INTERFERENCE WITH THE BIOLOGIC TRANSMISSION OF *ANAPLASMA MARGINALE* BY TARGETING THE TICK-PATHOGEN INTERFACE

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By

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INTERFERENCE WITH THE BIOLOGIC TRANSMISSION OF *ANAPLASMA MARGINALE* BY TARGETING THE TICK-PATHOGEN INTERFACE

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DEDICATION

This work is dedicated to my parents who raised me to be here and to pursue this degree, my wife who supported me throughout, and my kids, Zion, and Zofia, who are my motivation for the better each day.

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ABBREVIATIONS

$	Dollar
%	Percentage
µm	Micrometer
1° Ab	Primary Antibody
2° Ab	Secondary Antibody
AAAP	*A. marginale* appendage associated protein
Ab	Antibody
ACUC	Animal Care and use committee
ADJ	Adjuvant
AFA	Artificial Feeding Apparatus
Ag	Antigen
Avg	Average
BC	Before Christ
BF	Baseline feeding
bp	Base pairs
BP	Before present (Before radiocarbon dating)
ºC	Degrees Centigrade
CA	California
cc	Cubic centimeter
CDC	Centers for Disease Control and Prevention
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate)
cm	Centimeter

CO	Colorado
CO_2	Carbon Dioxide
dMG	Denatured midgut
DNA	Deoxyribonucleic acid
dpi	Days post inoculation
dSG	Denatured salivary gland
E. coli	*Escherichia coli*
e.g.	“Exempli gratia” (for example)
ECF	East coast fever
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EST	Expressed sequence tag
et. al.,	*“et alia”* (and others)
ºF	Degrees Fahrenheit
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FDR	False discovery rate
g	Grams
H_2O	Water
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric Acid
HAT	Human African trypanosomiasis
H	Hour

HRP	Horse raddish peroxidase
i.e.	“id est” (that is)
IgG	Immunoglobulin G
IL	Illinois
IM	Intramuscular
IN	Indiana
IPTG	Isopropyl β- d-1-thiogalactopyranoside
Kg	Kilogram
L:D	Light: Dark
lbs	Pounds
LC-MS	Liquid chromatography-Mass spectrometry
LNo_2	Liquid Nitrogen
m.o.	Months
MA	Massachusetts
MG	Midgut
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
MO	Missouri
NC	North Carolina
nm	Nanometer
nMG	Native midgut

No.	Number
nSG	Native salivary gland
NTC	Non template control
NY	New York
OD	Optical density
OIE	Office international des Epizooties
P	Probability
PA	Pennsylvania
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline tween
PCR	Polymerase chain reaction
PCV	Packed Cell volume
PPE	Percent Infected erythrocytes
PPP	Prepatent period
PVDF	Polyvinylidene fluoride
qPCR	Quantitative Polymerase chain reaction
pI	Isoelectric point
RBCs	Red blood cells
RT	Room temperature
S-D	Shine-Dalgarno
SD	Standard Deviation
SE	Standard Error
SG	Salivary Gland

S.O.C	Super optimal broth with catabolite repression
Spp.	Species
SUB	Subolesin
TBEV	Tick-borne encephalitis virus
TBP	Tick-borne pathogens
TF	Transmission Feeding
TMB	Tetra methyl benzidine
TPS	Thrombin protease recognition site
USA/US	United States of America
v	Volume
VBDs	Vector-borne diseases
w	Weight

ABSTRACT

Ticks are globally renowned vectors for numerous zoonoses. Ixodid ticks transmit a variety of viral, bacterial, and protozoan pathogens to their vertebrate hosts. Because of the expansion of the geographic ranges of both ticks and pathogens, increasing tick populations, emerging tick-borne pathogens and continued challenges of achieving effective and sustained tick control, ticks and tick-borne diseases have increased in importance from medical and veterinary public health perspectives. Host immune resistance to ticks has been studied since the first half of the 20th century. This dissertation addresses (i) adaptation of a naturally coevolved large-animal model to investigate immunological underpinnings of resistance to tick challenge, (ii) interference with experimental biologic transmission of a naturally coevolved tick-borne pathogen, *Anaplasma marginale,* to cattle, (iii) preliminary observations of effects of tetracycline on acquisition and transmission of *A. marginale* by tick vectors and (iv) preliminary optimization of artificial feeding conditions for ixodid ticks with different apparatuses. Several tick proteins were identified, which were reactive to antisera of tick-extract-immune cattle that were associated with reduced tick performance. The proteins identified here are important for future experiments to determine their posited roles in reduction of tick feeding. *Dermacentor andersoni* and *D. variabilis* ticks, natural biologic vectors of *A. marginale* in the United States, did not experimentally transmit this pathogen to susceptible hosts immunized with crude homogenates of tick tissues. In addition to the main emphasis of this dissertation, preliminary observations included reductions in infection levels acquired by ticks and effects

of tetracycline on tick acquisition and transmission of *A. marginale*. Collectively, the work described in this dissertation suggested that interventions with acquisition and transmission of *A. marginale* and other tick-borne pathogens are not necessarily dependent on tick mortality.

CHAPTER 1

Introduction

The major vector-borne diseases of cattle worldwide

The first known example of the domestication of animals for food by human beings was around 11,000 BC, with sheep in Southwest Asia, followed by the domestication of goats around 8,000 BC (Lear, 2012). Since then, people have domesticated over 40 animal species that have directly or indirectly contributed to agriculture by providing food or products (Taberlet *et al*., 2008). The domestication of animals has been essential in the development of human society (FAO and UNEP, 2000). One of the most important domesticated animals is, arguably, cattle.

The ancestor of domestic cattle was a group of races of *Bos primigenius*, which had a very wide geographic distribution (Medugorac *et al*., 2009). There are two types of cattle, those with a prominent thoracic hump, known as zebu or indicine cattle (*Bos taurus indicus*), and cattle without a hump, known as taurine or European cattle (*Bos taurus taurus*) *(Senczuk et al*., 2020). The domestication of zebu and taurine cattle was around 6,000-5,500 BC, in the Indus Valley, and 8,500-8,000 BC in Anatolia, respectively (Senczuk *et al*., 2020; Medugorac *et al*., 2009; Tapio *et al*., 2006). Currently, it is estimated that more than 1.4 billion cattle are kept worldwide (Michalk *et al*., 2019), which contribute significantly to food and agriculture. The ten countries with the highest bovine ratios per capita are described in **Table 1.1**.

Table 1.1 Countries with the highest cattle production per capita

Rank	Country	Cattle Population	Human Population	Per Capita
1	Uruguay	11,946,000	3,461,734	3.45
2	New Zealand	10,063,000	4,783,063	2.10
3	Argentina	53,831,000	44,780,667	1.20
4	Brazil	252,700,000	211,049,527	1.20
5	Australia	23,217,000	25,203,198	0.92
6	Belarus	4,300,000	9,452,411	0.45
7	Canada	11,150,000	37,411,047	0.30
8	United States	93,595,000	329,064,917	0.28
9	India	305,500,000	1,366,417,754	0.22
10	European Union	85,545,000	447,700,062	0.19

Source: FSA/USDA (head/people)

Vector-borne diseases have been a continuous danger to people and animals, including the human plagues like the “Black Death” in Europe (Benedictow, 2006) and yellow fever that impacted the new world (Monath and Vasconcelos, 2015). Nagana is a major reason for lack of development in Africa (Gubler, 2009). Vector-borne diseases are usually triggered by eukaryotic parasites, bacteria or viruses spread by blood-sucking arthropods like ticks, fleas and mosquitoes (Beugnet and Marié, 2009). Among hematophagous arthropods, ticks can also directly affect the host, but arguably the most important effect ticks have on human and animal health is through transmission of infectious disease agents or “pathogens,” and ticks transmit a greater variety of etiologic agents than other blood-feeding arthropods (Jongejan and Uilenberg, 2004; Sonenshine and Roe, 2013; Telford and Goethert, 2004). Globally, there are five major vector-borne diseases of cattle, which cause significant production and economic losses to the cattle industry. Ticks transmit etiologic agents of four of these major vector-borne diseases. Examples of the primary etiologic agents and major vectors of these disease agents are listed in **Table 1.2**

Table 1.2. Five major vector-borne diseases of cattle, worldwide.

Disease	Etiologic Agent(s)	Vector(s)*	Reference
African Trypanosomiasis (Nagana)	*Trypanosoma brucei*	Tsetse Flies *(Glossina spp.)*	(Latif *et al*., 2019)
Theileriosis (East Coast Fever)	*Theileria parva*	*Rhipicephalus spp.*	(Mukhebi, 1992)
Babesiosis (Redwater)	*Babesia bovis, Babesia bigemina*	*Boophilus spp.*	(Bock *et al*., 2004)
Cowdriosis (Heartwater)	*Ehrlichia ruminantium*	*Amblyomma spp.*	(Cowdry, 1925a)
Anaplasmosis (Gall Sickness)	*Anaplasma marginale*	Rhipicephalinae and Biting Flies	(Kocan *et al*., 2010)

1. Nagana

Protozoa are considered the most primitive and diverse group of organisms among the eukaryotic kingdoms (Auty *et al*., 2015). Members of the kinetoplastid family Trypanosomatideae (*e.g.*, *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania* spp.), Amoebae (*e.g., Entamoeba* spp.) and the Apicomplexa (*e.g.,* genera *Plasmodium*, *Cystoisospora*, *Eimeria*, *Babesia*, *Theileria* and *Toxoplasma*) cause diseases in human beings as well as wild and domestic animals (Tyler and Engman, 2000). The parasitic unicellular flagellated protozoa of the genus *Trypanosoma* include etiological agents of human and animal diseases, which are found globally but mostly in the southern hemisphere (Steverding, 2008). All *Trypanosoma* spp. described to date are parasitic, affecting a wide range of metazoan hosts, including mammals (Ley *et al*., 1988). Trypanosomiasis is considered one of the most common infectious diseases of cattle in sub-Saharan Africa, and species related to the etiologic agents of this disease have also spread to Asia and South America (Stephen, 1986).

Importance

Trypanosomiases among domestic livestock are also denoted as Nagana or Surra (Waruri *et al*., 2021). African Trypanosomiasis, or Nagana, is a devastating disease with huge economic losses resulting from decreased livestock production and productivity in sub-Saharan Africa (Steverding, 2008). Trypanosomiasis also causes huge economic losses worldwide. In Central and South America, an estimated 300 million cattle, 1.8 million buffaloes and 16 million horses are at risk

of infection by *T. vivax* and *T. evansi,* which are closely related to *T. brucei*, causing a Nagana-like wasting disease in horses and cattle, but are adapted to vampire bats and tabanid flies as biologic or mechanical vectors, respectively (Auty *et al*., 2015). Although they are of major medical importance among people and companion animals, *T. cruzi* infections are not considered to be of economic importance among livestock (Hodo *et al*, 2018).

Transmission

The etiological agents of African Trypanosomiasis among domestic livestock in Africa include the *T. brucei* complex, *T. vivax*, *T. evansi*, *T. simeae*, *T. equiperdum* and *T. theileri*, while, in South America, *T. cruzi*, *T. theileri*, *T. equiperdum*, *T. evansi* and *T. vivax* are the major agents of Trypanosomiasis (Chadenga, 1994). Generally, *T. brucei* complex infections that threaten African livestock have a 100- to 150-fold higher prevalence than those that cause human African trypanosomiasis (also known as sleeping sickness) (Baral, 2010).

The *T. brucei* complex has been described as digenetic pathogens because they affect two hosts during their life cycle, *i.e*., the tsetse fly and the mammalian host (Chadenga, 1992). The life cycle has two phases in the invertebrate host / insect biologic vector and a third phase in the vertebrate / mammalian host (Hoare, 1972). The development of *T. vivax* is confined to the proboscis of *Glossina* spp. (**Figure 1.1**). In vertebrate hosts, salivarian trypanosomes such as *T. brucei* multiply as trypomastigotes. The infective stages that tsetse flies inject into vertebrate hosts are metacyclic forms that go into the blood stream and divide by binary fission as trypomastigotes. When a competent invertebrate biologic vector

(*Glossina* spp.) feeds on an infected vertebrate host, the *T. brucei* transform to the procyclic forms in the midgut of the fly and, after leaving the midgut, these parasites will transform to epimastigotes. After moving to salivary glands of the fly, epimastigotes transform to metacyclic forms that are infectious to vertebrate hosts (Shimelis and Melkamu, 2015).

Control

Trypanosomiasis is a significant challenge to livestock production and productivity, and further has a noticeable impact on land utilization in sub-Saharan Africa. *Glossina* spp. (tsetse flies) are the main vectors of *T. brucei* and *T. vivax* from infected to susceptible animals (Stephen, 1986). There are multiple methods in practice to control trypanosomiasis.

a) Parasite control

Chemotherapy is used to control trypanosomes of veterinary importance. So far only six compounds are currently licensed. In Africa most used compounds are, diminazene aceturate and isometamidium chloride (Holmes *et al*., 2004)

b) Tsetse fly control

Vector control is another effective measure to prevent the vector-borne disease. Therefore, attempts were made to control tsetse flies by using insecticides. This control method showed some advantages in cost or speed of action over other methods to control tsetse flies (Hocking *et al*., 1963). In the 1940s, tsetse fly eradication was also attempted, after the discovery of dichloro-diphenyl-

trichloroethane (DDT). The following three methods were introduced to control the vector (Dransfield *et al*., 1991)

I. Ground spraying
II. Aerial spraying
III. Odor-baited traps and targets

c) Applications of insecticides to cattle by dipping or pour-on formulations

Insecticide dips or pour-on formulations are used to target the tsetse flies (Chadenga, 1994). In controlled experiments, it was shown a single treatment with deltamethrin, as a wash or pour-on, killed almost 100% of alighting tsetse flies for 2 weeks (Chadenga, 1992). For 8 weeks, 60% of alighting tsetse flies were immobilized or "knocked down." It was estimated that 95% of knocked-down flies are predated upon and fail to survive in the natural environment (Shimelis and Melkamu, 2015).

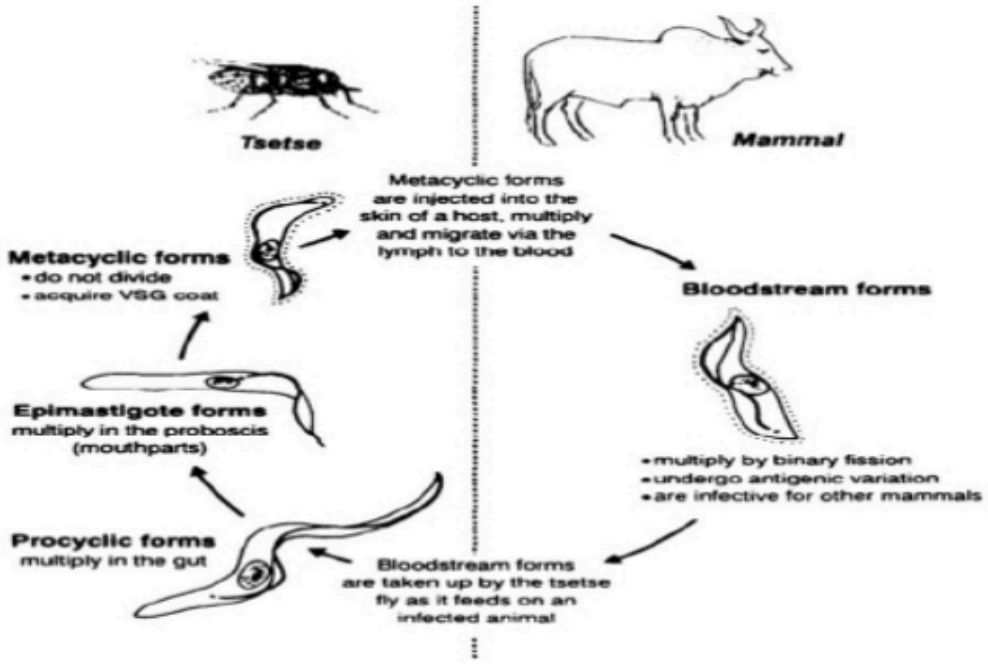

Figure 1.1. A general summary of the life cycle of trypanosomes showing the stages involved in tsetse flies and mammals (Authié, 1994 ; Nagagi *et al*., 2018).

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2. Babesiosis

Babesiosis is a tick-borne disease caused by intraerythrocytic protozoan parasites of the genus *Babesia,* which as a genus parasitize a wide variety of domestic and wild animals (Bock *et al*., 2004). Genus *Babesia* was initially discovered in bovine erythrocytes, in association with hemoglobinuria, by Babes in 1888, who later saw a similar parasite in sheep erythrocytes (Uilenberg, 2006). In 1893, Smith and Kilborne named this pathogen *Pyrosoma bigeminum* (Smith and Kilborne, 1893). Within the same year these parasites were renamed *Babesia bovis*, *Babesia ovis* and *Babesia bigemina* (Taboada and Merchant, 1991).

Importance

Babesiosis is considered the most economically important tick-borne disease of cattle worldwide (Minjauw and McLeod, 2003). This disease greatly impacted the US cattle industry during the 19th through the mid-20th century, and even provoking conflicts as cattlemen took up arms to stop the movement of southern herds into Missouri (Todorovic, 1974). In 1893, Smith and Kilborne published a report of transmission of *B. bigemina,* the etiological agent of Texas cattle, by ticks that are now known as *Rhipicephalus (Boophilus) annulatus*, one of several one-host cattle tick species now known to transmit *B. bigemina* and *B. bovis*. This work had significant scientific and medical impact because it was the first conclusive demonstration of transmission of a pathogen by an arthropod and of a non-contagious agent of infectious disease (Smith and Kilborne, 1893). Furthermore, perhaps even more importantly, this report of experimental tick

transmission of *B. bigemina* had a significant historical impact, leading to (1) elimination of endemic bovine babesiosis in the US through eradication of vector ticks and (2) lateral transfer of this discovery to experimental transmission of numerous other vector-borne infections; most notably, mosquito-borne pathogens, yellow fever virus and human malaria parasites (*Plasmodium* spp.), which in turn led to implementation of effective vector control and thus disease prevention strategies that made possible construction of the Panama Canal (Soper, 1967). Although these etiologic agents and their vectors are eradicated from the US, a permanent tick quarantine zone is maintained and continually monitored in Southwest Texas (Tidwell *et al*., 2018).

It is estimated that most of the 1.2 billion cattle in the world are exposed to *Babesia* spp., but an even greater number of cattle are at risk (Bock *et al*., 2004). Cattle indigenous to *Babesia*-endemic areas are often naturally premune to babesiosis, while previously unexposed non-indigenous, thus non-premune, cattle suffer from severe hemolytic anemia when infected with these parasites upon introduction to enzootic regions (Castro and Newson, 1993). Premune is a term used for infection-based immunity, it is a host response that protects the host against high numbers of pathogens and illness without eliminating the infection. The vector population also plays an important role in exposure after infection. For example, in tropical areas with high vector populations, natural exposure usually occurs at an early age and these cattle therefore become premune to subsequent disease (Emerson and Wright, 1968). Costs due to babesiosis are not only due to mortality, but also due to treatment, reduced milk or meat production, abortion, and

preventive measures like the use of acaricides (Norval, *et al*.,1991). The estimated cost of babesiosis prevention and control in different countries is as follows **(Table 1.3).**

Transmission

Babesia sporozoites, the infective stages transmitted to vertebrate hosts by ticks, directly infect erythrocytes (Mehlhorn and Schein, 1985). The sporozoites transform into trophozoites from which two merozoites develop by the process of merogony (Waruri *et al*., 2021). *Babesia* digest hemoglobin to the degree that no residual body remains in the cytoplasm of erythrocytes, suggesting that, unlike other piroplasms such as those of mosquito-transmitted *Plasmodium* spp*., Babesia* spp. are non-pigment-producing parasites (Mehlhorn and Schein, 1985). The merozoite, which are diploid in case of *B. bigemina*, can be a gamont precursor (Riek, 1964). These gamont precursors do not become gamonts until they are ingested by ticks (Mackenstedt *et al*., 1995).

Ticks feed on infected animals and infected erythrocytes are ingested by ticks, *Babesia*-infected erythrocytes contain gamonts, also known as gametocytes, which eventually fuse in the tick midgut lumen to form zygotes that multiply and enter different tick organs, including ovaries, for transovarian passage to the next generation (Mehlhorn and Schein, 1985). After sporogony in tick salivary glands, subsequent generations of *Babesia* spp. are ready to infect the next vertebrate host parasitized by their acarine host (Jongejan and Uilenberg, 2004; Bock *et al*., 2004). (**Figure 1.2**)

Table 1.3. Estimated cost of bovine babesiosis prevention and control.

Country	Amount /Year in US Dollars (millions)
Australia	23.3
Kenya	5.1
Zimbabwe	5.4
Tanzania	6.8
South Africa	21.6
China	19.4
India	57.2
Indonesia	3.1
Philippines	0.6

Source: (Alvarez *et al*., 2020)

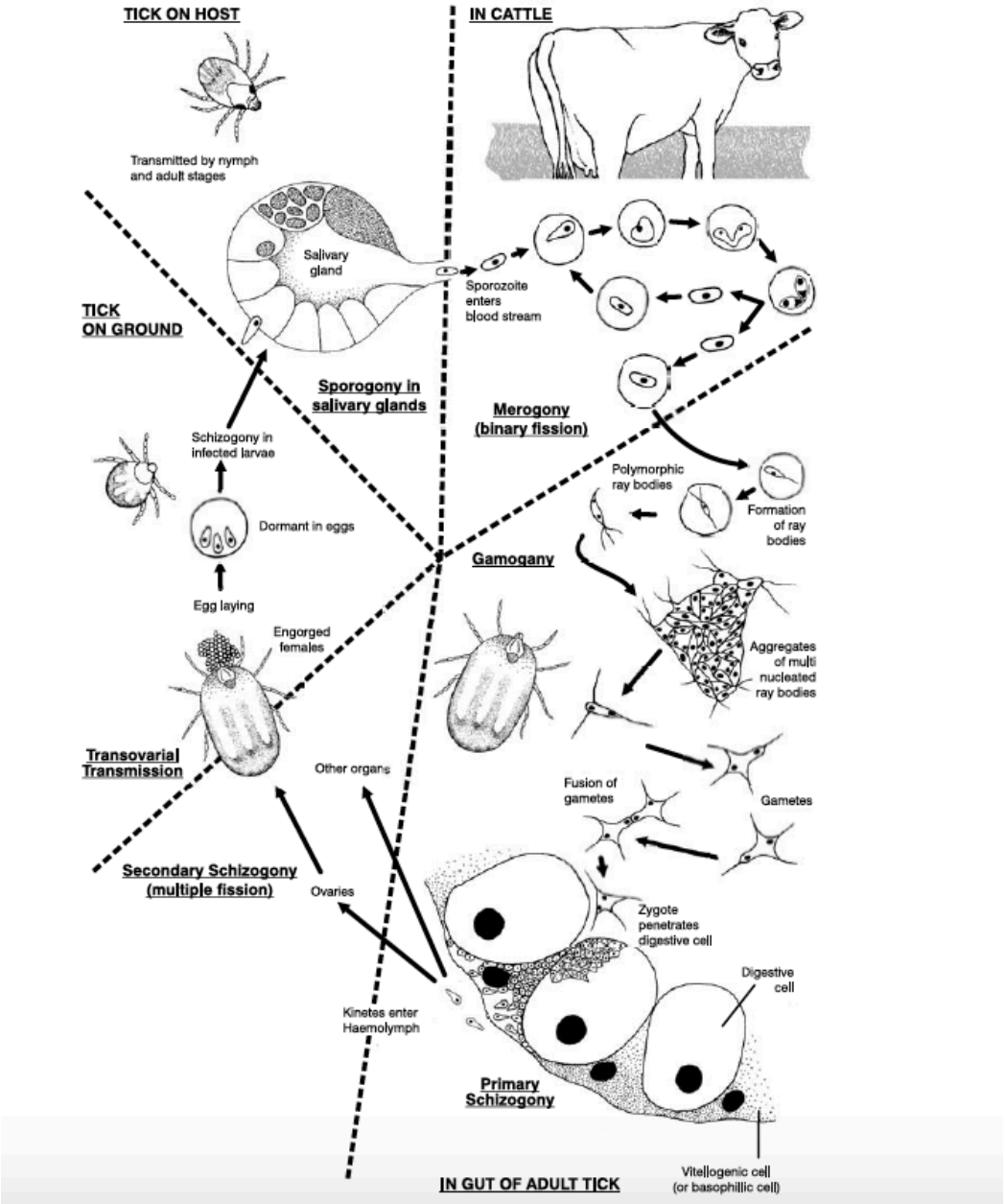

Figure 1.2. Life cycle of *Babesia bigemina* in cattle (Bock *et al*., 2004)

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Control

In *Babesia*-endemic regions, disease control is currently considered a better option than vector eradication (Uilenberg, 2006). Three main strategies are in use.

a) Anti-*Babesia* Vaccines

Live, attenuated, blood-based *Babesia* vaccines are in use in many countries, including Australia, Israel and Argentina (Bock *et al*., 2004; Brown *et al*., 2006; Florin-Christensen *et al*., 2014), but more modern methods for preparation of live vaccines have been developed (Rathinasamy *et al*., 2019).

b) Tick Control Strategies

Vector control is another way to mitigate babesiosis. This strategy has been employed for a long time, and acaricides have been used to control ticks (Gubler, 2009). There are some notable disadvantages to the use of acaricides, including irritation of animal skin, contamination of animal products and the environment, and the emergence of acaricide-resistant ticks (de la Fuente *et al*., 2007; Florin-Christensen *et al*., 2014).

c) Drug-based *Babesia* Control Strategies

The most employed drugs to treat clinical babesiosis are imidocarb dipropionate or diminazen acetate. Both drugs have documented efficacy in controlling clinical babesiosis; however, these drugs are expensive and can leave

residues in milk and meat products (Kuttler and Johnson, 1980; Mosqueda *et al*., 2012).

3. Theileriosis

Theileria spp. are tick-borne protozoan parasites that infect wild and domestic animals globally. They are classified in the phylum Apicomplexa, class Aconoidasida (=Sporozoa), order Piroplasmida (Levine *et al*.,1980). This genus has two important stages in the vertebrate host, a form of asexual reproduction known as schizogony in lymphocytes of the vertebrate host, and a piroplasm stage that divides by binary fission in erythrocytes (Mehlhorn and Schein, 1985). Some piroplasms develop into gametes that fuse within the tick midgut to form zygotes known as ookinetes; thus, sexual reproduction of *Theileria* spp. occurs in the tick.

