TARGET VALIDATION OF A MYOKININ RECEPTOR FROM THE

SOUTHERN CATTLE TICK *Boophilus microplus* (CANESTRINI)

A Thesis

by

MARIA ADYLIA BLANDON

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

MASTER OF SCIENCE

May 2006

Major Subject: Entomology

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Approved by:

Chair of Committee,	Patricia V. Pietrantonio
Committee Members,	Pete D. Teel
	Michael Longnecker
Head of Department,	Kevin M. Heinz

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ABSTRACT

Target Validation of a Myokinin Receptor from the Southern

Cattle Tick *Boophilus microplus* (Canestrini). (May 2006)

Maria Adylia Blandon, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Patricia V. Pietrantonio

A novel approach to control *Boophilus microplus* is to disrupt the physiological function of an endogenous myokinin receptor of this tick that was previously cloned in our laboratory. To test the hypothesis that this myokinin receptor might be a suitable target for development of a novel acaricide, this target was validated by immunological disruption.

A mixture of peptides, corresponding to the sequence of the extracellular loops of this receptor which were synthesized and linked to a carrier protein, was injected into Hereford cattle to induce an immunological response. Immunological tests (ELISA) were developed to test the sera of these animals for antibody titers. The data were analyzed using a randomized block split plot design and were compared between the control (calves numbers 407, 408, 427, 436, and 438) and peptide-injected calves (calves numbers 417, 420, 421, 426, and 435). A gradual increase of antibody production was observed with the peptide-injected calves with bleed 4 showing the highest absorbances.

Control calves and peptide-injected calves with high antibody titers were challenged with approximately 20,000 tick larvae at the USDA Cattle Tick Research laboratory. The tick challenge test determined that disruption of the receptor function produces a detrimental effect on tick physiology (development, feeding and

iv

reproduction) by looking at percentage of molting, time of survivorship, number of ticks

dropped, weight of fed females, weight of egg masses, and blood meal conversion. The

results, which were analyzed by a contingency table and a 2 sample T-test, did not

support the hypothesis that the sera ingested from the peptide-injected cattle would cause

a detrimental effect on tick physiology. There was no statistical significance in the

percentage of metanymphs molting from peptide-injected calves versus control calves (p

= 0.282) and in the time of adult survivorship. A statistical inference could be made

about the number of ticks that dropped since four calves died of bovine babesiosis after

the metanymphal collection. There was no statistical significance in the weight of fed

females (p = 0.061), weight of egg masses (p = 0.885), and bloodmeal conversion (p =

0.312) from peptide-injected calves versus control calves.

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My sincerest gratitude goes to the members of Dr. Pietrantonio's laboratory: Chris Jagge, Terry Junek, and Suparna Taneja-Bageshwar. They have helped me along the way with technical knowledge, encouragement, and emotional support. They were always ready to lead an ear or a shoulder to cry on, and I am proud to call them my friends.

TABLE OF CONTENTS

Page

LIST OF FIGURES

FIGURE   Page

LIST OF TABLES

CHAPTER I

INTRODUCTION

Overview

The Southern Cattle tick, *Boophilus microplus* (Canestrini), is a worldwide pest found in tropical and subtropical areas around the world (Nunez et al., 1985). This species is a one- host tick, this means that it feeds and undergoes the larval, nymphal, and adult stages on one host, preferably cattle (Oliver, 1989). Once the engorged adult female is ready to oviposit, she detaches from the host and lays one large egg mass, then death occurs. Once the eggs hatch, the larvae start questing for a host. The amount of time usually required to develop from unfed larvae to engorged adult female is approximately three weeks (Jongejan & Uilenberg, 2004).

This tick is a primary vector of *Babesia bigemina* (Bock et al., 2004), a protozoan that is the causative agent of bovine babesiosis, also known as Texas Tick Fever. Some of the main symptoms of Texas Tick Fever may include a high fever (>40ºC), urinating blood, anorexia, anemia, listlessness, and death (Bock et al., 2004). When a tick feeds on an infested animal, the *Babesia* parasites cross the midgut of the tick entering the hemolymph. Three types of transmission can transmit this protozoan: (1) direct, tick to host through tick salivary glands; (2) transovarial, from mother to eggs, and (3) transtadial, one life stage to another.

This thesis follows the style and format of *Insect Molecular Biology.*

The southern cattle tick is a major economical pest, causing tremendous financial loss to cattle producers worldwide (Angus, 1996). The prevalent areas of tick-related economic loss include Australia, Mexico, Central and South America, southern Africa, and parts of Asia (Baxter & Barker, 1999). In Queensland, the cost of cattle tick infestation was about $141 million in 1999 (Jonsson et al., 2001). This financial loss is also contributed by the cost of pesticides needed to prevent infestations and the decrease in milk and beef production caused by ticks when the pesticides are ineffective. This tick also damages the hides of cattle, since they tend to feed where there is good leather potential (Jongejan & Uilenberg 2004). This tick is also a major concern at the Mexico-Texas border. Since this tick is "endemic to Mexico" (Li et al., 2005) there is a threat of reintroduction of this pest to Texas. In Texas, the estimated cost of physical losses and cost associated with controlling this species by means of mandatory dipping programs is $1,322,724,132 (Dietrich & Adams, 2000).

The method used to control *Boophilus microplus* employed by the United States Cattle Fever Tick Eradication Program is the application of chemical acaricides such as organophosphates, specifically coumaphos at a concentration of 0.3% (George, 2000) in dipping vats. There are tick riders that inspect the cattle that enter Texas for ticks. If ticks are found then the cattle will have to be dipped in the vats and quarantined for at least ten days. If after ten days there are no ticks present then the cattle will be allowed into the U.S. If there are ticks present, the cattle will be dipped a second time but will not be allowed in the U.S. (Davey et al., 2004).

Other methods of control include using other classes of acaricides, and breeding cattle that have naturally acquired resistance to tick infestations. Other classes of acaricides that are commonly used in other places include carbamates, synthetic pyrethroids, amidine compounds, and acarine growth regulators. These and experimental acaricides are routinely tested by means of various bioassays for efficacy. The acaricidal activity of benzimidazole-carbamate was compared *in vitro* and *in vivo* against permethrin and amitraz (White et al., 2005). This benzimidazole-carbamate was more potent than permethrin in some species of ticks but less potent that permethrin in others. The efficacy of eprinomectin, a pour-on that could be a possible alternative to dipping, was tested for control of *Boophilus microplus* (Aguirre et al., 2005). The main effect of this acaricide was a reduction in the number of engorged females that dropped. Since most acaricides have oil adjuvants and are applied to cattle topically or in dipping vats, one problem that arises is the presence of residues in the milk and meat of the treated cattle (de la Fuente et al., 1998).

Acaricide resistance

The problem with using acaricides is that *Boophilus microplus* is known to develop resistance to these acaricides. Resistance to two organophosphates, coumaphos and diazinon was reported in Mexican strains of *Boophilus microplus* (Li et al., 2003). The bioassay was the standardized Food and Agriculture Organization (FAO) larval packet test, consisting of Whatman filter paper permeated with different dilutions of pesticide to which larvae are exposed (Miller et al., 1999). Probit analysis showed

significant cross-resistance between coumaphos and diazinon. Amitraz resistance was also detected and characterized in this tick (Li et al., 2004). Amitraz has been used in countries where high resistance to organophosphates and pyrethroids exist. A FAO larval packet of different dilutions of amitraz was used to test resistance. It was found that 11 out of the 15 Mexican strains tested showed a low-order resistance to amitraz (Li et al., 2004). Recently resistance to carbaryl was diagnosed in the Mexican Strain of the *Boophilus microplus* (Li et al., 2005). A FAO packet test with different dilutions of carbaryl was performed here as well. It was concluded that there was more susceptibility to carbaryl than to coumaphos or diazinon in the tick strains from the quarantine zones from Texas. The growing problem of resistance needs to be addressed by developing acaricides against novel targets in these ticks.

Need for novel acaricide targets

One target that merits validation is the myokinin receptor from *Boophilus microplus*. Previous cloning, transcriptional expression, and functional analysis of this receptor was conducted by Steven P. Holmes at Texas A&M University in Dr. Patricia V. Pietrantonio's laboratory (Holmes, 2003). This receptor is a G protein-coupled receptor located on the plasma membrane of cells that is hypothesized to mediate the signal transduction cascade involved in myotropic and diuretic activity (Holmes et al., 2000). This myokinin receptor belongs to the Family A of G protein- coupled receptors known as the Rhodopsin/B2 adrenergic receptor-like family (Holmes et al., 2003).

G protein-coupled receptors have a predicted structure of seven transmembrane regions with the N-terminus and three loops outside the cell. Inside the cell are the C-terminus and two loops. Previous studies have provided evidence that the N-terminus and extracellular loops are involved in the binding of peptide ligands (Strader et al. 1994; Gudermann et al. 1995; Schwartz & Rosenkilde 1996). When the ligand binds to the G protein-coupled receptor, the receptor undergoes a conformational change that activates a G protein. The G protein α subunit dissociates from the βγ dimer and interacts with either adenylate cyclase or phopholipase C inducing a signal cascade such as production of cAMP or release of intercellular calcium stores, respectively (Sakmar et al., 2002). This system uses second messengers to amplify the signal transduced by the receptor upon ligand binding.

The *B. microplus* receptor cloned is a leucokinin-like myokinin receptor. The insect neuropeptide kinin and kinin analogs produced a functional response in the receptor (Holmes et al., 2003). As of now, the endogenous ligand has not been identified in the ticks but research on kinins has been done in insects and other invertebrates (Torfs et al., 1999). Attempts to identify the endogenous ligand resulted in the identification of a different neuropeptide, periviscerokinin (Neupert et al., 2005). The size of leucokinins is 6-15 amino acids in length, and were first discovered in *Leucophaea maderae*, a cockroach (Holman et al., 1986a,b). The leucokinin's function was to stimulate the hindgut contractions of the cockroach, and it was concluded that it has myotropic activity. It has been tested electrophysiologically that the paracellular permeability to chloride in mosquito Malpighian tubules increased when leucokinins

were added (Wang et al., 1996). Using a leucokinin photoaffinity analogue technique, a leucokinin-like binding protein in the Malpighian tubule of the *Aedes aegypti* mosquito was characterized (Pietrantonio et al., 2000) and the kinin receptor from mosquito was cloned, expressed and shown to respond to the three *Aedes aegypti* kinins (Pietrantonio et al., 2005).

Tick physiology

Water balance is an important physiological process involved in tick feeding, development, and reproduction (Sonenshine, 1991). The main organs of osmoregulation in ticks are the salivary glands, excreting 70% of the water from a bloodmeal (Bowman & Sauer, 2004). This water is eliminated through the salivary glands by "transformed "F" cells in the type III acini" (Sonenshine, 1991). As the tick is feeding, the rate of fluid secretion of the salivary gland increases allowing the tick to concentrate the bloodmeal by returning excess water and ions to the host (Sauer et al., 1995). The salivary glands are hypothesized to be controlled by the neurotransmitter dopamine (Kaufman, 1976). Following the application of dopamine to isolated salivary glands from partially fed female adult ixodid ticks, fluid secretion of the salivary glands was induced (Kaufman, 1976).

Osmoregulation across the gut wall supplies the excretory organs with excess water and ions (Agbede & Kemp, 1987). *Boophilus microplus* ticks that dropped from cows after a bloodmeal were collected, dissected, and fixed for study under an electron microscope. After dehydrating and re-hydrating the gut dissected fragments, there was

evidence to suggest it is the basophilic cells of the gut epithelium that draw water from the lumen and pass of into the hemolymph.

The structure of the ovary has been morphologically and histologically studied by removing the ovaries, fixing, and staining them with bromophenol blue. This confirmed the structure of the ovary as a "large U-shape" structure (Saito et al., 2005). Most of the bloodmeal of ixodid females is digested rapidly and is utilized for egg production (Oliver, 1989). The oogenesis process in ticks begins during or immediately following the last nymphal feeding (Soneshine, 1991). In Dr. Pietrantonio's lab, the myokinin receptor transcript was found in the synganglion, salivary glands, ovaries, midgut, hindgut, and Malpighian tubules (Holmes et al., 2003) and this suggested the receptor could be a promising target to disrupt the function of various organs.

Receptor target validation

Some of the main approaches used for target validation for pesticides and medicines are pharmalogical, genetic, and immunological. The pharmalogical approach for target validation uses agonists and antagonists to produce either a normal response from the target when using agonists, or blocking the normal response when using the antagonist. The genetic approach uses genetic mutations such as deletions or insertions to knockout the target gene (prevent transcription of mRNA) that is being validated, such as it is routinely done in *Drosophila melanogaster*. This approach can also use RNA interference, which elicits the degradation of the messenger RNA, which in turns prevents translation of proteins (Blank & Bind, 2005).

The approach chosen to validate the tick myokinin receptor as a potential target for acaricide development was the immunological disruption of this receptor (Willadsen et al., 1989). This approach elicits the host immune response and the production of antibodies against the receptor. We chose receptor fragments that correspond to the predicted extracellular regions and partial transmembrane regions of the receptor as antigens. It is expected that these antibodies when ingested by the tick will bind to the cell membrane-bound receptors and cause a disruption in the tick physiology (Willadsen & Kemp, 1988). In the past, the strategy of inducing resistance of cattle to ticks has included vaccinating the cattle with tick antigens (Opdebeeck et al., 1988). One such vaccine currently utilized is GAVAC™, (Heber Biotec S.A., Havana Cuba). It is comprised of a recombinant Bm86 glycoprotein midgut antigen preparation (Redondo et al., 1999). The myokinin receptor and the Bm 86 receptor are cell surface receptors, found embedded in the plasma membrane. The extracellular regions of the receptor are in contact with the hemolymph while the intracellular regions are in contact with the cytoplasm. After the tick has ingested the antibodies, the tick gut cells lyse (Gonzales et al., 2004). The population of the tick decreases due in part to the reduction of fecundity that this vaccine induces (Rodriguez et al., 1995).

Research objectives

The goal of this research was to validate the *B. microplus* myokinin receptor as a novel target for acaricide development by means of immunological disruption. The two specific objectives were to: (1) Induce an immunological response in the primary host of

this tick (*Bos taurus,* Hereford) to antigens corresponding to the extracellular loops and partial transmembrane regions of the myokinin receptor and; (2) Determine whether receptor disruption affects tick feeding, development, or reproduction by performing a tick challenge on immunized cattle.

CHAPTER II

MYOKININ RECEPTOR ANTIGEN SYNTHESIS AND APPLICATION

OF ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) TO

DETECT BOVINE ANTIBODIES AGAINST RECEPTOR FRAGMENTS

Introduction

This project aims at validating the myokinin receptor as a target for acaricides by disrupting the receptor's normal function by immunological means. One way to achieve validation is to interfere with the ligand binding site. In this project, antibodies against the receptor will serve as the antagonists. The hypothesis is that blocking of the binding site will prevent the normal physiological processes of the receptor to occur, thus causing detrimental damage to the tick. This tick receptor is expected to produce an immunological response in cattle since this receptor is invertebrate-specific (Nässel, 1996).

Peptides corresponding to the receptor extracellular regions and to fragments of the transmembrane regions will be utilized as the antigen for antibody synthesis (Fig. 1). The antigen synthesis for antibody production in this experiment was modeled after the antigen synthesis and antibody production against the neuropeptide Y receptor, which is similar to the myokinin receptor (both in the Family A of G protein-coupled receptors) (Wieland et al., 1998). In the aforementioned example there were 18 peptides designed corresponding to the extracellular loops of the Y-Y1 receptor which were conjugated

with bovine serum albumin (BSA) and injected into rabbits (Wieland et al., 1998), and

an ELISA was performed to test the antibody titers.
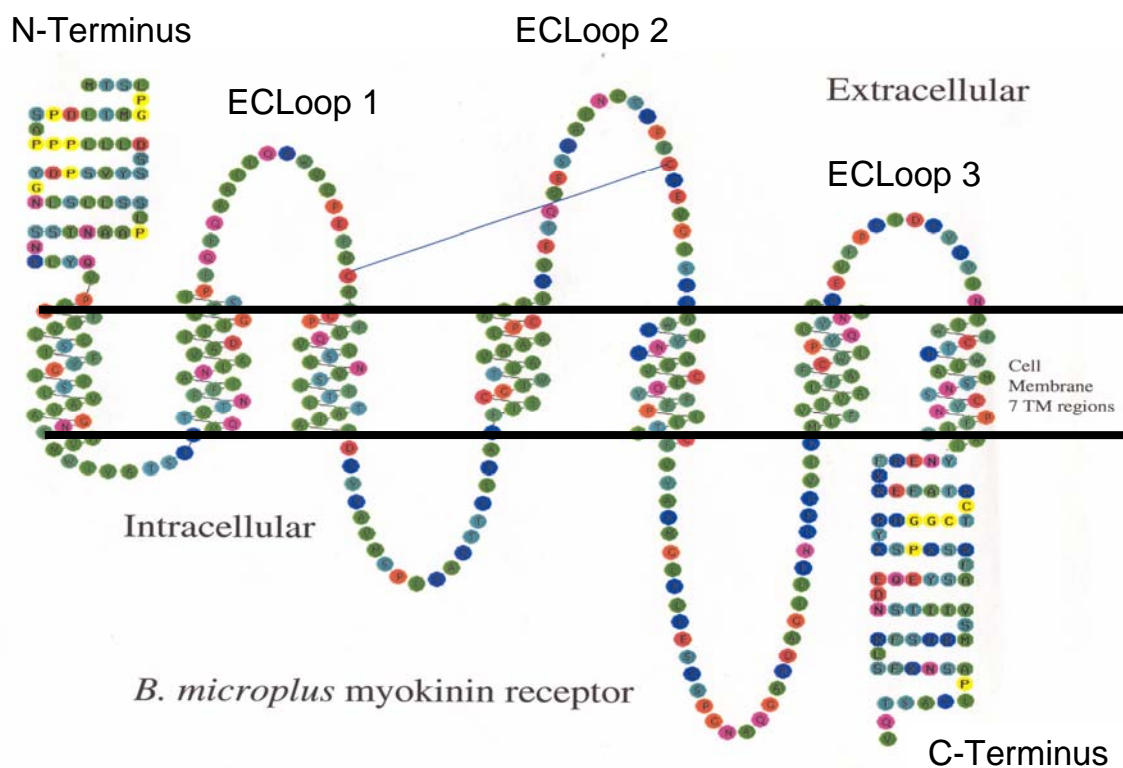
Figure 1. A "snake diagram" of the *Boophilus microplus* myokinin receptor. Adapted after Steven Holmes used Residue-based Diagram editor (RbDe), a web application found at http://icb.med.cornell.edu/services/rbde to create the predicated structure of the receptor. There are seven transmembrane regions. The N-terminus and three extracellular loops are outside the membrane, while the C-terminus and two intracellular loops are inside the membrane. EC codes for extracellular.

This chapter presents the hypothesis that myokinin receptor fragments are immunogenic in cattle. For this, the antibody titers in calf serum will be estimated by ELISA. The statistical analysis indicates that some, but not all of the receptor fragments are immunogenic in the calves.

Methods

Experimental animals

Twelve *Bos taurus* castrated calves of 6-9 months in age were purchased by Dr. Chris Skaggs (Dept. of Animal Science, TAMU, Beef Center, manager) from Sutor Hereford Farms in Kansas, were transported to the Texas A&M Beef Center utilizing procedures discussed under AUP number 2003-0227, and maintained at this location from December 2004 to May 2005. The calves were purchased in Kansas to obtain cattle that was naïve with respect to contact with *B. microplus*.

