c}
BM86	5	56.2(±10.02)^{b}	0.247(±0.05)^{b}	0.120(±0.02)^{b}	48.6(±2.5)^{a}	40.7(±14.2)^{a}	0.21(±0.03)^{b}
Control	5	121.1(±10.02)^{a}	0.524(±0.05)^{a}	0.287(±0.02)^{a}	51.1(±1.8)^{a}	62.5(±14.2)^{a}	0.33(±0.03)^{a}

*Column means with a different letter are significantly different (p<0.5).

Figure 17 depicts the daily IF (±SE) values of the ticks observed on cattle treated with either Bm86, moxidectin, or a combination of Bm86+moxidectin. All moxidectin treated groups remain 100% efficacious until 5 June. From 5 June through 29 June, only the Bm86+cydectin treatments remain essentially 100%. After 3 July, there appears to be no significant differences in any treatment group for the remainder of the study. The 24-day gap between Bm86+moxidectin-treated cattle versus moxidectin-only treated cattle correlates well to earlier findings by Kemp et al. (Unpublished) discussed earlier. Bm86 was highly efficacious for 116 days and remained the lowest calculated mean IF for 3 of 5 observations until day 148 of the study.

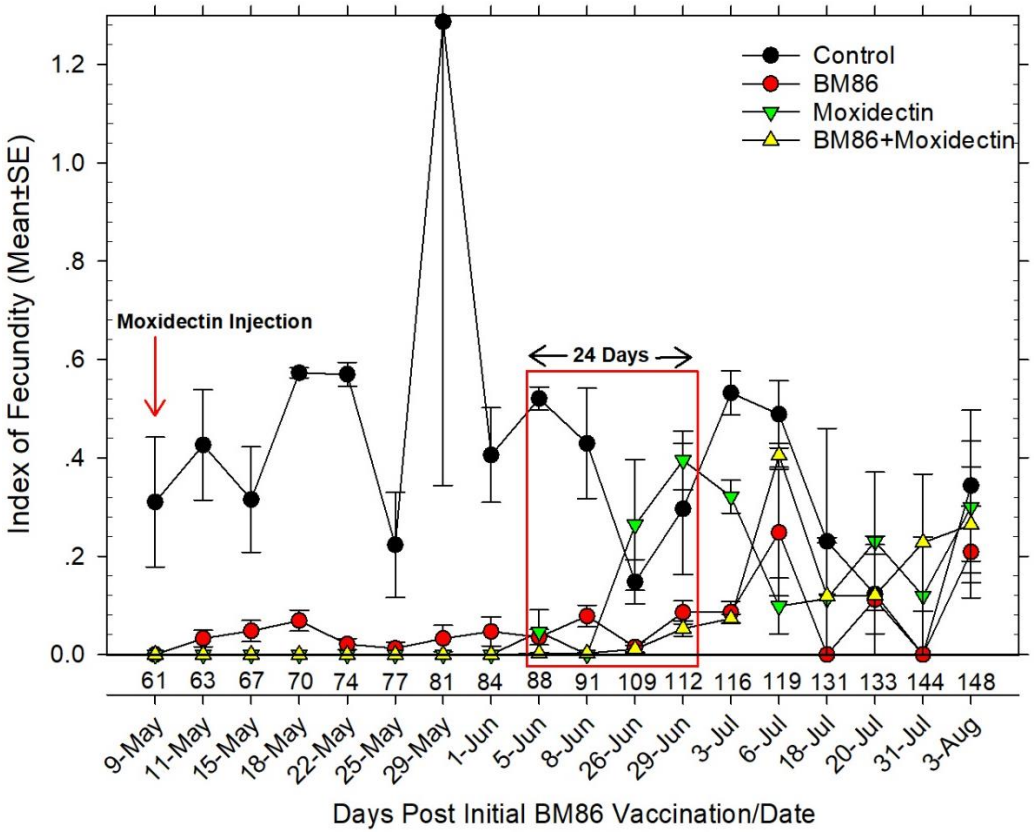

Figure 17. Index of Fecundity (±SE) of ticks observed on cattle treated with either Bm86, moxidectin, or a combination of Bm86+moxidectin under field conditions. All moxidectin

treated groups remain 100% efficacious until 5 June. From 5 June through 29 June, only the Bm86+cydectin treatments remain essentially 100%. After 3 July, there appears to be no significant differences in any treatment group for the remainder of the study. The 24-day gap (red box) between Bm86+moxidectin-treated cattle versus moxidectin-only treated cattle corresponds to earlier findings by Kemp et al. discussed earlier. Bm86 was highly efficacious for 116 d.