Importance

At least two *Theileria* spp. are economically important. *Theileria parva*, transmitted by *R. appendiculatus* ticks, causes a lymphoproliferative disease known as East Coast fever (Neitz, 1957), and was responsible for economic losses of 170 million USD in 1989 alone (Mukhebi *et al*., 1992). *Theileria annulata*, transmitted by several *Hyalomma* spp., uses the Asian water buffalo (*Bulbulus bubulis*) as a vertebrate reservoir (Dolan, 1989) and this species is responsible for tropical theileriosis from Southern Europe to China, a vast geographic region in which an estimated 250 million cattle are at risk (Gubler, 2009). Both *T. parva* and *T. annulata* induce a transformation-like phenotype in nucleated mammalian host cells, which is the major cause of pathology (Kuttler and Kreier, 1986).

In September 2017, *Theileria orientalis* (Ikeda strain) was diagnosed in seven cattle from a herd in Albemarle County, VA, US (USDA, 2020). Adults and nymphs of *Haemaphysalis longicornis* ticks, also known as the Asian longhorned tick, were sampled from the habitat of these cattle. In a preliminary report, an experimental transmission trial was performed by USDA's Agricultural Research Service (ARS), in collaboration with the Virginia Tech Animal Laboratory Services (ViTALS) laboratory, which confirmed the vector competence of *H. longicornis* for a highly virulent nonlymphoproliferative agent of bovine theileriosis, *Theileria orientalis* Ikeda (Dinkel *et al*.,2021). Some *Theileria* spp. (*T. parva* and *T. annulata*) are reportable to the World Organization for Animal Health (OIE); however, *T. orientalis* currently is not reportable to the OIE.

Transmission

Theileria spp. that infect cattle and small ruminants are transmitted by Ixodid ticks of the genera *Rhipicephalus*, *Amblyomma*, *Hyalomma* and *Haemaphysalis* (Binnington and Kemp, 1980; Shahzad *et al*., 2015). *Theileria* spp. have complex life cycles in both vertebrate and invertebrate (acarine) hosts, with sexual reproduction occurring in the tick (Waladde *et al*., 1993).

In vertebrate hosts, the life cycle of *T. parva* starts with secretion of infective sporozoites from the infected tick salivary glands, into the tick-feeding lesion. The sporozoites enter lymphocytes of the vertebrate host and divide by schizogony, developing into schizonts containing merozoites. Host cells and schizonts both divide simultaneously (Hawa *et al*., 1981). As a result of schizogony, lymphocytes eventually burst, releasing merozoites that enter erythrocytes and form piroplasms, which replicate by binary fission, some of which develop into

microgametocytes or macrogametocytes within vertebrate erythrocytes that can be ingested by subsequent generations of ticks.

In the acarine host, the gametocytes fuse in the tick midgut lumen, resulting in the formation of motile zygotes known as ookinetes, which penetrate the tick midgut epithelium and travel via the hemolymph to salivary gland acini (Binnington and Kemp, 1980; Fawcett *et al*., 1982; Mehlhorn and Schein, 1985). *Theileria* reaches the salivary glands and undergoes sporogony to transform into vertebrate-infective sporozoites (Dolan, 1989) (**Figure 1.3**).

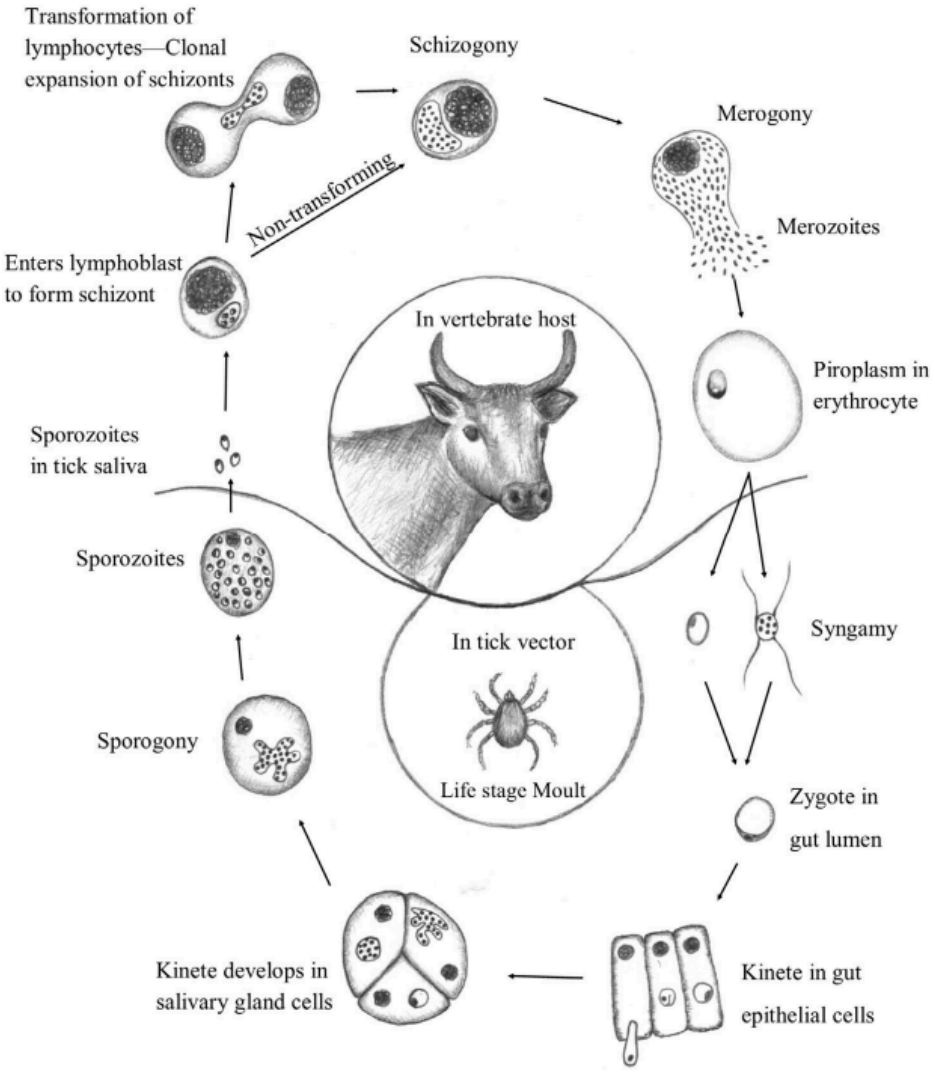

Figure 1.3. Life cycle of *Theileria parva (*Mans *et al*., 2015)

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Control

Three common methods are in practice for the control of bovine theileriosis (Dolan, 1989).

a) Chemotherapy

Commonly used chemotherapeutic agents are buparvaquone, imidocarb and oxytetracyclines to treat *T. parva* and *T. annulata* infections (Merck Manual, 9th edition). Buparvaquone treats *Theileria* infections with great efficacy when used in the early stages of disease. Imidocarb and oxytetracyclines reportedly performed well for cases of mild theileriosis (Coetzer and Tustin, 2004).

b) Acaricides

In enzootic areas, tick control is an important option for theileriosis control, because indigenous animals can be subclinical carriers of the infection (Mukhebi *et al*., 1992). The control of theileriosis is mainly achieved by prevention of tick infestation, as described for vectors of *Babesia* spp. (above). Unfortunately, the use of acaricides selects for tick resistance to these chemicals (Irvin *et al*., 2012).

c) Vaccination

Immunization against *Theileria* was first attempted in South Africa in 1956 (Henning, 1956). Several immunization methods have been investigated for control of theileriosis. Many cattle were immunized with *T. parva*-infected lymph node and spleen homogenates in Africa (Neitz, 1957). However, the preparation of fresh immunogen from sick cattle was difficult and could be contaminated with other infectious disease agents. Inactivated vaccines are not yet available against

Theileria. However, reliable live vaccines of known efficacy have been developed for *T. parva* and *T. annulata*. The method used parasitized cell lines in which the parasite had been attenuated by up to 200 passages *in vitro* (Pipano and Tsur, 1966, Pipano, 1989, Irvin and Morison, 1989; Lightowler, 1994). The limitation of this vaccine was to keep it frozen until shortly before administration. An alternative live vaccine against *T. parva* was developed in the 1970s. This involved infection of cattle with sporozoites and simultaneous treatment with oxytetracycline to delay parasite development, resulting in mild, transient infections followed by recovery of recovered animals that demonstrated a robust immunity to homologous challenge, which usually lasted for the lifetime of the animal (Radley *et al*., 1975). This method is called the "infection and treatment protocol," and the immunized animals usually become carriers of the immunizing parasite stock(s). Experimental subunit vaccines are being developed for ECF, and ideally will contain antigens from both sporozoites (*e.g.,* p67 protein) and schizont stages (OIE, 2020).

4. Cowdriosis

Cowdriosis, or heartwater, was first recognized in South Africa in the 19th century (Uilenberg, 1983). *Ehrlichia ruminantium,* the etiological agent of heartwater, was first described in 1925 as *Cowdria ruminantium* . The first vaccine against this pathogen was developed in 1945 (Oberem and Bezuidenhout, 1987).

Importance

Heartwater is reportable to the World Organization for Animal Health (OIE). *Ehrlichia ruminantium* is a serious problem in southern Africa, and high-yielding

European livestock breeds are reportedly more susceptible to severe cowdriosis than traditional stock breeds, such as zebu (Simpson *et al*., 1987). Due to poverty or unwillingness, farmers are usually hesitant to test for definitive diagnosis of heartwater, which leads to difficulty in measuring the economic impact of this disease. According to the multinational organization known as the Southern African Development Community (SADC), annual animal production losses were estimated at $48 million (USD) (Perry *et al*., 1998; Minjauw *et al*., 2000).

Transmission

Ehrlichia ruminantium is an obligate intracellular gram-negative prokaryotic parasite, classified in the alpha-proteobacterial order *Rickettsiales*, family *Anaplasmataceae*. *E. ruminantium* is the etiological agent of cowdriosis and is transmissible by several *Amblyomma* spp. ticks, including *A. maculatum,* which is indigenous to the USA (Uilenberg, 1982). Notably, the USDA Foreign Animal Disease Preparedness and Response Plan includes guidance for responding to introduction of two of the most competent vectors of *E. ruminantium* to the US: *A. variegatum* and *A. hebraeum* (USDA, 2013). *Amblyomma variegatum*, the tropical bont tick, has spread from Africa to Caribbean Islands near the southeastern US (Pegram *et al*., 2004). *E. ruminantium* can infect many cloven-hoofed vertebrate hosts, including cattle, sheep, goats and wild ruminants, and primary infections are frequently fatal (Allsopp, 2010).

Control

Three methods are in practice to control heartwater.

a) Antibiotics

Oxytetracycline is the drug of choice for *E. ruminantium* infection, acute infection is normally treated with two successive doses of 20 mg/kg (Allsopp, 2015). Prophylactically, long-acting oxytetracycline is widely used (Purnell *et al*., 1989).

b) Resistant livestock

In heartwater-endemic regions, some livestock breeds have become less susceptible to clinical disease because of long-term artificial selection. For instance, *B. taurus* (European) cattle are more susceptible than *B. indicus* (Zebu) breeds. Zebu can still be infected, but the prognosis is typically not as severe as it is for infected *B. taurus* (Uilenberg, 1995). An unfortunate limitation with the more resistant cattle breeds is that these breeds are less productive than *B. taurus* breeds (Simpson *et al*., 1987).

In 1941, a vaccination approach was first described for control of Cowdriosis (Neitz and Alexander, 1945). This approach was in part based on the natural resistance of young calves to severe clinical cowdriosis. *E. ruminantium*-infected ovine blood was administered intravenously, and then animals were treated with antibiotics upon first appearance of clinical signs. Although this vaccination method was successful, it has the following limitations:

- A cold chain is required for cryopreserved host cells (sheep blood or cell cultures) infected with virulent *E. ruminantium*.
- Personnel typically require training to properly administer an intravenous inoculation.

- Inoculated animals require monitoring for several days.
- Antibiotics need to be administered upon observation of clinical signs of heartwater.

Research is still underway to develop a safe, effective, and cost-efficient vaccine against heartwater (Allsopp 2009; Vachiéry *et al*. 2013).

c) Tick Control

Intensive use of acaricides can control ticks; however, as previously stated, acaricide resistance is an increasingly important problem. Strategic tick control through regular dipping to control tick numbers present on the livestock is still in use (Tice *et al*., 1998; George *et al*., 2004; De Meneghi *et al*., 2016).

5. Bovine Anaplasmosis

Clinical bovine anaplasmosis is associated with nonhemolytic anemia, with the lack of hemoglobinuria distinguishing bovine anaplasmosis (or “gall sickness”) from babesiosis (or “redwater”). *Anaplasma marginale*, the primary etiologic agent of bovine anaplasmosis, is an obligate intracellular prokaryotic parasite classified in the alpha-proteobacterial order *Rickettsiales,* family *Anaplasmataceae* (Aubry and Geale, 2011), the same family as the etiologic agent of heartwater, *E. ruminantium* (Dumler *et al*., 2001).

Anaplasmosis was first described, as a distinct infectious disease, in South Africa by Sir Arnold Theiler (Theiler, 1910). Theiler ascribed the genus *Anaplasma* to the intraerythrocytic etiologic agent, due to the lack of what was presumed to be protozoal cytoplasm, and the species *marginale* was proposed due to the marginal

location of these organisms within bovine erythrocytes. Theiler also noted that Smith and Kilbourne (1893) had also reported similar inclusions on margins within bovine erythrocytes, but that these authors thought the marginal bodies they observed represented an early developmental stage of *B. bigemina* (Smith and Kilborne, 1893).

As previously suggested, the acute phases of clinical anaplasmosis and clinical babesiosis both result in severe anemia, and these distinct diseases can be distinguished clinically by erythrolysis during babesiosis and the lack of hemoglobinuria due to anaplasmosis alone, probably due to distinct immunopathogenic mechanisms (Akel and Mobarakai, 2017). However, in regions where both pathogens are enzootic, these two diseases frequently occur together and often exacerbate each other (Obregón *et al*., 2019), resulting in their designation as the two most economically significant vector-borne diseases of cattle on a global scale (Minjauw and McLeod, 2003).

Importance

Anaplasma marginale is one of the most widely distributed vector-borne pathogens described to date. Thus, anaplasmosis has a major impact on the cattle industry on a global scale, especially in tropical and subtropical regions (Jabbar, *et al*., 2015). Anaplasmosis is not a reportable disease in most of the US, thus contributing to challenges in estimating the economic impact of this disease on cattle production in the US. The more recently quoted estimate of $300 million USD annual loss to cattle production (now, likely higher) was re-calculated in 1999 from an earlier publication in 1976 (Kocan and Fuente, 2003). However, due to

lack of ongoing information on the incidence of anaplasmosis and its economic impact on cattle production, these estimates do not allow accurate assessment of production losses in the US cattle industry .

The widespread economic importance of bovine anaplasmosis has led to extensive investigation of this disease, resulting in a predictable experimental model system that allows reliable observation of pathogen, host and vector interactions.

Transmission

Anaplasma marginale can be mechanically transmitted among cattle by blood-contaminated fomites and hematophagous arthropods, but only certain ticks of the family Ixodidae are known to transmit the organism biologically (Howell, 1957; Reeves and Swift, 1977; Ewing, 1981; Scoles, Broce, and Lysyk, 2005). Mechanical transmission by biting flies, especially the brachyceran family *Tabanidae*, are more likely to transmit *A. marginale* from cattle in the acute phase of clinical anaplasmosis, when the percentage of infected erythrocytes is relatively high, while transmission from subclinical carrier cattle is likely to require a biologic (tick) vector that also serves as an amplification host in which the pathogen will replicate.

It is documented that *Ixodes* and *Amblyomma* spp. are not competent vectors of *A. marginale* (Ewing, Dawson, and Kocan, 1995). However, *Dermacentor* spp. indigenous to the US are competent biologic vectors of *A. marginale*, including *D. variabilis*, *D. andersoni*, *D. occidentalis* and *D. albipictus* (Kocan *et al*., 2004). Both male and female ticks can transmit infection; however,

the intermittent feeding of male metastriate ticks makes them particularly important for the transmission of the infection to susceptible cattle (Kocan *et al*., 1992). Because male ticks and bovine hosts become persistently infected with *A. marginale*, they can also serve as reservoirs of the infection (Aubry and Geale, 2011). Ticks can transmit *A. marginale* transstadialy (*i.e.,* pathogen passage between different developmental stages of the same tick) or intrastadially (*i.e.,* both acquire and transmit a pathogen during the same tick development stage) (Stich *et al*., 2008). Thus, male *Dermacentor* ticks can continuously transmit *A. marginale* among hosts without the additional acquisition of infection or molting periods (Kocan *et al.,* 1992).

In one report, a single tick was capable of transmitting *A. marginale* to a susceptible bovine host (Rozeboom and Moe, 1940). This male *D. andersoni* was removed from an infected animal, held for 1 day and subsequently fed on a naïve host for 5 days resulting in the host developing clinical anaplasmosis, with observation of *A. marginale* in blood smears. The author did not list the conditions in which these cattle were housed, thus it is unknown if these cattle were open to mechanical transmission by biting flies. In 2005, adult male ticks were acquisition fed on infected cattle, held in the laboratory for 24 hours, and subsequently transmission fed on susceptible cattle. In this experiment, as few as 3 ticks transmitted the infection to susceptible animals (Scoles *et al*., 2006). Another study tested transstadial tick transmission by placing 50 pairs of adult ticks, previously fed on an infected host as nymphs, in a sack around the scrotum. These ticks reportedly transmitted *A. marginale* to this bull, but only 16 female and 20 male

ticks were recovered (Rees, 1933). The limitation of this study is that it was not reported how long the 50 pairs could feed before they died and thus resulting in low recovery numbers. It was later reported that *A. marginale* was transstadialy transmitted by ticks to naïve calves after 6-7 days of feeding (Kocan *et al*., 1978). Multiple reports demonstrated that 25 pairs of ticks were used to transstadialy transmit *A. marginale* to hosts (Howarth and Hokama, 1973; Kocan *et al*., 1985; Kocan and Fuente, 2003). Multiple studies have reported on the conditions of intrastadial and transstadial transmission of *A. marginale* to susceptible animals, which are summarized in (**Table 1.4**).

After infecting the tick midgut, *A. marginale* replicates within a vacuole, as what is referred to as the reticulate (vegetative) form. Afterward, the organism changes to a electron-dense form that is associated with transmission to the vertebrate host (Kocan *et al.,* 1990; Kocan and de la Fuente, 2003). This form leaves the midgut epithelium through the basal membrane and enters the hemocoel to migrate to the salivary glands (Kocan *et al*., 1990; Stich *et al*., 1993). Entry into the salivary glands is subsequent; however, the mechanism of entry is unknown. Replication then occurs again in the tick salivary glands (Kocan *et al*., 1985), and *A. marginale* is secreted in tick saliva after 6 days of feeding (Kocan *et al*., 2009; Stich *et al*., 1993; Ueti *et al*., 2007).

Control

The control of Bovine anaplasmosis depends on level of endemicity and region. It can be controlled by

- Maintaining *A. marginale*-free herds

- Proper vaccination
- Tick control
- Treatment with tetracycline during acute disease and administration of low-level tetracycline to prevent relapse of clinical disease.

a) Tick control

Tick control is practiced globally by using different means. Successful tick control can reduce the number of engorged females which ultimately leads to less progeny (*i.e.*, a smaller number of eggs or larvae). This measure can help in prevention of bovine anaplasmosis. The following measures can be taken to control ticks.

i. Pasture Spelling

Rotational grazing is also known as “Pasture Spelling.” This practice has been used effectively in Australia for the control of *Boophilus microplus*.

ii. Habitat Modification

Ticks are usually found in humid places. Leaf litter removal, mechanical brush removal, mowing and burning vegetation demonstrate effective means of tick control in residential areas. Removing leaf litter and underbrush also eliminates tick habitats and reduces the density of small mammal hosts, like deer mice and meadow voles. Leaf litter provides the suitable microhabitats that provide the necessary environmental conditions for survival, such as high relative humidity. Mowing the lawn and removing bushes also reduces the occurrence of all stages of ticks.

iii. Chemical Control of Ticks

Tick control is mostly dependent upon use of acaricides. Several factors are associated with successful acaricidal tick control, including:

- Type of acaricide
- Ambient temperature
- Dosage
- Penetrability of canopy
- Extent of coverage
- Susceptibility of the target tick species
- Tick life stage and physiologic condition

Two methods of tick control by acaricides are possible:

I. Application of acaricide to the surface

II. Application of acaricides to livestock as dips or sprays

b) Chemotherapy

The most widely used control measure for bovine anaplasmosis is chemotherapy with antibiotics. Tetracycline is the predominant treatment method. Oxytetracycline is used for acute anaplasmosis and chlortetracycline in feed is used for prevention (Kuttler *et al*., 1980).

c) Vaccination

An most important aspect to anaplasmosis control is vaccination. It is the control method used since the description of bovine anaplasmosis in 1911 in

South Africa (Theiler, 1911). Both Live and killed vaccines are used to control bovine anaplasmosis (Kocan *et al*., 2010). *A. marginale* from infected erythrocytes is the source of antigen in both live and killed vaccines. Both types of vaccines can induce protective immunity that prevents clinical disease, but neither type prevents cattle from becoming persistently infected with *A. marginale* (Kocan and Fuente, 2003).

Sir Arnold Theiler isolated *A. centrale* during the early 20th century and used it as a live vaccine that remains the most widely spread vaccine for the control of bovine anaplasmosis (Kocan and de la Fuente, 2003). Theiler observed that *A. centrale* is less pathogenic than *A. marginale* and it provides protection against *A. marginale* challenge. This strain is being used for vaccine production in Australia, Africa, Israel and Latin America (Kocan *et al*., 2010). An attenuated form of *A. marginale* was also considered for live vaccine production (Kuttler, 1967) and sheep or deer were used to generate this attenuated strain (Kuttler *et al*, 1988).

Vaccination is a long-standing objective of the cattle industry to control bovine anaplasmosis (Kocan *et al.,* 2000). In the 1960s, a killed vaccine was developed in the US and was marketed until 1999. This vaccine contained partially purified *A. marginale* antigens from infected bovine erythrocytes and it was marketed in the US for heterologous protection against clinical anaplasmosis (Brock *et al.*, 1965). However, a significant drawback for this vaccine was the production of erythrocytic isoantibodies which resulted in hemolytic anemia in certain calves (Dennis *et al*., 1970). Another drawback of blood-derived vaccines is the risk of transmission of other pathogens (Kocan *et al*., 2003). This vaccine

was reportedly withdrawn from the marketplace due to company restructuring, and killed vaccines continue to be tested (Rodrıguez *et al.*, 2000). A less expensive and safer method to extract *A. marginale* antigens from cell culture was also used in the US; this method reportedly provided broader protection against anaplasmosis in all endemic areas of the US (Kocan *et al*., 2001). Vaccination with live-attenuated organisms can also cause persistent infection in cattle.

Currently, a fully licensed anaplasmosis vaccine is not available in the US. Vaccines based on molecular technologies would likely be an improved control method, but such products have not been developed (Kocan and Fuente, 2003).

Table 1.4. Reported studies of experimental anaplasmosis.

Tick species used	Transmission Route	Number of Ticks used	Results	Reference
D. variabilis, D. andersoni	Transstadial	Not listed	+	(Rees, 1932)
D. andersoni	Transstadial	Multiple with lowest at 50 pairs	+	(Rees, 1933)
D. variabilis	Transstadial	100 Nymphs and 150 pairs of adults	+	(Stich *et al*., 1989)
D. andersoni, D. occidentalis, D. albipictus	Unknown	Not listed	+	(Boynton *et a*l., 1936)
D. andersoni	Unknown	1 male or 4 Larvae clutches for 3 cows	+ Adult male/ - Transovarial	(Rozeboom and Moe, 1940)
D. variabilis	Unknown	2 males	+	(Anthony and Roby, 1962)
D. albipictus	Transovarial	10 egg clutches	-	(Beugnet and Marié, 2009)
D. andersoni	Transstadial	Variable (lowest 25)	+	(Bram, 1971)
D. occidentalis	Transstadial & Transovarial	25 pairs	+ Transstadial/ - Transovarial	(Howarth and Hokama, 1973)
D. variabilis, D. andersoni	Transstadial	600 adults/cow	+	(Kocan *et al*., 1981)

Table 1.4. Reported studies of experimental anaplasmosis (continued).

Tick species used	Transmission Route	Number of Ticks used	Results	Reference
D. andersoni	Transstadial	25 pairs	+	(Kocan *et al*., 1985)
D. andersoni	Transstadial	25 pairs	+ for VAM	(Wickwire *et al*., 1987)
B. annulatus	Transstadial & Intrastadial	100 and 220 Nymphs; 100 & 300 adults	+	(Samish *et al*., 1993)
D. andersoni	Transstadial & Intrastadial	Up to 50 pairs	+	(Eriks *et al*., 1993)
B. microplus	Transstadial	10, 20, 40 & 80 Nymphs	+	(Aguirre *et al*., 1994)
D. andersoni	Transstadial	50 ticks total	+	(Kocan *et al*., 1996)
D. andersoni	Intrastadial	3 ticks and 30 male ticks only	+	(Scoles *et al*., 2005)
D. andersoni	Intrastadial	10 and 35 male ticks only	+	(Ueti *et al*., 2007)
D. variabilis, ***D. andersoni***	Intrastadial	90 *D. variabilis*, *D. andersoni* as low as 3 up to 48	+	(Lankester and Scandrett, 2007)
D. reticulatus	Intrastadial	30 adults total	+	(Zivkovic *et al*., 2007)
D. andersoni, ***B. microplus,*** ***B. annulatus***	Intrastadial	120 *B. microplus*, 66 *B. annulatus*, 59 *D. andersoni,* total	+	(Scoles *et al*., 2007)

Past, Present, and Future

Globally, tick-borne diseases are a grave danger to the livestock industry because of substantial losses in forms of death, decreased productivity, and lowered working efficiency (Uilenberg, 1995). The Food and Agriculture Organization (FAO) reported that 80% of the cattle population worldwide is exposed to ticks, with economic losses to the cattle industry (FAO, 2004). Heavy infestations of ticks can cause up to a 65% reduction in cattle body weight (Seebeck *et al*., 1971). *Rhipicephalus* (*Boophilus*) *microplus*, the "Southern Cattle Tick," causes economic losses to the Brazilian cattle industry estimated at 3.24 billion USD per year (Grisi *et al*., 2014).

Current approaches to the mitigation of tick-borne diseases

Tick-borne diseases a major health concern for human and animals globally. The incidences of tick and mosquito-borne diseases have increased two-fold from 2004 -2016 in the US (Rosenberg *et al*., 2018). However, control strategies used to date have had limited success. Vectors and parasites have developed resistance to a few insecticides and drugs that were effective in the past (Nolan, 1990). These facts emphasize the need to devise novel strategies for disease control. Artificial immunization against tick infestation may be the most attractive alternative application to control ticks (Barriga, 1994; Kay and Kemp, 1994).