Antigen synthesis

A total of 13 peptides were designed that correspond to the extracellular regions of the cloned *Boophilus microplus* leucokinin-like peptide receptor (GeneBank/EMBL accession number AF228521) (Fig. 2, Table 1). The peptides were 12- to 16- residues long with 3 residues overlapping (Wieland et al., 1998). Some factors taken into consideration to design these peptides were the percentage of hydrophobic residues of the peptides (needed to be less than 50%), that the peptides themselves did not make secondary structures, and that there should be a least one charged residue for every five

amino acids (Collawn & Paterson, 1989). A peptide calculator found on the Sigma

Genosys webpage was utilized for those calculations. A cysteine residue was added to

the C-terminus of peptides 1-7, and 12, and to the N-terminus of peptides 9 and 11 to

ensure linkage to Keyhole limpet hemocyanin (KLH). KLH is a non-immunogenic

carrier protein; a carrier protein is needed for the immune system of the calf to recognize

these peptides, since these peptides are too small to be immunogenic without being

presented by a carrier protein.
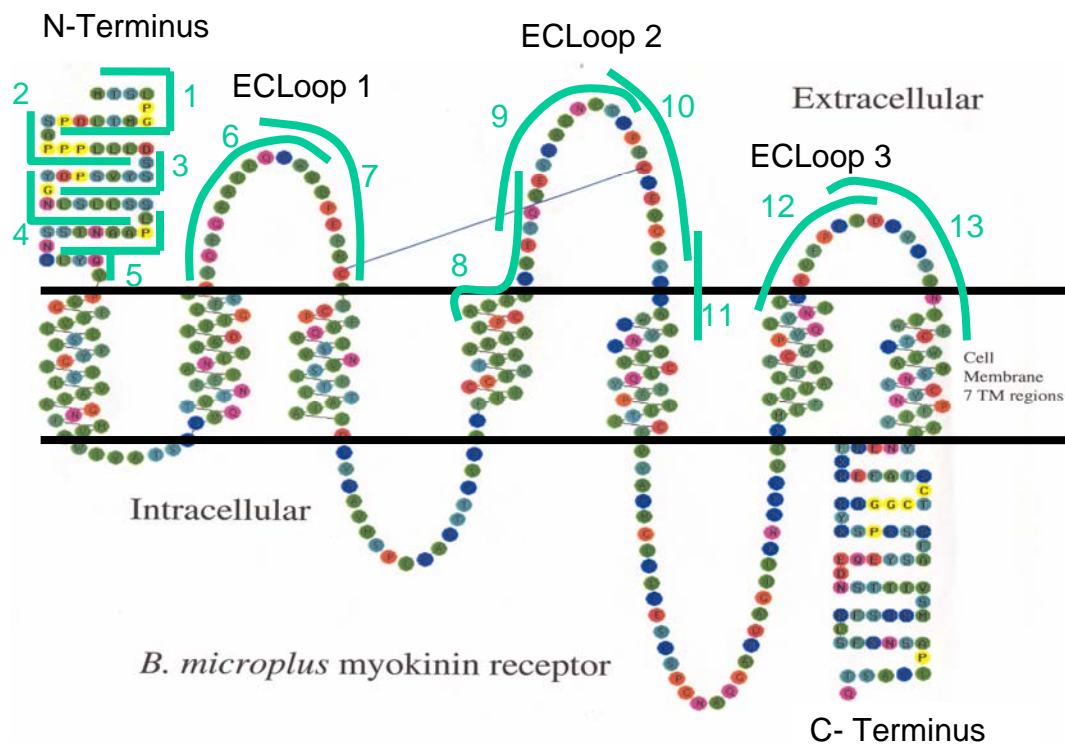
Figure 2. A "snake diagram" of the *Boophilus microplus* myokinin receptor illustrating
the 13 synthesized peptides corresponding to the extracellular regions. The regions are
shown as green lines and numbered respectively. The ligand is hypothesized to bind to
the extracellular regions.

Table 1. Sequences of the 13 peptides designed that correspond to the extracellular regions and partial transmembrane regions of the tick myokinin receptor. The underlined residues are the overlapping residues between two contiguous peptides. The C in bold are the added cysteine residues needed for KLH conjugation.

Name	Peptide Number	Sequence Location	**Sequence**
N-Terminus 1	Peptide 1	1-12	MTSLPGMTLDPS**C**
N-Terminus 2	Peptide 2	10-21	DPSAPPPLLLDS**C**
N-Terminus 3	Peptide 3	19-30	LDSSYVSPDYGN**C**
N-Terminus 4	Peptide 4	28-39	YGNLSLLSSLPA**C**
N-Terminus 5	Peptide 5	37-50	LPAANISSNKLYQV**C**
Loop 1-1	Peptide 6	111-122	FQFQAALLQRWV**C**
Loop 1-2	Peptide 7	118-129	LQRWVLPEFMCA**C**
Loop 2-1	Peptide 8	188-199	CALALRVETQVE
Loop 2-2	Peptide 9	196-207	**C**TQVESHALNLTK
Loop 2-3	Peptide 10	205-216	LTKPFCHEVGIS
Loop 2-4	Peptide 11	214-225	**C**GISRKAWRIYNH
Loop 3-1	Peptide 12	295-306	YNILREVFPKID**C**
Loop 3-2	Peptide 13	304-319	KIDKYKYINIIWFCTH

The peptides were purchased from Sigma Genosys (The Woodlands, TX) at 95% purity. A total amount of 5mg per peptide was purchased, of which 2 mg was conjugated with KLH, from Sigma Genosys (The Woodlands, TX). Each conjugated peptide was reconstituted using 80% acetonitrile, 0.01% trifluoroacetic acid. Peptides were aliquoted 500 µg in respective Eppendorf tubes. Peptides were dried in a SpeedVac Plus (Savant SC110A) at low temperature (ambient) and stored at -80 ºC until use. To coat the ELISA plates, 20ml of coating buffer was needed for one plate for each 96 well to have 100 µl of buffer. One 500 µg aliquot tube was resuspended with 80% acetonitrile, 0.01% trifluoroacetic acid and was re-aliquoted 20 µg in respective Eppendorf tubes and dried the same as before.

Immunization regime

Immunizations for animals consisted in injecting a mixture of the 13 peptides, each conjugated to KLH for the treatment animals, and for control animals in injecting KLH only. Once the peptides were synthesized, cattle injections were with the conjugated peptides or KLH only (controls) in adjuvant. The first injection used Ribi's adjuvant that induces a humoral immune response and has a minimal inflammatory response (Rudbach et al. 1988). The subsequent injections used Freund's Incomplete adjuvant. The amount of peptide injected gradually increased so the calves' immune system had time to adjust (Table 2).

The first immunization of the peptide-injected calves consisted of 100μg of KLH-conjugated peptide (8μg of each peptide and 8μg of carrier protein) mixed in Ribi's adjuvant (Corixa Co., Seattle, WA) injected into 6 calves. The other 6 calves (controls) were injected with 100μg of KLH (Sigma; St. Louis, MI) only mixed in Ribi's adjuvant. The Ribi's adjuvant was reconstituted with saline. The immunizations were given intramuscularly in the neck using an 18-gauge, 1-inch hypodermic needle.

The second immunization consisted of 100μg of KLH-conjugated peptide (8μg of each peptide) in saline mixed 1:1 with Freund's Incomplete adjuvant (Sigma, St. Louis, MI) to treatment animals and 100 μg of KLH only to control animals. The third immunizations consisted of 300μg of KLH-conjugated peptide (23μg of each peptide) in saline mixed 1:1 with Freund's Incomplete adjuvant to treatment animals and 300μg of KLH only to control animals. The fourth, fifth, and sixth immunizations consisted of

500μg of KLH-conjugated peptide (38μg of each peptide) in saline mixed 1:1 with

Freund's Incomplete adjuvant to treatment animals, and 500μg of KLH only to control

animals. The intervals between immunizations were 4 weeks for all calves (Table 2).

One calf (Calf #415) did not receive immunizations four, five, and six due to unruly

behavior.

Before each immunization, the calves were bled by under the tail bleed technique

and blood was collected in Vacutainer™ (BD, Franklin Lakes, NJ) tubes. After

coagulation of the blood at 4°C overnight, the serum was decanted into 15 ml conical

tubes. The serum was spun 15 min at 500 Xg (1500 rpm) in an IEC Centra CL2 table

centrifuge, sodium azide was added to obtain a 15mMolar concentration in serum, and

the serum was filtered manually using a .2μm syringe filter, and stored at -80 °C.

Table 2. Schedule of immunization regime. The procedure, date
of procedure, the amount of peptide (mixture of all 13 peptides only, not
including KLH), and the location where the procedures took place is shown.

Procedure	Date	Amount of Peptide	Location
Bleed 0	1/10/05		A&M Beef Center
Injection 1	1/10/05	100 μg	A&M Beef Center
Bleed 1	2/17/05		A&M Beef Center
Injection 2	2/17/05	300 μg	A&M Beef Center
Bleed 2	3/24/05		A&M Beef Center
Injection 3	3/24/05	300 μg	A&M Beef Center
Bleed 3	4/28/05		A&M Beef Center
Injection 4	4/28/05	500 μg	A&M Beef Center
Bleed 4	5/12/05		A&M Beef Center
Injection 5	5/12/05	500 μg	A&M Beef Center
Injection 6	6/09/05		USDA Tick Lab
Bleed 5	6/16/05	500 μg	USDA Tick Lab

Note: An equal amount of KLH is used to conjugate the peptides; thus
the total amount of protein is double the number on the table.

Enzyme-linked immunosorbent assay (ELISA)

Sera from the peptide-injected and control calves was tested for antibody production against the peptides using ELISA. ELISAs were conducted using Costar 96-Well Flat-Bottom EIA plates (Bio-Rad; Hercules, CA). Controls for each step of the ELISA process were performed on every plate (Crowther, 2001). The controls were as follows: one row with no conjugated peptide, one row with no serum, and one row with no secondary antibody (Fig. 3A). Each plate was coated with 1μg of KLH conjugated peptide mixture/ml in a 50 mM carbonate coating buffer containing 15.01 mM Na_2CO_3, 34.88 mM $NaHCO_3$ pH 9.5 applying 100μl per well and incubating overnight at 4°C in a humid chamber. The plates were washed twice with 1X phosphate buffer saline (PBS) containing 136.9 mM NaCl, 2.68 mM KCl, 1.01 mM Na_2HPO_4, 1.76 mM KH_2PO_4 pH 7.4. A blocking buffer of PBS with 5% rabbit serum (Jackson ImmunoResearch Laboratories, West Grove, PA) (200μl/well) was added to the plates and incubated at room temperature for 2 h. Four dilutions of the serum in blocking buffer (1/50, 1/100, 1/500, 1/1000) were added to the plate (200μl/well) and incubated overnight at 4°C in a humid chamber. The plates were washed three times with PBS containing .05% Tween 20 (PBST). One hundred μl of rabbit anti-bovine IgG conjugated with alkaline phosphatase (Jackson ImmunoResearch Laboratories,West Grove, PA) diluted in 1/10000 in blocking buffer were added to each well. Incubation was at room temperature for 2 h. The plates were washed three times with PBST. One hundred μl of Alkaline Phosphatase Yellow (p-NPP) Liquid Substrate for ELISA (Sigma; St. Louis,

MI) was added to each well. The optical density at 405 nm was measured with the ELISA VERSA Max plate reader from Molecular Probes (Eugene, OR) and the readings were for 30 min at 2 min intervals. A positive result yielded a yellow color on the plate (Fig. 4).

ELISA to detect calves titer toward individual peptides

In order to determine if all peptides or only few of them were antigenic in cattle a different type of ELISA was conducted. The ELISA plates were coated with 1 μg of each single KLH- linked peptide instead of 1 μg of the 13 KLH-linked peptide mixture to which KLH-linked peptide(s) were involved in the binding of the anti-receptor antibodies from the calves. The control for the ELISA experiment was one column with no serum. Only one dilution of serum (1:100) of each of the peptide-injected calves (#417, #420, #421, #426, and #435) from two bleeds (Bleed 4 and Bleed 0) was used for primary antibody binding step (Fig. 3B). Bleed 4 was selected for this because it showed a higher antibody titer against the peptide mixture than Bleed 5.

A)

	Control Bleed 0	Control Test Bleed	Peptide Bleed 0	Peptide Test Bleed
No peptide				
No serum				
No secondary antibody				
1:50 serum dilution				
1:100 serum dilution				
1:500 serum dilution				
1:1000 serum dilution				

B)

	No serum	Peptide Bleed 0	Peptide Bleed 4
Peptide 1			
Peptide 2			
Peptide 3			
Peptide 4			
Peptide 5			
Peptide 6			
Peptide 7			
Peptide 8			

Figure 3. A schematic of an ELISA plate for testing antibody production. (A) A peptide mixture was used as coating antigen. The no peptide, no serum, and no secondary antibody rows are the controls for the ELISA process. The Bleed 0 and Control Bleeds are the negative controls for this project. The serum dilutions for Bleeds 2 and 3 were 1:10, 1:100, and 1:1000. The serum dilutions for Bleeds 4 and 5 were 1:50, 1:100, 1:500 and 1:1000. Three replications of each plate for each of the bleeds were done. (B) Single peptides were used as coating antigens and the serum from Bleed 4 and Bleed 0 peptide-injected calves was the primary antibody. Bleed 0 is control for specific binding for the peptides and no serum lane is the control for the ELISA process. Peptides 9-13 were coated on a separate plate because there are only 8 rows in the plates.
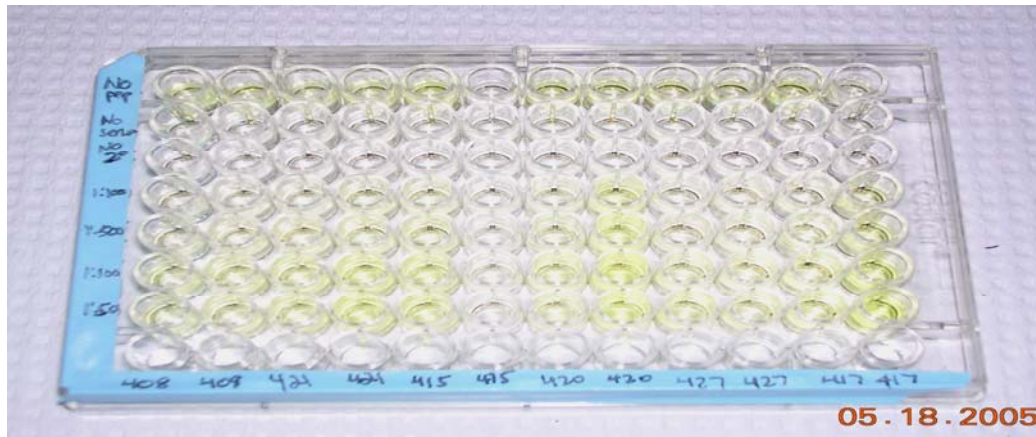
Figure 4. An ELISA plate after the detection step. A yellow color indicates a positive result.

Data analyses

Treatments were (1) control bleed 0; (2) control test bleed; (3) peptide-injected bleed 0, and (4) peptide-injected test bleed. Data for these 4 treatment levels were arranged as a "randomized block split plot design" with plates as the blocking variable. The treated vs. control was the whole plot factor and the specific bleed number to be tested vs. control (bleed 0) was the split plot factor. ANOVA procedure in the Statistical Package for the Social Sciences (SPSS) program was used to test the following 3 null hypothesis: (H_o 1) there was no statistical difference between control bleed 0 and control test bleed, (H_o 2) the absorbances of peptide-injected test bleed were equal to those of the peptide-injected bleed 0 and (H_o 3) the absorbances of peptide-injected test bleed were equal to those of the control test bleed. The means and 95% confidence intervals for each treatment were compared for significant statistical differences. For the analysis of the ELISA for single peptides the treatments were (1) peptide-injected bleed 0 and (2) peptide-injected bleed 4. For the statistical analysis of the absorbances of ELISAs from

the peptide-injected Bleed 0 and Bleed 4 sera using the 13 individual KLH-bound

peptides as coating antigens, ANOVA was used to testing the following 2 null

hypothesis: (H_o 1) there was no statistical difference between peptide-injected bleed 0

and peptide-injected bleed 4 and (H_o 2) there was no difference between the thirteen

different peptides for bleed 4. The means and the 95% confidence intervals for each

treatment were compared for significant statistical differences. For these tests the effect

of individual animals has not taken into account as a source of variation so that this

analysis answers which peptides across animals were more antigenic.

Results

To test the hypothesis that the calves were producing antibodies to these

conjugated peptides, an indirect ELISA (Enzyme-Linked ImmunoSorbent Assay) test

was conducted. The theory of an indirect ELISA test is that when an antigen is bound to

a plate a primary antibody will attach to the antigen, and a secondary antibody that is

linked with an enzyme will bind to the primary antibody. A substrate that will react to

the enzyme will produce a response, such as a color change and that response will be

measured.

Tables 3, 4, 5, and 6 show the mean value of absorbance and the 95% confidence

intervals of the absorbances from the ELISAs from the control and peptide-injected

calves. There is no statistical evidence to indicate that the absorbances of the Control

calves, Bleed 0 and the Peptide-injected Bleed 0 (pre-immune) calves differ. In Bleed 2,

we cannot reject the null hypotheses, that the absorbances of Bleed 2 are equal to Bleed

0, and that the absorbances of peptide-injected sera are equal to those of the control sera for all serum dilutions. In Bleeds 3 and 4, we can reject the null the hypotheses that the absorbances of Bleeds 3 and 4 are equal to their respective Bleed 0, and that absorbances of sera from peptide-injected calves are equal to the control sera for all serum dilutions. Bleed 3 and Bleed 4 present statistical evidence that the absorbances of the peptide-injected test bleeds are greater than the peptide-injected bleed 0. For Bleed 5 we can only reject the null hypotheses that the absorbances of Bleed 5 are equal to Bleed 0, and absorbances of peptide-injected sera are equal to the control sera for sera dilutions 1:50 and 1:100. This evidence shows that the antibody production in the peptide-injected calves in the test bleeds (after several immunizations) was produced against the conjugated peptides that were injected and not due to an innate humoral response of the calf.

The sera of Bleeds 2, 3, 4, of the peptide-injected cattle showed a gradual increase in antibody production with an increase of conjugated peptide injected. For Bleed 2, 300µg of conjugated peptide was injected resulting in no statistical difference in the absorbances of the sera with respect to the control animals. Similarly, 300µg of conjugated peptide was injected for Bleed 3, resulting in a statistical difference between the absorbances of the sera from peptide-injected cattle and the control calves. For Bleed 4, 500µg of conjugated peptide was injected resulting in the highest absorbances of the peptide-injected test bleed. The absorbances for the sera of the peptide-injected Bleed 5 cattle were higher than Bleed 3 but lower than Bleed 4. This leads to the conclusion that the time of highest antibody production was four months (5/12/05) after

the initial injection of 100µg (1/10/05), and after 2 injections of 300µg (2/17/05 and

3/24/05), and an injection of 500µg (4/28/05). Figures 5, 6, 7, and 8 provide a graphic

analysis of tables 3, 4, 5, and 6.

Table 3. Mean absorbances and 95% confidence intervals of Bleed 0 and
Bleed 2 at three different dilutions.