3.4 Discussion

Treatment of animals with a combination of Bm86 and moxidectin appeared to be safe and effective in controlling *R. microplus*. Vaccination with Bm86 under field conditions in South Texas provided 54% control of engorging adult female ticks for at least 87 days, 2-5 months (63-150 days) after the initial vaccination with Bm86. Additionally, the control of IF was 92% for at least 56 days, 2-5 months (63-119 days) after the initial vaccination with Bm86. The combination of Bm86 vaccination with moxidectin treatment increased the control of females by 3% and the control of IF by 6%. Vaccination with Bm86 in combination with moxidectin treatment was 9 and 16% more effective than using moxidectin alone for the control of standard females and control of IF, respectively.

Proper cattle handling, nutrition, and health monitoring were three main factors that helped to develop a good behavior in the cattle during scratching. Good animal behavior translated to a better tick count during scratching, because the animal allowed the scatcher to check ticks in all parts of the animal without risking injury. The 20 animals in this study did not present any injury or health problems during the project. No deficiencies in nutrition were observed, and all the animals were in good condition at the end of the study.

Larval eclosion has been measured by visual observations for years but has been criticized by peer reviewers as this method is thought to introduce bias into the dataset. In some published studies, the larval hatch is actually by weight or counting. One technique is the Drummond method where 15 of 50 squares displayed on the bottom of a petri dish are selected at random and hatched egg shells are counted. From this an estimation is created a formula to determine how many of larvae hatched. In this study both techniques were followed. The data show a correlation of 0.6 between visual and Drummond methods suggesting the visual larval hatching measurement to be a moderately acceptable technique to estimate percent eclosion. Therefore, visual estimation could be considered for large data sets where observation numbers and replicates are high. In smaller studies a direct counting method or weight measurement method should be used.

Bm86 alone or in combination with moxidectin reduced the number of observed standard females as well as moxidectin alone. The cumulative number of engorged standard females throughout the study was reduced by over 50% in both the Bm86 and Bm86+moxidectin treatment groups. This was slightly and not significantly better than the 48% drop in females counted from the moxidectin-only treatment group. Products that “knock-down” the production of adult females are desirable as it is this life stage that causes the most hide damage and weight loss in cattle leading to loss in profit for producers.

The combination of Bm86+moxicectin reduced the number of live larvae produced in the study by 81% when compared to the control group. This was significantly better than the 71 and 78% control observed from Bm86 and moxidectin groups, respectively. Interestingly, the same 24 days increase in persistent efficacy from the combination of Bm86+moxidectin over moxidectin alone was observed in this study as was observed in the unpublished work completed

in Australia. Further, it was observed that after it appeared the moxidectin treatment was losing efficacy, the efficacy of the vaccine + ML combination remained on par with the efficacy Bm86-only treatment until day116 while the moxidectin only group was no different than the control group by day 109.

Vaccination with Bm86+moxidectin or Bm86 alone provided significant and persistent control of engorging female ticks for at least 4.3 months and greatly reduced IF for 4 months. This study was started in March around the time of the "spring rise" of ticks. This is a time in South Texas where warmer temperatures and some rain traditionally leads to an increase in tick numbers as overwintering eggs eclose and climatic conditions generally favor tick population growth. The break down in significant differences between treatment groups from day 131 until day 148 is likely to be a combination of loss in treatment efficacy (ELISA and moxidectin pharmacokinetics data pending) and possibly harsh late summer weather conditions where temperatures rise to above 100 degrees and humidity drops reducing tick survivability.

These data support observations made on working dairy and beef farms in Puerto Rico where cattle were vaccinated with Bm86 and treated with a macrocyclic lactone. In the Puerto Rican farms, the addition of Bm86 vaccination greatly reduced the requirement for acaricide treatments from every two weeks or 12 treatments every 6 months to 4 or fewer treatments for the same period of time, a 67 to 100% reduction in acaricide use. Similar observations have also been made in Mexico, Australia, Cuba, and Venezuela, but this is the first controlled study to investigate these observations. Additionally, this work lends support to model predictions indicating that anti-tick vaccination would significantly reduce the carrying capacity for ticks in the ecological system (Miller et al 2012).

4. SUMMARY AND CONCLUSION

4.1 Summary

The Cattle Fever Tick (CFT), *Rhipicephalus (Boophilus) microplus*, has been the root of large economic losses for cattle breeding industries, particularly in tropical and subtropical regions of the world. In the United States alone, losses were estimated at $130.5 million/year, before the eradication program in Texas began in 1906. Vaccination against ticks using the gut protein Bm86 has been shown to be somewhat effective against this malady, however much remains to be studied to develop a procedure that can completely eradicate the CFT.

This study integrated anti-tick vaccination, with systemic acaricides that may generate additive or synergistic effect that can prove effective in the elimination of the CFT. The main objective of this study was to determine if an interaction exists between anti-tick vaccination with Bm86, and the use of the systemic acaricide moxidectin. The second objective of this study was to compare the efficacy of Bm86 vaccine, for suppression of *R. microplus* using an *in vitro* technique.