Immune protection against ticks can be challenging, because ticks have co-evolved with their natural hosts. The arthropod provides a sustainable environment

for microbes, and multiple pathogens have adapted to use tick feeding as a means of transmissions between vertebrate hosts. It would be ideal to consider these vectors as epidemiologically significant pathogen transmitters instead of only as pests (Billingsley, 1994). It appears that host antibodies in tick bloodmeals can be used to target tick performance and, more importantly to disrupt the acquisition and transmission of pathogens between ticks and vertebrates (Nogge and Giannetti, 1980; Kemp *et al*., 1986; Agbede and Kemp, 1986; Kemp , *et al*., 1989; Wikel, 1980). Thus, anti-tick vaccines might provide protection against pathogens transmitted by ticks, as previously reported when rabbits immunized against *D. variabilis* were protected from transmission of *Francisella tularensis* (Bell *et al.*, 1979).

Vaccinology

Vaccinology is defined as the discovery and development of biologic products (vaccines) used to safely induce a protective response to an etiologic agent of disease (Pollard and Bijker, 2021). Resolution of acute phases of infectious diseases usually results in acquired immune protection to subsequent infection with the same etiologic agent (Raffel, 1949; Dougan *et al*., 2011; Muraille, 2016). Protection of vertebrate hosts against infectious diseases can be achieved by using a biologic product to safely induce protective immune responses. “Vaccine,” the term describing such products, was derived from the latinized name of the cowpox virus (*Variolae vacinnae,* meaning “smallpox of the cow”), which was used to devise Europe’s first smallpox vaccine (Baxby and Jenner, 1981). “Vacca” is the Latin noun for “cow.”

Historically, some authorities have reported that the world's first vaccines may have been used in China, India or Africa, seemingly as early as 1,000 AD (Boylston, 2012). However, it was also reported that people in the ancient Middle East and Central Asia intentionally inoculated inconspicuous locations of the skin of children with exudates from lesions known as oriental sores, to protect these inoculated hosts from what is now known to be cutaneous leishmaniasis (Boelaert, and Sundar. 2014; Steverding, 2017). This process, more recently referred to as "leishmanization," is reportedly at least 90% protective against reinfection with the protozoan etiologic agent of cutaneous leishmaniasis (Row, 1912; Marzinowsky and Schurenkowa, 1924).

The first European vaccine was produced by Edward Jenner in 1796, when Jenner inoculated an 8-year-old boy with a lesion of cowpox from a milkmaid (Riedel, 2005). Since development of this vaccine, according to the CDC, 58 vaccines against 26 pathogens have been fully licensed in the US (CDC, 2012; Meissner *et al*., 2018).

Research strategies for vaccine discovery

Over the past 200 years, scientists have made multiple vaccines to protect people and animals from lethal infectious diseases. Two strategies are in use for the development of vaccines: "forward" and "reverse." In forward vaccinology, live, attenuated or killed pathogens are used to induce protective immunity in naïve animals. Both vaccinated and unvaccinated animals are then challenged with live, virulent pathogen. Upon challenge, vaccinated animals may demonstrate improved immune response and ability to survive challenge with fewer side effects

than control animals. Conversely, for reverse vaccinology, known gene sequences are characterized and selected vaccine candidate(s) are formulated to generate prototype immunogens to immunize naïve hosts prior to challenge with live, infectious agent (pathogen) (Small *et al*., 2016). Both technologies are described in more detail below.

Forward Vaccinology

Historically, two methods have been used for forward vaccine development, (1) attenuation of pathogens that are then used for vaccination and (2) identification of antigens that elicit protective immunity, which then are used as defined subunit vaccines (Masignani, *et al*., 2002). For the identification of these protective antigens, pathogens are first grown in a laboratory then potentially protective components are determined with biochemical, immunologic and/or genetic methods (Sette and Rappuoli, 2010). This approach is time-consuming, and a specific quantity of identified antigens is required for immunization (Rappuoli, 2000).

The approach described in this dissertation uses the vertebrate host immune system to target tick molecules, to determine if such responses are capable of interference with the tick-pathogen interface. Forward vaccinology relies on millions of years of coevolution between infectious organisms and their hosts, potentially working around inadequately understood mechanisms of host protection and parasite countermeasures such as immune evasion.

Reverse Vaccinology

Reverse vaccinology has yielded important targets. In the 1990's, Rappuoli *et al*, developed a genome-based approach to identify meningococcal vaccine candidate antigens, which was then termed "reverse vaccinology" (Rappuoli, 2000), analogous to the field of "reverse genetics." Whole-genome sequencing revolutionized the field of biology. Essentially, reverse vaccinology is often a computer-based approach for the discovery of candidate vaccine antigens. In a reverse vaccinology approach, a target such as a gene product is typically identified as a potential "Achilles' heel," often with *in silico* analysis of a genome, to identify vaccine candidate antigens. Animals can be subsequently immunized with recombinant proteins, for example, and tested through host challenge and by using the antisera for molecular analysis with different strains as a correlate of efficacy (Sette & Rappuoli, 2010). Those vaccines that are determined to be efficacious in animal models proceed to clinical trials or field studies (Lew-Tabor and Valle, 2016). The most recent example is COVID 19 vaccines (Andreadakis *et al*., 2020).

Anti-tick vaccines

In 1918, Johnston and Mackerras observed the development of resistance to tick feeding among naturally infested cattle (Johnston and Mackerras, 1918). These authors also reported resistance to tick feeding in association with cattle breed. Tick resistant cattle vary from the more tick-resistant *Bos taurus indicus* breed to the less tick-resistant *B. t. taurus* (George *et al*., 1985; Rechav *et al.*, 1991; Mattioli *et al*., 2000). Tick resistance is a multi-factorial trait, mostly host related such as age, sex, age, lactation, grooming behavior, skin composition, host

surface area, coat length and environmental factors (Wharton and Utech, 1970; Seifert, 1971; Doube and Wharton, 1980; Binta and Cunningham, 1984; Ali and Castro, 1993; Meltzer, 1996; Martinez *et al*., 2006; Kongsuwan *et al*., 2010).

In 1939, Trager experimentally documented the phenomenon of host resistance to ticks after multiple infestations of rabbits and guinea pigs with *D. variabilis* larvae and nymphs (Trager, 1939). Notably, Trager also reported limited *D. variabilis* survival after feeding on guinea pigs immunized with homogenized tick larvae, and multiple studies since have revealed similar phenomena of reduced tick performance after immunization of hosts with crude tick homogenates or extracts (Allen and Humphreys, 1979; Jittapalapong *et al*., 2004a; Jittapalapong *et al.,* 2004b). More studies showing the same phenomena are documented in **Table 1.5.**

Immunization against the tick vector is a well-documented approach that results in reduced tick performance after feeding on immunized hosts (Trager, 1939; Allen and Humphreys, 1979; Willadsen *et al*., 1989; Jittapalapong *et al*., 2000; Jittapalapong *et al*., 2004). The *R.(B.) microplus*-derived molecule, Bm86, is a midgut membrane-bound recombinant protein of *R. microplus* (Willadsen *et al*., 1995). Immunization with Bm86 protects cattle against tick feeding by impacting the fecundity of ticks (Willadsen, 1987). Orthologs of Bm86 in other tick species like *R. annulatus* and *Hyalomma anatolicum* subsp. *anatolicum* also confer some protection to immunized animals against tick infestations. Immunization of cattle with Bm86 ortholog BA86 in *R. annulatus* protects not only against *R. annulatus,* but also offers cross-protection against *R. microplus* (Canales *et al.,* 2008).

Moreover, immunization with tick molecules like 64TRPP, a putative tick cement protein had negatively impacted the transmission of tick-borne encephalitis virus (TBEV) to the mice by *I. ricinus* (Labuda *et al*., 2006). A further study by Jittapalapong *et al.* found that immunization of hosts with denatured *R.* (*Boophilus*) *microplus* tissues negatively impacts *B. bigemina* transmission to uninfected cattle (Jittapalapong *et al*., 2004). In this current study, our findings coincide with other studies that immunization with tick tissues or molecules can negatively impact tick-transmission of a tick-borne pathogen.

Table 1.5. Early examples of reports of host resistance to ticks.

Tick species	Host species	Resistance (+/-)	Reference
	guinea pigs	+	(Trager, 1939)
D. variabilis			
R. sanguineus	dogs	+	(Pogoielyi, 1966)
B. microplus	cattle	+	(Wharton and Utech, 1970)
D. andersoni	guinea pigs	+	(Wikel and Allen, 1976)
B. microplus	British cattle	+	(Kemp *et al*.,1976)
B. microplus	cattle calves	+	(Roberts and Kerr, 1976)
B. microplus	cattle	+	(Brossard, 1976)
D. andersoni	guinea pigs and cattle	+	(Allen and Humphreys, 1979)
I. ricinus	rabbits	+	(Brossard and Girardin, 1979)
D. variabilis	rats	+	(Ackerman *et al*., 1980)
D. andersoni	rabbits	+	(Wikel, 1980)
D. andersoni	guinea pigs	+	(Wikel, 1983)
D. variabilis	BALB/c mice	+	(Aleen, 1985)
D. variabilis	mice	+	(Allen, 1985)
B. microplus	cattle	+	(Johnston *et al*.,1986)
B. microplus	cattle	+	(Kemp *et al*., 1986)
I. ricinus	rabbits	+	(Girardin and Brossard, 1990)
A. americanum	sheep	+	(Barriga *et al*., 1991)
D. andersoni	guinea pigs	+	(Barriga *et al*., 1993)
I. ricinus	rabbits	+	(Schorderet and Brossard, 1993)
D. andersoni	guinea pigs	+	(Whelen and Wikel, 1993)
R. sanguineus	dogs	+	(Inokuma *et al*., 1997)
R. sanguineus	dogs	+	(Jittapalapong *et al*., 2000)
B. microplus	cattle	+	(Jittapalapong *et al*., 2004a)

Immunization with whole-tick homogenates

Ixodid ticks are ectoparasites which feed slowly on the host and leave the host only to molt and to oviposit. Ixodid tick feeding generally lasts for 3-5 days in immature stages and 7 or more days in female adults (Balashov, 1984). After feeding, the female body weight can increase by more than 100 times their original weight (Balashov and Raĭkhel', 1976). Most ixodids are three-host ticks.

The tick midgut and salivary glands are the primary organs for pathogen acquisition and transmission, respectively. Specifically, the midgut is the first organ to have contact with pathogens during the bloodmeal uptake, while salivary glands along with their secretions can play a crucial role in pathogen transmission to the host (Binnington and Kemp, 1980; Ribeiro, 1987a; Ribeiro, 1989; Nuttall *et al*., 1994).

Several studies demonstrated that crude extracts of tick tissue induce immune protection to tick infestations (**Table 1.6**). Trager (1939) protected guinea pigs against *D. variabilis* larvae challenge by immunizing guinea pigs with extract of whole larvae subcutaneously. Tortoises were also protected from challenge feeding of *Amblyomma testudines* by immunization with an extract of unfed nymphs (Schneider *et al*, 1971). Calves immunized with salivary glands of partially engorged female *Boophilus microplus* subsequently allowed fewer ticks to engorge (Brossard, 1976).

Due to acaricide resistance, an alternative strategy to control ticks and tick-borne diseases is to vaccinate the host against ticks. The idea of anti-tick vaccines has been around for some time, but the progress in making effective vaccines is

slow (Trager, 1939). Currently, vaccines derived from Bm86 (a midgut membrane-bound protein of the southern cattle tick, *B. microplus*), are the only ectoparasite vaccines commercially available (Willadsen *et al*., 1995). The concept of exposed and concealed antigenic targets was reviewed by Nuttall (Nuttall *et al*., 2006). During normal tick feeding, the host is immune system is exposed to tick antigens secreted in saliva, thus these are known as exposed antigens. Exposed antigens are taken up at the tick feeding site by host dendritic cells, which process and present them to T lymphocytes, priming a cell- or antibody-mediated immune response (Allen *et al*., 1985). The antigens which the host is not usually exposed to during normal tick feeding are called concealed or novel antigens.

Table 1.6. Reports of host immunization with tick homogenates.

Tick species used	Host species	Tick Homogenate	Immune protection +/-	Purified Antigen/detected Proteins	Approach	Reference
D. variabilis	guinea pigs	Larvae	+	Not purified	Forward	(Trager, 1939)
R. sanguineus	guinea pigs and rabbits	SG extracts	+	Not purified	Forward	(Garin and Grabarev, 1972)
A. testudinis	tortoises	Nymph extract	+	Not applicable	Forward	(Schneider *et.,* 1971)
B. microplus	cattle	SG extracts	+	Not applicable	Forward	(Brossard, 1976)
I. holocyclus	guinea pigs	Larvae	+	Not purified	Forward	(Bagnall and Doube, 1975)
D. andersoni	guinea pigs	MG / Reproductive Tract	+	Not purified	Forward	(Allen and Humphreys, 1979)
D. variabilis	rats	MG	+	Not applicable	Forward	(Ackerman *et al.,* 1980)
D. andersoni	guinea pigs	SG Antigens	+	Not purified	Forward	(Wikel, 1981)
A. americanum	guinea pigs	SG extracts	+	Not purified	Forward	(Brown and Shapiro, 1984)
A. americanum	guinea pigs	SG extracts	+	Purified	Forward	(Brown and Askenase, 1986)

Table 1.6. Reports of host immunization with tick homogenates (continued).

Tick species used	Host species	Tick Homogenate	Immune protection +/-	Purified Antigen/detected Proteins	Approach	Reference
R. appendiculatus	rabbits	Whole ticks	+	9 proteins detected by immune sera	Forward	(Mongi *et al*., 1986)
B. microplus	cattle	Whole ticks	+	Proteins detected by immune sera	Forward	(Johnston *et al*., 1986)
B. microplus	cattle	MG extracts	+	Not purified	Forward	(Agbede and Kemp, 1986)
B. microplus	cattle	MG extracts	+	Not applicable	Forward	(Kemp *et al*., 1986)
A. americanum	guinea pigs	Gut extract	+	Not applicable	Forward	(Wikel *et al*., 1987)
A. americanum	rabbits	SG	+	3 proteins detected by immune sera	Forward	(Brown, 1988)
R. appendiculatus	guinea pigs	Larvae and nymphs	+	2 proteins detected by immune sera	Forward	(Varma *et al*., 1990)
R. sanguineus	dogs, hamsters, and guinea pigs	Whole tick	-for dogs, + guinea pigs and hamsters	Not applicable	Forward	(Bechara *et al*., 1994)

Table 1.6. Reports of host immunization with tick homogenates (continued).

Tick species used	Host species	Tick Homogenate	Immune protection +/-	Purified Antigen/detected Proteins	Approach	Reference
R. sanguineus	dogs and guinea pigs	Gut extract	+	Not applicable	Forward	(Szabó and Bechara, 1997)
H. marginatum marginatum	cattle	SG and MG extracts	+	Not applicable	Forward	(Sahibi *et al*., 1997)
R. sanguineus	rabbits	Larvae and Nymph	+	Not applicable	Forward	(Tripathi *et al*., 1998)
B. microplus	cattle	MG	+	Bm86	Forward	(Willadsen *et al*., 1989), (Willadsen *et al*., 1995)
B. microplus	cattle	MG and SG	+	Bm91	Forward	(Riding *et al*., 1994) (Willadsen *et al*., 1996)
B. microplus	cattle	MG	+	Bm95	Forward	(García-García *et al*., 2000)
B. microplus	Sheep	Whole ticks	+	vitellin	Forward	(Tellam *et al*., 2002)
B. microplus	cattle	Whole ticks	+	BmPRM	Forward	(Ferreira *et al*., 2002)

Table 1.6. Reports of host immunization with tick homogenates (continued).

Tick species used	Host species	Tick Homogenate	Immune protection +/-	Purified Antigen/detected Proteins	Approach	Reference
H. longicornis	rabbits	recombinant HLS1	+	HLS1	Reverse	(Sugino *et al*., 2003)
H. longicornis	rabbits	recombinant HLS2	+	HLS2	Reverse	(Imamura *et al*., 2005)
H. longicornis	Mice	rP27/30	+	P27/30	Reverse	(You, 2005)
I. scapularis	Sheep	r4D8	+	4D8	Forward	(Almazán *et al*., 2005)

Immunization with tick midgut

Tick midgut and salivary glands directly interface with host tissues during tick feeding, and most tick-borne pathogens are adapted to both of these tick organs. When ticks ingest host blood containing effector molecules reactive to tick molecules, this can result in necrosis of tick midgut epithelium, reduction in tick engorgement weights and lower fecundity (Wikel *et al*., 1987; Jittapalapong *et al*., 2000; Jittapalapong *et al*., 2004a).

In 1979, Allen and Humphreys immunized guinea pigs and cattle with midgut or all other tissues from partially engorged *D. andersoni* (Allen and Humphreys, 1979), which resulted in tick mortality, and reduced female tick engorgement weight, egg-laying, egg cluster weight, and larval yields. In 1980, a different study found similar reductions in tick performance after feeding on rats immunized with *D. variabilis* midgut (Ackerman *et al*., 1980). Immunization of cattle with *Hyalomma marginatum* tick midgut was also reported to impact tick fertility (Sahibi *et al*., 1997). Jittapalapong *et al*. reported the same phenomenon, that immunization with midgut reduced the reproductive performance of *R. sanguineus* ticks from canine hosts (Jittapalapong *et al*., 2000) and of *R. (Boophilus) microplus* from cattle (Jittapalapong *et al*., 2004a).

In 1988, a research group in Queensland, Australia was able to isolate antigenic material from the midgut of partially engorged cattle tick *B. microplus,* by recombinant DNA technology. This antigen was able to elicit the protective immunological response in bovine host (Willadsen, McKenna, and Riding, 1988). For the isolation of the antigen, partially engorged *B. microplus* were homogenized

in a minimum volume of buffer; after preparation of the antigen, cattle were immunized with three injections. The first injection was in Freund's complete adjuvant, the second was intramuscularly in the rump and the last was antigen alone without adjuvant over the shoulder subcutaneously. All three injections were 4 weeks apart. After vaccination, tick performance was measured and the number of ticks collected, and the weights of ticks from the immunized cattle were significantly lower than of the control cattle. Although this vaccine was discovered in 1988, it was not introduced commercially until 1994 (TickGARD, Hoechst Animal Health, Australia). At the same time another group isolated recombinant Bm86 antigen in Latin American countries (de la Fuente, *et al.,* 1995) (Gavac, HeberBiotec S.A., Havana, Cuba). Field trials for Gavac were performed in Cuba (Rodriguez *et al*., 1995), Brazil *(*de la Fuente *et al*., 1999), Argentina, Colombia and Mexico (Canales *et al*., 1997) while the Australian group also successfully showed the negative impact on *B. microplus* fertility in Australia (Cobon *et al*., 1995). Bm86-specific IgG, in combination with the complement cascade, was shown to mediate damage to the midgut epithelium (Willadsen, *et al*., 1993). Bm86-specific IgG protects against ticks by reducing the number of engorging females, female engorgement weights and tick fecundity (de la Fuente *et al*., 2007). A study in Cuba documented reduction in the number of clinical babesiosis cases, while reducing the use of acaricides, through vaccination of dairy calves with one of these products (CLSI 2003). It was suggested that vaccination with Bm86/Bm90 reduced incidence of clinical babesiosis by reducing tick fecundity (Rand *et al*., 2013).

Immunization with tick salivary glands

Ixodid salivary glands have a critical role in the adaptation to feed on vertebrates for extended periods of time (Šimo *et al.*, 2017). In addition to maintenance of water balance in the tick, the saliva secreted into the feeding lesion is a complex milieu of molecules that facilitate tick feeding through analgesic, anticoagulant and immunomodulatory effects (Ribeiro *et al*., 1985; Ribeiro and Spielman, 1986; Ribeiro, 1987b; Ribeiro, 1987a). The components of tick saliva can change as the tick feeds and according to the host on which the tick is feeding (Wang and Nuttall, 1994).

Although adapted to the vertebrate immune system, tick saliva can be immunogenic, and it is a likely source of antigen to induce host resistance to tick feeding due to repeated infestation (Ribeiro, 1987a; Ribeiro, 1989; Brossard and Wikel, 2004; Kotál, and Andersen, 2015; Šimo *et al*., 2017; Ren *et al*., 2019). Brown and Gleich, described salivary gland immunogenicity (Brown *et al*.,1982). Guinea pigs were immunized with salivary gland extract from partially fed *A. americanum* ticks*,* and performance parameters like engorgement weight and the survival rate were measured. The engorgement weights of these ticks were reduced, and fewer female ticks were recovered. Since then, several studies recorded similar findings with different tick species on different vertebrate hosts (Wikel, 1980; Wikel, 1981; Sahibi *et al*., 1997; Nikpay and Nabian, 2016).

In 1997, Sahibi *et al*. documented contrasting results from immunization with midgut versus salivary glands, because tick feeding performance was more reduced with cattle immunized with salivary gland extract (Sahibi *et al*., 1997).

Jittapalapong *et al*. reported similar results in 2000 and 2004 (Jittapalapong *et al*., 2000; Jittapalapong *et al*., 2004a). Immunization with tick salivary gland extract provides both concealed and exposed antigens, with secreted exposed molecules at significantly higher quantities in salivary gland extracts. Thus, immunization with salivary gland extract allows for the vertebrate host to produce a strong response to all the molecules they would normally encounter in addition to concealed antigens associated with the tick salivary gland acini.

Jittapalapong *et al.,* reported that immunization with tick salivary gland extract was associated with reduced numbers of ticks that were PCR-positive for *B. bigemina* and further reduced the number of cattle presenting with clinical babesiosis (Jittapalapong *et al*., 2004b)

Recombinant vaccine targets

Several studies have been conducted to identify recombinant targets by using different tick tissues (Brock, *et al*., 2019; Xu *et al*., 2020; Wei *et al*., 2020; Rahman *et al*., 2020). It was documented that tick feeding and reproductive potential were reduced by using recombinant vaccine antigens (Lew-Tabor and Valle, 2016). Multiple tick recombinant proteins have been shown to reduce tick-pathogen infection (Merino, Alberdi, and Lastra, 2013). For instance, vaccination of cattle with Subolesin significantly reduced *R. microplus* survival and infection by *B. bigemina* or *A. marginale* (Merino, Almazán, Canales, and Villar, 2011). Moreover, another salivary gland molecule, Salp15 was used to immunize mice, which resulted in a lower levels of *Borrelia burgdorferi* infection in immunized mice (Dai *et al*., 2009). This work collectively indicated that immunization of vertebrate

hosts against the tick can negatively impact tick survival, feeding and transmission of tick-borne pathogens.

The bovine anaplasmosis model system

In the United States, *Dermacentor* spp. ticks are well-known biologic vectors of *A. marginale* (Kocan *et al*., 2010). The reliable, well-characterized bovine-*A. marginale-D. andersoni* transmission model chosen for this work is a vertebrate host-pathogen-tick interaction that exists in nature. Importantly, this model system provides predictable measures of infection (prepatent period, pathogen burden and percent infected host cells) and disease (clinical and hematologic signs in association with non-hemolytic anemia) in vertebrate hosts as well as measures pathogen acquisition, maintenance and transmission by tick vectors (infection rates and infection levels) (Stich *et al*., 1989; Ueti *et al*., 2007).

Research problem

The goal of anti-tick vaccine development is to develop sustainable interventions to decrease the incidence of tick-borne disease and to provide cost-effective and environmentally friendly control measures for ticks. Paradoxically, direct interference with the tick-pathogen interface is frequently overlooked as the *primary* criterion when screening for protective antigens. There is strong evidence that host immunization with tick antigens can be detrimental to tick feeding, development and fecundity (Barriga, 1994; Willadsen *et al*., 1996; Jittapalapong *et al*., 2000; Jittapalapong *et al.*, 2004a; Jittapalapong *et al*.,, 2004b; Almazán *et al*., 2005; Labuda *et al*., 2006; Fuente, Kocan and Blouin, 2007; Jittapalapong and

Phichitrasilp, 2008; Havlíková *et al*., 2013; Merino *et al*., 2013; Alvarez *et al.*, 2020). Some investigations led to identification of antigenic targets for interference with the tick life cycle, but interference with tick-borne pathogens has often been a secondary parameter for such work. The overall objective of this dissertation was to determine the feasibility of direct interference with an infectious disease agent in ticks. Previous work demonstrated that immunization with denatured and reduced tick salivary gland extracts resulted in lower incidence of naturally occurring babesiosis among dairy cows, and distinct effects were observed upon tick feeding and fecundity performance parameters when bovine and canine hosts were immunized with denatured and reduced salivary gland or native midgut extracts (Jittapalapong *et al*., 2000; Jittapalapong *et al.*, 2004a; Jittapalapong *et al*., 2004b).

To achieve the overall objective of this project, we co-adapted two established model systems, the experimental *A. marginale* transmission model and immunization with tick homogenates, to test the central hypothesis that immunization with tick tissue extracts which was compared by transmission of infection by ticks fed on cattle immunized with different immunogen preparations.

The first study (Chapter 2) was conducted to test the feasibility of host immunization with tissue extracts of *D. andersoni,* a tick that is indigenous to the US. The objective of the next study (Chapter 3) was to determine the feasibility of immunization with similarly prepared tick tissue extracts for intervention with biologic tick transmission of *A. marginale*, the primary etiologic agent of bovine anaplasmosis. A subsequent study (Chapter 4), based on findings from Chapter

3, used *D. variabilis,* another species indigenous to the US, to determine if immunization with different tick tissue extracts would have measurable differences in transmission of *A. marginale*. Future investigations examining the vertebrate response to tick tissue immunization are expected to aid the field in the development of anti-tick vaccines for protection against tick-borne pathogens.

In Chapter 5, preliminary observations about the infection level of *A. marginale* in *D. andersoni* ticks are discussed while in Chapter 6, the effect of tetracycline on the transmission of *A. marginale* by *D. andersoni* is discussed. Hard ticks are slow feeding ectoparasites and it is very expensive to study host vector or vector pathogen interaction using large animal models. Therefore, an effort was made to feed the Ixodid ticks on the laboratory bench. Chapter 7 will elaborate preliminary results for different optimization conditions to feed different stages of Ixodid ticks in the laboratory. The topics mentioned in the research problem section will be discussed in detail in the remaining chapters of this dissertation.