Treatment	Mean Absorbance	95% Confidence Interval of Absorbances
1:10 dilution of serum		
Control Bleed 0	0.785	(0.628, 0.943)
Control Bleed 2	0.813	(0.656, 0.970) ns
Peptide-Injected Bleed 0	0.862	(0.717, 1.008)
Peptide-Injected Bleed 2	1.148	(1.002, 1.293) ns
1:100 dilution of serum		
Control Bleed 0	0.556	(0.434, 0.678)
Control Bleed 2	0.660	(0.538, 0.783) ns
Peptide-Injected Bleed 0	0.597	(0.484, 0.710)
Peptide-Injected Bleed 2	0.844	(0.731, 0.958) ns
1:1000 dilution of serum		
Control Bleed 0	0.409	(0.295, 0.523)
Control Bleed 2	0.513	(0.399, 0.627) ns
Peptide-Injected Bleed 0	0.413	(0.307, 0.519)
Peptide-Injected Bleed 2	0.643	(0.537, 0.748) ns

ns = Means are non significantly different from each other.
There was no significant difference between the peptide-injected Bleed 0 and the
peptide-injected Bleed 2 as there are no overlapping values of the confidence
intervals.

A)

B)

Figure 5. Histogram showing the average absorbance values at 405nm (Abs_{405}) of Bleed 2 in comparison to Bleed 0 at several dilutions. (A)1:10 serum dilution (B)1:100 serum dilution (C)1:1000 serum dilution. Black vertical bars represent the 95% confidence intervals of Bleed 2 and Bleed 0.

C)
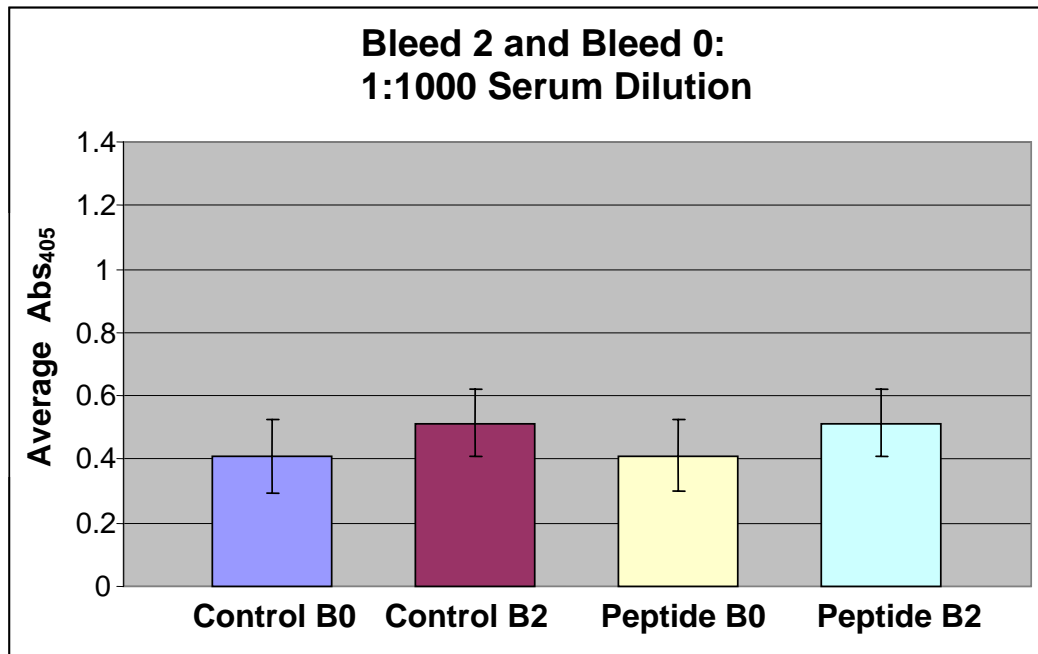
Figure 5. Continued.

Table 4. Mean absorbances and 95% confidence intervals of Bleed 0 and Bleed 3 at three different dilutions.

Treatment	Mean Absorbances	95% Confidence Interval of Absorbances
1:10 dilution of serum		
Control Bleed 0	0.872	(0.687, 1.058)
Control Bleed 3	0.821	(0.628, 1.013) ns
Peptide-Injected Bleed 0	0.934	(0.763, 1.106)
Peptide-Injected Bleed 3	1.625	(1.453, 1.797)**
1:100 dilution of serum		
Control Bleed 0	0.557	(0.371, 0.743)
Control Bleed 3	0.644	(0.452, 0.837) ns
Peptide-Injected Bleed 0	0.602	(0.429, 0.774)
Peptide-Injected Bleed 3	1.348	(1.176, 1.520)**
1:1000 dilution of serum		
Control Bleed 0	0.376	(0.249, 0.503)
Control Bleed 3	0.523	(0.392, 0.654) ns
Peptide-Injected Bleed 0	0.387	(0.269, 0.504)
Peptide-Injected Bleed 3	0.949	(0.832, 1.067)**

** 95% Confidence Intervals were significant.

ns = Means are non significantly different from each other.

There is statistical evidence that the absorbances of peptide-injected Bleed 3 are greater than peptide-injected Bleed 0 in all dilutions. Control Bleed 0 and Control Bleed 3 were not statistically different in all dilutions.

A)
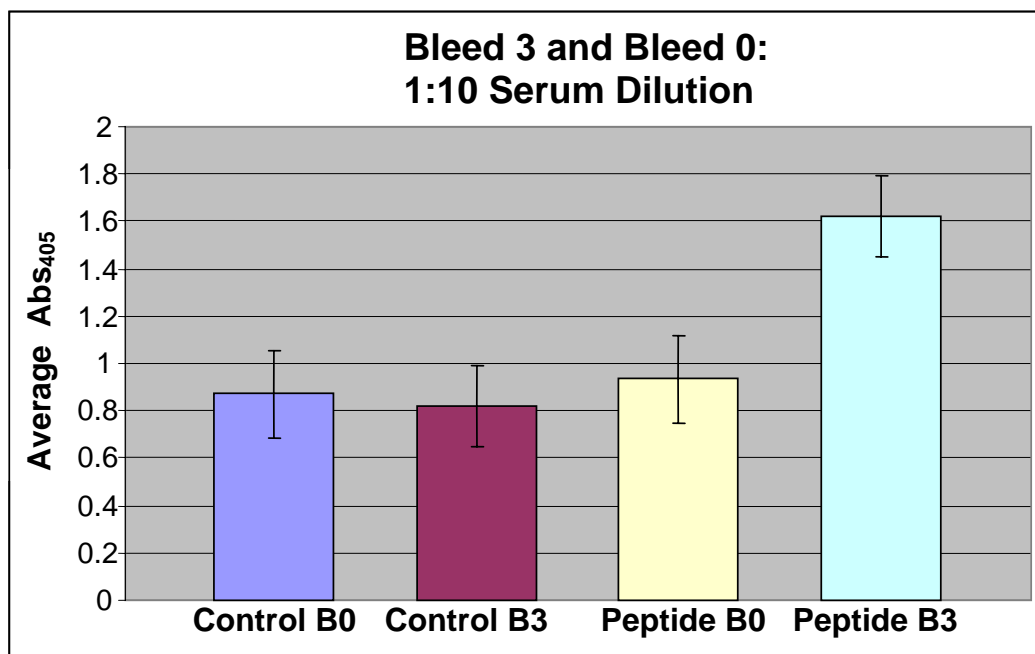
B)
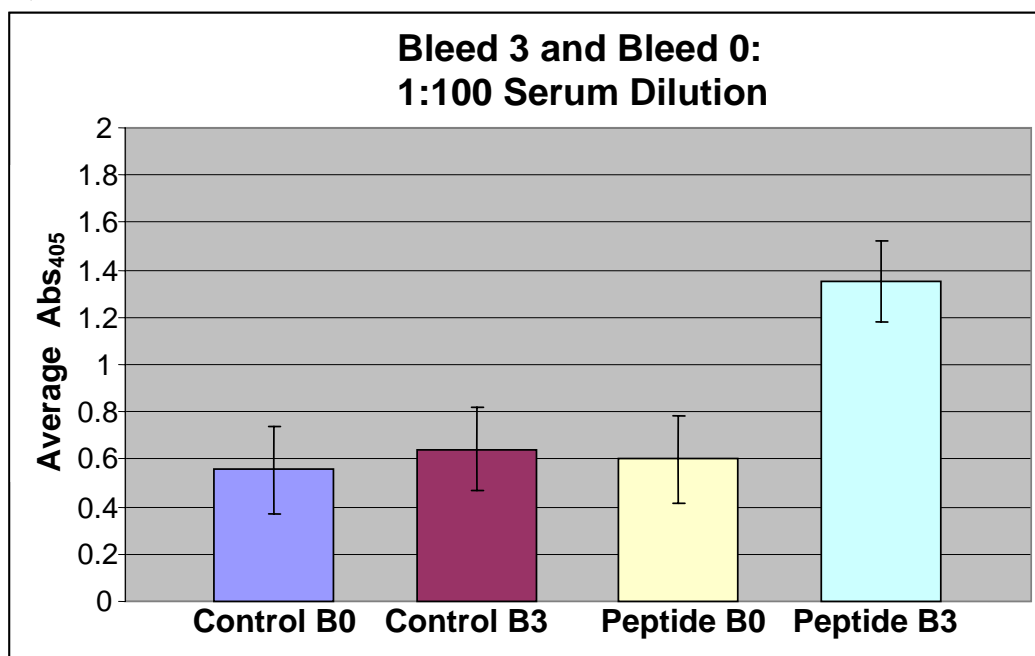
Figure 6. Histogram showing the average absorbance values at 405nm (Abs_{405}) of Bleed 3 in comparison to Bleed 0 at several dilutions. (A)1:10 serum dilution (B)1:100 serum dilution (C)1:1000 serum dilution. Black vertical bars represent the 95% confidence intervals of Bleed 3 and Bleed 0.
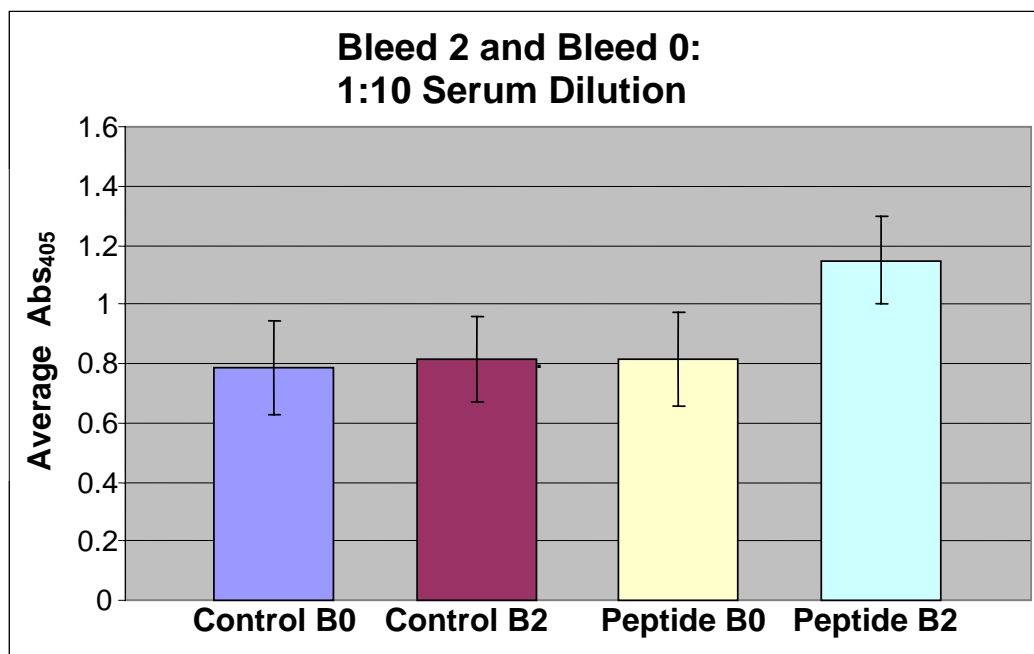
C)
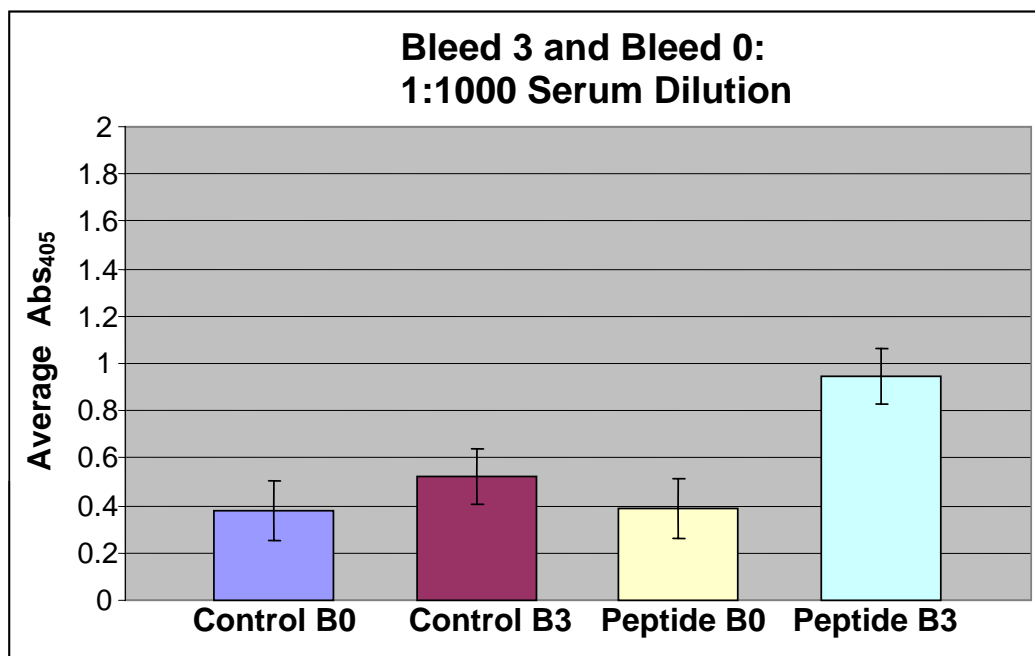
Figure 6. Continued.

Table 5. Mean absorbances and 95% confidence intervals of Bleed 0 and Bleed 4 at four different dilutions.

Treatment	Mean Absorbances	95% Confidence Interval of Absorbances
1:50 dilution of serum		
Control Bleed 0	0.898	(0.731, 1.065)
Control Bleed 4	1.040	(0.873, 1.207) ns
Peptide-Injected Bleed 0	1.075	(0.925, 1.224)
Peptide-Injected Bleed 4	1.684	(1.535, 1.833)**
1:100 dilution of serum		
Control Bleed 0	0.730	(0.584, 0.877)
Control Bleed 4	0.966	(0.819, 1.112) ns
Peptide-Injected Bleed 0	0.935	(0.805, 1.066)
Peptide-Injected Bleed 4	1.490	(1.360, 1.621)**
1:500 dilution of serum		
Control Bleed 0	0.517	(0.360, 0.674)
Control Bleed 4	0.806	(0.649, 0.963) ns
Peptide-Injected Bleed 0	0.652	(0.511, 0.792)
Peptide-Injected Bleed 4	1.235	(1.095, 1.376)**
1:1000 dilution of serum		
Control Bleed 0	0.379	(0.210, 0.547)
Control Bleed 4	0.584	(0.416, 0.753) ns
Peptide-Injected Bleed 0	0.503	(0.353, 0.654)
Peptide-Injected Bleed 4	0.975	(0.824, 1.126)**

** 95% Confidence Intervals were significant.

ns = Means are non significantly different from each other.

There is statistical evidence that the absorbances of peptide-injected bleed 4 are greater than those of peptide-injected bleed 0 in all dilutions. Control Bleed 0 and Control Bleed 4 were not statistically different for all dilutions.
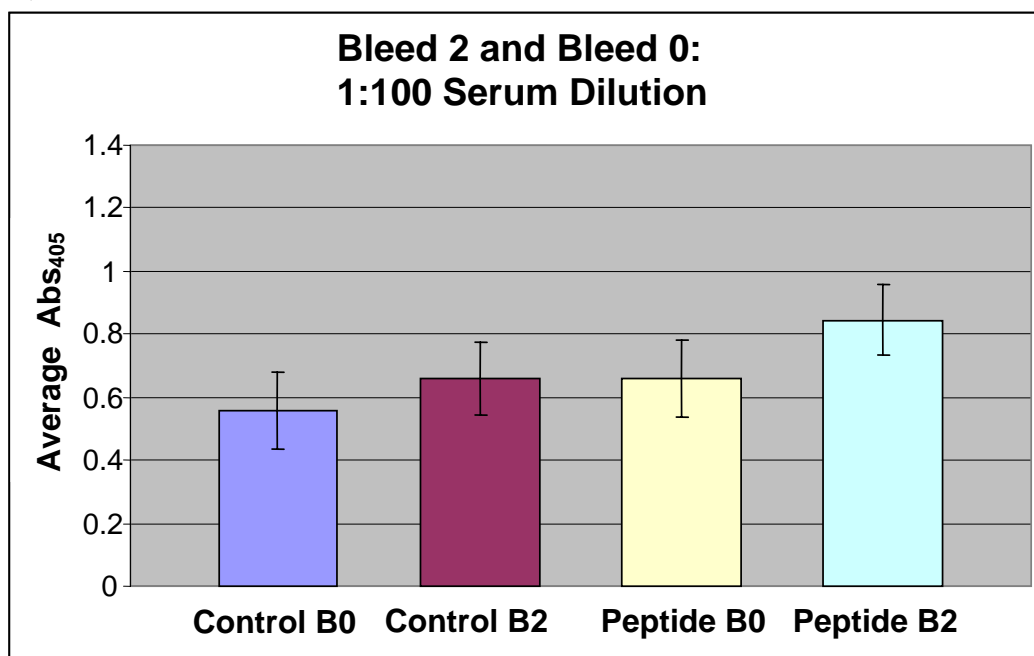
A)
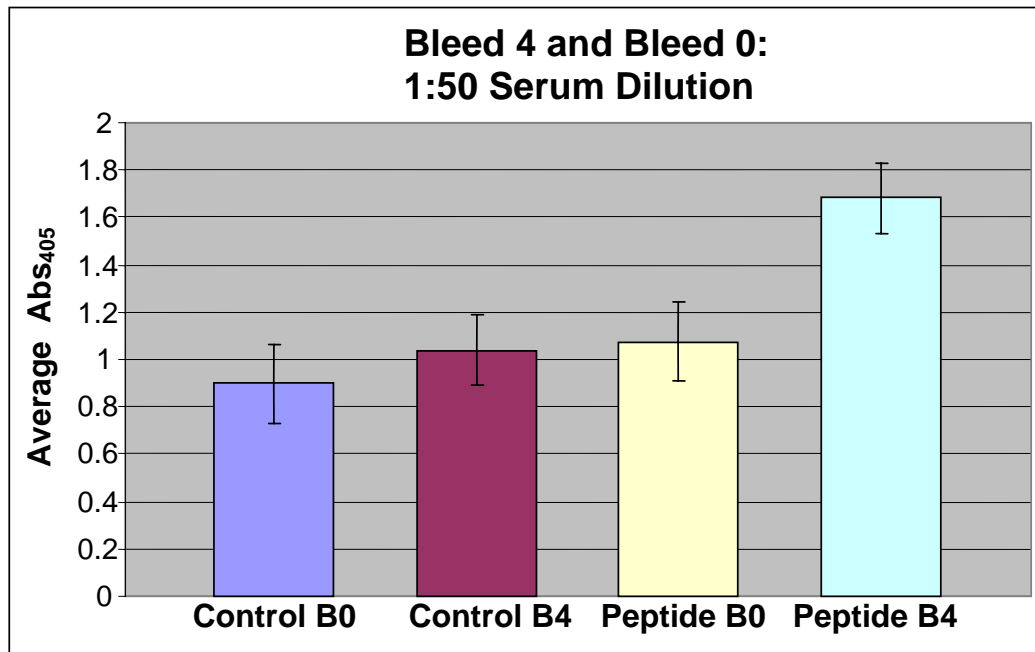
B)
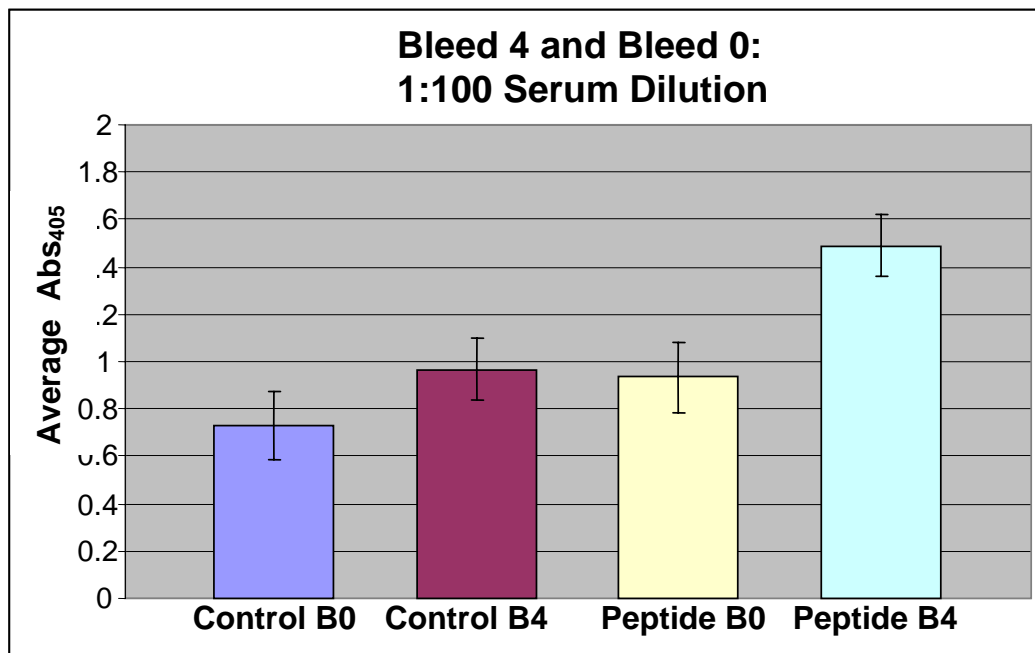
Figure 7. Histogram showing the average absorbance values at 405nm (Abs_{405}) of Bleed 4 in comparison to Bleed 0 at several dilutions. (A)1:10 serum dilution (B)1:100 serum dilution (C)1:1000 serum dilution. Black vertical bars represent the 95% confidence intervals of Bleed 4 and Bleed 0.