The first component of this work consisted of a field study with twenty calves, 6-12 months old (*Bos taurus*), randomly divided into four treatment groups of five animals each, and held in a 40-acre pasture in South Texas. The four treatment groups were divided as follows: group 1 recieved Bm86 vaccine; group 2 received moxidectin injections; group 3 received a combination of Bm86 vaccine and moxidectin injections; and finally, group 4 served as the control experiment (non-treated). All animals were infested with 125 mg of *R. microplus* larvae, twice a week. Standard females ticks (0.4 to 8 mm diameter) were counted from the left side of each animal, twice per week. Blood was collected from all the study groups, to determine the

pharmacokinetics of moxidectin. Additionally, half of the serum extracted was used to determine Bm86 antibody levels by ELISA test.

We observed a 91% decline in the index of fertility of standard females with Bm86 treatment alone, in comparison with the control group. The index of fertility was reduced by 81% for moxidectin and 97% for the combination of the vaccine and acaricide injections across the entire period of observation of this part of the study. There appears to be an added benefit between anti-tick vaccination, in combination with moxidectin. The additional 6% control observed in the vaccine-moxidectin combination was accounted for in the reduced the hatch of oviposited eggs.

For the second component of this study, *in vitro* feeding of ticks with IgG from vaccinated and non-vaccinated animals with and without the presence of acaricide, was conducted for comparison to the field study. For this part, 15 groups gave results that could be measured statistically. Four groups that were compleated to measure Bm86 in comnination with acaricide need to be repeated because the mortality in the control groups was too high. In all the groups, ticks that were fed 200 µL of heparinized blood showed a high survival rate compared to the ones that were fed 400 µL or 200 µL of EDTA blood. All ticks that were fed 100ppm fluazuron died with only 11% of the ones that were fed 1ppm of fluazuron survived. Ticks fed with moxidectin in concentrations of 0.6, 6, 60, and 600ppm. The 0.6ppm group showed a mortality of 50% with only 77% of the egg mass hatched. The moxidectin 6ppm group showed a survival of 70% with only 21% of the egg mass hatched. The last group that present any hatchability or survival results was the 6ppm where only 10% of the ticks survive and 3% of the egg mass hatch. There is a clear reduction of blood intake by ticks when the concentration of moxidectin is increased in the blood.

The results of this project provides the Cattle Fever Tick Eradication Program with an alternative method to combat CFT zone in South Texas. With the suspension of spray-box use, the combination of systemic acaricide with anti-tick vaccination can become a viable and economical means to help farmers in the cattle industry. Cattle fever ticks are one of the biggest problems the US cattle industry is facing right now and this research will help maintain the eradicaton program for future years.

4.2 Conclusion

For the in-vitro feeding objective, it is possible conclude that the ticks on the day 5 and 6 of engorgement of 40 mg or more are the adequate size of *R. microplus,* to complete in vitro feeding. The mouth parts of the ticks need to be clean of debris, serum residues and blood before placing them upsidedown on the tape. The most effective anticoagulant tested in this study was heparin fed in 200 ul of blood. Acetone worked as an acaricide carrier in fresh blood and did not affect tick health, fecundity or blood consumption.

To measure the effect of fluazuron on *R. microplus* by bioassays a good concentration is 1ppm. This group had a female survival rate of 60% and the effect as growth regulator was observed in the 11% egg hatch. In terms of moxidectin, a good concentration was 0.6 ppm, were 50% of the ticks tested survived with 60% egg hatch. The moxidectin effect in the GABA-A and glutamate-gated chloride ion channels was observed with the reduction of blood intake on the ticks when the concentration of moxidectin was increased in the blood.

The main objective of this study was to determine if a synergistic interaction exists between anti-tick vaccination with Bm86 and the use of acaricide moxidectin injectable. In this part of the study all 20 animals used remained in good health. In terms of total standard females counted on the cattle, females collected, and female weight, no significance difference was

observed between the treated groups. In cumulative numbers of engorged females throughout the entire study higher control was observed with Bm86 and the combination of Bm86/moxidectin followed by moxidectin treatment. The cumulative number of live larvae throughout the study was a significantly different between treatment groups. A control of 81.3% of larvae was observed with the combination of Bm86/moxidectin, a synergistic interaction exists between anti-tick vaccine Bm86 and moxidectin. This was expressed in the lavae production, the combination of both treatments affected the fecundiy of the eggs and resulted in a lower number of larvae produced. This combination can be used as a new technique in the cattle fever tick quarantine zone in Texas and in the cattle farms of Puerto Rico for the suppression of ticks on cattle.

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