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CHAPTER 2

Utility of a bovine model for experimental immunization with *Dermacentor andersoni* midgut and salivary gland extracts

Abstract

Ticks impact livestock production worldwide, and tick control is dependent on chemical acaricides that may not be sustainable. Immune protection of vertebrate hosts is an alternative approach to tick control, and most of these efforts involve ticks indigenous to tropical and subtropical climates. This report describes adaptation of a bovine model to evaluate immunization with midgut (MG) and salivary gland (SG) extracts from *Dermacentor andersoni,* a tick indigenous to temperate climes of western North America. The immunized hosts seroconverted, as evidenced by increasing antibody titers to tick MG and SG antigens. Detached female ticks collected from immunized calves had reduced feeding and reproductive performance parameters compared to cohorts fed prior to immunization, with the greatest decreases in performance among ticks fed on SG-immune calves. Two-dimensional polyacrylamide electrophoresis (2D-PAGE) followed by Western blotting was used to localize 46 salivary gland protein spots uniquely reactive with antisera from SG-immunized hosts. 2D-PAGE gel regions containing these proteins were processed for shotgun proteomics and preliminary identification of candidate sequences. Five proteins encoded by transcripts in *D. andersoni* SG datasets, but not in MG transcriptome datasets, were chosen to test for reactivity with SG-immune sera. The results of this study indicated that

immunization of cattle with *D. andersoni* tissue extracts is practicable as a model system for identification and testing of anti-tick vaccine candidate antigens.

Key Words: *Dermacentor andersoni;* tick midgut; tick salivary glands; bovine immunity; tick performance

Introduction

Ticks are obligate blood-feeding ectoparasites that impact animals and people worldwide (Jongejan and Uilenberg, 2004). Immunization with crude tick homogenates can result in decreased tick feeding and reproduction (Allen and Humphreys, 1979; Willadsen, 1987; Rand *et al*., 1989; Sahibi *et al*., 1998; Jittapalapong *et al*., 2000; Jittapalapong *et al*., 2004a), and this phenomenon was also demonstrated with cattle (Kemp *et al*., 1986; Banerjee *et al*., 1990; Khalaf-Allah and el-Akabawy, 1996). Much of this work with has focused on tick species that parasitize cattle in tropical and subtropical climates.

The purpose of this study was to test the feasibility of host immunization with tissue extracts of *Dermacentor andersoni,* an ixodid ectoparasite of cattle in temperate regions of western North America (Bishopp and Trembley, 1945). Calves were immunized with *D. andersoni* midgut (MG) or salivary gland (SG) extracts, followed by observation of the performance of ticks experimentally fed on these hosts, host seroconversion to MG and SG antigens and the reactivity of recombinant tick proteins to the different immune sera. The results of this work indicated that immunization with *D. andersoni* extracts can elicit protective

immunity in cattle, and that this experimental model system is practicable for discovery and development of anti-tick vaccine candidates.

Materials and Methods

Ticks

Adult *D. andersoni* were obtained from the Oklahoma State University Medical Entomology Laboratory. Ticks were stored at room temperature (RT; 20 to 25°C), >90% relative humidity, with a 12 h:12 h (L:D) photoperiod. Twenty-five pairs of male and female *D. andersoni* were fed on each calf before immunization, and identical sets of adult ticks were fed on the same calves 14 days after their second booster immunizations (**Figure 2.1**).

Immunization of calves

MG and SG tissues were collected from male and female *D. andersoni* fed for 3, 4 and 5 days, and MG and SG were dissected free of other tissues and placed in sterile 0.01M Phosphate Buffer Saline (PBS) (pH 7.4) on ice before processing as previously described elsewhere (Jittapalapong *et al*., 2000; Jittapalapong *et al*., 2004a).

Four 3 to 6-month-old Holstein steers were housed in an enclosed building at the University of Missouri (MU) Middlebush Farm and maintained in accordance with MU Animal Care and Use Committee protocol #8981. For each experimental calf, tick MG or SG extract (0.5 ml, 2 mg/ml of protein) was mixed with an equal volume of complete (primary) or incomplete (booster) Freund's adjuvant H37Ra

(Fisher Scientific, Pittsburg, PA), immediately prior to intradermal immunization at 10 sites with 0.1 ml per injection site (1 mg total protein per host) of MG or SG extract. The second and third booster immunizations were given at two-week intervals, followed by tick challenge infestation 14 days after the final booster (**Figure 2.1**).

Tick performance

Male and female *D. andersoni* (25 pairs) were fed on each calf before and after immunization (**Figure 2.1**), and the ticks were checked daily so that detached female ticks could be recovered, weighed, and maintained in humidity chambers. Tick performance parameters included feeding period, mortality, detached female tick weight and egg cluster weight. Egg mass weights were determined after oviposition was complete. The engorgement period was measured as the time from application of the ticks to the host until the day of removal of individual detached female ticks. These performance parameters were used to determine feeding and fertility efficiency indices as previous described elsewhere (Barriga *et al.*, 1991; Jittapalapong *et al.*, 2000; Jittapalapong *et al.*, 2004a, Jittapalapong *et al.*, 2010).

ELISA

Indirect ELISAs were used to titrate bovine immune sera with *D. andersoni* MG and SG homogenates. 96-well high-binding ELISA microplates (Greiner Bio-One, Monroe, NC) were coated overnight at 4 °C with 75 µl/well of protein antigen

(1.0 µg/ml) in 0.1M carbonate coating buffer (pH 9.6), followed by washing five times with PBS and 0.5% (v/v) Tween-20 (PBS-T), and blocking with PBS-T (30 min) followed by 2 h with 100 µl/well of 3% (w/v) bovine serum albumin (2h) (Sigma-Aldrich, St. Louis, MO) in PBS (Xiao *et al*., 2012). After blocking, 60 µl/well of antisera (serially diluted from $1x10^{-2}$ to $1x10^{-8}$) were incubated for 3 h at 37 °C, the wells were washed five times with PBS-T, and then incubated with 50 µl/well of rabbit anti-bovine IgG (heavy and light chain, Invitrogen, Carlsbad, CA; $1x10^{-4}$ in PBS-T) for 15 min at RT. The wells were washed five times with PBS-T before the assays were developed at RT for 15 min with 50 µl/well of 3,3,5,5-tetramethylbenzidine (TMB) solution (1 mM TMB, 0.1 M citric acid, 0.03 M sodium citrate dihydrate and 0.01% (v/v) H_2O_2) and then were stopped with 50 µl/well of 2M H_2SO_4 prior to measuring A_{450} values with a Molecular Devices SpectraMax-Plus (San Jose, CA).

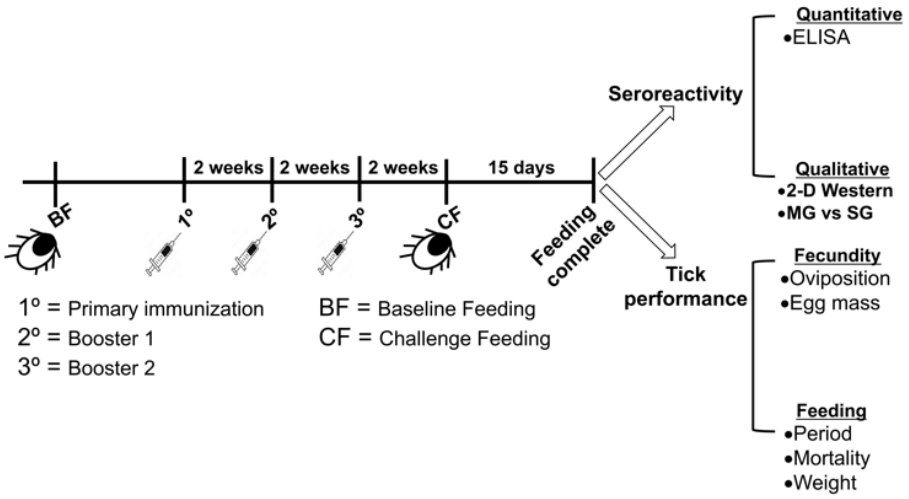

Figure 2.1. Experimental design.
Uninfected ticks were fed on calves before (baseline feeding, BF) and after immunization (challenge feeding, CF). Calves were immunized with *D. andersoni* SG or MG every two weeks, indicated by 1° , 2° or 3°. Female ticks were monitored for performance parameters and calves were tested for seroreactivity.

Statistical analysis

Tick performance data were collected for baseline and challenge infestations, before and after immunization, respectively, to compare observations from pre-immune and immune hosts with the student's t-test. A two-way analysis of variance (ANOVA) with a Holm-Sidak *post-hoc* analysis was used to compare ELISA titers to MG and SG antigens. Analyses were performed with SigmaPlot (Systat Software, San Jose, CA). A P-valve ≤ 0.05 was considered statistically significant.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Unfed *D. andersoni* MG or SG tissues were mixed with loading buffer (10% (v/v) glycerol, 60 mM Tris/HCl pH 6.8, 2% (w/v) SDS, 1.25% (v/v) β-mercaptoethanol, 0.002% (w/v) bromophenol blue) and heated to 90-100°C for 10 min prior to 12% SDS-PAGE with a Mini-PROTEAN Tetra Cell (Bio-Rad, Hercules, CA) at 125 V, until the dye front reached the bottom of the gel. For staining, gels were fixed with 25% (v/v) isopropanol and 10% (v/v) acetic acid for 15 min, rinsed three times with water (10 min each) and stained overnight with PageBlue (ThermoScientific, Waltham, MA) followed by de-staining overnight in ddH_2O.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

MG and SG tissues were collected from unfed male and female *D. andersoni* and stored at 4 °C in lysis solution (8 M urea, 2 M thiourea, 2% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate [CHAPS]); long-

term storage of these tissues was at -20 °C. After protein concentrations were calculated with the Bradford method (Quick Start^{Tm} Bradford 1X Dye Reagent, Hercules, CA), male and female *D. andersoni* MG or SG (20 µg each) were combined and diluted in rehydration solution (8M urea, 2M thiourea, 2% (w/v) CHAPS, 0.5% (v/v) ZOOM carrier ampholytes pH 3-10 (Invitrogen, Carlsbad, CA) and 0.002% (w/v) bromophenol blue) for overnight rehydration of 7 cm, 3-11 NL immobilized pH gradient (IPG) strips (GE, Pittsburg, PA). Isoelectric focusing was performed with the ZOOM IPGRunner System (Invitrogen, Carlsbad, CA) at 4 °C and 200V for 20 min, followed by 450V for 15 min, 750V for 15 min and 2000V for 1.5 h. These IPG strips were used for 12% SDS-PAGE.

Western blots

After SDS-PAGE, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Sigma-Aldrich, St. Louis, MO) at 4 °C with 350 mA for 70 min in a Mini Trans-Blot Electrophoretic Transfer Cell (BioRad). The membranes were incubated while rocking for 1 h with sera (1:400) in TBS (150 mM NaCl, 50 mM Tris-HCl, pH 7.6) with 0.1% (v/v) Tween-20 (TBS-T), washed three times for 5 min with TBS-T, incubated 60 min at RT with rabbit anti-bovine IgG (H & L chain; Invitrogen) in TBS-T ($1x10^{-4}$), washed another three times for 5 min in TBS-T and developed for 15 min with diaminobenzidine (DAB) solution (0.8 mg/ml DAB, 100 mM Tris-HCl pH 7.5, 0.4 mg/ml $NiCl_2$, 0.1% H_2O_2) before stopping development with ddH_2O.

Mass spectrometry (MS)

Gel regions expected to contain proteins of interest were excised from stained gels, digested with trypsin (Promega, Madison, WI) and purified with C18 tips according to the manufacturer instructions (Pierce, Appleton, WI). Samples were lyophilized, resuspended in 10 µl of 5% (v/v) acetonitrile and 0.1% (v/v) formic acid, and MS was performed at the MU Gehrke Proteomics Center with a Bruker timsTOF-PRO mass spectrometer (Bruker Daltonics, Billercia, MA). MS data were collected in positive-ion data-dependent parallel accumulation-serial fragmentation (PASEF) mode, over a range of 100 to 1700 m/z, with trapped ion mobility spectrometry (TIMS). These samples were run five days after the most recent calibration.

Database searches and bioinformatic analysis

The TIMS time-of-flight (timsTOF) data were converted to mascot generic format (Bruker Compass Hystar software) and submitted to the Proteome Discoverer (Sequest HT) search engine with the *Dermacentor silvarum* genome database (26,821 open reading frames (ORFs), GenBank GCA_013339745.1). An automated decoy database search was conducted, in which all sequences were reversed and added to the search, to generate a false discovery rate (FDR) of 1% for protein/peptide matches. Data were searched with trypsin as the protease, two missed cleavages allowed, carbamidomethyl cysteine as a fixed modification and oxidized methionine and deamidation of asparagine/glutamine as variable

modifications. Search result files were filtered for “high” confidence (1% FDR for protein matches).

The MS dataset was used to assess deduced protein sequences from *D. silvarum* ORFs, and tBLASTn (Nadkarni *et al.,* 2020) was used to search sequence read archive (SRA) datasets of *D. andersoni* SG and MG transcriptomes, which included SRX540759, SRX540760, SRX540761 (Mudenda *et al*., 2014), SRX608533, SRX608563, SRX608565 and SRX608566. Deduced amino acid sequences with coverage in *D. andersoni* SG transcriptomic data sets were then built to create a full-length transcript that matched the *D. andersoni* mRNA data. If a partial sequence was available, then a chimera was designed using the closely related *D. variabilis* SRA data set SRX001955 (Jaworski et al., 2010), or, if no data was present from *D. variabilis,* the complete sequence was assembled using the deduced *D. silvarum* proteome. Three of the five overexpressed proteins were chimeric sequences. Protein 1 contained amino acids deduced from *D. andersoni* (AAs 1-237) and *D. variabilis* (AAs 238-287); protein 4 contained residues deduced from *D. variabilis* (AAs 1-34 and 162-192) and *D. andersoni* (AAs 35-161); protein 5 contained residues deduced from *D. andersoni* (AAs 1-147) and *D. variabilis* (AAs 148-197) (**Figures S2.1 and S2.2**).

Results

Tick performance

Previous reports indicated significant reductions in tick feeding and reproductive performance on various hosts immunized with tick MG or SG extract (Barriga *et al*., 1991; Barriga *et al*., 1993; Jittapalapong *et al*., 2000; Jittapalapong *et al*., 2004a), so it was postulated that *D. andersoni* performance would also be reduced after feeding on similarly immunized calves. Baseline tick performance on pre-immune hosts was measured to control for variation due to tick feeding on different hosts, and ticks were fed again after immunization with MG or SG.

Female tick survival decreased 37.5 and 35% with SG-immunized calves in trials 1 and 2, respectively, while 18.3 and 16% decreases in tick survival were observed among ticks fed on the respective MG-immune calves. *D. andersoni* oviposition decreased 50 and 35.3% from SG-immune calves of trials 1 and 2, respectively, while 27.3 and 24% decreases in oviposition were observed among *D. andersoni* fed on MG-immunized calves. A significant difference ($P < 0.01$) was observed in the average decrease in female tick survival after feeding on SG-immune (36.2 ± 1.8%) and MG-immune (17.1 ± 1.6%) hosts, while the difference was not significant for average decreases in the number of female ticks that oviposited after feeding on SG-immune (25.7 ± 2.3%) and MG-immune (42.7 ± 10.4%) calves.

Female tick feeding periods increased on all of the immunized calves. In trial 1, the mean female *D. andersoni* feeding period for the baseline to MG-immune host (calf 2249) increased from 9.4 to 13.6 days ($P < 0.001$; 45% increase)

and from 9.1 to 12.1 days (P < 0.001; 33% increase) on the SG-immune host (calf 2335) (**Figure 2.2A and B**). In trial 2, the average female *D. andersoni* feeding periods for the baseline to MG-immune host (calf 2324) increased from 9.2 to 11.6 days (P < 0.001; 26% increase) and from 9.8 to 15 days (P < 0.001; 53% increase) on the SG-immune host (calf 2468) (**Figure 2.2C and D**).

Detached female *D. andersoni* weights decreased among ticks fed on immunized calves. In trial 1, baseline detachment (*i.e.,* engorgement on pre-immune hosts) weights decreased from a mean of 592 to 461 mg with MG-immune calf 2249 (P < 0.039; 22% decrease) and from 610 to 297 mg (P < 0.001; 51% decrease) with SG-immune calf 2335 (**Figure 2.2A and B**). In trial 2, baseline engorgement weights decreased from a mean of 591 to 421 mg on MG-immune calf 2324 (P < 0.001; 28% decrease) and from 603 to 262 mg on SG-immune calf 2468 (P < 0.001; 56% decrease). Detached female tick weights were significantly lower, with a higher percent decrease, among ticks fed on SG-immune (279.5 ± 17.5 mg; 53.5 ± 2.5%) compared to MG-immune (441 ± 20 mg; 25 ± 3%) calves (P < 0.05) (**Figure 2.2C and D**).

Egg cluster weights were significantly reduced among ticks fed on immunized calves**.** In trial 1, the mean egg cluster weight from *D. andersoni* fed on pre-immune hosts was reduced from 317 to 194 mg on MG-immune calf 2249 (P < 0.001; 41% decrease) and from 335 to 141 mg on SG-immune calf 2335 (P < 0.001; 57% decrease) (**Figure 2.2A and B**). In trial 2, egg clusters were reduced from 336 to 223 mg with MG-immune calf 2324 (P < 0.001; 34% decrease) and from 336 to 118 mg with SG-immune calf 2468 (P < 0.001; 64% decrease). Egg

cluster weights were significantly lower, with a higher percent decrease, among ticks fed on SG-immune calves (129 ± 11.4 mg; 60.5 ± 3.5%) compared to MG-immune calves (208.5 ± 14.5 mg; 37.5 ± 3.5%; $P < 0.05$) (**Figure 2.2C and D**).

The feeding efficiency indices were reduced among female *D. andersoni* fed on hosts immunized with either SG or MG extract. In trial 1, baseline indices with pre-immune hosts fell from 63 to 35 mg/day with MG-immune calf 2249 ($P < 0.001$; 43% decrease) and from 67 to 24 mg/day with SG-immune calf 2335 ($P < 0.001$; 63% decrease) (**Figure 2.2A and B**). In trial 2, baseline indices fell from 64 to 37 mg/day with MG-immune calf 2324 ($P < 0.001$; 42% decrease) and from 62 to 17 mg/day with SG-immune calf 2468 ($P < 0.001$; 71% decrease). Feeding efficiency indices were significantly lower, with a higher percent decrease, among ticks fed on SG-immune (20.5 ± 3.5 mg/day; 67 ± 4%) compared to MG-immune (36 ± 1 mg; 42.5 ± 0.5%) calves ($P < 0.05$).

The reproductive efficiency indices were reduced among *D. andersoni* fed on hosts immunized with either tissue. For trial 1, baseline reproductive efficiency indices with pre-immune hosts fell from 55 to 40% for MG-immune calf 2249 ($P < 0.001$; 27% reduction) and from 54 to 40% for SG-immune calf 2335 ($P < 0.001$; 26% reduction) (**Figure 2.2A and B**). In trial 2, baseline indices fell from 57 to 50% with MG-immune calf 2324 ($P < 0.046$; 12% decrease) and from 50 to 42% for SG-immune calf 2468 ($P < 0.001$; 16% decrease). Although significant reductions in reproductive efficiency indices were measured for ticks fed on every immunized calf, a significant difference was not indicated between ticks fed on SG-immune

(45 ± 5% index; 21 ± 5% decrease) compared to MG-immune (41 ± 1% index; 19.5 ± 7.5% decrease) calves (**Figure 2.2C and D**).

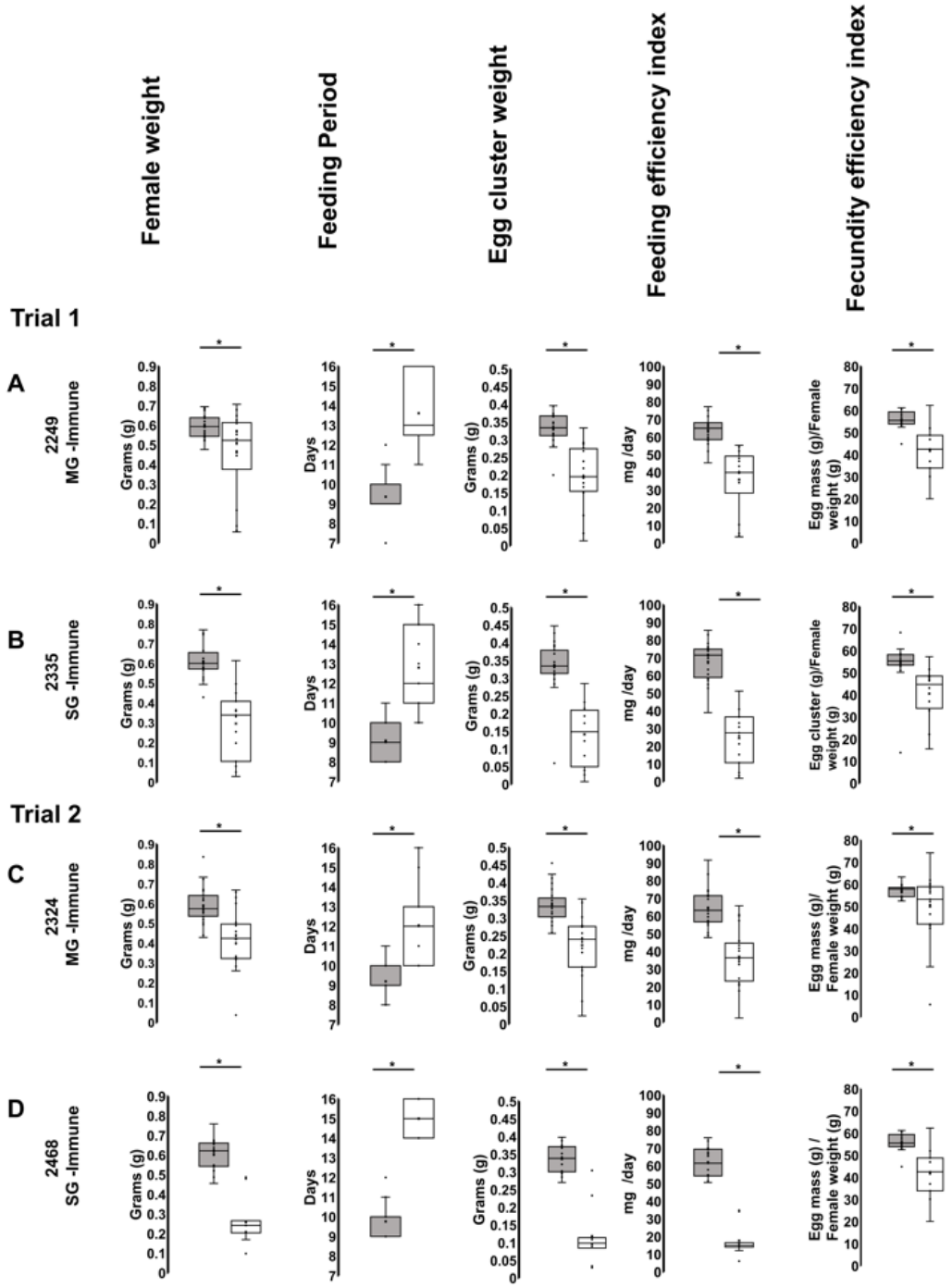

Figure 2.2. Performance of engorged female *D. andersoni* fed on pre-immune and immunized calves.

Female *D. andersoni* were fed on calves before and after immunization with MG or SG extract. Boxes indicate the mean, 1st and 3rd quartiles. Whiskers extend to the high and low values in the range of values. An asterisk (*) indicates a significant difference ($P \leq 0.05$) based upon the student's t-test.

ELISA

Immunization with tick tissues elicits protective antibodies to tick tissues, which have been passively transferred to naïve guinea pigs, rabbits and cattle (Wikel and Allen, 1986, Whelen and Wikel, 1993; Sahibi *et al.*, 1997; Nikpay and Nabian, 2016; Garcia *et al*., 2017; Roberts and Kerr, 1976; Brossard and Girardin, 1979). Thus, ELISA-based titers for cattle immunized in this study were measured to confirm seroconversion to *D. andersoni* MG and SG antigens.

Increasing titers were observed as the immunization schedule progressed (**Figure 2.3**). In the first trial, sera from MG-immune calf 2249 (**Figure 2.3A**) had a detectable titer on Day 9 post-immunization, and titers to both MG and SG homogenates increased during the immunization period. SG-immune calf 2335 also showed increasing titers to the same antigens (**Figure 2.3B**). In the second trial, sera from MG-immune calf 2324 showed increasing titers with greater cross-reactivity to SG antigens (**Figure 2.3C**). SG-immune calf 2468 serum titers to both antigens also increased through the course of immunization (**Figure 2.3D**).

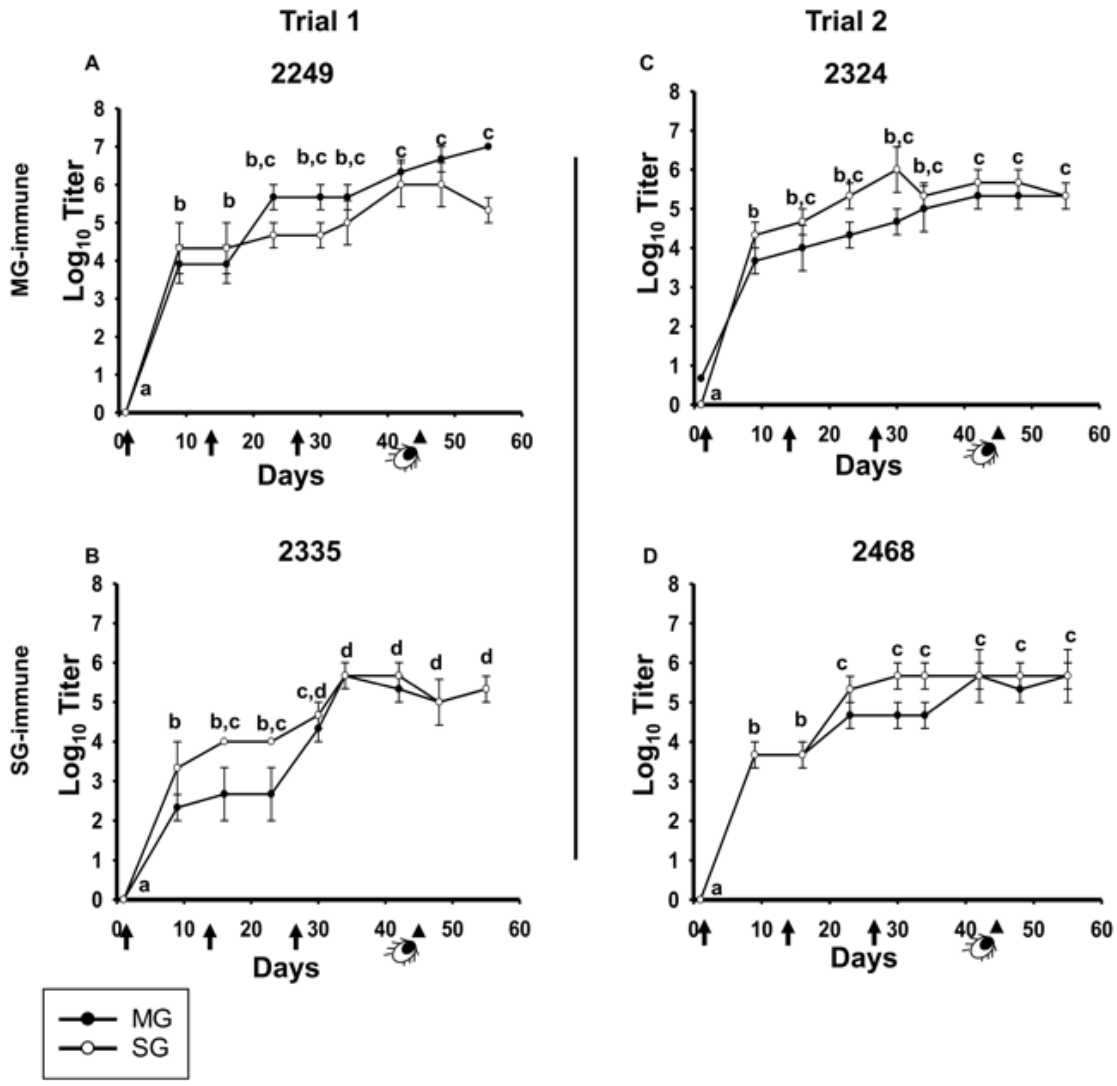

Figure 2.3. Antibody responses of calves immunized with *D. andersoni* MG or SG extracts.