C)
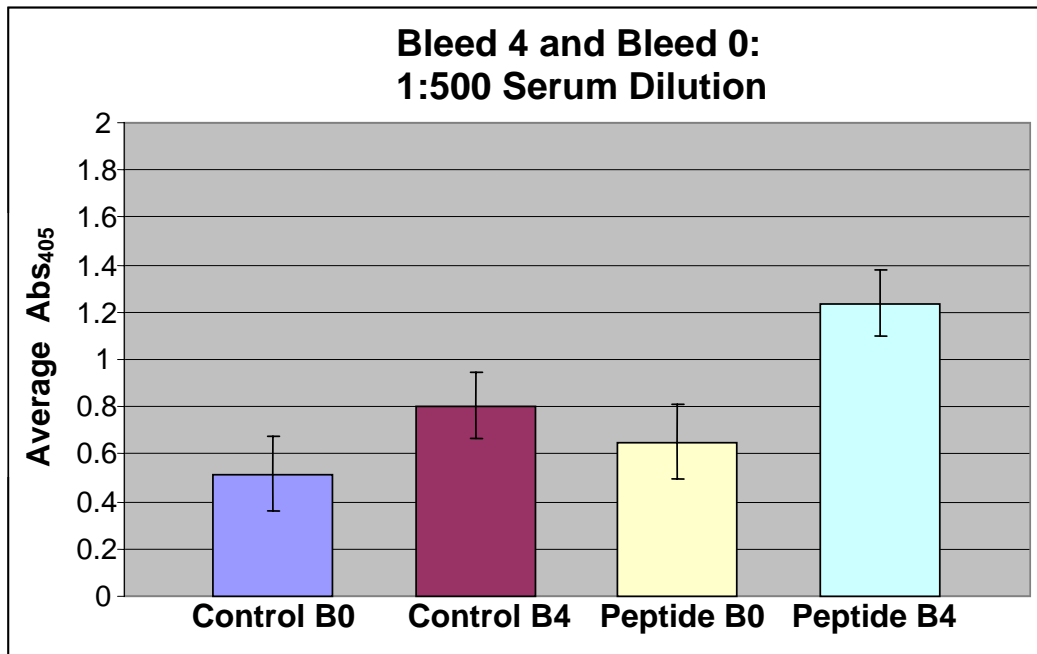
D)
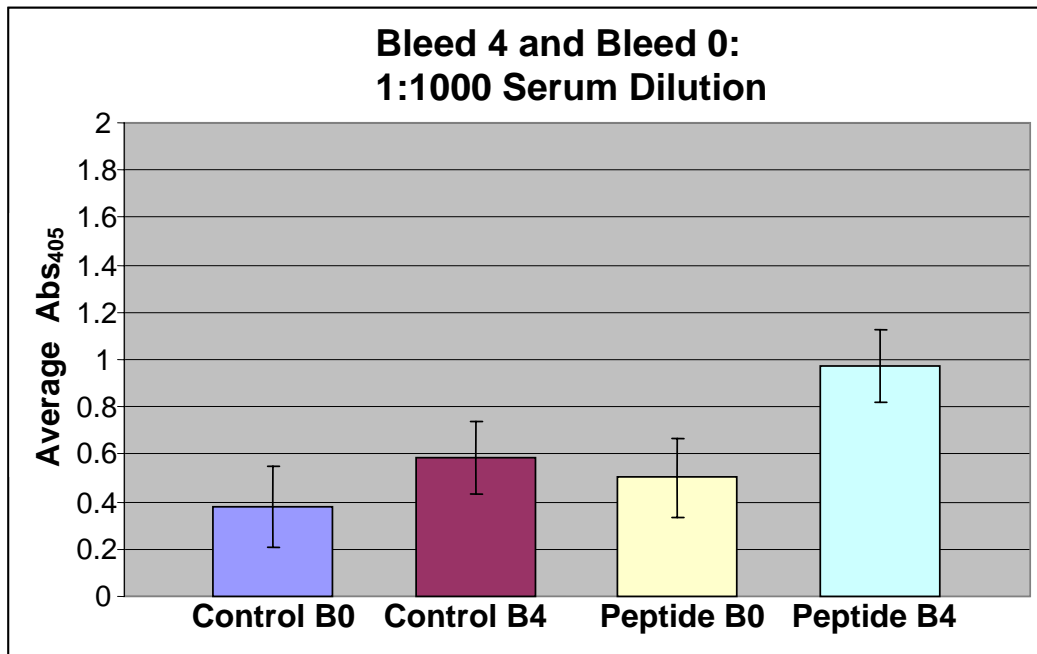
Figure 7. Continued.

Table 6. Mean absorbances and 95% confidence intervals of Bleed 0 and Bleed 5 and four different dilutions.

Treatment	95% Confidence Interval of Absorbances	Mean of Absorbances
1:50 dilution of serum		
Control Bleed 0	(0.745, 1.012)	0.878
Control Bleed 5	(0.638, 0.904) ns	0.771
Peptide-Injected Bleed 0	(0.744, 1.011)	0.878
Peptide-Injected Bleed 5	(1.213, 1.480)**	1.346
1:100 dilution of serum		
Control Bleed 0	(0.589, 0.785)	0.687
Control Bleed 5	(0.516, 0.711) ns	0.614
Peptide-Injected Bleed 0	(0.663, 0.859)	0.761
Peptide-Injected Bleed 5	(1.031, 1.227)**	1.129
1:500 dilution of serum		
Control Bleed 0	(0.475, 0.736)	0.606
Control Bleed 5	(0.375, 0.637) ns	0.506
Peptide-Injected Bleed 0	(0.406, 0.668)	0.537
Peptide-Injected Bleed 5	(0.687, 0.948)**	0.817
1:1000 dilution of serum		
Control Bleed 0	(0.304, 0.536)	0.420
Control Bleed 5	(0.250, 0.482) ns	0.366
Peptide-Injected Bleed 0	(0.263, 0.495)	0.420
Peptide-Injected Bleed 5	(0.488, 0.720) ns	0.604

** 95% Confidence Intervals were significant.

ns = Means are non significantly different from each other.

There is statistical evidence that the absorbances of peptide-injected Bleed 5 are greater than peptide-injected bleed 0 in dilutions 1:50, 1:100, and 1:500 dilutions. Control Bleed 0 and Control Bleed 5 were not statistically different in all dilutions.

A)
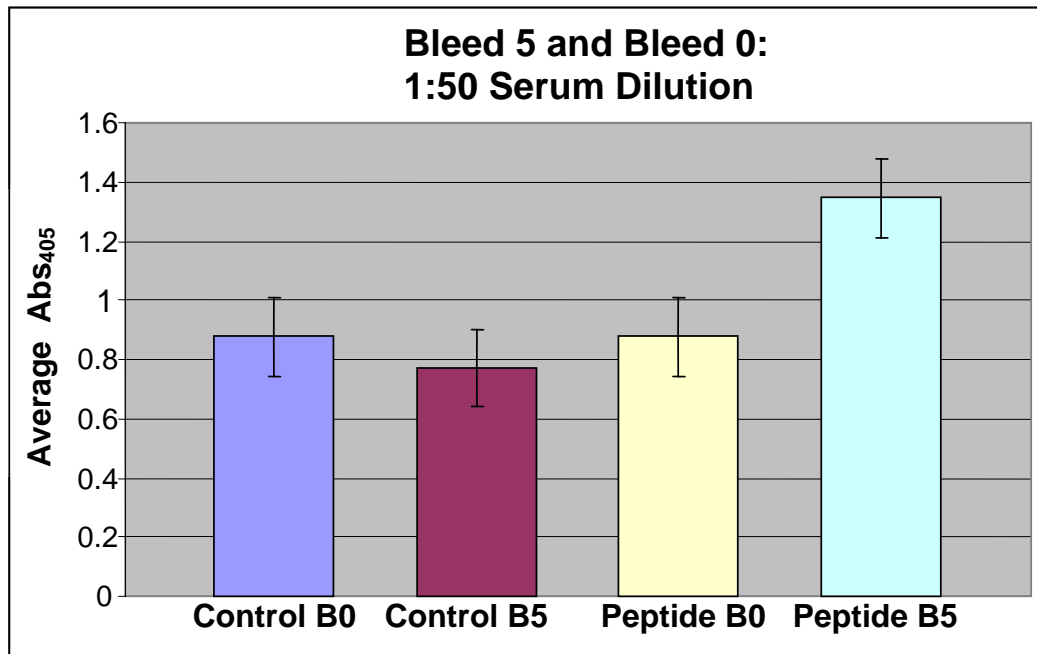
B)
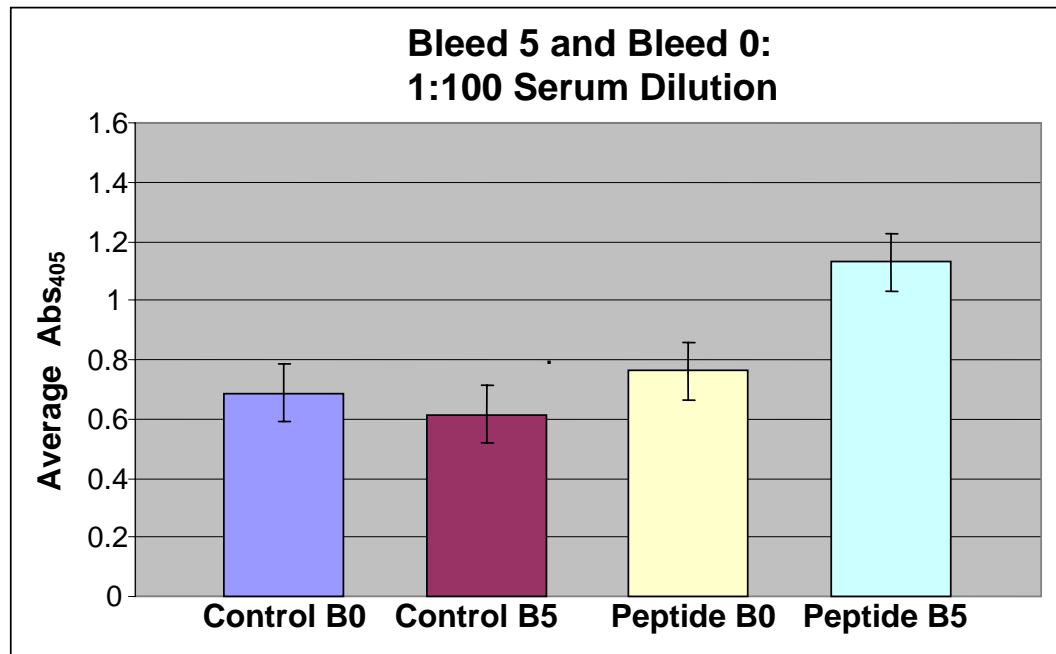
Figure 8. Histogram showing the average absorbance values at 405nm (Abs_{405}) of Bleed 5 in comparison to Bleed 0 at several dilutions. (A)1:10 serum dilution (B)1:100 serum dilution (C)1:1000 serum dilution. Black vertical bars represent the 95% confidence intervals of Bleed 5 and Bleed 0.

C)
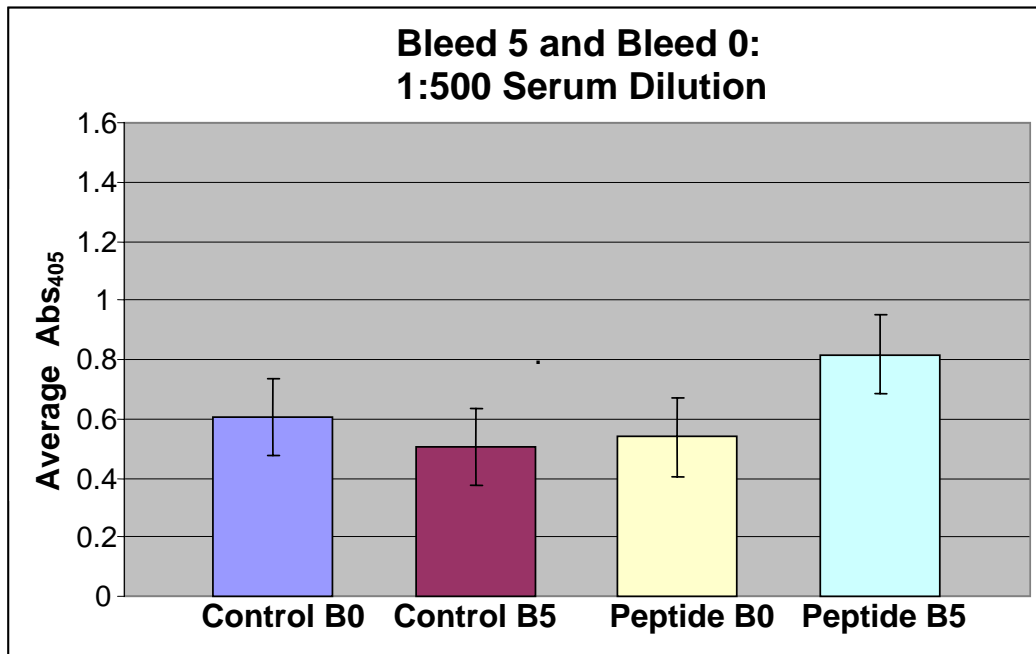
D)
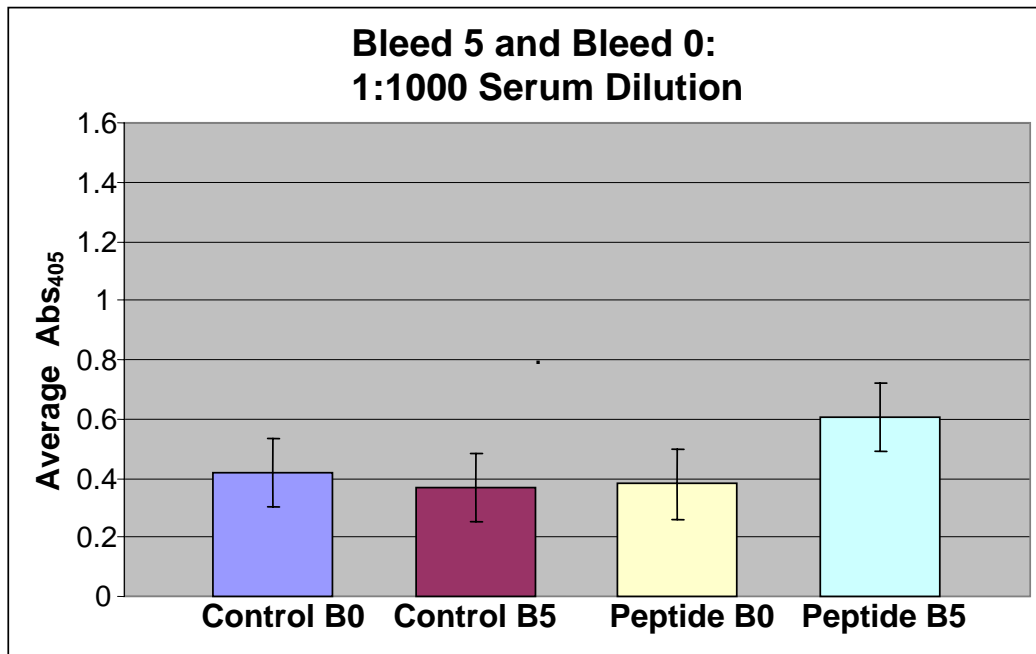
Figure 8. Continued.