Arrows indicate biweekly immunizations with *D. andersoni* SG or MG, *D. andersoni* challenge infestation is indicated by the symbol resembling a tick (). ELISAs were used to calculate titers to tick SG (open circles) or MG (closed circles) as antigens. Samples denoted with different superscripts (a, b, c or d) were statistically different based on a two-way ANOVA with a Holm-Sidak *post-hoc* analysis ($P \leq 0.05$).

Antigenic specificity

All calves seroconverted to both MG and SG antigen after immunization with either extract. Although titers to both tissues were similar for each calf (**Figure 2.3**), tick performance parameters were more significantly impacted after feeding on SG-immunized hosts (**Figure 2.2**), suggesting molecules uniquely reactive to SG-immune antisera were associated with higher reductions in female tick feeding performance and egg cluster weight. Thus, 2D-PAGE followed by Western blotting were used to localize proteins uniquely reactive to SG-immune antisera (**Figure 2.4**). SG-immune sera from calves 2335 and 2468 were uniquely reactive with 19 and 25 SG antigen protein spots, respectively.

Gel regions containing proteins reactive with SG-immune sera, but not reactive with anti-MG sera, were collected from five regions of similar pI and size (**Figure 2.5**), for preliminary identification of SG proteins posited to be uniquely reactive to SG-immune sera.

Sequences identified by searching the *D. silvarum* genome with timsTOF results derived from gel regions D and E were chosen for further analysis, reducing the number of putative sequences from 258 to 58 (**Figure 2.6**); 12 of these sequences were shared between gel regions D and E, leaving 46 candidate sequences for searching *D. andersoni* MG and SG transcriptomic data sets. The majority of *D. silvarum* sequences (36) were eliminated due to strong read coverage and sequence identity to *D. andersoni* MG transcripts. Another two *D. silvarum* protein sequences were eliminated due to limited read coverage and sequence identity to *D. andersoni* MG transcriptomic data. There were eight

remaining *D. silvarum* protein sequences without detectable coverage and sequence identity to *D. andersoni* MG transcripts, three of which had complete coverage and high sequence identity and two of which had partial coverage and sequence identity to *D. andersoni* SG transcripts. Thus, these five protein sequences were chosen for further analysis (**Table 2.1**).

Immunoreactivity of recombinant proteins

Sequences encoding five *D. andersoni* SG proteins were optimized for bacterial expression, synthesized, and inserted into pTwist vectors for overexpression and Western analysis. MG and SG homogenates were used as positive controls, while negative controls included expression host lysate and an irrelevant protein with the same purification tags (inactive tetanus neurotoxin binding domain, GenBank Accession No. WP_035141397) and expressed in the same *E. coli* strain. All five recombinant tick proteins were seroreactive to anti-SG sera **(Figure 2.7)**. Recombinant proteins expressed with clones 1 and 3 were also reactive to anti-MG sera. Clone 4 was chosen for further analysis, and the recombinant protein expressed from clone 4 had comparatively more reactivity to SG-immune sera than to MG-immune sera **(Figure 2.8)**.

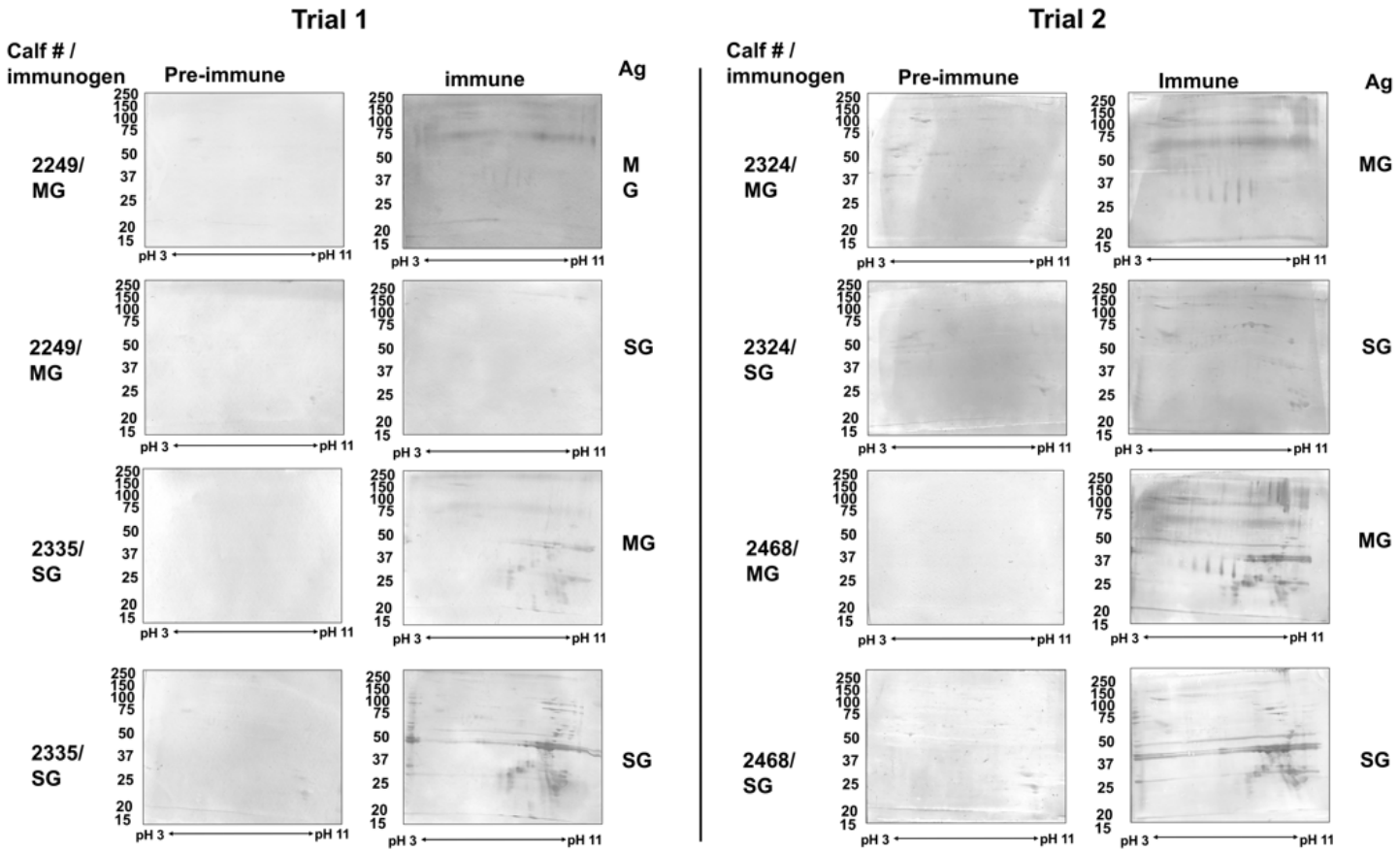

Figure 2.4. *D. andersoni* antigens uniquely recognized by sera from SG-immune calves.

D. andersoni SG and MG resolved by 2D-PAGE and transferred to PVDF. MG-immune calves 2249 and 2324 of trials 1 and 2, respectively; SG-immune calves 2335 and 2468 of trials 1 and 2, respectively. Approximate IEF pH and SDS-PAGE molecular size (kDa) ranges are indicated at the bottom and on the left of each image.

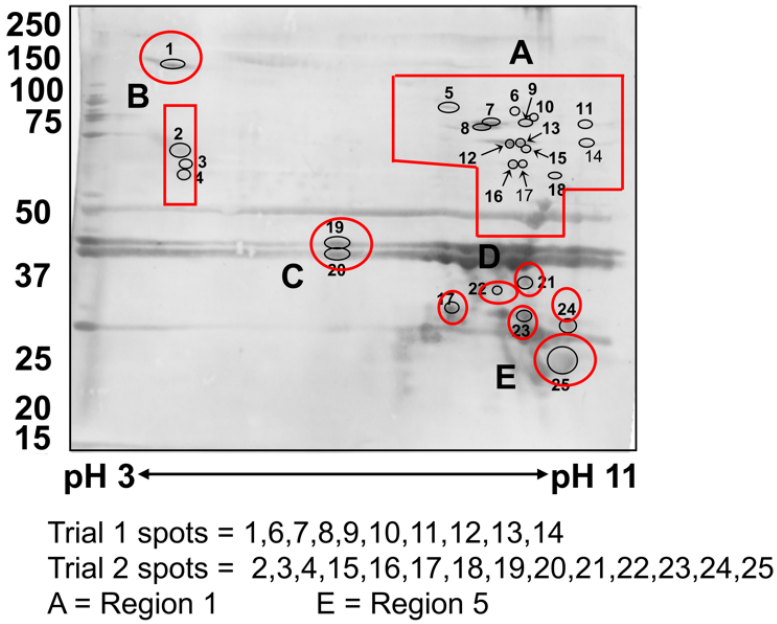

Figure 2.5. *D. andersoni* proteins uniquely recognized by SG-immune sera.

D. andersoni SG resolved with 2D-PAGE and stained with PageBlue. *D. andersoni* SG spots considered uniquely reactive to anti-SG sera are circled, with five regions (A-E) indicated.

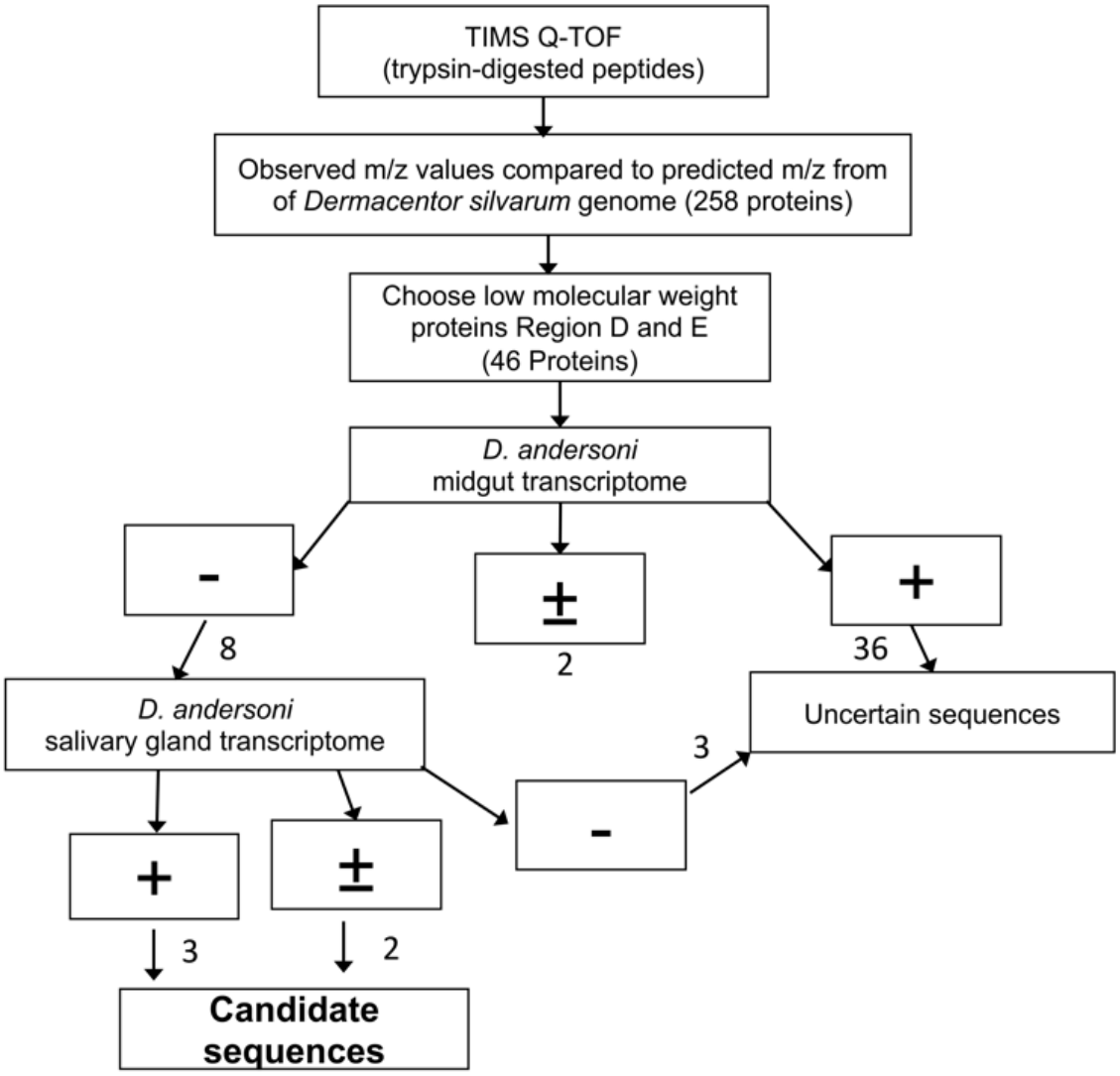

Figure 2.6. Schematic diagram for identification of uniquely seroreactive SG protein candidates.

Table 2.1. *D. silvarum* sequences encoding candidates for uniquely reactive SG proteins (Figure 2.6).

Clone No.	Region	Mr (kDa)	pI	*D. silvarum* (Accession No.)	Annotation	Fusion Protein ORF (bp)	Fusion Protein MW (kDa)	Fusion Protein Mr (kDa)
1	D	37	7.3	XP_037554463.1	Transcription EF-A-protein1- like	867	32.0	36.5
2	E	25-30	8.7	XP_037582046.1	Uncharacterized protein LOC119465317	702	30.4	30.7
3	E	25-30	9.8	XP_037573424.1	Uncharacterized protein LOC119455948	696	29.2	29.7
4	E	25-30	9.3	XP_037582267.1	Uncharacterized protein LOC119465860	579	25.7	25.4
5	D	37	9.0	XP_037581354.1	Shematrin-like protein 2 isoform X1	594	25.1	24.8

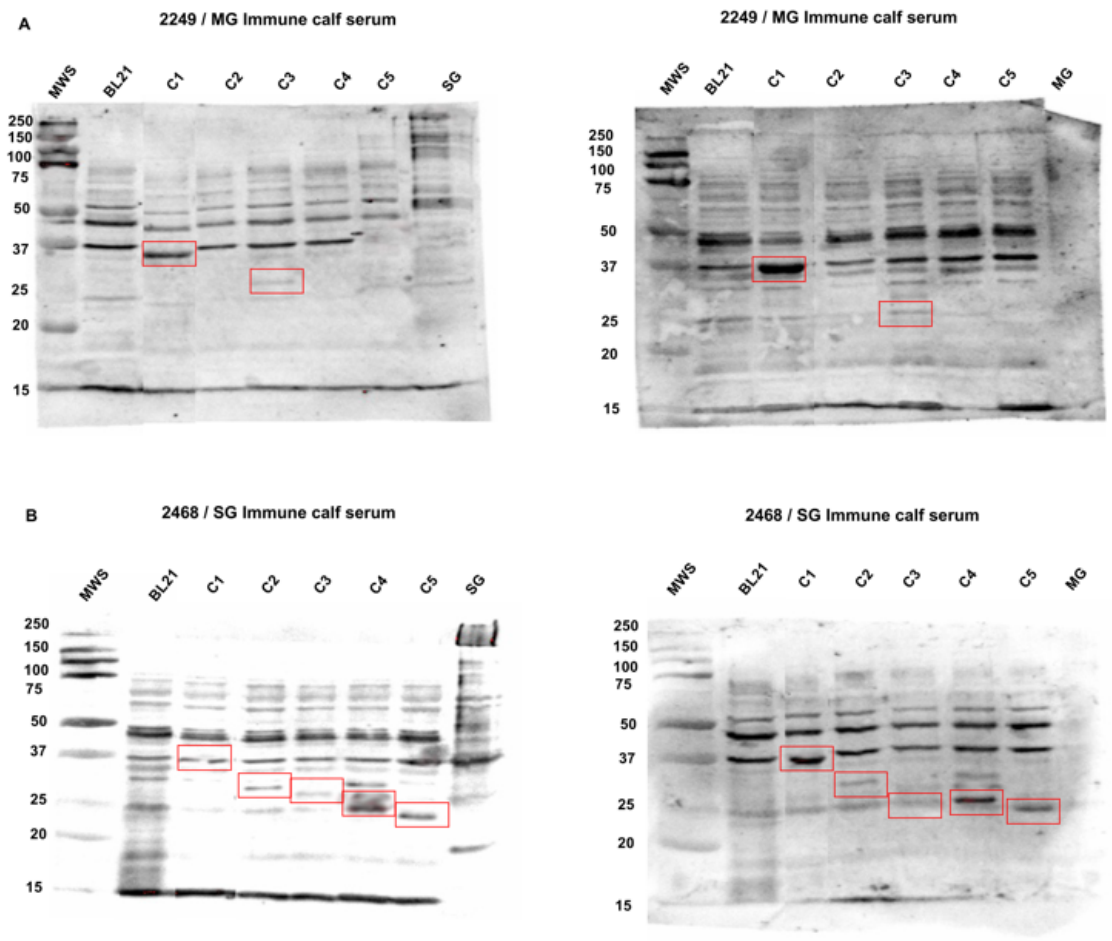

Figure 2.7. Immunoreactivity of proteins expressed by clones 1-5.

Proteins from clones 1-5 (C1-C5) were separated by SDS-PAGE and transferred to PVDF membranes for Western analysis with MG-immune (A) and SG-immune (B) sera. SG or MG homogenates served as positive controls. Seroreactivity of target bands are indicated by red boxes. Abbreviations include MWS – Molecular weight standard (kDa), SG – salivary gland, MG – midgut, C1-5 – Clones 1-5.

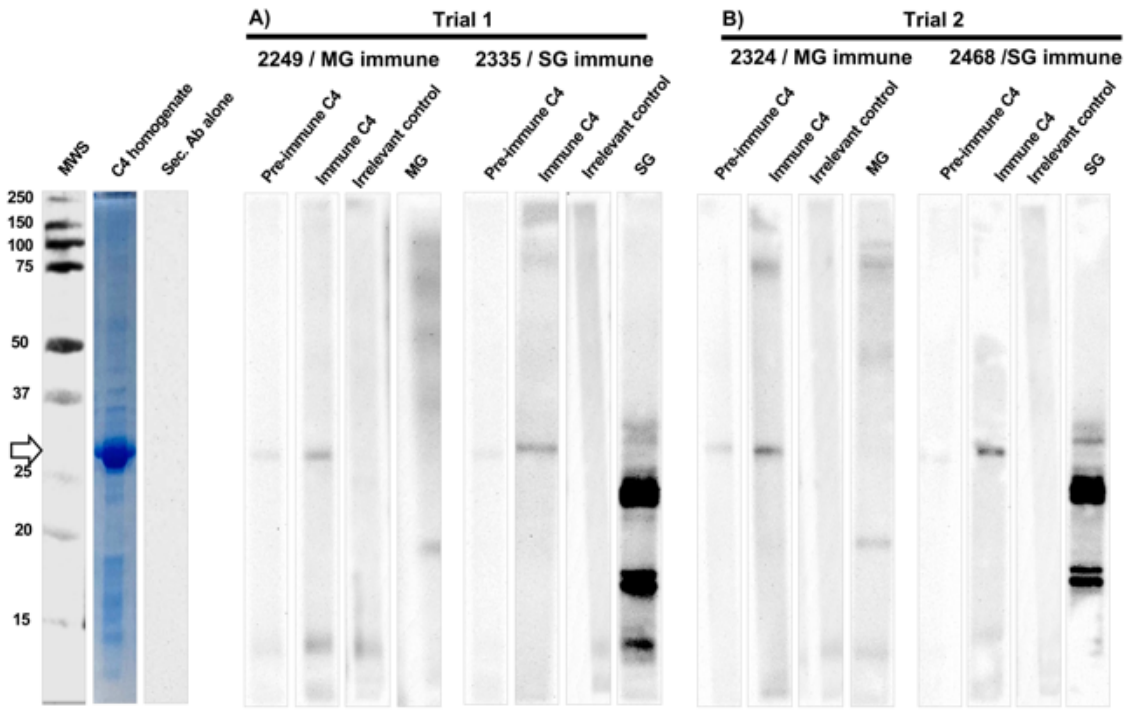

Figure 2.8. Immunoreactivity of recombinant protein expressed by clone 4 (C-4).

Clone 4 was overexpressed and separated by 12% SDS PAGE and transferred to PVDF before development with host sera. Blots were developed with pre-immune and immune sera from (A) calves 2249 and 2335, immunized with MG or SG, respectively, in trial 1 and (B) calves 2324 and 2468, immunized with MG or SG, respectively, in trial 2. SG or MG homogenates were positive controls. An irrelevant protein (tetanus neurotoxin binding domain, GenBank accession no. WP_035141397) with the polyhistidine purification tag and development without antisera (secondary antibody only) were negative controls. Abbreviations include MWS – Molecular weight standard (kDa), SG – salivary gland, MG – midgut, C4 – Clone 4 and sec Ab – secondary antibody.

Discussion

The results of this study demonstrated reductions of female *D. andersoni* feeding and reproductive performance values after feeding on calves immunized with either MG or SG extract. The greatest effect on both feeding performance and egg cluster weight was observed among ticks fed on SG-immune hosts, while immunization with either SG or MG extract appeared to have similar effects on fecundity efficiency indices. Seroconversion of calves immunized with *D. andersoni* SG or MG extracts was observed, as described in previous reports (Barriga *et al*., 1991; Barriga *et al*., 1993; Jittapalapong *et al*., 2000; Jittapalapong *et al*., 2004a). As expected, these antisera contained both cross-reactive and tissue-specific immunoglobulins, and 2D-PAGE followed by Western analysis indicated that several SG proteins were uniquely reactive with anti-SG sera.

A 2D-PAGE-based shotgun proteomics approach was employed to identify SG proteins uniquely reactive to anti-SG immune sera. *D. andersoni* peptide sequences were used to find matching ORFs encoded by the *D. silvarum* genome, which were then used to search *D. andersoni* MG or SG transcriptome datasets, eventually leading to five sequences that were only found in the *D. andersoni* SG transcriptome. These five genes encoded proteins that, when expressed as recombinant proteins, were seroreactive with anti-SG sera.

The reduced tick performance observed in this investigation confirmed results of previous studies involving immunization of mammalian hosts with other tick species (Banerjee *et al*., 1990; Khalaf-Allah and el-Akabawy, 1996; Sahibi *et al*., 1997; Jittapalapong *et al*., 2000; Jittapalapong *et al*., 2004a; Jittapalapong *et*

al., 2010; Nikpay and Nabian, 2016). In the current study, immunization with either SG or MG extract reduced both tick feeding and fecundity, which was consistent with similar reports involving *R. sanguineus, R. (Boophilus) microplus* and *Hyalomma marginatum* (Sahibi *et al*., 1997; Jittapalapong *et al*., 2000; Jittapalapong *et al*., 2004a). Although all these studies reported reductions in both groups of parameters after immunization with either MG or SG extract, previous reports suggested immunization with MG extract reduced tick fecundity indices to a greater extent than immunization with SG extract, while the present study did not demonstrate a difference in fecundity index for ticks fed on MG-immune compared to SG-immune calves. In the current study, and in all the previous reports comparing immunization with SG and MG extracts, the greatest decreases in feeding parameters were observed among ticks fed on SG-immune hosts.

Western analysis indicated numerous cross-reactive proteins, as well as several 2D-PAGE protein spots that were uniquely reactive to SG-immune or MG-immune sera. Immunoblots of 2D-PAGE-fractionated MG appeared less reactive to anti-MG sera, possibly due to denaturing SDS-PAGE resulting in linear epitopes less reactive to antisera against native MG extract, while antisera to denatured SG extract immunogen were more reactive to proteins subjected to denaturing electrophoresis. Conversely, the ELISA results indicated all the antisera were similarly reactive to native MG and SG antigens.

To the best of our knowledge, this is the first report of immunization of cattle with separate *D. andersoni* SG and MG extracts, as previously used with *R. sanguineus* and *R. (B.) microplus* (Jittapalapong *et al.* 2000; Jittapalapong *et*

al. 2004). In an early report, repeated infestation of guinea pigs with *D. variabilis* larvae resulted in subsequently reduced larval and nymphal engorgement (Trager, 1939). A similar phenomenon was reported with *D. andersoni,* in reports of repeated infestations of guinea pigs resulting in reduced engorgement of nymphs and female adults, which was passively transferrable to naïve guinea pigs (Wikel and Allen, 1986, Whelen and Wikel, 1993). Allen and Humphreys (1979) immunized guinea pigs against whole female *D. andersoni* extracts or extracts of midgut and reproductive organs, and immunized cattle with MG and reproductive organ extract, reporting significantly reduced feeding and reproductive performance of female *D. andersoni* subsequently fed on these immunized hosts.

Immune sera collected during this study were used to identify SG proteins posited to be uniquely reactive with anti-SG immune sera. Recombinant forms of these proteins were reactive with sera from SG-immune hosts. Recombinant protein overexpressed with clone 4 was more reactive to anti-SG than to anti-MG immune sera, while other recombinant proteins were reactive to both groups of immune sera. Thus, in our hands, this approach to identification of uniquely reactive antigens is fallible.