Figure 9 shows the Abs_{405} of Bleed 4 at 1:50 dilution for all the peptide-injected calves. The serum from Bleed 4 Calf #435 had the highest average absorption of 2.0. The serum from Bleed 4, Calf #412 had the lowest average absorption of 1.0. The absorbance of the serum from Bleed 4, Calf #435 was double of that of Bleed 4, Calf# 412. The immune system of Calf #412 was not producing antibodies against the injected conjugated peptides. This could suggest that the immune system of this calf was not as efficient as the other calves. Calf #412 was not utilized in the tick challenge. Only the calves in the tick challenge were bled for Bleed 5.
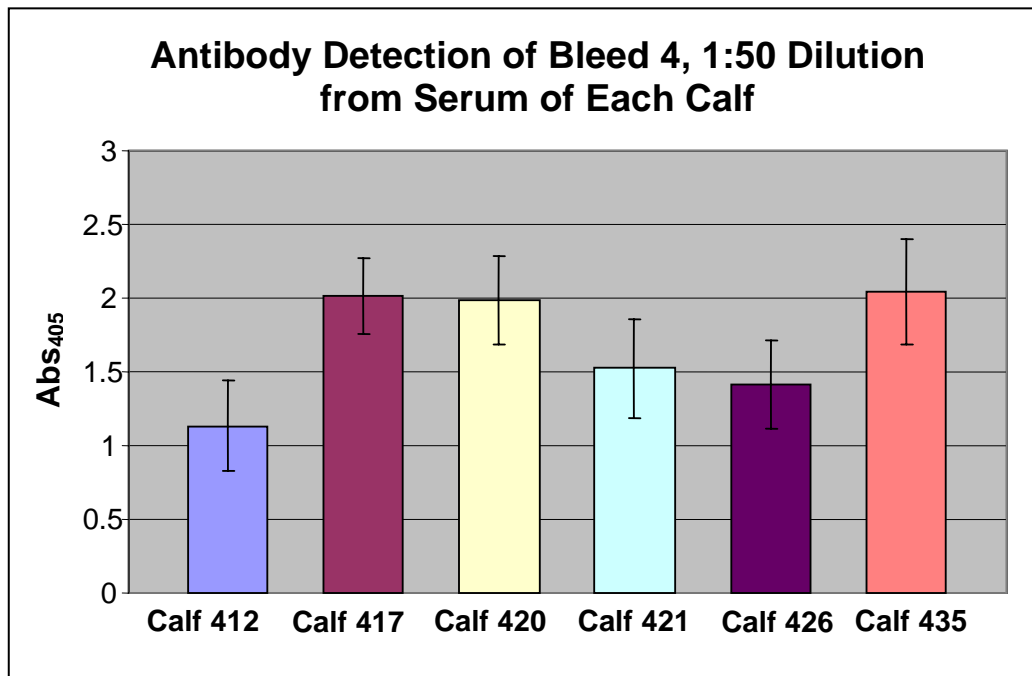
Figure 9. Histogram showing the average value of absorbances for peptide-injected calves sera Bleed 4. The vertical black bars represent the 95% Confidence Intervals of the Abs_{405}. Calf #435 showed the highest mean value, followed by Calf #417, Calf #420, Calf #421, and Calf #426. Calf #412 showed the lowest mean value.

Table 7 and Figure 10 show the mean absorbance at 405 nm and the 95% Confidence Intervals of ELISAs from peptide-injected for Bleed 0 sera and Bleed 4 sera, 1:100 dilution using the 13 individual KLH-bound peptides as coating antigen. The null hypothesis is that the mean absorbances of Bleed 4 sera in ELISA using the individual 13 peptides as coating antigen will be equal to the mean absorbance of Bleed 0 sera in that ELISA. ELISA testing reveals antiserum reacted more strongly to wells containing peptides 2 and 9. There is a statistical significance to show that the absorbances of the peptide-injected Bleed 4 towards Peptides 2, 3, 4, 5, 7, 8, 9, 11, 12 are greater than the absorbances of the peptide-injected Bleed 0 sera for the peptides 1-13. The absorbances of peptide-injected Bleed 4 Peptides 2 and 9 are the highest of out of all 13 peptides with average absorbances of 1.549 and 1.746 respectively. Peptides 2, 3, 4, 5 are found on the N-terminus, peptides 8, 9, and 11 are found on loop 2, and peptide 12 on loop 3. Peptide 2 corresponds to the N-terminus and Peptide 9 corresponds to loop 2 (Fig. 2). Peptides 1 corresponds to the N-terminus, Peptides 6 and 7 to loop 1, and Peptides 10 to loop 2, and Peptide 13 to loop 3 (Fig. 2). Table 8 shows the individual mean absorbances of the ELISAs from each peptide-injected Bleed 4 calf using the 13 individual KLH-bound peptides as coating antigen.

When the 13 individual peptides were used as antigens, there was no statistical evidence that any of the peptides produced a differential absorbance when sera from Bleed 0 of treatment animals was tested in ELISA. There was no statistical significance to show that the ELISA absorbances of the peptide-injected Bleed 4 when Peptides 1, 6, 10, 13 were used as coating antigens are greater than the absorbances of the peptide-

injected Bleed 0 sera for the peptides 1-13. Peptide 1 is found on the N-terminus,

peptides 6 and 7 are found on loop 1, peptide 10 is found on loop 2, and peptide 13 on

loop 3.

Table 7. Mean absorbances and 95% confidence intervals of Abs_{405} of ELISAs from peptide-injected Bleed 0 and Bleed 4 sera using the 13 individual KLH-bound peptides as coating antigens. Sera dilution of 1:100 and was used. Peptides 2 and 9 showed the highest absorbances. Peptides 1,6,7,10,13 showed the lowest absorbances.

Peptide	Mean of Absorbances		95% Confidence Intervals of Absorbances	
	Bleed 0	**Bleed 4**	**Bleed 0**	**Bleed 4**
Peptide 1	0.272	0.419	(0.153, 0.392)	(0.300, 0.539) ns
Peptide 2	0.363	1.549	(0.243, 0.482)	(1.429, 1.668) **
Peptide 3	0.480	0.937	(0.361, 0.600)	(0.817, 1.056) **
Peptide 4	0.461	0.836	(0.342, 0.581)	(0.717, 0.956) **
Peptide 5	0.231	0.639	(0.112, 0.351)	(0.519, 0.758) **
Peptide 6	0.234	0.442	(0.115, 0.354)	(0.322, 0.561) ns
Peptide 7	0.210	0.495	(0.090, 0.329)	(0.375, 0.614) **
Peptide 8	0.305	1.095	(0.185, 0.424)	(0.975, 1.214) **
Peptide 9	0.300	1.746	(0.181, 0.420)	(1.627, 1.866) **
Peptide 10	0.232	0.417	(0.113, 0.352)	(0.298, 0.536) ns
Peptide 11	0.251	0.929	(0.131, 0.370)	(0.809, 1.048) **
Peptide 12	0.282	0.897	(0.163, 0.420)	(0.778, 1.017) **
Peptide 13	0.238	0.318	(0.119, 0.358)	(0.198, 0.437) ns

** 95% Confidence Intervals were significant.
ns = Means are non significantly different from each other.

Table 8. Individual mean absorbances of Abs$_{405}$ of ELISAs from each peptide-injected Bleed 4 calf using the 13 individual KLH-bound peptides as coating antigen. Sera dilution of 1:100 was used.

Peptide	Calf 417	Calf 420	Calf 421	Calf 426	Calf 435
1	0.314	0.489	0.505	0.375	0.412
2	1.147	2.314	1.170	1.378	1.735
3	0.511	0.970	0.883	0.716	0.903
4	0.618	1.225	0.822	0.593	0.925
5	0.409	0.957	0.664	0.453	0.711
6	0.295	0.426	0.528	0.460	0.500
7	0.281	0.883	0.469	0.381	0.461
8	0.926	1.672	1.081	1.292	1.204
9	1.925	2.397	1.522	1.373	1.514
10	0.298	0.408	0.464	0.353	0.562
11	0.644	1.322	0.972	0.796	0.909
12	0.761	1.076	0.950	0.660	1.039
13	0.255	0.336	0.360	0.304	0.333
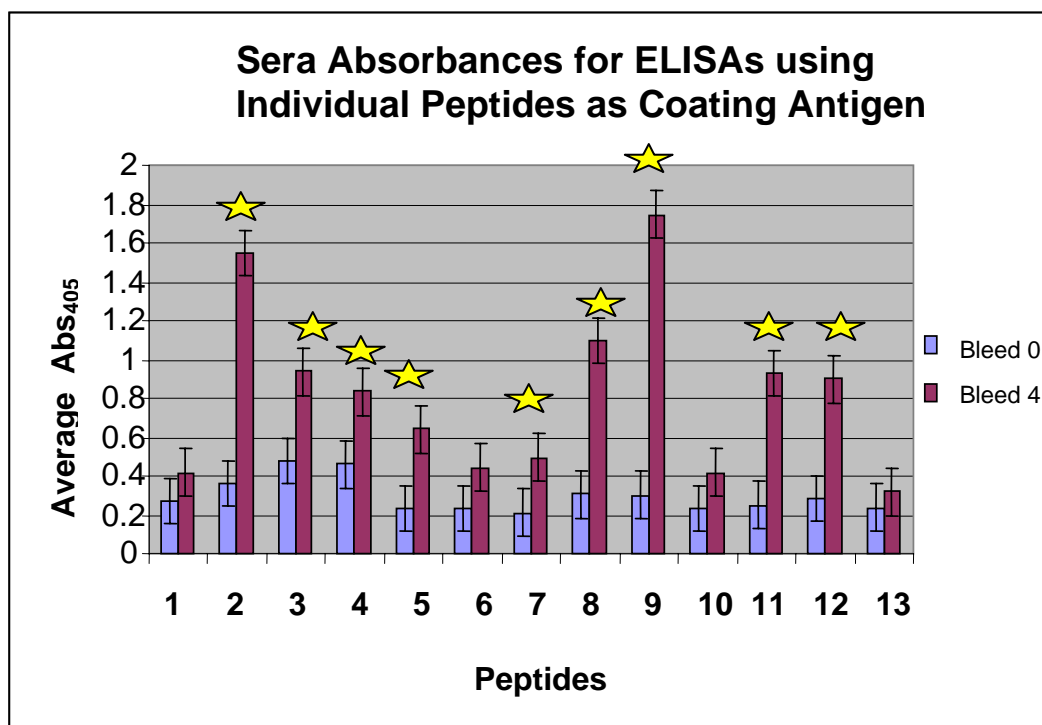
Figure 10. Histogram showing the mean absorbance in ELISA using the individual KLH-bound peptides as coating antigen. The black vertical bars represent the 95% Confidence Intervals of Abs_{405} of peptide-injected Bleed 0 and Bleed 4, 1:100 sera dilution (Table 7). The yellow stars represent a significant difference between Bleed 4 and Bleed 0 for each peptide.

Discussion

The hypothesis that the cattle would produce an immunological response to the antigens corresponding to the extracellular regions of the Southern cattle tick myokinin receptor was supported by the results. The absorbances of sera from Bleed 4 Peptide-Injected calves as a group (calves #417, #420, #421, #426, and #435) were statistically different from Bleed 0 (the pre-immune) Peptide-Injected calves and Bleed 0, Bleed 4 Control calves as a group (calves #407, #408,#427,#436 and #438) indicating anti-receptor antibody production in the Bleed 4 Peptide-Injected calves. There was

differences within the calves with Calf #435 showing the highest absorbance in Bleed 4 followed by Calf #417, Calf #420, Calf #421 and Calf #426 (Fig. 9). Similarly, the extracellular region of plasma membrane receptors have been found to be immunogenic (Wieland et al. 1998).

In previous studies, peptides have been utilized with success as antigens for antibody production in animals (Vordermeir et al., 2005). A strong cellular immune response against bovine tuberculosis was induced by a synthetic peptide vaccine against it (Vordermeir et al., 2005). The combination of the appropriate peptides and the suitable adjuvant formulation chosen enhances the immunogenicity of the peptides. The results from the ELISA experiments support the hypothesis that peptides can be used as antigens given that the peptide-injected cattle did produce antibodies against the injected conjugated peptides. The Bleed 4 Peptide-Injected sera bound to Peptides 2, 8, and 9 showed the highest response (Fig. 10). The absorbance rank order for the thirteen peptides was not similar in all five peptide-injected calves that were producing antibodies indicating variance between the calves (Table 8).

The amino acid sequence of all 13 peptides was tested for hydrophobicity with the peptide calculator, found on the Sigma Genosys webpage, to detect a correlation between reduced hydrophobicity and immunogenicity. The less hydrophobic the peptide, the higher the absorbance to the peptide-injected Bleed 4 sera. For example, Peptide 2 (mean absorbance of 1.549) was 38.46% hydrophobic while Peptide 7 (mean absorbance of 0.495) was 53.33% hydrophobic. However, "peptide immunogenicity

depends on complex interactions with various elements of the host immune system"
(Van Regenmortel, 2001).

The calves that did not produce antibodies were not going to be utilized for the tick challenge. The absorbance of the sera from Calf #412 Bleed 4 was not statistically different from Calf #412 Bleed 0 (Pre-Immune) sera, reason for excluding that calf in the tick challenge. The precaution of injections for a fifth time had to be taken to prevent a fall in antibody titer in serum from the time of the last injection in College Station (5/12/05) to the time of transfer of the calves to the USDA Cattle Fever Tick Laboratory (6/09/05). The cattle were delayed when transferred to the Cattle Fever Tick laboratory so Injection 6 was given to ensure that the antibody was still being produced in the peptide-injected calves when they were challenged with *Boophilus microplus* ticks.

CHAPTER III

Boophilus microplus CHALLENGE ON ANTI-MYOKININ

RECEPTOR VACCINATED *Bos taurus* CALVES

Introduction

The tick challenge of receptor fragments-inoculated cattle is designed to test if the anti-myokinin receptor antibodies cause a detrimental effect on the tick. This is determined by examining tick physiological functions such as feeding, development, and reproduction. This receptor is hypothesized to be involved in diuresis and water balance (Holmes et al., 2000). The myokinin receptor messenger RNA was detected in all life stages of the tick by semiquantitative RT-PCR (Holmes et al., 2000) so the tick challenge included testing for impairments in tick feeding, development, and reproduction.

Evidence suggests that antibodies ingested by ticks are not digested but pass through the midgut barrier of the ticks (Tracey-Patte et al., 1987). The host's immunoglobin G (IgG) may disrupt essential biochemical functions leading to the tick's death or inhibit its reproduction (Elvin & Kemp, 1994). Anti-tick vaccines have shown a significantly reduced number of ticks engorging, reduction in weight after feeding, and reduction of conversion of engorged weight into eggs (Willadsen, 2004). For example, the physiological response induced by the vaccine GAVAC is a reduction in the average fertility of the adult ticks (de la Fuente et al., 1998). In the adult female soft tick

Ornithodoros moubata, mouse monoclonal antibodies against hemocytes given through an artificial membrane system had a tickcidal effect (Matsuo et al., 2004).

A radioimmunoassay technique to measure the concentration of IgG in the hemolymph has been established and host IgG has been found in the tick hemolymph after ingestion of vaccinated blood (Ben-Yakir, 1989). Immunoglobulins have been quantified by competitive ELISA in *Boophilus microplus* hemolymph (da Silva Vaz et al., 1996). It was shown that 2% of host IgG was present in the engorged tick hemolymph and is "present for at least 48 h after completing the parasitic life cycle" (da Silva Vaz et al., 1996). Undigested host IgG has also been found in hemolymph during nymphal development of *Dermacentor variabilis* and *Ixodes scapularis* ticks (Vaughan et al., 2002). The host IgG acquired by the fed larvae was detected by ELISA in the hemolymph of the non-fed nymphs that had molted from these larvae.

Methods

Tick challenge

Five control and five peptide-injected calves were transported to the USDA Cattle Fever Tick Laboratory in Edinburg, TX. The tick challenge consisted of a "natural" whole body infestation of larvae that could produce engorged females on each of the peptide-injected and control calves. In addition, there was a confined subset of ticks on the flanks of each calf to possibly produce metanymphs to be detached to evaluate survivorship without a host, i.e., unfed adults. The two calves not taken were Calf #415

(too unruly to be handled and could not be injected as per protocol) and Calf #412 (did

not show an immune response against the receptor antigens).

At the USDA laboratory, the calves were put into individual stanchions. Each calf

was infested with approximately 20,000 larvae produced from 1 gram of eggs

(Drummond et al., 1973) of *B. microplus* of the Muñoz strain. Two vials (each 500 mg

of larvae) were glued to the middle of the back of the calves (Fig. 11). Since

metanymphs are $\leq$ 8mm in length (Nunez et al., 1985) and difficult to collect on the calf

when they are free to move around, a patch was glued on one of the flanks of the all the

calves. Once the larvae were placed in this patch enclosure, it was stapled closed.

Figure 11. Tick challenge setup. The calf in its stanchion with two vials containing
500mg of larvae glued on its back. The cotton plug in the vials in the picture was taken
out of the vials to allow the larvae to attach to the hide of the calf.

Ixodid ticks rely upon active and passive water uptake mechanisms to maintain homeostasis (Sauer et al., 1995). The insect growth regulator pyriproxyfen has been shown to negatively affect water balance in *Amblyomma americanum*, lonestar ticks and reduce their survival rate off the host (Strey et al., 2001). Reduced survivorship of unfed adult lone star ticks emerging from pyriproxyfen-treated engorged nymphs held under constant conditions of temperature and relative humidity was a principle criteria to compare water homeostasis to untreated control ticks (Donahue et al., 1997).

In the present study, water homeostasis of newly molted adult *B. microplus* was determined by comparative molting and survivorship of ticks from treated and control cattle. When metanymphs were detected from the general body infestations, the patch was cut open with scissors. Thirty metanymphs were manually detached with tweezers from the patches of each calf and put into vials (Fig.12). The vials were incubated at 28ºC and 92.5% humidity. On a daily basis, the thirty metanymphs in the vials were inspected for molting into adults. The adults that had molted from the metanymphs were inspected daily for mortality.
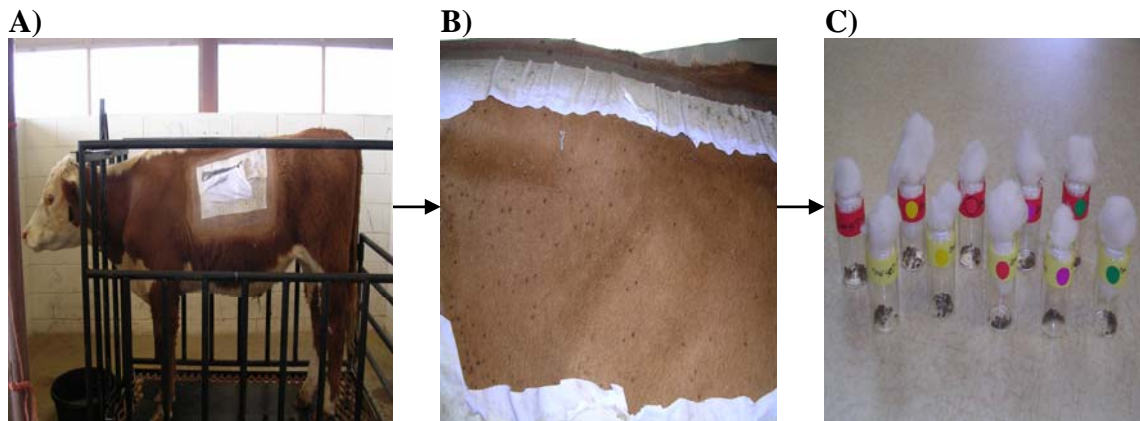
Figure 12. Metanymph collection setup. (A) The calf in its stanchion with a patch glued on. The patch was cut open, the larvae placed inside, and the patch stapled closed. (B) After two weeks the patch was cut open to detach the metanymphs, seen here as dark dots. (C) Thirty metanymphs from each calf were put into vials and incubated at 28ºC and 92.5% humidity.

Adult engorged females that dropped from the calves were collected daily to test for any disruption in tick feeding. After three weeks of infestation, the engorged females that dropped were collected by washing the stalls with water and letting the water flow into filters that caught the ticks. The ticks were placed in disposable containers that were taken to the tick lab (Fig 13). Then they were washed, counted, and weighed individually on previously tared filter paper. A Metler electronic balance was used for weight measurements. The weight for each individual engorged female tick (a sample size of thirty engorged females total collected over four days) that dropped from each calf (calves with the ear-tag numbers 408, 417, 426, 427, 436, and 438) and the date of the weight taken was recorded.

A)   **B)**
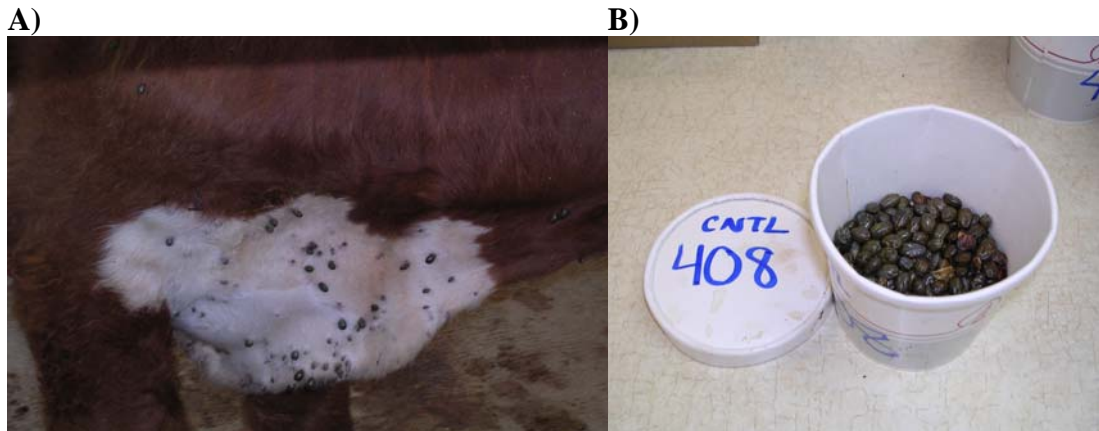
Figure 13. Collection of engorged females setup. (A) The calf infested with *Boophilus microplus*. (B) Once the engorged *B. microplus* females dropped from the calves they were collected, washed, and placed in plastic containers for counting.

To test for any disruption in tick reproduction those thirty ticks from each calf were individually placed in vials, labeled with a number and the calf number they dropped from, and incubated at 28ºC and 92.5% humidity (Fig. 14). Three weeks after they dropped, the date, the weight of the egg masses, and the weight of ticks after egg laying were recorded. To inspect the ticks for any physical damage that incurred while blood feeding, the color of the tick after egg laying was recorded. Engorged females are normally brown to reddish brown and any other coloration is considered abnormal.

Figure 14. The female engorged ticks were collected in vials and incubated in a humid chamber that is maintained at 28ºC and 92.5% humidity.

Data analyses

Statistical significances between the number of ticks that molted from metanymphs into adults from the peptide-injected versus the control calves were evaluated by developing a 2x2 contingency table (Ott & Longnecker, 2001). A 2 sample T-test was performed to test for statistical significance of the survivorship and the number of days to achieve 50% and 100% death of adult ticks that molted from the metanymphs from the peptide-injected calves versus the control calves. The SPSS-PC program was used to perform the contingency table and the 2 sample T-tests. A general statistical inference of the difference of ticks dropped from control calves versus the peptide-injected calves could not be made due to a limited sample size of calves. After the ticks had molted on the calves but before the ticks had dropped, three peptide-injected calves (Calf #420, Calf #421, Calf #435) and one control calf (Calf #407) died as result of bovine babesiosis. In order to perform any statistical analysis of the measurements from the ticks from the remaining calves, the measurements from the 30

ticks per peptide-injected calf were pooled to compare with the pooled measurements of the ticks from control calves. To test for a statistical significance in weight, egg mass weight, and bloodmeal conversion (the weight of the egg mass divided by the weight of the engorged female after she has dropped from the calf but before oviposition) between the ticks fallen from the peptide-injected calves versus the control calves a 2 sample T test was performed.

Results

Table 9 shows the comparison of the number of metanymphs that molted into adults after being detached from the peptide-injected and control calves, respectively. The null hypothesis that the number of metanymphs that molted into adult from peptide-injected calves and from the control calves are equal, cannot be rejected (p = 0.282). That there is no difference between the percent survival of the adults from the peptide-injected calves and the control calves is confirmed in Figure 15 showing the percent survivorship of the newly molted adults from the metanymphs that had been detached. In order to confirm that indeed there was no difference in the survivorship of the 2 groups, the number of days to achieve 50% and 100% death was compared between groups, tables 10 and 11 show those comparisons. The null hypotheses that the date of 50% cumulative death occurrence and 100% cumulative death occurrence of the peptide-injected calves are similar from the control calves cannot be rejected (p = 0.865 and p = 0.291 respectively).

Table 9. Comparison of the number of metanymphs that molted into adult from the peptide-injected calves and the control calves. A 2x2 contingency table is shown. There is no statistical difference in the number of ticks molted from the peptide-injected calves versus the control calves (p = 0.282).

	Peptide-Injected	Control
Number of ticks molted	128	121
Number of ticks not molted	22	29
Total	150	150

n=300, d.f.=1, χ^2 = 1.158, p = 0.282
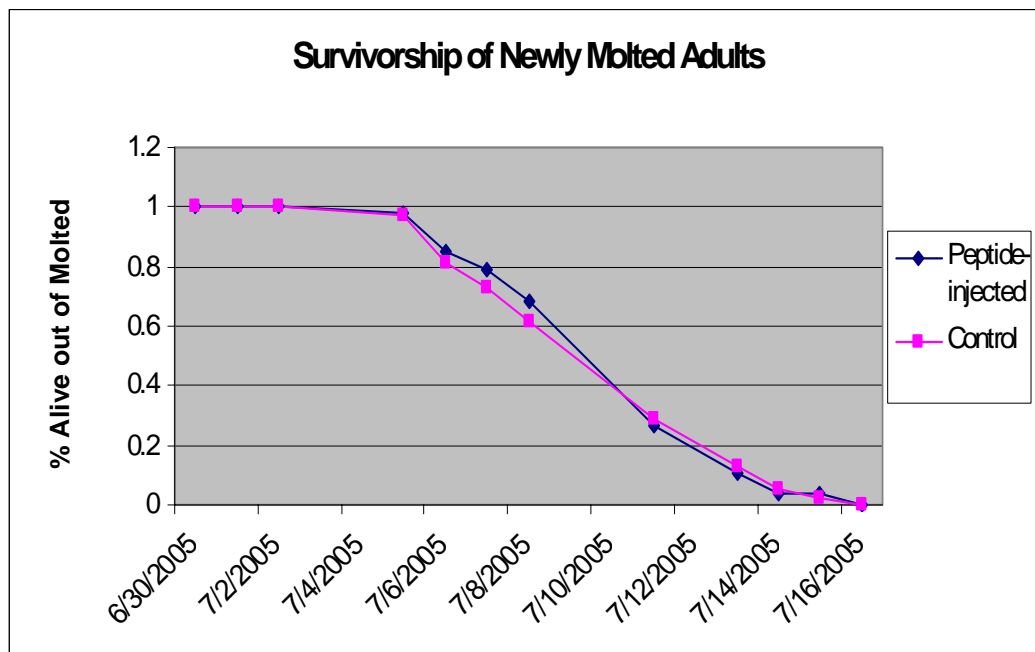
Figure 15. The survivorship of newly molted adults. The percentage alive for each day for ticks obtained from control calves and peptide injected calves is graphed. The lines are parallel; there is no difference in the percentage alive from the newly molted adults detached from the peptide-injected calves versus control calves.

Table 10. Comparison of date of 50% cumulative death occurrence of ticks collected from control and peptide-injected calves. There is no statistical difference between the date of 50% cumulative death occurrence of newly molted adults collected from peptide-injected calves versus control calves (p = 0.865).

Calf	Date of 50% cumulative death	Days from initial detachment (6/30/05)
Control:		
Calf #407	7/11/05	12
Calf #408	7/11/05	12
Calf #427	7/07/05	8
Calf #436	7/11/05	12
Calf #438	7/08/05	9
Peptide-Injected:		
Calf #417	7/11/05	12
Calf #420	7/11/05	12
Calf #421	7/08/05	9
Calf #426	7/11/05	12
Calf #435	7/08/05	9

n=10, d.f.= 8, t= -0.175, p= 0.865

Table 11. Comparison of date of 100% cumulative death occurrence of ticks collected from control calves and peptide-injected calves. There is no statistical difference between the date of 100% cumulative death occurrence of newly molted adults picked from the peptide-injected calves versus control calves (p = 0.291).

Calf	Date of 100% cumulative death	Days from initial detachment (6/30/05)
Control:		
Calf #407	7/15/05	16
Calf #408	7/16/05	17
Calf #427	7/13/05	14
Calf #436	7/14/05	15
Calf #438	7/16/05	17
Peptide-Injected:		
Calf #417	7/16/05	17
Calf #420	7/16/05	17
Calf #421	7/14/05	15
Calf #426	7/16/05	17
Calf #435	7/16/05	17

n=10, d.f.= 8, t= -1.131, p= 0.291

Table 12 and Figure 16 show the frequency distribution of ticks that dropped daily from peptide-injected calves 417 and 426, and from control calves 408, 426, 436, and 438. No statistical inference can be made from the data due to the low numbers of calves. Four calves (control Calf #417, and peptide-injected calves #420, #421, and #435) died from bovine babesiosis. The distributions of the ticks that dropped from all the calves (except Calf #427) appear skewed to the left. Calf #427 dropped late in the drop, with a long period of dropping but a small number of drops continuously.

Figures 17, 18, and 19 show the pooled data of the average weight of engorged female ticks, the average weight of the their egg masses and the conversion of bloodmeal that dropped from peptide-injected calves (60 ticks) and control calves (120 ticks), as well as the individual averages for each calf. The null hypothesis that the average weight of fed females from peptide-injected calves is similar from those of the control calves cannot be rejected ($p = 0.061$). The null hypothesis that the average weight of their egg masses from peptide-injected calves is similar than that of the control calves cannot be rejected ($p = 0.885$). The null hypothesis that the conversion of the bloodmeal from peptide-injected calves is similar from that of the control calves cannot be rejected ($p = 0.312$).

Table 12. Tick challenge experiment: frequency distribution of ticks that dropped from calves on a daily basis. Calves 408, 427, 436, and 438 are control calves. Calves 417 and 426 are peptide-injected calves. The first drop was on 7/8/05 from Calf #408 and Calf #438. The last drop was on 8/02/05 from Calf #427. With the exception of the ticks from Calf #427, there was a short time of dropping with a large number of drops in the middle of the drop time. The ticks from Calf #427 dropped with small numbers over a longer period of time. Not an expected result from a control calf.

Date	#408	#427	#436	#438	#417	#426
		CONTROL CALVES			PEPTIDE INJECTED CALVES	
7/08/05	8	0	0	5	0	0
7/09/05	158	0	0	52	0	3
7/10/05	398	0	19	256	0	22
7/11/05	809	0	196	588	1	146
7/12/05	491	1	351	763	20	350
7/13/05	394	1	576	668	76	496
7/14/05	270	3	484	379	155	390
7/15/05	101	12	170	83	364	443
7/16/05	50	13	210	107	224	247
7/17/05	19	33	104	97	285	262
7/18/05	6	45	69	81	268	129
7/19/05	3	76	27	15	232	99
7/21/05	3	80	40	19	99	31
7/22/05	0	101	10	10	92	29
7/23/05	0	74	6	1	25	16
7/24/05	0	52	2	3	21	4
7/25/05	0	76	2	0	7	6
7/26/05	0	47	0	0	12	3
7/27/05	0	54	0	0	5	2
7/28/05	0	33	0	2	3	0
7/29/05	0	20	0	0	2	0
7/30/05	0	15	0	0	1	0
7/31/05	0	15	0	0	0	0
8/01/05	0	13	0	0	0	0
8/02/05	0	3	0	0	0	0
Total	2710	767	2266	3129	1892	2678
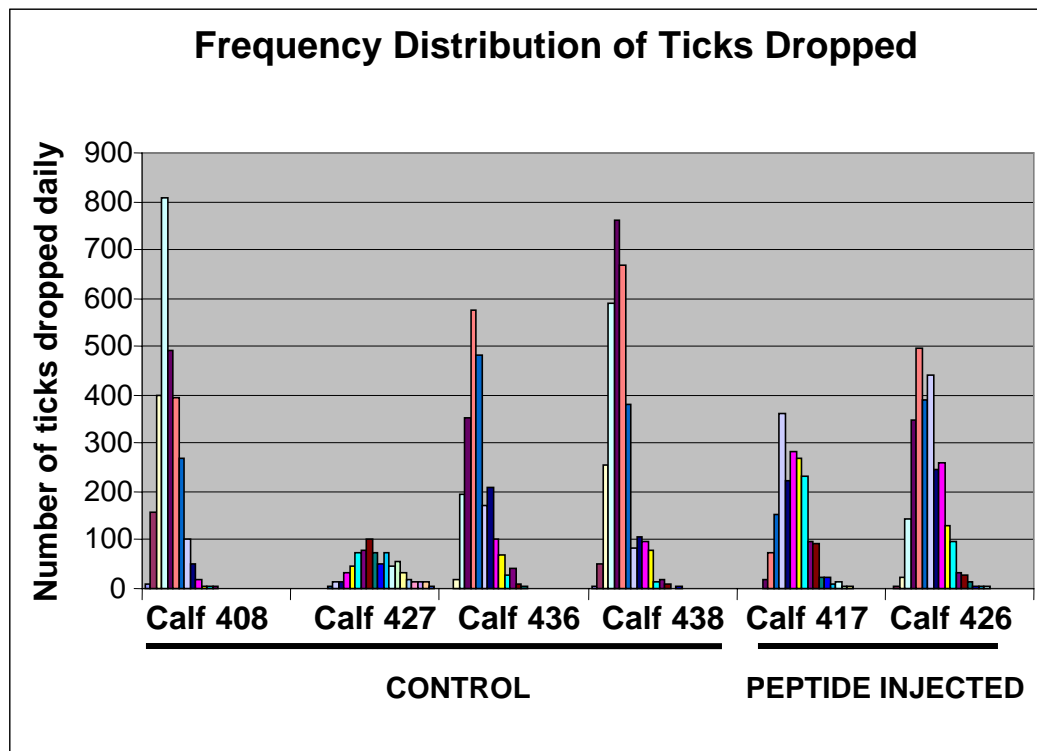
Figure 16. Frequency distribution of the ticks dropped from the tick challenge experiment (Table 11). Bars represent the number of ticks that dropped daily. The control calves are #408, #427, #436, #438 and the peptide-injected calves #417 and #426.

(A)
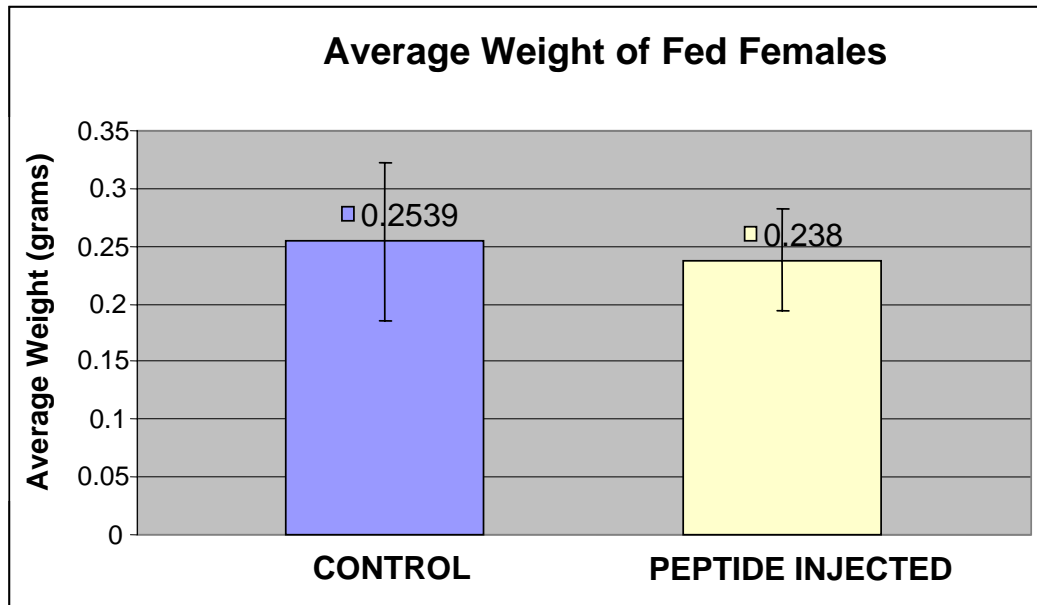
n=30, d.f.= 166.47, t= 1.888, p = 0.061

(B)
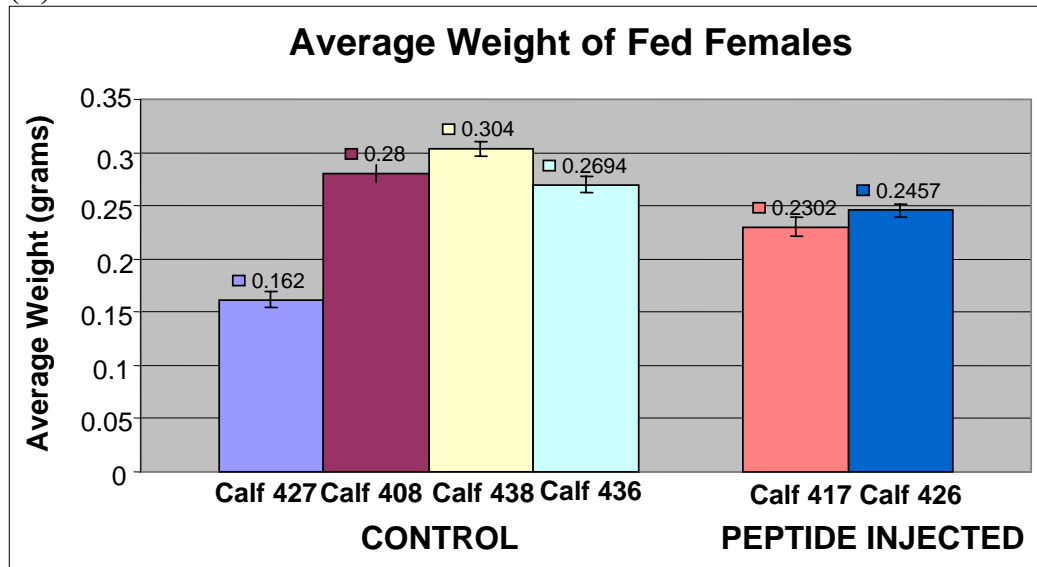
Figure 17. Comparison of the average weight of engorged *Boophilus microplus* (Canestrini) females dropped from control calves and peptide injected calves. (A) The weights of ticks from control cattle and peptide-injected cattle were pooled among calves in these two treatments for statistical analysis. There is no statistical difference in the average weight of fed ticks dropped from the peptide-injected calves versus the control calves (p = 0.061). (B) Descriptive statistics of average weight of ticks for each individual calf.