In conclusion, this study demonstrated the feasibility of adapting the *D. andersoni*-bovine model system for host immunization with tick tissues and the utility of this model system for anti-tick vaccine candidate antigen discovery and development. Immunization against *D. andersoni* SG extract appeared to have a greater impact on tick performance than immunization with MG extract. A 2D-PAGE proteomics approach had limited utility for identification of proteins uniquely

reactive to SG-immune sera, although only a limited number of candidates were eventually evaluated. Future work will involve adaptation of this model system for experimental intervention with pathogen transmission by *D. andersoni.*

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Supplemental Information (Chapter 2)

Expression of recombinant proteins

Sequences encoding recombinant proteins of interest were synthesized and inserted into pET-28a(+) expression vectors by Twist Biosciences (San Francisco, CA). *E. coli* BL21 (DE3) were used as expression hosts. Competent cells were thawed on wet ice and mixed gently, then 50 µl aliquots were mixed with 1−5 µl of expression vector and incubated on ice for 30 min. Heat shocked cells (42°C for 30 sec) were incubated in 250 µl of super optimal broth with catabolite suppression (SOC+ glucose) at RT before shaking at 225 rpm for 1 h (37°C) and plating 20 or 200 µL from each transformation on separate LB plates containing kanamycin (30 µg/ml). Before induction, an overnight culture was diluted and grown at 37°C with shaking to OD_{600} ≥ 0.3 and expression conditions were optimized **(Figure S2.2 and S2.3)**

Nucleotides encoding clone 1:

ATGGGTTGTGAGGACGAGGTCATGAAGATCGCGAAGCGTTTAGATAAGATGGCATCGGGGAGCAGTGATGACCAAACGCAGGCTTTAGAC
CTTTTAAAGAACCTTCAGGAACTTCCTATTACCCTGGAGATCCTGCAACGTACACGCATTGGAATGACTGTGAACAGCCTTCGCAAGAGTTCTA
ATGATGAGGAAGTCATCACACTGGCCAAGGTTTTGATCAAGTCCTGGAAGAAGCTCTTATCCGGGACACCGAGCAGTAAGGAAGCTCCGGC
GAAAGAGCCAGAGCCAAAACCCAGCGCCTCAGCAAAGCACTCGGCAGCTGCCGCAGGTCGCCCAAACGCAAAGCAGACTAGCTTTCCCGCT
GACACCACTAACGCTGTTCGCTTAAAGTGCCGCGAGTTGCTGAGCAGCGCGCTGAAGTGTGAGGACATGCCAGAGGACTGTGACGTGGATG
GCCTGGCAGCGGCAAAGATCGAGGAGAGTATTTATAATGAGTTCGGCGACACCAACAATAAGTATAAGAACCGTGTGCGCTCGCGTGTTAGT
AACTTAAAGGATAGCAAGAATCCAGCGTTACGCTTAAATGTTCTGCACGGCGCGATTGAACCAGAGCGCATCGCGCGTATGACCGCCGAGGA
AATGGCCAGTGATGATATGAAGCAATTACGCCAGCGCCTTACAAAAGAAGCCATCAATGACCATCAGATGGCAACGACCGGTGGGACAAAG
ACTGACCTGCTGAAGTGCGGTAAGTGCCGTAAGAATAACTGTACCTACAACCAAGTCCAGACGCGCAGCGCCGACGAGCCTATGACGACCTT
CTGCTTCTGCAATGAGTGTGGCCATCGTTGGAAGTTTTGTTAA

Nucleotides encoding clone 2:

ATGGAACAAGTTGGTCGCTATCTTGCCTGCGTTACACTGGCCCTTGCGGCCGGATCGTTCTGCGACGCGAGCCCTCGCGACTTTTATCCACAT
GTGTGTAGCGGTTTCTTCTTCGGGCCAACGCTGGAGAGCCCGTGCGGGTACTTTTGTTATACGTACGAGGGCTCCTTTCAGGGCACGCTTCCT
GATGGCGCTCGCTGTCCCCTGCGTACGTTCCCGCCACGTTCCGGTCGTTGTATTCGTGGTGTATGTATCGCTGTTTCACGTCCCAAGCCCCGTG
AATGCGACGGGATCTACCGTGGTTCCGGCTATGCCCCGACGTGTACCTACGATTGTACGGGCCCGTACGGGCGTCCCATCTTGCGTCCGTATC
GTGACGGGACCCCGTGTCTCAACATCAATCGTCGCGGTAATCCAGTGGGAGCGGCGGGCGTATGTAAGGCAGGTCAGTGTATTGAACACGA
CGATCTGGAAGTACGTAGTCCGTGGGTACGCGAGACCGTTTTCGGGTCTAAGTATCACCGCTGTCTGGCCAAGAAGCAACTGACGAAGAACT
ACTTATGGGACTGCCACCACTATTGTAAGCGTAATTCAGAGTGGTATTACGGTGTCTACGAGGACTGTCAACACCCGGACCAGCGCATCCCCG
GCTGGTGTTGTCATGGCCGTTGCCGTAGCAGTGATTGCCAACGTAAGGAATAA

Nucleotides encoding clone 3:

ATGCGCACCTTCGCGTTATTTGCCGCTGTTTTCGCATTCGCCGCGTATCAAGTTAATGGAGAAGCATGCAACTGTCACCTGCGCGAACTCGACC
TTTGTGCCGCAACATTATTATTATTCAACCAGAATCCATCGGGCGTAGCGACCACCGATGCCGAGGTAGATAAGCAATGTGGATTCTTGAAGG
AAAGCCAGGATTGCTTTCGCAATTTTACAACTCGCTGTGCAACCCCTTTACAACGTGAGTTAATTGGCTTCGTTGCAGAGGGTTCTCAGGAGTT
GTTCAAGCAGTTTTGTACTAAGGGGACGGAAGTACGTACGAATTATCTGAAGCATGCCCCGTGTTTAGGACAGACATTACCTGACCAGAAGA
AGTGTCTCACAGACATCCAAGCGGGTCTTGAGAAGGTATCGACCGTGGCTTTTAACGACCGCGTTCCTGCCGCATGTTGCATGTACAATCGCT
ATCAAGGTTGCACACGTAAAGCTGTGGCGTCAAAGTGCGGCGAAGAGGCCATTGAGTTTGGCGAGATCTTGGTTAAGATGGCGGCGTCCGA
CTTACCGAATGTGGTTTGCACTTCGTATGGTGAGAGCAATGCACGCTGCAATTCCTTACTCCCTCCTCCAGGAACTAAGCCCAGCGGCAAGCC
GACGAGCGTTCTGTCTCGCTTGTTCAGCGCATACCTTGGAAACTGA

Nucleotides encoding clone 4

ATGACATCGTCCTATCGCGTGCGCACAATTCCGACCCGCACCGTCACTACAGTTCGTCGTCAACTGATCGACGACGATGGCGACTTCTTTGATT
TCCCGAAGGCCCGTTCTGGCGGAAGCGAGTTCACGACAGTTCGCCATCGTGTAACGCCAGGTGGTGGAATTGTGACCACGACGACCACCACT
ACCACTACACCGCGTCGCGTTATTCGTGACGTGAGCTTGGACGGAATGGACGCGGATCCCTTCTTCGCTCGTGACGTCAGCCGTCCGAGTCGT
ACTAAGGATTTAGAGGAGTTCAAGGTTAGCTTCGACATCGGTCTGCAGTTCAAACCGGACGACCTGAGCGTGAAAACACGTAACGGTATGGT
GTTTATTACTGCAAAGCGTACGGACGGAAACATCACGCGCGAGTTTGATCGTAAGGTCCCACTGCCCGATGGCGTGGACCCCTTGACCGTAC
GCGCGCTGTTAAGCCCTACAGGTGTTCTGACAATTCGCAGTCCGCGCAATCTGAGCAGCCGCTACACGTCATCAATCTACGAGCGCCACGTTC
CGGTGCGTATGAGCCTGTACTGA

Nucleotides encoding clone 5

ATGGTAAAGCGCAATTACCCAGTAGACCCGTATTATGGCGGTGACGCTCGCTGTGTCTTATACGAGCGCATCGGGCCATTCCGTAACTTTACC
ATTCCCGCCCGCTTTAGCTGGTGTCAAGACGGGTCCGGTCGTGGATGCGGATCGGTTGTTGGTAGCTATGTACTCAGTTCAACACCAGGCTAT
TGGGCCCGCAACCTGTACACATTCTATCCCAACAATGGCCCCGCGGAATGGCAACAGCATACTATCTATAAGGACTGCTCCACCTGTTTTATTG
GCCGCTACCGCTACGCGCTTAATGGCTATGGTTGTGCAATGTGGCGCCGCACTTCACACCTGCACAAGCGTGCGGACTACTGCGACTTCATTT
TCAACATCTTTTGTGGCGGCGTCCCAAAGTACCAGGTTTTCGAGCCATCATGTATTCGCTTATTCGGTGGTGGCTACGGCGGTGGCTTTGGTG
GTGGTCTTGGTGCAGGCTTAGGCGGCGGCCTGGGTGGGCTGGGTGGCCTGGGCGGTGGATTGGACGGCGGATCGGGTGGTGGGTTAGGC
GGTGGCTTAGGCTCAAAAGGTGGTCACATGTGGAAGTGA

Figure S2.1. Nucleotide sequences of clones 1-4 used for expression of recombinant proteins.

Nucleotides encoding clones 1-5, including the plasmid leader sequence and purification tag.

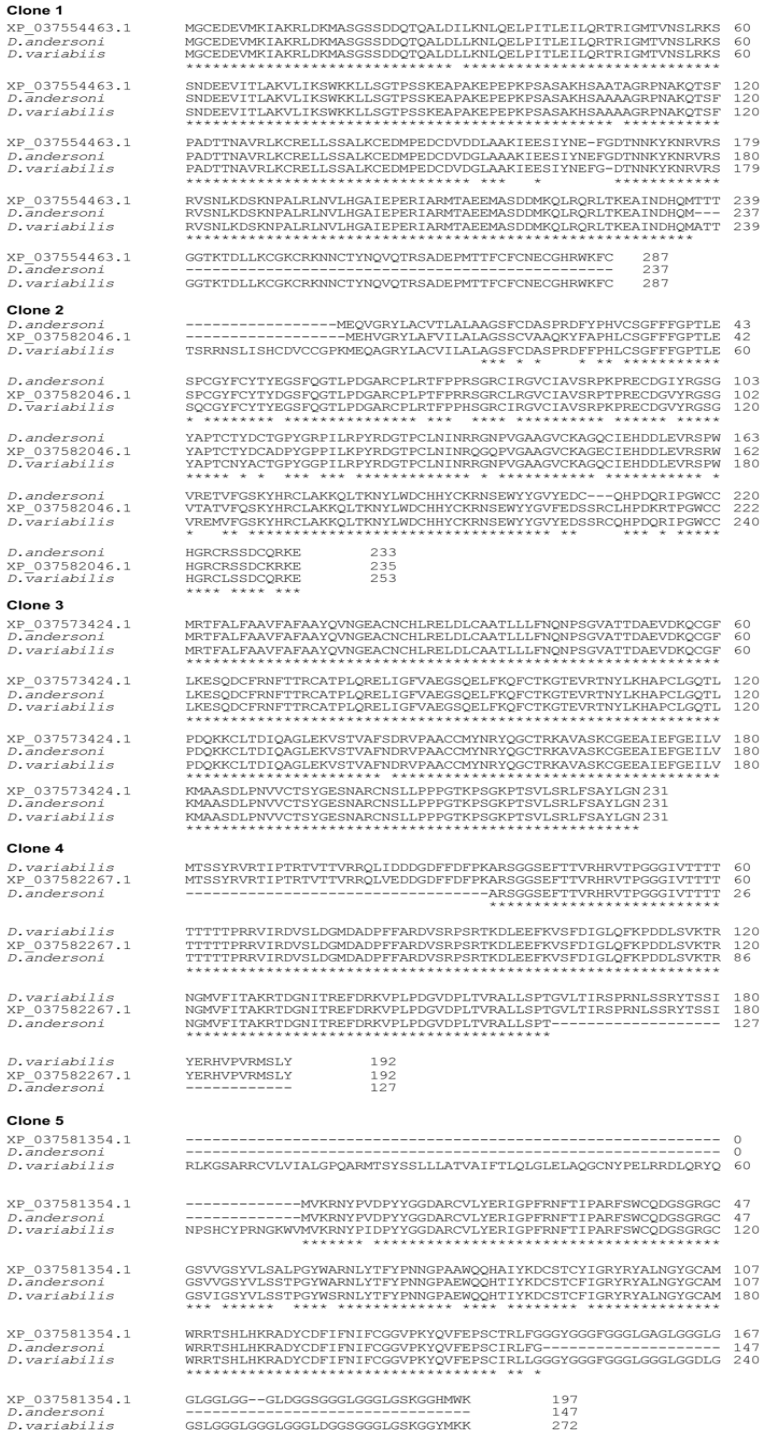

Figure S2.2. Alignments of deduced amino acid sequences encoded by recombinant portions of clones 1 – 5.

ClustalO alignment of amino acids encoded by clones 1-5 from the available sequences from *D. andersoni*, *D. variabilis* and *D. silvarum*.

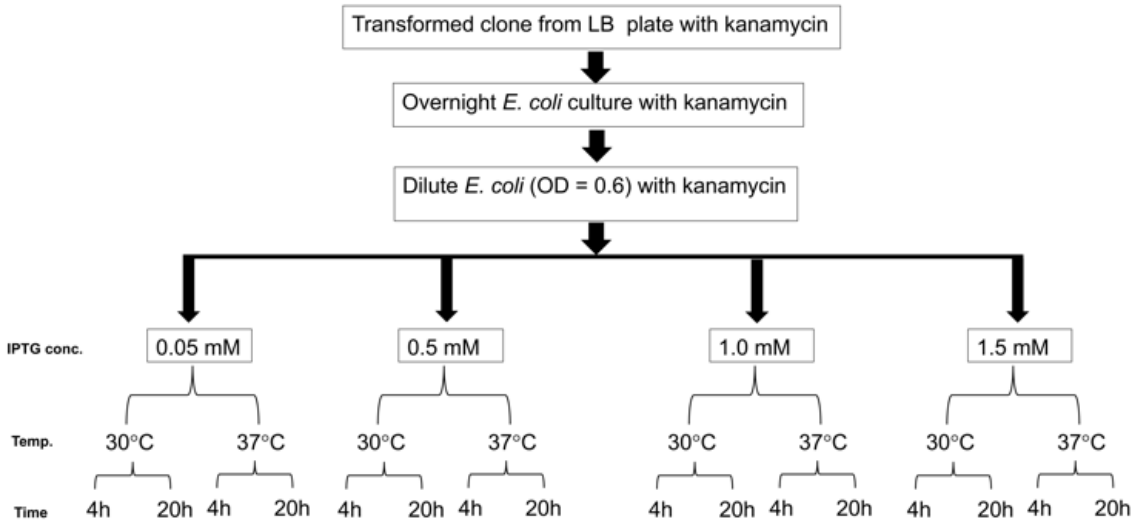

Figure S2.3. Flow illustrating optimization of different parameters for IPTG induction of clones 1-5.

IPTG concentration, temperature of induction and time for induction were optimized for clones 1-5 after optimizing optical density (OD), IPTG concentrations and temperature and time for induction for clone 5.

Table S2.1. Optimum conditions for overexpression of recombinant proteins.

Clone No.	IPTG (mM)	Temp. (°C)	Incubation (hr)
1	1.0	30	20
2	1.0	30	4
3	1.0	30	20
4	1.0	30	20
5	0.5	37	20

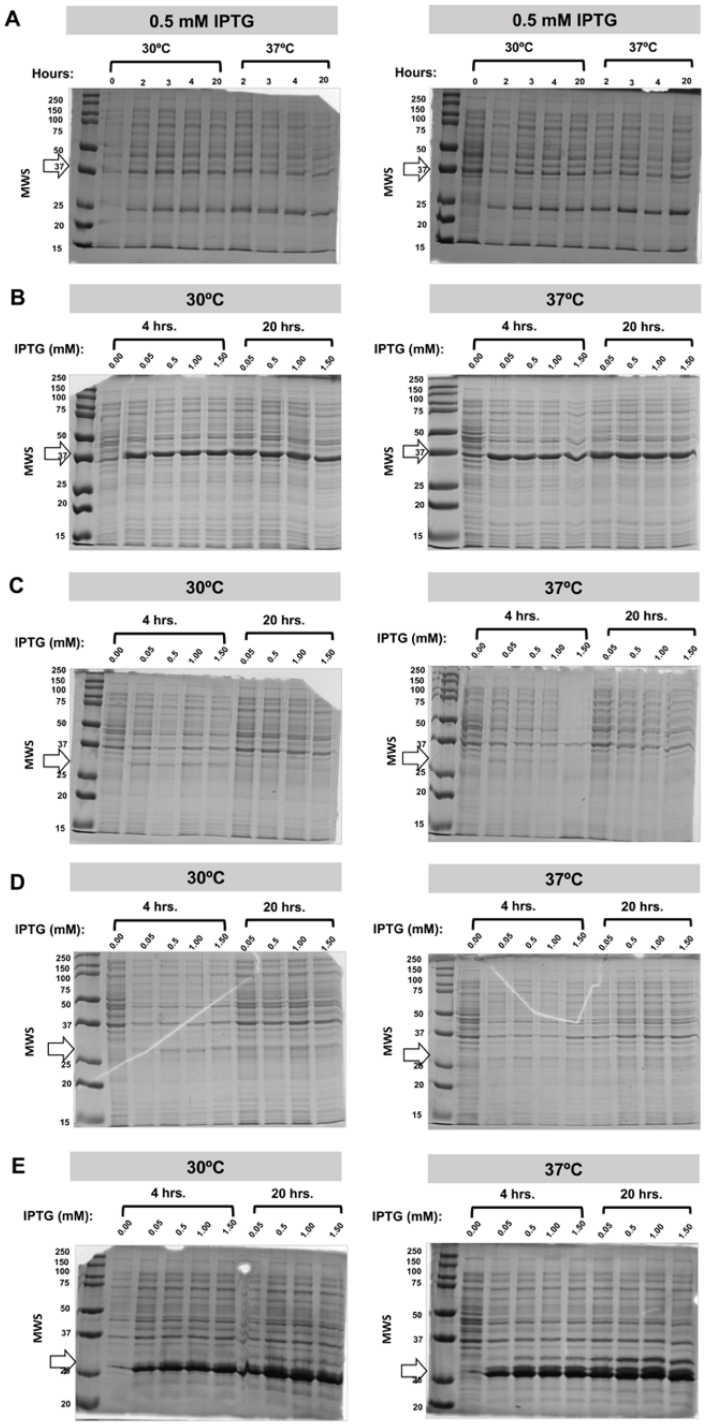

Figure S2.4. Induction of protein overexpression by clones 1 - 5.

Sequences encoding SG proteins were synthesized and cloned by Twist Biosciences into the pET-28a (+) expression vector. Expression of clones 5 and 2-4 are depicted by panels A and B-E, respectively

CHAPTER 3

Interference with the transmission of *Anaplasma marginale* by *Dermacentor andersoni* ticks fed on cattle immunized with tick tissue homogenates

Abstract

The goal of anti-tick vaccine research is the development of sustainable interventions to decrease the incidence of tick-borne diseases. The overall objective of this study was to determine the feasibility of immunization with tick tissue extracts for intervention with biologic tick transmission of *Anaplasma marginale*, the primary etiologic agent of bovine anaplasmosis. *D. andersoni* midgut (MG) and salivary glands (SG) were dissected and pooled from female and male ticks, and native or denatured homogenates were used to immunize calves prior to challenge with adult *D. andersoni* infected with *A. marginale* as nymphs. Denatured MG and SG extracts from experimentally infected *D. andersoni* were inactivated by denaturation and used to immunize another group of calves. Seroconversions of all the immunized calves were confirmed. *D. andersoni* ticks transmitted *A. marginale* to calves immunized with denatured tissue extracts, resulting in microscopically detectable infections and clinical anaplasmosis. However, calves immunized with native tick tissue homogenates did not show signs of infection nor clinical disease, and susceptibility to infection was confirmed by intravenous inoculation of *A. marginale*-infected carrier blood. These results

suggested that immunization of hosts with *D. andersoni* tissues can interfere with experimental biologic transmission of *A. marginale*.

Keywords: Anti-tick immunity; *Dermacentor andersoni;* tick salivary glands; tick midgut; *Anaplasma marginale;* anaplasmosis.

Introduction

Ticks are obligate ectoparasites that are highly adapted to vertebrate hosts (Sauer *et al*., 1995), and, because of tick feeding habits, divergent pathogens have adapted to use ixodid ticks as biologic vectors (Wikel and Whelen 1986). Tick-borne disease control relies extensively on the use of acaricides, but their success is limited by acaricide-induced resistance, contamination of food products and contamination of the surrounding environment (Newton 1967, Keating 1983, Nolan 1990, Soloman 1983 Rego *et al*. 2019).

Immunization of hosts with tick-derived molecules can be detrimental to subsequent tick feeding, development and fecundity (Barriga *et al*., 1991; Barriga *et al*., 1993; Jittapalapong *et al*., 2000; Jittapalapong *et al*., 2004a; Jittapalapong *et al*., 2010). Furthermore, reduced incidence of clinical babesiosis was observed among dairy cows naturally infested the highly adapted one-host southern cattle tick, *Rhipicephalus (Boophilus) microplus*, after immunization with tick salivary gland (SG) extracts (Jittapalapong *et al*., 2004b). Recently, immunization of cattle with *D. andersoni* tissues extracts resulted in reduction of tick survival, engorgement weight and oviposition, as reported in the second chapter of this dissertation (Hoffman *et al.*, 2022).

The goal of anti-tick vaccine research is to develop sustainable interventions to decrease the incidence of tick-borne diseases. Paradoxically, interference with pathogen transmission is often overlooked during primary screening for anti-tick vaccine candidate antigens. The objective of this study was to determine the feasibility of the *D. andersoni*-bovine host model system for immunization with tick tissue extracts and homogenates for interference with biologic tick transmission of *Anaplasma marginale*, the primary etiologic agent of bovine anaplasmosis. This transmission, infection and disease model system is based on a well-characterized, naturally coevolved host-pathogen-tick interaction, involving the primary etiologic agent of what has been described as one of the two most economically important tick-borne diseases of cattle worldwide (Morley 1985; Kocan *et al*., 2003; Minjauw 2003; Jongejan and Uilenberg 2004; Waruri et al, 2021).

Previous studies resulted in observations of different effects of immunization with denatured SG (dSG) or native midgut (nMG) on tick feeding and possibly pathogen transmission (Barriga *et al*., 1991; Barriga *et al*., 1993; Jittapalapong *et al*., 2000; Jittapalapong *et al*., 2004a & b; Jittapalapong *et al*., 2010), suggesting these effects were due to immunizations with these different tick tissues or due to a denaturation step in preparation of the SG immunogens. The current work involved immunization of calves with native SG (nSG), nMG, dSG, denatured midgut (dMG) or denatured MG and SG, combined, collected from *A. marginale*-infected ticks (dIT). Immunized calves were challenged by site infestation with *D. andersoni* infected with *A. marginale as* nymphs. The results of

this study indicated that immunization with native *D. andersoni* MG or SG interfered with experimental tick transmission of *A. marginale,* confirming the utility of this model system for discovery and development of transmission-blocking anti-tick vaccine candidates and refuting the hypothesis that denatured tick immunogens would be more protective against biologic transmission of *A. marginale*.

Materials and Methods

Ticks

Adult *D. andersoni* ticks were obtained from the Oklahoma State University Medical Entomology Laboratory. Ticks were stored at room temperature in tick humidity chambers with >90% relative humidity and a 12 h:12 h (L:D) photoperiod. *D. andersoni* were fed in orthopedic stockinettes attached to each host, as described elsewhere (Kocan *et al*., 1980; Stich *et al*., 1989). During tick feeding, calves were checked daily for engorged, detached female ticks, which were recovered, counted, and maintained in tick humidity chambers. Twenty-five pairs of adult male and female *D. andersoni* were fed on each calf before immunization, which was designated as baseline feeding (BF), and 10 pairs of infected male and female ticks were fed on each calf seven days after the second booster immunization, which was designated as transmission feeding (TF) **(Figure 1)**.

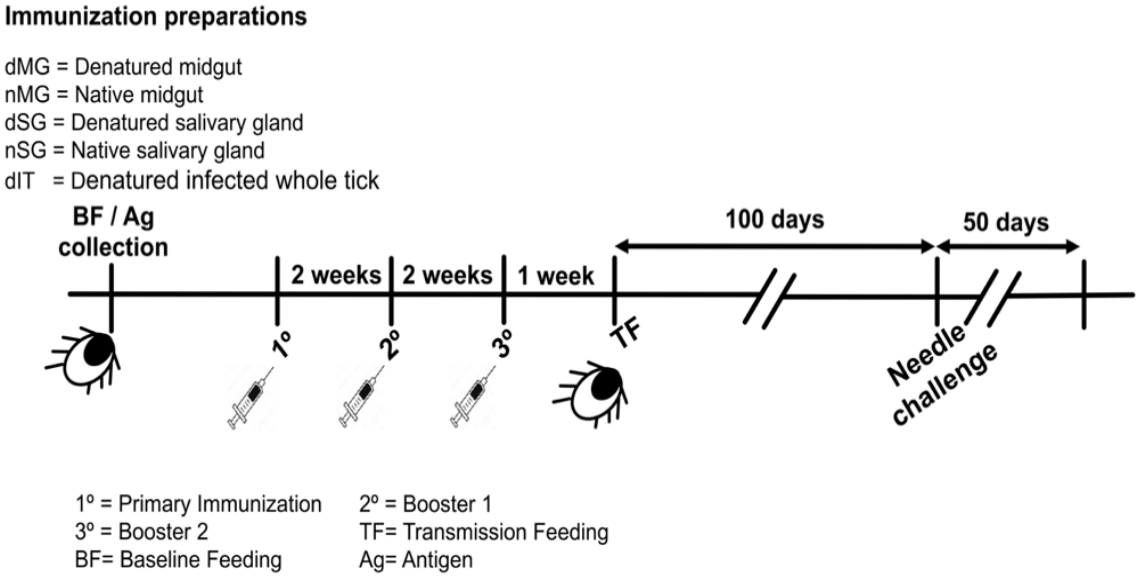

Figure 3.1. Experimental design.

Uninfected ticks were fed on calves before (baseline feeding, BF) and infected ticks were fed after immunization (Transmission feeding, TF). Calves were immunized every 2-3 weeks with denatured or native *D. andersoni* SG or MG, indicated by 1° , 2° or 3°. Female ticks were monitored for performance parameters and calves were tested for seroreactivity and signs of *A. marginale* infection and anaplasmosis. All calves were challenged 100 days after exposure to *A. marginale*-infected ticks by intravenous inoculation with blood from an *A. marginale* carrier.