(A)
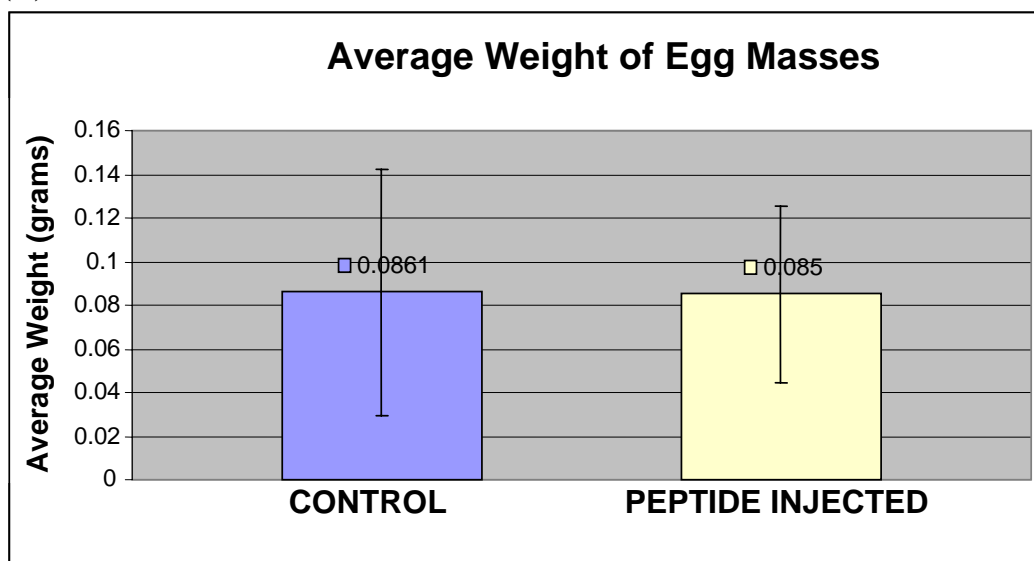
n=30, d.f.= 155.95, t= 0.145, p = 0.885

B)
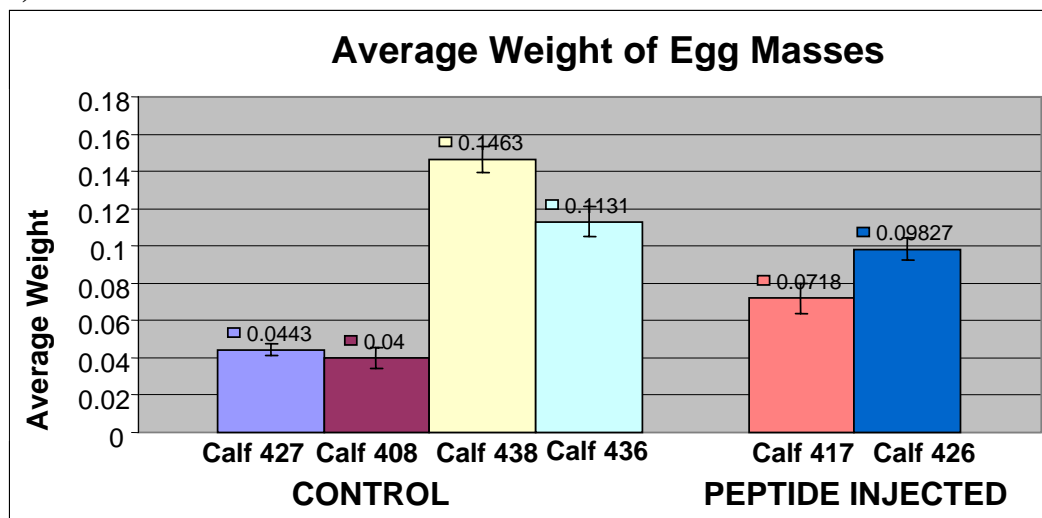
Figure 18. Comparison of the average weight of the egg masses oviposited by the *Boophilus microplus* (Canestrini) females that dropped from control calves and peptide injected calves. (A) The weights of the egg masses from ticks that dropped from control cattle and peptide-injected cattle were pooled among calves in these two treatments for statistical analysis. There is no statistical difference in the average weight of fed ticks dropped from the peptide-injected calves versus the control calves (p = 0.885). (B) Descriptive statistics of average weight of the egg masses of the ticks for each individual calf.

(A)

n=30, d.f.= 178, t = -1.015, p = 0.312

(B)
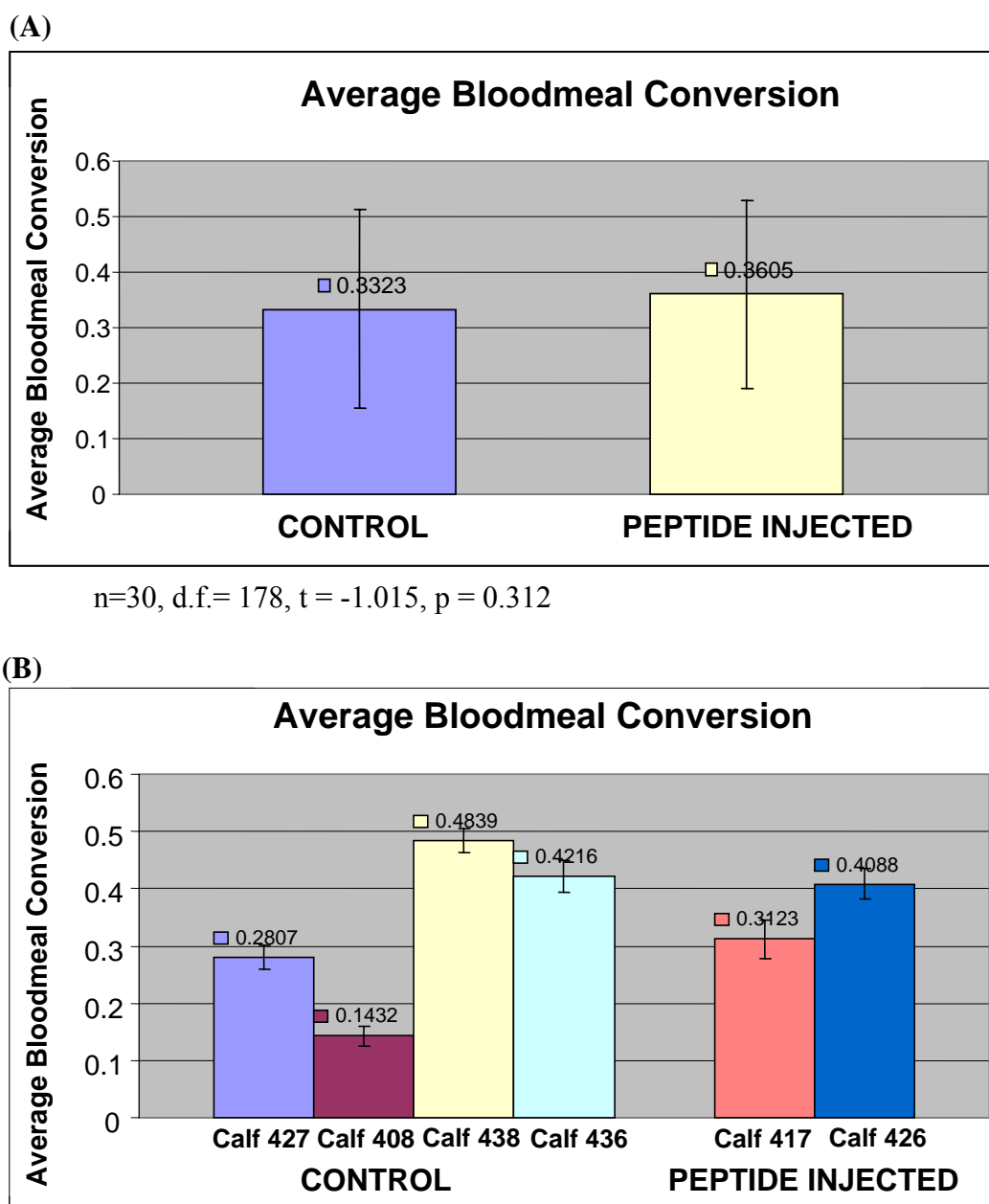
Figure 19. Comparison of the average bloodmeal conversion (weight of egg mass divided by the weight of the engorged female after she has dropped but before oviposition) of the *Boophilus microplus* (Canestrini) females that dropped from control calves and peptide injected calves. (A) The bloodmeal conversion from ticks that dropped from control cattle and peptide-injected cattle were pooled among calves in these two treatments for statistical analysis. There is no statistical difference in the average bloodmeal conversion of the ticks dropped from the peptide-injected calves versus the control calves (p = 0.312). (B) Descriptive statistics of average bloodmeal conversion of ticks for each individual calf.

Discussion

The objective was to validate the myokinin receptor by immunological means as a suitable target for development of novel acaricides. In previous studies, immunization of cattle with Tick-GARD (Bm86 vaccine) resulted in reduction in number of larvae and adult ticks found on the host (Pipano et al., 2003). The results of this research do not concur. The hypothesis that antibodies presented in cattle against the myokinin receptor would disrupt tick physiology in development of the *Boophilus microplus* tick was not supported by the results of these experiments. There was no statistical difference in the number of metanymphs that molted into adults detached from the peptide-injected calves versus those from control calves. The numbers were almost the same for both treatments. There was no statistical significant difference between groups in survivorship, the time to achieve 50% death, and the time to achieve 100% death of the newly molted adults from the peptide-injected calves versus those from the control calves. The lines for the survivorship (Fig. 15) were parallel for both treatments. The results were not what was expected since in a previous study, *Amblyomma americanum* adults that molted from larvae and nymphs that were in glass vials treated with the acaricide pyriproxyfen were lethargic and short-lived compared with adults that molted from larvae and nymphs in untreated glass vials (Donahue et al., 1997).

From 07/03/05 to 07/05/05, four calves (Control Calf #407 and Peptide-injected Calves #420, #421, and #435) died due to bovine babesiosis. Investigations into the cause of the disease suggested that the laboratory Muñoz colony was the source of *Babesia bigemina* since four animals died almost simultaneously, suggesting a

simultaneous infection. It is possible that the immune systems of the peptide-injected calves were more compromised than those of the control calves to overcome the protozoan infection because the three peptide-injected calves that died were producing high levels of the antibody (shown by high absorbances in the ELISA experiments).

The hypothesis that the myokinin receptor disruption affects feeding of the *B. microplus* tick was not supported by these results. The data collected on the number of engorged female *B. microplus* ticks that dropped was limited to 4 control calves and 2 peptide-injected calves. The number of ticks that dropped from each calf was collected but no statistical analyses of this drop could be performed because there were only 2 peptide-injected calves left, therefore a reliable estimate of variance cannot be computed. However, one observation can be made from Table 11 and figure 16. The ticks from control Calf #427 did not drop as uniformly as those from the other five calves. The time to drop was delayed but extended with respect to that of the other five calves, and the number of ticks that dropped each day were less than any of the ones dropped from the other five calves. One reason in this could be that the ticks did not prefer Calf #427 as a host. For the statistical analysis, the weights of the ticks dropped from the peptide-injected calves were pooled (30 weights from two calves for a total of 60 weights) and compared to the pooled weights of the ticks dropped from the control calves (30 weights from four calves for a total of 120 weights). There is no statistical significant difference in the average weight of the females from the control calves versus the peptide-injected calves. There was also no color change to indicate physiological distress in any ticks that dropped.

The hypothesis that the myokinin receptor disruption affects reproduction of the *B. microplus* tick was not supported by these results. There is no statistical significant difference in the average weight of the egg mass and the average bloodmeal conversion of the pooled data from the control calves versus the peptide-injected calves.

One reason for the absence of any effect to the physiological processes of the ticks could be that the calf antibodies are not binding to the myokinin receptor of the cell membrane of the ticks. The myokinin receptor in the cell membrane could have a tertiary structure different from that of the injected peptides. One way to test for antibody binding is through western blot or histochemistry. Membranes containing the myokinin receptor can be isolated and tested by western blots with sera from the 5 calves. If a band that is of the expected size of the myokinin receptor is detected in the western blot, then the antibody was binding to the myokinin receptor. If the antibody does bind to the receptor, then an alternative reason for the null effect of the tick challenge could be not enough concentration of the antibody in the bloodmeal.

CHAPTER IV

CONCLUSION

This thesis demonstrated that peptides designed to correspond to the N-terminus (Peptides 2, 3, 4, and 5), extracellular loops (Peptides 7, 8, 9, 11, and 12), and part of the transmembrane regions of the myokinin receptor from the *Boophilus microplus* tick are antigenic in cattle. There was no statistical evidence to suggest that there was a physiological detrimental effect on ticks that fed on immunogenic cattle. The death of four calves from babesiosis impeded part of the tick challenge experiment. Further investigation is needed for target validation of this myokinin receptor.

A mixture of 13 peptides (12-16 amino acids in length) linked to KLH (Keyhole limpet hemocyanin) was injected into six calves following a defined immunization regime. Six control calves, following the same immunization regime, received KLH only injections. An indirect ELISA (Enzyme-Linked ImmunoSorbent Assay) was developed to determine antibody titers on the sera of these calves. There was statistical evidence to indicate that sera from Peptide-injected Bleed 4 calves have the highest absorbance out of five bleeds. Generally, absorbances increased with successive bleeds, but the absorbance decreased by a small amount for Bleed 5. The absorbance of sera from Calf #412 Bleed 4 was not significantly different from Calf #412 Bleed 0, thus this calf was not used for the tick challenge. The sera from Calves #417, #420, #421, #426, and #435 Bleed 4 showed the highest absorbance when bound to Peptides 2, 8, and 9, and the lowest absorbance when bound to Peptides 1, 6, 7, 10, 13.

Peptide-injected calves (#417, #420, #421, #426, and #435) and control calves (#407, #408, #427, #436, and #438) were transported to the USDA Cattle Fever Tick Laboratory in Edinburg, TX for the tick challenge experiments. Approximately 20,000 larvae were infested on each calf to test the anti-myokinin receptor antibodies for any detrimental effects on tick physiology to validate this receptor as a novel target for acaricides. There was no statistical evidence that the percentage of survival and the time to achieve cumulative 50% and 100% death of adults molted from the metanymphs collected from the peptide-injected calves was different from those from the control calves.

A week after the metanymphs were collected, four calves (peptide-injected calves #420, #421, #435, and control calf #407) died due to bovine babesiosis. The female engorged ticks that dropped from the remaining surviving calves (peptide-injected calves #417, #435, and control calves #408, #427, #436, and #438) were collected to measure their weight, the weight of their egg masses and their bloodmeal conversion. For statistical analysis, the weights of the engorged females, egg masses, and bloodmeal conversions from all the peptide-injected calves were pooled to compare with all the pooled data of the control calves. There was no statistical evidence to indicate that there was a difference in the average of weights of the ticks, the average weights of their egg masses and the average bloodmeal conversions of ticks that dropped from the peptide-injected calves versus those that dropped from the control calves.

The sera of Peptide-injected calves (except Calf #412) are a resource for future studies to validate the myokinin receptor as an acaricidal target. Future studies include

injecting the sera directly into the engorged female to test for any difference in weight of the egg masses. The sera can be used in western blots, immunocytochemisty and in-vivo cell assays for direct visualization of antibody-receptor binding in the first two and for blocking of the myokinin-induced intracellular calcium response in the later.

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APPENDIX I

Example of SPSS output for analyzing ELISA data.
I) Test to determine if the peptide-injected calves were producing antibodies. This is an example of Bleed 4 and Bleed 0, 1:50 dilution data output. Refer to Chapter II Table 6 and Figure 7a.

A)
Between-Subjects Factors

		Value Label	N
TRT	1.00	Peptide-Injected	36
	2.00	Control	30
BLEED	1.00	Bleed 0	33
	2.00	Bleed 4	33
PLATE	1.00	Plate 1	12
	2.00	Plate 2	10
	3.00	Plate 3	12
	4.00	Plate 4	10
	5.00	Plate 5	12
	6.00	Plate 6	10

Note: N stands for the number of wells in the plate for three ELISA experiment repetitions corresponding to the categories of the value label.

B)
Tests of Between-Subjects Effects

Dependent Variable: ABS

Source		Type III Sum of Squares	Df	Mean Square	F	Sig.
Intercept	Hypothesis	88.228	1	88.228	51.893	.001
	Error	8.511	5.006	1.700(a)		
PLATE	Hypothesis	8.581	5	1.716	16.665	.004
	Error	.515	5	.103(b)		
TRT	Hypothesis	2.695	1	2.695	26.181	.004
	Error	.525	5.096	.103(c)		
TRT * PLATE	Hypothesis	.515	5	.103	2.863	.068
	Error	.396	10.993	.036(d)		
BLEED	Hypothesis	2.259	1	2.259	61.741	.000
	Error	.424	11.592	.037(e)		
TRT * BLEED	Hypothesis	.874	1	.874	23.896	.000
	Error	.424	11.592	.037(e)		
TRT * BLEED * PLATE	Hypothesis	.349	10	.035	.355	.959
	Error	4.126	42	.098(f)		

a .990 MS(PLATE) + .010 MS(Error)
b MS(TRT * PLATE)
c .990 MS(TRT * PLATE) + .010 MS(Error)
d .983 MS(TRT * BLEED * PLATE) + .017 MS(Error)
e .973 MS(TRT * BLEED * PLATE) + .027 MS(Error)
f MS(Error)

C) Analysis of interaction of bleed and treatment
BLEED * TRT

Dependent Variable: ABS

BLEED	TRT	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
Bleed 0	Peptide-Injected	1.075	.074	.925	1.224
	Control	.898	.083	.731	1.065
Bleed 4	Peptide-Injected	1.684	.074	1.535	1.833
	Control	1.040	.083	.873	1.207

II) Test to determine which calves have developed immunoreactive serum. Refer to Chapter II Figure 9.

A)
Descriptives

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Calf 412	3	1.1333	.52697	.30425	-.1757	2.4424	.55	1.57
Calf 435	3	2.0403	.61941	.35762	.5016	3.5790	1.36	2.58
Calf 426	3	1.4180	.51429	.29693	.1404	2.6956	.92	1.95
Calf 421	3	1.5217	.58095	.33541	.0785	2.9648	.86	1.97
Calf 420	3	1.9807	.51962	.30000	.6899	3.2715	1.40	2.40
Calf 417	3	2.0107	.44600	.25750	.9027	3.1186	1.55	2.44
Total	18	1.6841	.57551	.13565	1.3979	1.9703	.55	2.58

Note: N is the three absorbance repetitions of the Bleed 4, 1:50 dilution

B)
ANOVA

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	2.166	5	.433	1.501	.261
Within Groups	3.465	12	.289		
Total	5.631	17			

III) Test to determine which peptide(s) bind to the Bleed 4 sera from the peptide-injected calves the strongest: Refer to Chapter II Table 7 and Figure 10.

A)
Between-Subjects Factors

		Value Label	N
Bleed	1.00	Bleed 0	195
	2.00	Bleed 4	195
Peptide	1.00	Peptide 1	30
	2.00	Peptide 2	30
	3.00	Peptide 3	30
	4.00	Peptide 4	30
	5.00	Peptide 5	30
	6.00	Peptide 6	30
	7.00	Peptide 7	30
	8.00	Peptide 8	30
	9.00	Peptide 9	30
	10.00	Peptide 10	30
	11.00	Peptide 11	30
	12.00	Peptide 12	30
	13.00	Peptide 13	30
Plate	1.00	Plate 1	80
	2.00	Plate 2	50
	3.00	Plate 3	80
	4.00	Plate 4	50
	5.00	Plate 5	80
	6.00	Plate 6	50

Note: Note: N stands for the number of wells in the plate for three ELISA experiment repetitions corresponding to the categories of the value label.