Antigen preparation

Tick tissues were dissected from 360 normal, unfed female and male *D. andersoni* in addition to cohorts collected while feeding on normal calves for 1 to 6 days (30 male and female ticks per day). SG and MG were removed, dissected free of other tissues, and placed in sterile 0.01M phosphate buffered saline (PBS) (pH 7.4) with 1X protease inhibitor mini tablets (Thermo Fisher Scientific, Waltham, MA, Catalog No. A32953) on ice. Male and female MG and SG collected on each feeding day were ground in separate sterile Dounce homogenizers (Corning Inc., Corning, NY, Catalog No. 7727-2) and stored at - 80ºC. In addition, dIT immunogen was prepared from 186 male *D. andersoni* fed 10 days on a calf with acute experimental anaplasmosis, during the ascending parasitemia, followed by a 10-day holding period in a humidity chamber.

Protein concentrations were measured with the Bradford method (Quick Start Bradford 1X Dye Reagent, Hercules, CA, Catalog No. 5000205), and equivalent amounts of protein from male and female tissues were pooled for MG or SG from feeding days 0-6, and diluted with sterile PBS to a protein concentration of 2.5 mg/ml. A portion of each MG or SG homogenate pool was incubated overnight at 56 °C in 1% (w/v) sodium dodecyl sulfate (SDS) and 5% (v/v) β- mercaptoethanol, followed by heating to 90-100°C for 5 min, cooling to room temperature and removal of SDS (SDS-Out™ SDS Precipitation Kit, catalog No. 20308).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Native and denatured male and female MG or SG homogenates (15 μg each) were mixed with gel-loading solution (10% (v/v) glycerol, 60 mM Tris/HCl pH 6.8, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue, 1.25% (v/v) β-mercaptoethanol) and heated to 90-100°C for 10 min. These preparations were subjected to 12% SDS-PAGE in a Mini-PROTEAN Tetra Cell (BioRad, Hercules, CA) with running buffer (192 mM glycine, 3.45 mM SDS, 25 mM Tris-base pH 8.3), and separated at 125V (constant voltage) until the dye front reached the bottom of the gel. After electrophoresis, the gel was fixed with 25% (v/v) isopropanol and 10% (v/v) acetic acid for 15 min, rinsed with dH_2O three times (10 min per rinse) and stained overnight with PageBlue (Thermo Scientific, Waltham, MA) followed by destaining overnight in dH_2O.

Immunizations

Thirteen 3 to 6 month-old Holstein or Angus-cross steers were confirmed to be negative for *A. marginale* with light microscopy and a *msp5*-based PCR assay (Ueti *et al*., 2007), and were maintained indoors at the MU Middlebush farm in accordance with MU Animal Care and Use Committee protocol #8981. Prior to immunization, 0.4 ml each of dMG, nMG, dSG, nSG or dIT homogenates (1.0 mg total protein) was emulsified with 0.6 ml of Montanide ISA VG 61 (Seppic, Fairfield, NJ) with 5 ml syringes (Global Easy Glide Catalog No. EN302) attached with double female luer-lock adapters (Air-Tite Catalog No. MX494E), following the manufacturer's instructions of 20 slow followed by 60 rapid cycles. The calves were

shaved and disinfected with 70% ethanol on both sides of the vertebral column, and immunized intradermally at 10 sites (0.1 ml per site) along their backs. In trial 1, a total of three immunizations were performed every three weeks. In trial 2, three immunizations were performed every two weeks **(Figure 3.1)**. Recipients of each immunogen are shown in **Table 3.1**.

Tick performance

Male and female *D. andersoni* (25 pairs) were fed on each calf before immunization and 10 pairs of infected *D. andersoni* were fed on each calf after immunization (**Figure 3.1**). These ticks were checked daily so that detached females could be recovered, weighed and maintained in humidity chambers (Barriga *et al.*, 1991; Jittapalapong *et al.*, 2000; Jittapalapong *et al.*, 2004, Jittapalapong *et al.*, 2010). Tick performance parameters included feeding period, tick survival, detached female tick weight and egg cluster weight. Egg mass weights were determined after oviposition was complete. The engorgement period was measured as the time from application of the ticks to the host until the day of removal of individual detached female ticks.

ELISA

Indirect ELISAs were used to titrate bovine immune sera with MG and SG antigens collected from unfed female and male *D. andersoni*. 96-well high-binding ELISA microplates (Greiner Bio-One, Monroe, NC) were coated for 2 h at 37 °C with 75 µl/well of antigen (1.0 µg/ml of protein) in 0.1M carbonate coating buffer

(pH 9.6), followed by five washes with 0.5% (v/v) Tween-20 in PBS (PBS-T) and incubation with PBS-T at RT for 30 min. 60 ul/well of antisera (serially diluted from $1x10^{-2}$ to $1x10^{-8}$) were incubated for 1 h at 37°C. The wells were washed five times with PBS-T, before incubating 50 µl/well of rabbit anti-bovine IgG (heavy and light chain) conjugated to horse radish peroxidase (Invitrogen, Carlsbad, CA), diluted 1:20,000 in PBS-T, for 1 h at 37°C. The wells were again washed five times with PBS-T and developed at RT for 4 min with 50 µl/well of 3,3,5,5-tetramethylbenzidine (TMB) solution (1mM TMB, 0.1 M Citric Acid, 0.03 M Sodium Citrate Dihydrate and 0.01% (v/v) H_2O_2) and then were stopped with 50 µl/well of 2M H_2SO_4 prior to measuring A_{450} values with a Molecular Devices SpectraMax-Plus (San Jose, CA). Each serum sample dilution was assayed in singlet on separate plates in three independent experiments, with corresponding pre-immune serum sample dilutions in triplicate to determine cutoff thresholds as described elsewhere (Frey *et al*., 1998).

Transmission of *A. marginale*

Donor calf 635 was inoculated (IV) with heparinized blood from an *A. marginale* (Virginia strain) carrier. Calf 635 was monitored through the incubation, acute and post-acute phases of clinical anaplasmosis. For experimental infection of *D. andersoni*, 400 nymphs (Trial 1) were fed to repletion on calf 635 during the acute phase of experimental anaplasmosis and 500 nymphs (Trial 2) were fed to repletion during the post-acute, subclinical phase of

experimental anaplasmosis. Subsequently molted adult ticks were used for challenge infestations.

Male and female *D. andersoni* (10 pairs) infected with *A. marginale* as nymphs on calf 635 were transmission fed on each calf 7 days after the third immunization. 100 days after attachment of infected ticks, all calves, regardless of whether transmission had been observed, were challenged a second time by inoculation (IV) of 2 ml of heparinized blood from *A. marginale*-infected carrier 635.

After exposure to *A. marginale*-infected ticks or carrier blood, semiweekly blood samples were collected from each calf and monitored for *A. marginale* infections and reductions in packed cell volume (PCV) (Stich *et al*., 1989). Blood samples were monitored daily after observation of *A. marginale* in host erythrocytes. Prepatent periods were defined as the number of days from exposure to *A. marginale*-infected blood or ticks until measurement of 1% parasitemia in peripheral blood.

Statistical analysis

Tick performance data were collected for baseline and transmission feeding, using a random block design, before and after immunization, respectively. The student's t-test was used to compare baseline and transmission observations between trials. A two-way analysis of variance (ANOVA) with a Holm-Sidak *post-hoc* analysis was used to compare ELISA titers to MG and SG antigens. Associations were measured with the Fisher exact test. All analyses were

performed using SigmaPlot (Systat Software, San Jose, CA). A P-valve ≤ 0.05 was considered statistically significant.

Table 3.1. Cattle used for this study.

	Animal Number	Breed	Sex	Age at D0 (Months)	Treatment*
Trial 1	3371	Holstein	Male	3	dMG
	3241J	Angus	Male	3	dMG
	3275	Angus	Male	3	nMG
	3342	Angus	Male	3	dSG
	3248	Angus	Male	3	nSG
	3124	Holstein	Male	6	dIT
	3280	Angus	Male	7	dIT
	3124J	Holstein	Male	3	Control
Trial 2	648	Angus	Male	3.5	dMG
	3178	Angus	Male	3.5	nMG
	3239	Angus	Male	3.5	dSG
	3250	Angus	Male	3.5	nSG
Carrier	635	Angus	Male	4	AF

*****Treatments: AF, acquisition feeding of *D. andersoni* nymphs; dMG, denatured MG immunogen; nMG, native MG immunogen; dSG, denatured SG immunogen; nSG, native SG immunogen; dIT, denatured infected tick immunogen.

Results

Antigen preparation and Immunizations

A. marginale infection was experimentally established in donor calf 635, with a nadir of 11% in PCV (74.42% reduction) and a peak parasitemia of 8.59%. Adult *D. andersoni* exposed to *A. marginale* as nymphs tested PCR-positive for *A. marginale* **(Figure 3.2).**

The effect of immunogen denaturation was investigated by 12% SDS-PAGE of dMG, nMG, dSG and nSG preparations, resulting in similar band patterns among the denatured and native homogenates used to immunize these calves **(Figure 3.3).**

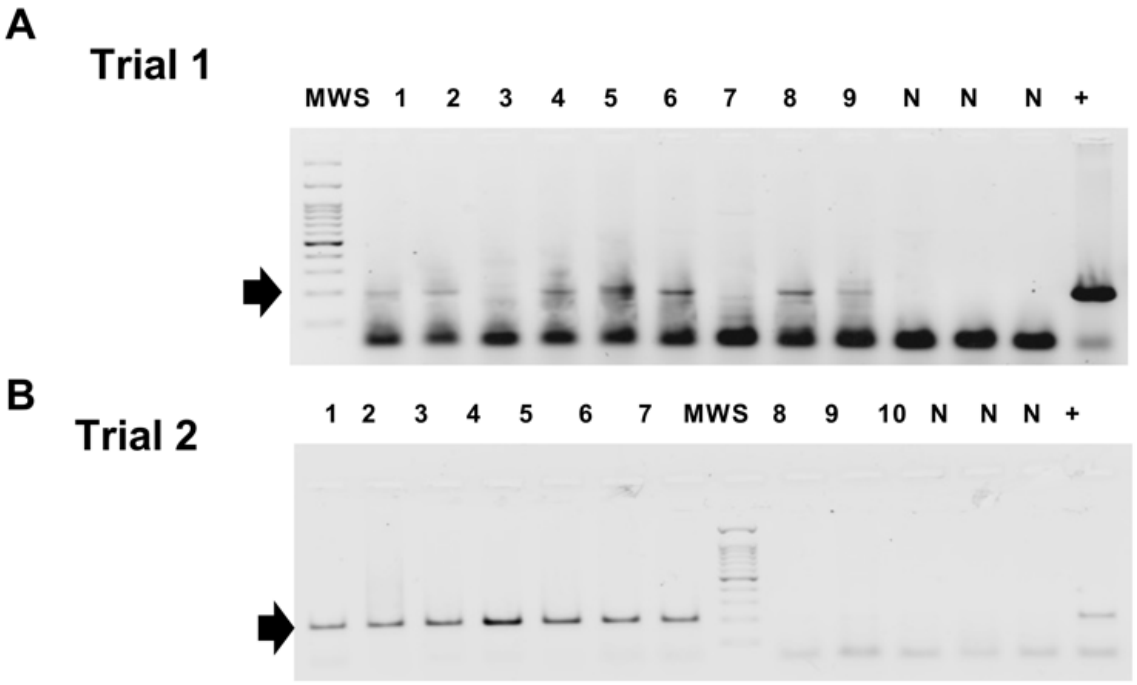

Figure 3.2. PCR assays of adult *D. andersoni* used for challenge infestations.

PCR products from whole tick samples amplified with the *A. marginale*-specific *msp*5-based assays. *D. andersoni* adult ticks in Trial 1 (1-9) and in Trail 2 (1-10), no-template controls (N), *A. marginale* Virginia strain-infected calf (+) were assayed and electrophoresed with a 100 bp molecular mass marker (M).

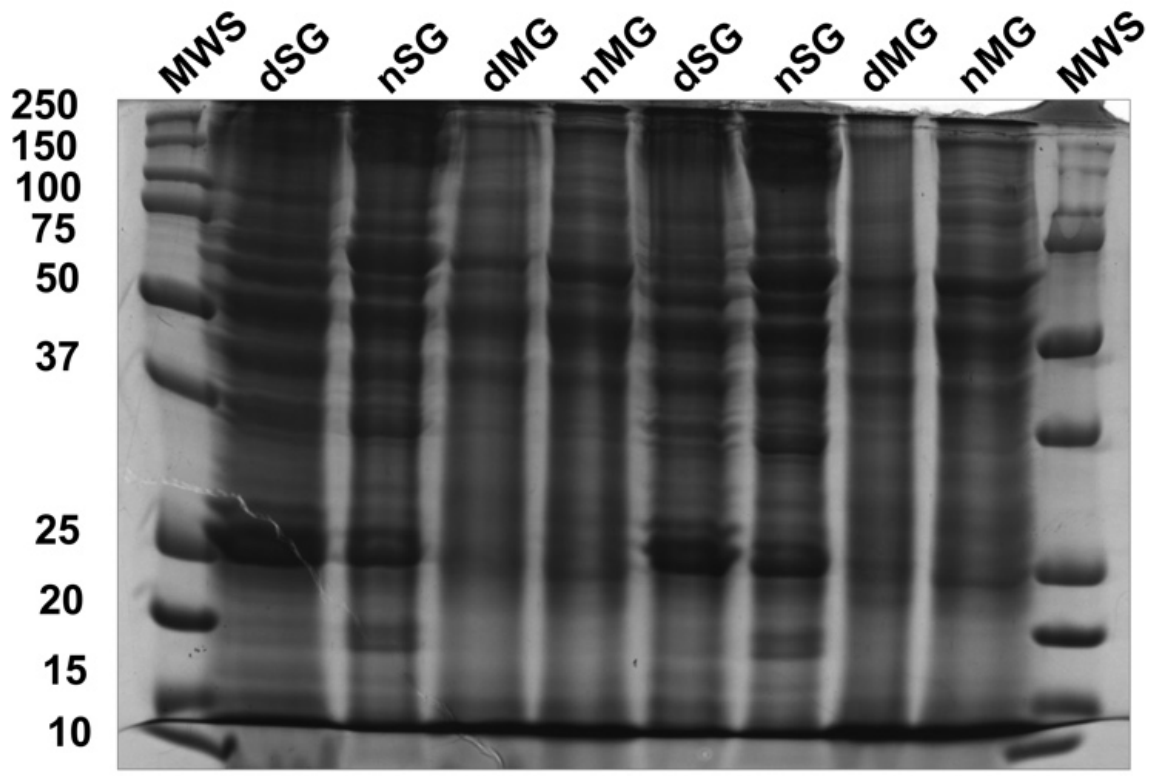

Figure 3.3. Effect of denaturation on tick tissue homogenates.

Denatured and native *D. andersoni* MG and SG homogenates were fractionated and compared by 12% SDS-PAGE. The different lanes show the molecular size standard (MWS), denatured salivary glands (DSG), native salivary glands (nSG), denatured midgut (dMG) and native midgut (nMG)

Tick performance

We recently reported adaptation of a bovine model for immunization with tick tissue extracts prepared from *D. andersoni*, in which reductions in female *D. andersoni* feeding and reproductive performance values were observed after immunization with dSG or nMG extracts (Hoffman *et al.*, 2022). Thus, these same parameters were measured in the current study to determine if such performance would be associated with biologic transmission of *A. marginale.*

Mixed results were observed with respect to female tick feeding periods during the transmission-feeding phases of trials 1 and 2 (**Table 3.2**). In trial 1, average female tick feeding periods in baseline feeding ranged from 19 days to 22 days. However, female *D. andersoni* infected with *A. marginale* as nymphs did not perform as expected when fed on these calves, including the untreated control calf, and all of these female and male tick pairs were removed by traction at 22 days after attachment. In trial 2, female *D. andersoni* feeding periods ranged from 10 to 19 days during baseline feeding phase, with calf 3178 removing the tick feeding cell before all but one of the specimens could be recovered. Female *D. andersoni* infected with *A. marginale* as nymphs fed more as expected during the transmission feeding phase of this trial 2, with the longest feeding period for ticks fed on the dMG-immune host (648; $P < 0.014$) followed by those fed on the nSG-immune host (3250) which fed longer than ticks fed on dSG-immune 3239 ($P < 0.05$) but not nMG-immune 3178.

Significant differences in weights of female ticks and egg masses were not observed among ticks fed before or after immunization of hosts. In trial 1, female

D. andersoni did not transmission feed as expected, so the weights of these detached female ticks and their egg masses were not considered. In trial 2, the weights of detached, transmission-fed female *D. andersoni* ranged from 159 to 431 mg and egg mass weights ranged from 88 to 301 mg; however, none of these values were significant due to the high levels of variance within the different groups of tick and egg mass weights.

Table 3.2. Performance of adult *D. andersoni* fed on immunized calves.

Calf	L & N fed	Trt	Feeding period (days)		Female weight (mg)		Survival (%)				Egg mass (mg)		Ovi-posited (%)		Tick TF*
							BF		TF						
			BF	TF	BF	TF	F	M	F	M	BF	TF	BF	TF	
Trial 1															
3371	No	dMG	19±3	22**	490±178	8±2	72	NR	100	100	216±106	ND	89	0	**ND**
3241J	No	dMG	19±4	22**	227±154	14±15	64	NR	100	100	109±80	ND	81	0	**+**
3275	No	nMG	19±5	22**	501±209	7±2	84	NR	100	80	255±130	ND	100	0	**ND**
3342	No	dSG	22±2	22**	485±203	8±3	72	NR	80	80	207±117	ND	94	0	**+**
3248	No	nSG	20±4	22**	392±212	9±3	100	NR	100	80	197±135	ND	96	0	**ND**
3124J	Yes	None	NA	22**	NA	520†	NA	NR	60	80	NA	210†	NA	17	**+**
3124	Yes	dIT	19±7	22**	410±396	340±170†	10	NR	50	70	280±325	110±71†	100	100	**+**
3280	Yes	dIT	21±2	22**	394±289	NR†	40	NR	70	100	335±89	ND†	57	0	**+**
Trial 2															
648	Yes	dMG	16±1	20±2	319±151	159±232	16	NR	80	40	90±26	301±286	75	38	**+**
3178	Yes	nMG	10‡	16±4	360‡	288±163	4	NR	80	100	80‡	88±29	100	80	**ND**
3239	Yes	dSG	19±2	15±5	315±182	431±296	28	NR	80	80	110±80	121±37	71	75	**+**
3250	Yes	nSG	16±3	17±4	286±244	211±249	24	NR	100	100	73±49	137±126	67	50	**ND**

Abbreviations: BF, baseline feeding; L, larvae; N, nymphae; NA, not applicable; ND, not detected; NE, none engorged; NR, not recorded; TF, transmission feeding; Trt, treatment group.

Treatment groups: dMG, denatured MG homogenate; nMG, native MG homogenate; dSG, denatured SG homogenate; nSG, native SG homogenate; dIT, denatured infected tick homogenate.

*Results of *A. marginale*-infected tick transmission feeding challenge

**Attached ticks collected after 22 days.

†Values were not recorded for apparently non-engorged female ticks; values shown represent 1 of 6 (3124J), 2 of 7 (3124) and 0 of 10 (3280) recovered specimens.

‡Single specimen recovered.

Seroconversion of immunized calves

Immunization with tick tissues has been used repeatedly to induce host antibody production and host resistance to ticks (Sahibi *et al.*, 1997; Nikpay and Nabian, 2016; Garcia *et al*., 2017; Roberts and Kerr, 1976; Brossard and Girardin, 1979). To confirm seroconversion of immunized calves, ELISA-based antibody titers to MG and SG were measured for weekly samples collected from each vertebrate host. Antibody titers measured for trials 1 and 2 **(Figure 3.4)**. In both trials, antibody titers of immunized calves increased to 1 x 10^5 against MG and SG homogenates. Among calves immunized with dMG, antibody titers reached 1 x 10^6 ± 0.33 to MG and 1 x 10^{6-} ± 0.66 to SG by days 25 ± 5 and 25 ± 5, respectively. Among calves immunized with nMG, antibody titers reached 1 x 10^7 ± 0.33 to MG and 1 x 10^6 ± 0.33 to SG by days 25 ± 5 and 27 ± 5, respectively. Among calves immunized with dSG, antibody titers reached 1 x 10^5 ± 0.33 to MG and 1 x 10^7 ± 0.33 to SG by days 35 ± 5 and 40 ± 5, respectively. Among calves immunized with nSG, antibody titers reached 1 x 10^5 ± 0.33 to MG and 1 x 10^7 ± 0.33 to SG by days 45 ± 5 and 47 ± 5, respectively. Lastly, calves immunized with dIT reached antibody titers of 1 x 10^4 ± 0.57 to MG and 1 x 10^4 ± 0.33 to SG by days 20 ± 5 and 35 ± 5, respectively.

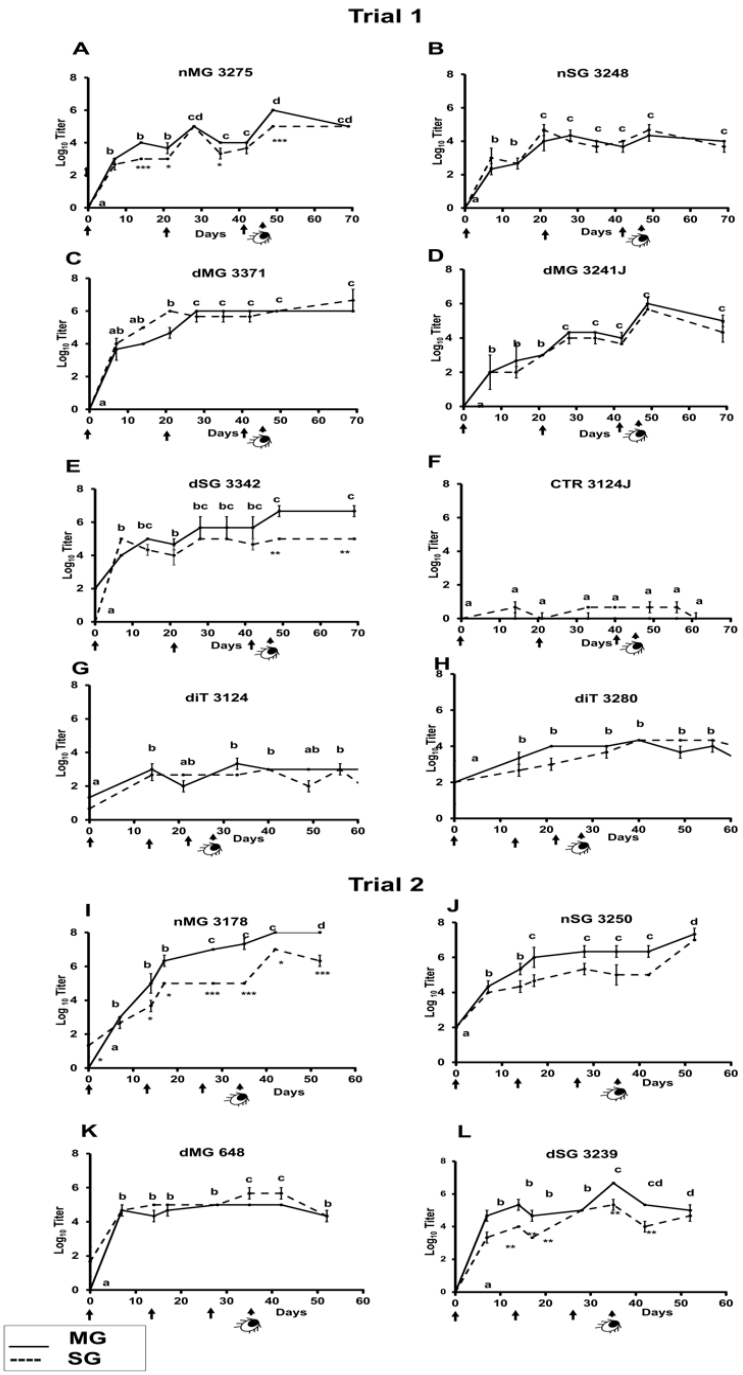

Figure 3.4. Antibody responses of calves immunized with *D. andersoni* MG or SG extracts.

Arrows indicate weekly immunizations with denatured or native *D. andersoni* SG or MG. *D. andersoni* challenge infestation is indicated by . ELISAs were used to calculate titers to tick SG (dotted line) or MG (solid line) as antigens. Superscripts a, b, c or d represent statistical differences based upon a two-way ANOVA with a Holm-Sidak *post-hoc* (P ≤ 0.05: *, ≤ 0.01: ** and ≤ 0.001: ***)

Transmission of *A. marginale*

Previous work indicated reduced incidence of babesiosis among dairy cows immunized with *R. (B.) microplus* dSG extract (Jittapalapong *et al*., 2004b), and it was hypothesized that immunization with similarly prepared immunogen could reduce experimental transmission of *A. marginale* by *D. andersoni*. Calves were immunized with dMG, nMG, dSG, nSG or dIT to challenge infestation with experimentally infected ticks **(Figure 3.1)**. Cohorts of the transmission-fed ticks tested PCR-positive for *A. marginale* **(Figure 3.2)**. Because denaturation with SDS inactivates infectious organisms, two calves were also immunized with denatured extracts of *A. marginale*-infected ticks, to determine if this treatment would induce protective immunity.

In both trials, infected *D. andersoni* did not transmit *A. marginale* to hosts immunized with nMG or nSG. Conversely, *A. marginale* infections were detected among all but one host immunized with denatured tick homogenates, as well as in the host used as a non-immunized control **(Table 3.3)**. The Fisher exact test indicated association between protection from transmission of *A. marginale* and immunization with native tick tissue homogenates ($P<0.02$; OR=4), but no association between antibody titer levels and protection against transmission of *A. marginale.*

To confirm susceptibility to infection, every host immunized with MG or SG was challenged a second time by inoculation (IV) with infected blood from an *A. marginale* carrier. *A. marginale* was detected in the tick-transmission-negative hosts after challenge with blood-inoculation; calves infected via tick feeding were not reinfected after the second challenge treatment **(Table 3.3** and **Figure 3.5)**.

Table 3.3. Infection and disease parameters of cattle exposed to *Anaplasma marginale*.