B)
Tests of Between-Subjects Effects

Dependent Variable: Absorbance

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	120.882	1	120.882	115.007	.000
	Error	4.205	4.001	1.051(a)		
PEPTIDE	Hypothesis	21.338	11	1.940	25.653	.000
	Error	1.664	22	.076(b)		
PLATE	Hypothesis	4.210	4	1.052	13.918	.000
	Error	1.664	22	.076(b)		
PEPTIDE * PLATE	Hypothesis	1.664	22	.076	.801	.699
	Error	2.454	26	.094(c)		
BLEED	Hypothesis	27.134	1	27.134	287.486	.000
	Error	2.454	26	.094(c)		
BLEED * PEPTIDE	Hypothesis	15.328	12	1.277	13.533	.000
	Error	2.454	26	.094(c)		
BLEED * PEPTIDE * PLATE	Hypothesis	2.454	26	.094	1.706	.019
	Error	17.257	312	.055(d)		

a .999 MS(PLATE) + .001 MS(PEPTIDE * PLATE)
b MS(PEPTIDE * PLATE)
c MS(BLEED * PEPTIDE * PLATE)
d MS(Error)

C) Analysis of interaction of bleed and peptide.
Bleed * Peptide

Dependent Variable: Absorbance

Bleed	Peptide	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
Bleed 0	Peptide 1	.272(a)	.061	.153	.392
	Peptide 2	.363(a)	.061	.243	.482
	Peptide 3	.480(a)	.061	.361	.600
	Peptide 4	.461(a)	.061	.342	.581
	Peptide 5	.231(a)	.061	.112	.351
	Peptide 6	.234(a)	.061	.115	.354
	Peptide 7	.210(a)	.061	.090	.329
	Peptide 8	.305(a)	.061	.185	.424
	Peptide 9	.300(a)	.061	.181	.420
	Peptide 10	.232(a)	.061	.113	.352
	Peptide 11	.251(a)	.061	.131	.370
	Peptide 12	.282(a)	.061	.163	.402
	Peptide 13	.238(a)	.061	.119	.358
Bleed 4	Peptide 1	.419(a)	.061	.300	.539
	Peptide 2	1.549(a)	.061	1.429	1.668
	Peptide 3	.937(a)	.061	.817	1.056
	Peptide 4	.836(a)	.061	.717	.956
	Peptide 5	.639(a)	.061	.519	.758
	Peptide 6	.442(a)	.061	.322	.561
	Peptide 7	.495(a)	.061	.375	.614
	Peptide 8	1.095(a)	.061	.975	1.214
	Peptide 9	1.746(a)	.061	1.627	1.866
	Peptide 10	.417(a)	.061	.298	.536
	Peptide 11	.929(a)	.061	.809	1.048
	Peptide 12	.897(a)	.061	.778	1.017
	Peptide 13	.318(a)	.061	.198	.437

a Based on modified population marginal mean.

D) Analysis of interaction of peptide and calf.
Between-Subjects Factors

		Value Label	N
Peptide	1.00	Peptide 1	15
	2.00	Peptide 2	15
	3.00	Peptide 3	15
	4.00	Peptide 4	15
	5.00	Peptide 5	15
	6.00	Peptide 6	15
	7.00	Peptide 7	15
	8.00	Peptide 8	15
	9.00	Peptide 9	15
	10.00	Peptide 10	15
	11.00	Peptide 11	15
	12.00	Peptide 12	15
	13.00	Peptide 13	15
Cow number	1.00	Cow 417	39
	2.00	Cow 420	39
	3.00	Cow 421	39
	4.00	Cow 426	39
	5.00	Cow 435	39

Cow number * Peptide

Dependent Variable: Absorbance

Cow number	Peptide	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
Cow 417	Peptide 1	.314	.165	-.012	.641
	Peptide 2	1.147	.165	.820	1.474
	Peptide 3	.511	.165	.184	.837
	Peptide 4	.618	.165	.291	.945
	Peptide 5	.409	.165	.082	.735
	Peptide 6	.295	.165	-.032	.621
	Peptide 7	.281	.165	-.046	.607
	Peptide 8	.926	.165	.599	1.253
	Peptide 9	1.925	.165	1.599	2.252
	Peptide 10	.298	.165	-.029	.624
	Peptide 11	.644	.165	.317	.971
	Peptide 12	.761	.165	.434	1.087
	Peptide 13	.255	.165	-.072	.582
Cow 420	Peptide 1	.489	.165	.162	.816
	Peptide 2	2.314	.165	1.987	2.641
	Peptide 3	1.673	.165	1.346	1.999
	Peptide 4	1.225	.165	.898	1.552
	Peptide 5	.957	.165	.630	1.283
	Peptide 6	.426	.165	.099	.752
	Peptide 7	.883	.165	.556	1.209
	Peptide 8	.970	.165	.643	1.297
	Peptide 9	2.397	.165	2.070	2.723
	Peptide 10	.408	.165	.082	.735
	Peptide 11	1.322	.165	.996	1.649
	Peptide 12	1.076	.165	.749	1.403
	Peptide 13	.336	.165	.009	.662
Cow 421	Peptide 1	.505	.165	.179	.832
	Peptide 2	1.170	.165	.843	1.497
	Peptide 3	.883	.165	.556	1.209
	Peptide 4	.822	.165	.495	1.148
	Peptide 5	.664	.165	.337	.990
	Peptide 6	.528	.165	.201	.854
	Peptide 7	.469	.165	.142	.796
	Peptide 8	1.081	.165	.755	1.408
	Peptide 9	1.522	.165	1.195	1.849
	Peptide 10	.464	.165	.137	.790
	Peptide 11	.972	.165	.645	1.298
	Peptide 12	.950	.165	.624	1.277
	Peptide 13	.360	.165	.034	.687

Cow number	Peptide	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
Cow 426	Peptide 1	.375	.165	.048	.702
	Peptide 2	1.378	.165	1.052	1.705
	Peptide 3	.716	.165	.389	1.042
	Peptide 4	.593	.165	.266	.919
	Peptide 5	.453	.165	.127	.780
	Peptide 6	.460	.165	.134	.787
	Peptide 7	.381	.165	.054	.708
	Peptide 8	1.292	.165	.966	1.619
	Peptide 9	1.373	.165	1.046	1.700
	Peptide 10	.353	.165	.026	.680
	Peptide 11	.796	.165	.470	1.123
	Peptide 12	.660	.165	.333	.987
	Peptide 13	.304	.165	-.022	.631
Cow 435	Peptide 1	.412	.165	.086	.739
	Peptide 2	1.735	.165	1.408	2.061
	Peptide 3	.903	.165	.576	1.230
	Peptide 4	.925	.165	.598	1.251
	Peptide 5	.711	.165	.385	1.038
	Peptide 6	.500	.165	.173	.827
	Peptide 7	.461	.165	.134	.788
	Peptide 8	1.204	.165	.878	1.531
	Peptide 9	1.514	.165	1.187	1.841
	Peptide 10	.562	.165	.236	.889
	Peptide 11	.909	.165	.582	1.235
	Peptide 12	1.039	.165	.713	1.366
	Peptide 13	.333	.165	.006	.660

Tests of Between-Subjects Effects

Dependent Variable: Absorbance

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	46.359(a)	64	.724	8.848	.000
Intercept	132.552	1	132.552	1619.125	.000
COW	5.174	4	1.293	15.800	.000
PEPTIDE * COW	41.185	60	.686	8.385	.000
Error	10.643	130	.082		
Total	189.553	195			
Corrected Total	57.001	194			

a R Squared = .813 (Adjusted R Squared = .721)

APPENDIX II

Example of SPSS output for analyzing data from the tick challenge.

I) Output for contingency table for the number of ticks molted collected from peptide-injected calves versus control calves. Refer to Chapter III Table 8.

A)
Treatment * Molt Crosstabulation

			Molt		Total
			1.00	2.00	
Treatment	1.00	Count	128	22	150
		Expected Count	124.5	25.5	150.0
		% within Treatment	85.3%	14.7%	100.0%
		% within Molt	51.4%	43.1%	50.0%
	2.00	Count	121	29	150
		Expected Count	124.5	25.5	150.0
		% within Treatment	80.7%	19.3%	100.0%
		% within Molt	48.6%	56.9%	50.0%
Total		Count	249	51	300
		Expected Count	249.0	51.0	300.0
		% within Treatment	83.0%	17.0%	100.0%
		% within Molt	100.0%	100.0%	100.0%

B)
2x2 Chi-Square Tests

	Value	Df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.158(b)	1	.282		
Continuity Correction(a)	.850	1	.356		
Likelihood Ratio	1.161	1	.281		
Fisher's Exact Test				.357	.178
N of Valid Cases	300				

a Computed only for a 2x2 table
b 0 cells (.0%) have expected count less than 5. The minimum expected count is 25.50.

II) Output for 2 sample T-tests for time to achieve 50% cumulative death and 100% cumulative death for the adults that molted from metanymphs that were detached from the peptide-injected calves and the control calves.

A) 50% Death: Refer to Chapter III Table 10.
Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	T	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
NUM	Equal variances assumed	.758	.409	-.175	8	.865	-.200	1.1402	-2.8292	2.4292
	Equal variances not assumed			-.175	7.777	.865	-.200	1.1402	-2.8424	2.4424

B)100% Death: Refer to Chapter III Table 11.
Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	T	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
NUM	Equal variances assumed	1.258	.295	-1.131	8	.291	-.8000	.70711	-2.43059	.83059
	Equal variances not assumed			-1.131	7.082	.295	-.8000	.70711	-2.46812	.86812

III) Output for the 2 sample T-tests for the average weight of fed females, the average weight of egg mass, and the average bloodmeal conversion of 30 ticks that dropped from 2 peptide-injected calves and 4 control calves. All the thirty measurements of the ticks that dropped from each peptide-injected calf were pooled for a total of 60 measurements to compare with the pooled measurements of the ticks that dropped from the control calves (120 measurements).

A) Average Weight of Fed Females: Refer to Chapter III Figure 17a.
Group Statistics

	TREATMENT	N	Mean	Std. Deviation	Std. Error Mean
WEIGHT	Control	120	.2539	.06809	.00622
	Peptide	60	.2380	.04402	.00568

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	T	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
WEIGHT	Equal variances assumed	14.601	.000	1.644	178	.102	.0159	.00967	-.00319	.03499
	Equal variances not assumed			1.888	166.470	.061	.0159	.00842	-.00073	.03253

B) Average Weight of Egg Mass: Refer to Chapter III Figure 18a.
Group Statistics

	TREATMENT	N	Mean	Std. Deviation	Std. Error Mean
EGGS	Control	120	.0861	.05636	.00514
	Peptide	60	.0850	.04053	.00523

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means					95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference		
									Lower	Upper
EGGS	Equal variances assumed	18.356	.000	.131	178	.896	.0011	.00817	-.01505	.01718
	Equal variances not assumed			.145	155.945	.885	.0011	.00734	-.01343	.01556

C) Average Bloodmeal Conversion: Refer to Chapter III Figure 19a.

Group Statistics

	TREATMENT	N	Mean	Std. Deviation	Std. Error Mean
CONVERSI	Control	120	.3323	.17905	.01635
	Peptide	60	.3605	.16953	.02189

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means					95% Confidence Interval of the Difference	
		F	Sig.	t	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference		
									Lower	Upper
CONVERSION	Equal variances assumed	1.765	.186	-1.015	178	.312	-.0282	.02782	-.08313	.02667
	Equal variances not assumed			-1.033	124.039	.303	-.0282	.02732	-.08230	.02584

APPENDIX III

This appendix describes how data was entered in the SPSS program.

I) ELISA Data:
A) Data analysis for bleeds 2-5 was entered analyzed as follows.

For each dilution, a separate file was done. The categories are as follows:
Plate   Treatment   Bleed   Absorbance   Calf

The variables for each of the categories are numbers, for example for the Chapter II
Figures 4-7 and Tables 3-6 this is how the data was entered:
Plate: 1= plate 1, 2 = plate 2 and so on. 6 plates all together
Treatment: 1= Control, 2 = Peptide-Injected
Bleed: 1= Bleed 0, 2 = Bleed that we are testing
Absorbance: enter the respective value of absorbance
Calf: 1= Calf 407, 2= Calf 412, 3= Calf 438, 4= Calf 435 , 5= Calf 436, 6= Calf 426, 7=
Calf 408, 8= Calf 421, 9= Calf 420, 10= Calf 427, 11= Calf 417

To analyze the data in the menu choose *Analyze* → *General Linear Model* →*Univariate*
In the Univariate option, Dependent Variable = Absorbance, Random Factor = plate, and
Fixed factors = treatment, bleed
Click on *Model*, choose *Custom* option. Drag plate from the left over to the right where
it says *Model* then move treatment to the right, choose plate and treatment at the same
time and move them to the right (make sure interaction is chosen in the middle). Then
bleed to the right. Bleed and treatment to the right. Plate, bleed, treatment to the right.
Click on *Continue*
Back in Univariate click on *Options* and move Bleed*treatment over to the right where it
says *Display Means For*. Click *Continue*
Back in Univariate click *OK*.

B) Data analysis for Bleed 4, 1:50 dilution antibody production, Chapter II Figure 8:
Categories are as follows:
Absorbance from Peptide Bleed 4   Calf

To analyze the data in the menu choose *Analyze* → *Compare Means* →*One-Way
ANOVA*
In the One-Way ANOVA, Dependent List = Absorbance, Factor = Calf
Click on *Options*, choose *Descriptives* →*Continue*
Click *OK*

C) Data analysis for Bleed 0 vs Bleed 4 Peptides 1-13, Chapter II Figure 9 and Table 7:

Categories are as follows:
Plate   Peptide   Calf   Bleed   Absorbance

Plate: 1= plate 1, 2= plate 2 and so on. 6 plates all together
Peptide: 1= Peptide 1, 2= Peptide 2, and so on. 13 Peptides all together
Calf: 1= Calf 417, 2= Calf 420, 3= Calf 421, 4= Calf 426, 5= Calf 435
Bleed: 1= Bleed 0, 2= Bleed 4
Absorbance: what the absorbance was.

To analyzes the data in the menu choose *Analyze → General Linear Model →Univariate*
In the Univariate option, Dependent Variable = Absorbance, Random Factor = plate, and
Fixed factors = peptide,bleed
Click on *Model*, choose *Custom* option. Move plate from the left over to the right where
it says *Model* then move peptide to the right, choose plate and peptide at the same time
and move them to the right (make sure interaction is chosen in the middle). Then bleed
to the right. Bleed and peptide to the right. Plate, bleed, peptide to the right. Click on
Continue
Back in Univariate click on *Options* and move Bleed*Peptide over to the right where it
says *Display Means For*. Click *Continue*
Back in Univariate click OK.

II) Tick challenge Data:

A) Data analyses for number of ticks molted from peptide-injected calves versus control
calves. A contingency table is performed.
Categories are as follows:
Trt   Molt   Count

Treatment: 1= Control, 2 = Peptide-Injected
Molt: 1 = Molted, 2 = Did Not Molt

To analyze data in the menu choose *Data → Weight cases*, click on *Weight cases by*,
Frequency variable = count, click *OK*
Then *Analyze →Descriptive Statistics →* Crosstab
In Crosstab option, Row = Trt, Column = Molt. Click on Percentage, click *OK*

B) Data analysis for time to achieve 50% and 100% cumulative death.
Categories are as follows:
Trt   number

Trt: 1= Control, 2 = Peptide-Injected

To analyze data in the menu choose *Analyze* → *Compare means* → *Independent Samples T Test*
Test variable = number, Grouping variable is Trt. Define Trt, Group 1=1, Group 2=2. Click *OK*

C) Data analysis for average weight of fed females, average egg mass, average bloodmeal conversion of the peptide-injected calves versus the control calves: Categories are as follows:
Trt   Weight/ Weight of Egg Mass/ Bloodmeal Conversion

Trt: 1= Control, 2 = Peptide-Injected

To analyze data in the menu choose *Analyze* → *Compare means* → *Independent Samples T Test*
Test variable = Weight/ etc., Grouping variable is Trt. Define Trt, Group 1=1, Group 2=2. Click *OK*

APPENDIX IV

I was bestowed the honor of being chosen as a fellow for the Hispanic

Leadership Program in Agriculture and Natural Resources (HLPANR) for the 2003-

2004 and 2004-2005 academic years. This great program focuses on research,

leadership, professional development, and policy analysis. My involvement in seminars,

workshops, field trips, my leadership project, and presenting my research to the program

has aided my goals of relating my research to the public and gaining leadership skills.

The seminars and workshops have given me the opportunity to understand policy

development, implementation, and analysis. The speakers and the presentations on such

topics of NAFTA and land-grant policies illustrated that policy development is a tool to

improve the lives of disadvantaged people.

The two field trips that I attended were to Uvalde, TX and Alice, TX. This was

an excellent opportunity to get to know the needs and experiences of the South Texas

farmers and ranchers, first hand. I was able to relate my research to the needs of the

ranchers. I observed how the farmers and ranchers apply their knowledge of

entomology for their use of agriculture.

My leadership project was to expose Hispanics to the study of entomology and

the opportunities that entomology has to offer by translating recruiting materials into

Spanish and attending college recruiting programs. The recruiting programs that I

attended were the February 26, 2005 Aggieland Saturday at Texas A&M and the April 9,

2005 Houston Hispanic Forum Career and Education Day. From this experience, I have
learned that recruiting for Hispanic college students is a tough job because some think
that college is not a possibility for them but at least the seed of higher education has been
planted in their minds and they were able to get information that they might not
otherwise have obtained.

I have presented my research to all of the fellows and administrators of the
program. That was an invaluable experience since most of the fellows studied such
diverse subjects such as English, Poultry science, and Agronomy. I developed the skills
to communicate and disseminate information in a manner understandable to diverse
audiences. I have been fortunate to have been part of this program.

List of activities that I attended:

August 28-29, 2003	HLPANR Orientation Program for AY 2003-2004 at Woodfield Suites, UTSA in San Antonio
October 18, 2003	Policy Analysis Workshop at Texas A&M in College Station
November 7, 2003	USFS Policy Workshop at San Antonio
March 5-6, 2004	HLPANR Uvalde Field trip in Uvalde, Texas
April 10-11, 2004	Fellows Research Presentations at UTSA, San Antonio
July 23-24, 2004	HLPANR meeting
August 27-28, 2004	HLPANR Orientation Program for AY 2004-2005 at Reed Arena, Texas A&M in College Station
October 22-23, 2004	Policy Workshop at Woodfield Suites, UTSA in San Antonio
November 12, 2004	HLPANR Meeting at Rudder Tower, Texas A&M in College Station
February 11-12, 2005	HLPANR Alice Field trip in Alice, Texas
April 15-16, 2005	Policy Workshop and Research Presentations at Rudder Tower, Texas A&M in College Station

VITA

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M.S., Entomology, Texas A&M University, 2006.

Experience: Technician I, Insect Toxicology Laboratory, Entomology
Department, Texas A&M University, May 2002-August 2003.

Laboratory Skills: Evaluate mammalian cell cultures for growth and contamination,
maintain the cultures by cell splitting and freezing aliquots.

Transfect *E. coli* cells with various vectors and culture them in
liquid and solid media.

Sequence DNA in various vectors by using DNA primers and the
PCR machine.

Calculate DNA and gene sizes by cutting the DNA with
restriction enzymes and perform DNA agarose electrophoresis.

Nucleic Acid manipulation: Purify DNA by using miniprep
procedures, extract DNA from agarose gels, and RNA extraction
from tissues.

In situ Hybridization

Design and handle peptide fragments.

Serum Collection

Enzyme-Linked Immunosorbent Assays (ELISA) /plate reader

Evaluating tick challenge experiments