Calf	Treatment	*A. marginale* transmission		Pre-Infection PCV (%)		Post-infection PCV Nadir (%)	Percent reduction PCV*	Peak Parasitemia (%)	Prepatent period (days)†
		Tick TF	Inoculation (IV)	$\bar{x} \pm SD$	N =				
Trial 1									
3371	dMG	**ND**	+	31.9 ± 2.7	13	11.5	63.9	8.0	43
3241J	dMG	**+**	ND	28.6 ± 2.6	80	13.0	54.5	10.2	24
3275	nMG	**ND**	+	27.2 ± 3.3	80	12.5	54.0	6.2	24
3342	dSG	**+**	ND	29.3 ± 2.7	13	11.0	58.1	6.1	51
3248	nSG	**ND**	+	27.0 ± 3.6	81	11.5	57.5	9.5	19
3124	dIT	**+**	NA	30.8 ± 2.8	6	18.5	40.0	2.7	43
3280	dIT	**+**	NA	28.7 ± 3.3	6	15.5	45.9	4.8	50
3124J	control	**+**	ND	31.1 ± 1.9	5	19.0	38.9	2.9	47
Trial 2									
648	dMG	**+**	ND	30.1 ± 2.7	8	20.0	33.6	1.2	49
3178	nMG	**ND**	+	28.6 ± 2.7	53	9.0	68.5	22.0	14
3239	dSG	**+**	ND	26.8 ± 2.4	8	10.0	65.1	7.5	43
3250	nSG	**ND**	+	28.7 ± 2.7	53	12.0	58.2	4.0	17

*((Mean pre-infection PCV – post-infection PCV nadir) ÷ mean pre-infection PCV) x 100

†Days from exposure to *A. marginale*-infected *D. andersoni* or bovine blood until observation of a parasitemia ≥1%.

Treatments: dMG, denatured MG; nMG, native MG; dSG, denatured SG; nSG, native SG; dIT, denatured infected tick homogenate.

Abbreviations: ND, not detected; NA, not applicable; TF, transmission feeding.

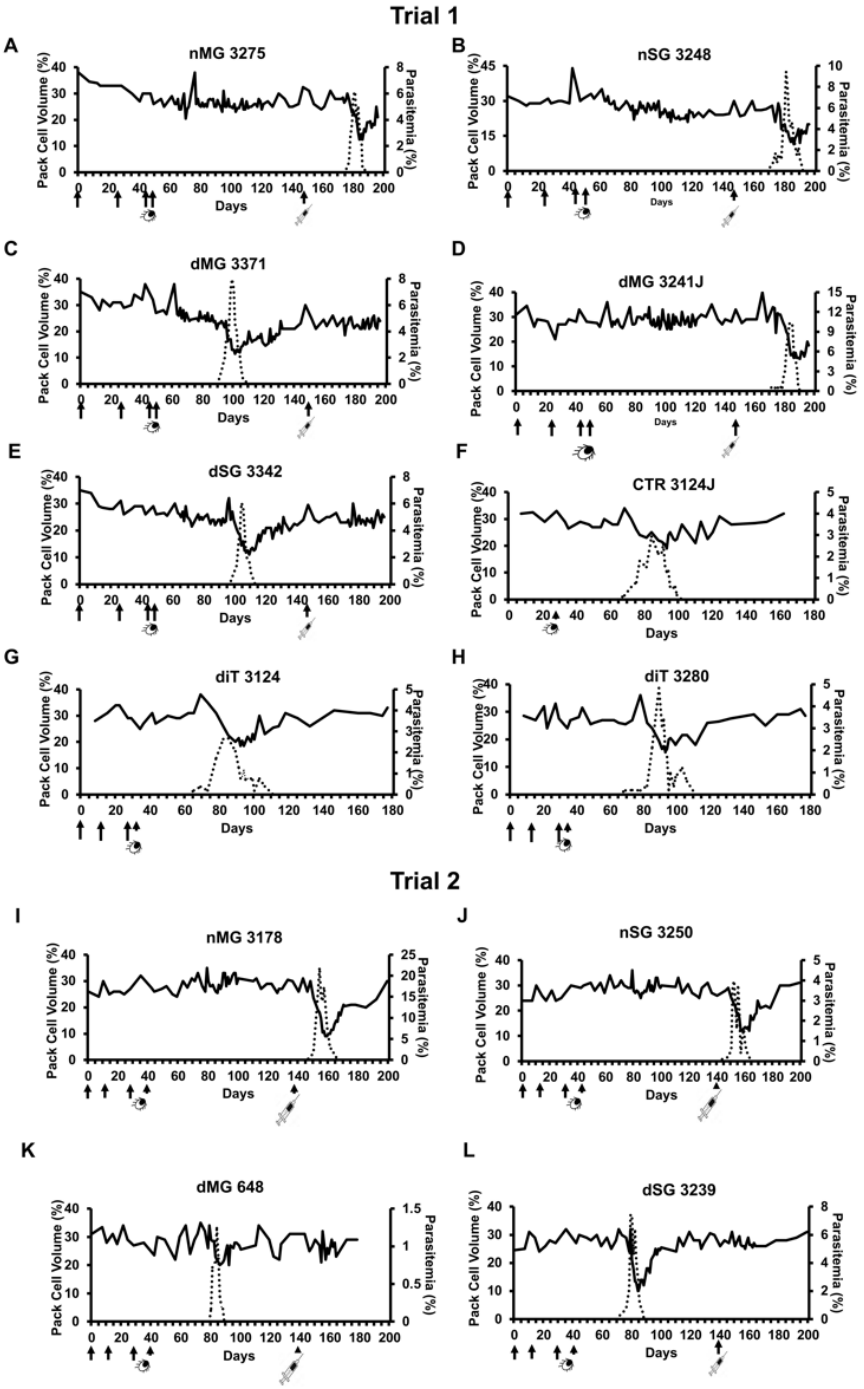

Figure 3.5. Challenge infections of calves immunized with tick homogenates.

Calves immunized with dMG, nMG, dSG, nSG or dIT were challenged by transmission feeding of *A. marginale*-infected *D. andersoni*. 100 days after tick transmission feeding, *A. marginale*-negative hosts and their cohorts were inoculated with blood from an *A. marginale* carrier to confirm susceptibility to the infection. The parasitemia (dotted line) and PCV (solid line) are illustrated.

Discussion

The results of this work indicated that immunization of cattle with native *D. andersoni* tissue homogenates elicited host immunity capable of interfering with biologic tick transmission of *A. marginale* to susceptible hosts. Both native and denatured homogenates of MG or SG resulted in seroconversion of all immunized hosts, as evidenced by several Log_{10}-fold increases in ELISA-based antibody titers. As expected, antisera from each immunized host were reactive to both MG and SG. Antibody titers to both MG and SG increased to similar titers after booster immunizations with every immunogen tested, but *A. marginale* was not transmitted by infected ticks fed on calves immunized with native tick-tissue homogenates.

Previous reports indicated that repeated tick infestations protected vertebrate hosts from tick transmission of *Francisella tularensis*, *Borrelia burgdorferi* and *Babesia* spp. (Bell *et al*., 1979; Francis and Little, 1964; Nazario *et al*., 1998; Wikel *et al*., 1997). Additionally, lower *B. bigemina* infection rates among *R. (B.) microplus* and reduced incidence of clinical babesiosis were observed among naturally infested dairy cows immunized with dSG (Jittapalapong 2004). In the current work, it was hypothesized that immunization with dSG may emulate the effect of repeated tick infestations through the gradual exposure of inaccessible epitopes as host immunoglobulins bind to and induce conformational changes to tick salivary antigens. Thus, it was predicted that immunization with dSG were more likely to evoke an adaptive humoral response that could interfere with tick transmission of *A. marginale* to a greater extent than native tick homogenate-specific antibodies. Although multiple immunizations with native homogenates also simulate repeated infestation, *A. marginale* was consistently

not transmitted by infected ticks fed on calves immunized with native tick-tissue homogenates in either trial, while the pathogen was transmitted by ticks fed on only one of seven hosts immunized with denatured tissues, including two calves immunized with denatured homogenates of infected ticks. Thus, refuting the hypothesis that immunization with denatured homogenates would be more efficacious in interfering with tick transmission of *A. marginale*.

Comparison of the denatured and native tissues with SDS-PAGE did not provide evidence to suggest that the denaturation step degraded immunogen to a degree to explain the differences observed among these immunization groups. This conclusion was corroborated by the observation of similar antibody titers among calves immunized with denatured versus native homogenates. Importantly, according to Fisher's exact test, immunization with native homogenate, but not antibody titer, was the only parameter associated with transmission interference of *A. marginale* ($P<0.02$).

Although somewhat speculative, based on the results of the current study, it now appears more likely that interference with pathogen transmission is likely to involve accessible conformational epitopes on tick molecules. Although immunization with concealed antigens can impact tick performance, taken together with past reports involving repeated tick infestations of hosts, it seems that antigenic determinants protective against transmission of *A. marginale* are perhaps more likely to be located on acarine molecules that are normally exposed to the vertebrate host during tick feeding.

In summary, to the best of our knowledge, this is the first report of interference with experimental biologic transmission of *A. marginale* by ixodid ticks through host immunization with tick tissue homogenates. This report supports the hypothesis that host immunization with tick tissues can induce immune responses capable of interference with transmission of *A. marginale* to cattle and refuted the hypothesis that denaturation of tick immunogens induces a more protective immune response with respect to blocking tick transmission of *A. marginale.* Previously, it was reported that ticks collected from SG-immunized cattle presented with damage type II and type III acini in association with reduced tick performance (Jittapalapong *et al.*, 2008)**.** Although transmission feeding of *D. andersoni* on immunized calves reduced tick performance, we did not observe an association between failure of *D. andersoni* to transmit *A. marginale* and reduced tick performance or raised anti-tick antibody titers, suggesting interference with biologic transmission of *A. marginale* was not due to detrimental effects on tick vectors and could be due to immune recognition immunoreactivity to specific tick molecules. This report also indicated that this model system can serve to develop novel approaches to interfere with tick transmission of *A. marginale* and other pathogens. Future efforts will involve identification of tick molecules reactive to protective immune sera and adaptation of this model to other experimental tick vectors of *A. marginale.*

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CHAPTER 4

Interference with biologic transmission of *Anaplasma marginale* to cattle immunized with *Dermacentor variabilis* salivary gland homogenates

Abstract

Anaplasma marginale is an obligate intraerythrocytic bacterium that causes severe economic loss in cattle worldwide. Ixodid ticks, including *Dermacentor* spp. in the US, are biologically transmit *A. marginale* from infected to susceptible cattle. Recently, we reported interference with biological transmission of *A. marginale* by *D. andersoni* fed on cattle immunized with native tick tissue homogenates. In the current study, *D. variabilis* midgut (MG) and salivary glands (SG) homogenates were used to immunize calves prior to challenge with adult *D. variabilis* infected with *A. marginale* as nymphs. Calves immunized with adjuvant alone served as controls. ELISAs were used to confirm seroconversion of the immunized calves. *D. variabilis* did not transmit *A. marginale* to calves immunized with SG, but *D. variabilis* did transmit *A. marginale* to calves immunized with MG homogenates, as well as with adjuvant alone, resulting in microscopically detectable infections and clinical anaplasmosis. The transmission-negative calves were confirmed to be susceptible to infection by inoculation with carrier blood. These results confirmed that host immunity to *D. variabilis* molecules can interfere with experimental

biologic transmission of *A. marginale,* and suggested that SG are a more abundant source of protective antigen.

Keywords: Anti-tick immunity; *Dermacentor variabilis;* tick salivary glands; *Anaplasma marginale;* anaplasmosis

Introduction

Bovine anaplasmosis has a broad and severe economic impact, with an estimated 3.6 % reduction in calving, and about 30% mortality in adults was recorded in a previously uninfected herd (Zabel and Agusto, 2018). In the US, *Dermacentor variabilis* is a competent vector of *A. marginale,* the etiological agent of bovine anaplasmosis (Kocan *et al*., 1981).

For decades, the host immune system has been stimulated to achieve resistance to tick vectors (Johnston and Mackerras, 1918; Trager, 1939; Wikel, Ramachandra, and Bergman, 1994; Wikel, 1996). Immunization of cattle against ticks can induce a protective host immune response which can damage ticks and reduce the tick population on the immunized host up to 70% (Johnston and Mackerras, 1918; Trager, 1939; Allen and Humphreys, 1979; Brossard and Girardin, 1979; Wikel, 1980; Allen, 1985; Johnston *et al*., 1986; Wikel *et al*., 1987; Shapiro *et al*., 1989; Wikel *et al*., 1994; Wikel, 1996; Jittapalapong *et al*,). Immunization of cattle with *D. andersoni* tissues extracts resulted in reduction of tick survival, engorgement weight and oviposition, as reported in the second chapter of this dissertation (Hoffman *et al.*, 2022).

More recently, due to the results reported in chapter 2 of this dissertation and previous reports of the different effects of immunization with salivary gland or

midgut extracts on tick feeding and pathogen transmission (Barriga *et al*., 1991; Barriga *et al*., 1993; Jittapalapong *et al*., 2000; Jittapalapong *et al*., 2004a & b; Jittapalapong *et al*., 2010), the work reported in chapter 3 of this dissertation reported that immunization of calves with native tick midgut (MG) or salivary gland (SG) homogenates can protect these hosts from experimental biologic transmission of *A. marginale* by *D. andersoni* ticks, while immunization with denatured extracts of tissues from the same ticks were not protective. However, the *D. andersoni* used to challenge immunized calves described in chapter 3 did not always feed as expected.

The purpose of this study was to test native tissue homogenates prepared from *D. variabilis,* a tick species found in the United States and a competent vector of *A. marginale* (Anthony and Roby, 1962). In addition, *D. variabilis* has been used extensively as an experimental vector of *A. marginale* (Anthony and Roby, 1962; Stich *et al*., 1989; Kocan *et al*., 1992; Ewing *et al*., 1995). Calves were immunized with *D. variabilis* SG, MG or with adjuvant alone, followed by site infestation with *D. variabilis* infected with *A. marginale as* nymphs. The results of this study indicated that immunization with *D. variabilis* SG alone interfered with biologic transmission of *A. marginale*.

Materials and Methods

Ticks

Dermacentor variabilis ticks were obtained from the Oklahoma State University Medical Entomology Laboratory. Ticks were stored at room temperature (RT; 20 to 25°C), over a saturated solution of potassium nitrate (relative humidity

>90%) and provided a photoperiod of 12h:12h (L:D). Fifteen pairs of adult male and female *D. variabilis* were fed on each calf before immunization and twenty pairs of adults ticks infected as nymphs were fed on each calf seven days after their second set of booster immunizations **(Figure 4.1).** Fresh containment cells were affixed to experimental cattle for each tick feeding. The ticks were checked every day and photographs were taken to keep record and monitor feeding of ticks, Tick feces, which was dried and accumulated in cells, were gently removed. The ticks were counted before and after their containment to ensure that none had escaped.

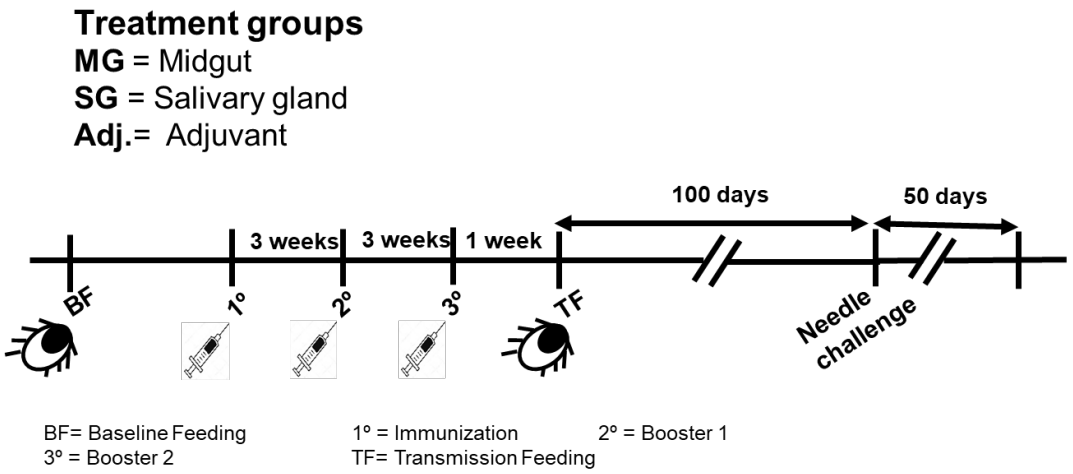

Figure 4.1. Immunization and challenge feeding of calves

D. variabilis were used for baseline feeding (BF) before the calves were immunized and boosted two times at three weeks intervals. One week after the final boost, these calves were challenged by transmission *D. variabilis* infected with *A. marginale* as nymphs. To confirm susceptibility to infection, each calf was challenged a second time by inoculation (IV) with blood from an *A. marginale* carrier.

Calves

Six Holstein or Holstein-Angus cross calves were obtained from the MU Foremost Dairy Research Center. All calves were of 5 months to 1 year of age, weighed between 300 - 500 lbs. and had no history of *Anaplasma* inclusions or evidence of anaplasmosis. The animals tested negative for anaplasmosis by *msp5* based PCR assay (Ueti *et al*., 2007). Calves were kept at the Middlebush Farm, University of Missouri containment facilities. The calves were housed under conditions of constant temperature (68°F) and light (12 hours of light and 12 of darkness per day) on heavy rubber mats in stalls equipped with headgates. The calves were observed, cleaned, fed, and given fresh water twice daily. The feed consisted of a pelleted ration (15% protein). Hay was provided *ad libitum*. Ticks were fed on a localized area on both sides of the vertebral column. Tick containment cells were made of a sleeve (10 cm in diameter) of a tubular elastic fabric (R4 Stockinette Cotton Blend; DeBusk, DeRoyal Industries, Powell, TN). Tick feeding cells were glued on the clipped and washed skin with Kamar adhesive glue (Heritage Animal Health, IA). The protocols for the experiments and animal care were approved by the Animal Care and Use Committee (ACUC # 8981) of the University of Missouri.

Antigen preparation

For immunogen, tick tissues were dissected from normal (uninfected) unfed female and male *D. variabilis* as well as cohorts collected while feeding on normal calves for 1-6 days (30 male and female *D. variabilis* pairs per day) (**Figure 4.2**).

All male and female ticks collected each day were bisected along their dorsal surface, SG and MG were removed, dissected free of other tissues, and placed in sterile 0.01M Phosphate Buffer Saline (PBS) (pH 7.4) with 3X protease inhibitor mini tablets (Thermo Fisher Scientific, Waltham, MA, Catalog No. A32953) on ice. Male and female SG or MG (Days 0-6) were ground in separate sterile Dounce homogenizers (Corning Inc., Corning, NY, Catalog No. 7727-2), and protein concentrations were measured using the Bradford assay (Quick Start^{Tm} Bradford 1X Dye Reagent, Hercules, CA, Catalog No. 5000205). After tick feeding Day 6, equal protein amounts of female and male SG or MG homogenates were pooled and diluted with sterile PBS to protein concentrations of 2.5 mg/ml. Proteins were mixed with Montanide ISA VG 61 (Seppic, Fairfield, NJ) following manufacturer instructions immediately before immunization of the hosts.

1. Tick Feeding

- Equal numbers of male and female *D. variabilis* ticks were fed on uninfected cattle

2. Tissue extraction

- Equal number of male and female ticks were removed from cattle from days 0-6
- Ticks were chopped and midgut (MG) and salivary gland (SG) tissues were extracted.
- MG and SG were kept separately in 0.1M PBS and 3X proteinase

3. Homogenization

- MG and SG were homogenized with Dunce homogenizer and store at -80℃.

4. Protein concentration

- Pool the SG tubes from 0-6 days into one tube.
- Pool the MG from 0-6 days into one tube.
- Protein concentration estimated by Bradford Assay for both SG and MG and stored at -80℃.

5. Immunogen preparation

- Just before immunization antigen and adjuvant were added to 5-cc syringes attached b ♀-♀ luer-lock adapter in ratio 2:3. Mix with 20 slow (~4 seconds/cycle) cycles and mix with 60 fast (~<1 second/cycle) cycles. Place (1 ml) of the emulsion into 1-cc syringe for each calf.

6. Immunization

- Calves were shaved on both sides of vertebral column, just before immunization, disinfect shaved area with 70% ethanol, Immunize calves intradermally in 10 sites (0.1 cc/ site) along their backs

Figure 4.2. Immunogen preparation and immunization

Immunization

Six 3-6-month-old Holstein or Angus-cross steers were used for this study. The calves were shaved on both sides of the vertebral column. The immunization site was disinfected with 70% ethanol. Calves were immunized with MG, SG or Adjuvant alone, intradermally in 10 sites (0.1 ml/ site) along their backs. In trial 1, calves B10, B4 and B3 were immunized with SG, MG and Adjuvant, respectively. In trial 2, calves 3356, 3255 and 8F were immunized with SG, MG and Adjuvant, respectively. Calves were immunized on days 0, 21 and 42. Immunization sites were documented with photographs before and after immunizations (**Figure 4.3**). Calves were monitored for 30 min after immunization, in case of labored breathing. Recipients of each immunogen are shown in **Table 4.1**.

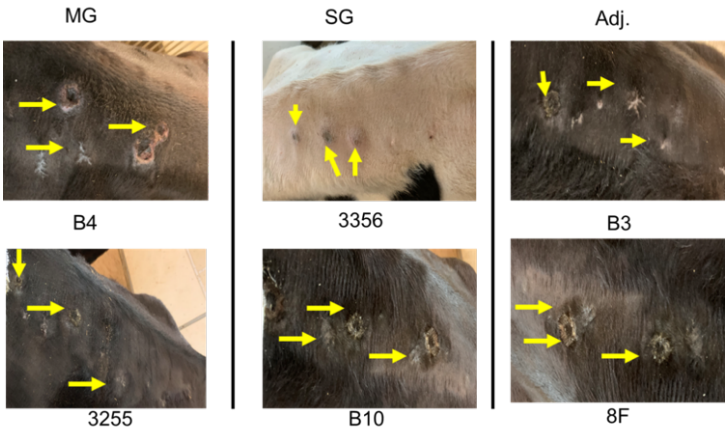

Figure 4.3. Immunization scars.

Calves were immunized intradermally with a 1 ml syringe on 10 different spots on both sides of the vertebral column. Yellow arrows are indicating the scars at immunization spots.

Table 4.1. Cattle used in this study

	Calf ID	Breed	Sex	Age at D0 (Months)	Treatment*
	3275F	Angus	Female	6	Carrier
Trial 1	B4	Angus	Male	9	MG
	3356	Holstein	Male	6.5	SG
	B3	Angus	Male	9	Adj.
Trial 2	3255	Angus	Male	6.5	MG
	B10	Angus	Male	9	SG
	8F	Angus	Female	7.5	Adj.

Tick performance

Male and female *D. variabilis* (15 pairs) were fed on each calf before immunization (**Figure 4.1**), followed by 20 *D. variabilis* pairs infected with *A. marginale* as nymphs. The ticks were checked daily so that detached female ticks could be recovered, weighed and maintained in humidity chambers as previous described elsewhere (Barriga *et al.*, 1991; Jittapalapong *et al.*, 2000; Jittapalapong *et al.*, 2004, Jittapalapong *et al.*, 2010, Shahzad 2022). Tick performance parameters included feeding period, tick survival, detached female tick weight and egg cluster weight. Egg mass weights were determined after oviposition was complete. The engorgement period was the time from application of the ticks to the host until the day of removal of individual detached female ticks.

ELISA

Sera from calves immunized with tick SG or tick MG were tested with indirect ELISAs with unfed *D. variabilis* MG or SG as antigen. 96-well high binding ELISA microplates (Greiner Bio-One, Monroe, NC) were coated for 2h at 37°C with 75 µl/well of protein antigen (1.0 µg/ml of protein) in 0.1 M carbonate coating buffer (pH 9.6), followed by washing five times with 0.5% Tween-20 PBS (PBS-T) and incubation with PBS-T at RT for 30 min. Antisera diluted in PBS-T (60 µl/well) were serially diluted from $1x10^{-2}$ to $1x10^{-8}$ and incubated for 1 h at 37°C. The plates were washed five times with PBS-T, before incubating 50 µl/well of rabbit anti-bovine IgG (H&L chain) conjugated to horse radish peroxidase (Invitrogen, Carlsbad, CA),

diluted 1:20,000 in PBS-T, for 1 h at 37°C. The plates were again washed five times with PBS-T and developed at RT for 4 min with 50 µl/well of 3,3,5,5-tetramethylbenzidine (TMB) solution (1mM TMB, 0.1 M Citric Acid, 0.03 M Sodium Citrate Dihydrate and 0.01% (v/v) H_2O_2). The reactions were stopped with 50 µl/well of 2M H_2SO_4 prior to measuring A_{450} values with a Molecular Devices SpectraMax-Plus. Each serum sample dilution was assayed in singlet on separate plates in three independent experiments, with corresponding pre-immune serum sample dilutions in triplicate to determine cutoff thresholds as described elsewhere (Frey *et al*., 1998).

A. marginale infection

An infected carrier was established by inoculation (IV) of calf 3275F with heparinized *A. marginale*-infected blood. Calf 3275F was monitored through the acute phase of clinical anaplasmosis. For experimental infection of *D. variabilis*, 200 nymphs were fed to repletion on calf 3275F during the subclinical phase of experimental anaplasmosis. Subsequently molted adult ticks were used for challenge infestations. Bovine blood samples were collected for monitoring packed cell volume (PCV) and percent infected erythrocytes (% parasitemia), as reported elsewhere (Stich *et al*., 1989). Prepatent periods were defined as the number of days from exposure to *A. marginale* to measurement of 1% parasitemia in peripheral blood. A commercial staining kit (Diff-Quick; Dade Behring, Newark, DE), was used to stain the blood smears. The staining procedure was including immersion of the slides 5 times (1s each time) in a methanol fixative, 5 times in an

eosin stain solution, 5 times in a solution of methylene blue, and 5 times in frequently changed water; the slides were then air-dried. The stained smear slides were examined by placing a coverslip on them. An oil immersion lens (100X magnification) compound microscope was used to read the smear. All animals were inoculated with 3 ml *A. marginale*-infected blood from carrier calf 3275F at 100 days after infected tick transmission, to confirm the susceptibility of calves to bovine anaplasmosis.

Statistical analysis

Tick performance data were collected for baseline feeding (BF) and transmission feeding (TF), using a random block design, before and after immunization, respectively. The student's t-test was used to compare BF and TF observations between trials. A two-way analysis of variance (ANOVA) with a Holm-Sidak *post-hoc* analysis was used to compare ELISA titers to MG and SG antigens. Associations were measured with the Fisher exact test. All analyses were performed using SigmaPlot (Systat Software, San Jose, CA). A P-valve ≤ 0.05 was considered statistically significant.

Results

Tick Performance

It was previously reported that host immunization with different tick tissues have different effects on tick performance parameters (Jittapalapong *et al*., 2002, Jittapalapong *et al*., 2004). More recently, in a study conducted involving *D. andersoni,* it was found that immunization of cattle with different tick tissue

extracts also reduced tick performance (Chapter 2), and a subsequent study did not find a direct link between female tick performance and transmission of *A. marginale* (Chapter 3). Performance of male and female *D. variabilis* was measured in the current study, to evaluate the similarity of the experimental hosts with respect to tick feeding during the baseline-feeding phase and to confirm changes in tick performance during the transmission-feeding phase of the study.

Differences in tick performance parameters associated with feeding on these six hosts were not observed during the baseline-feeding phases of these trials **(Table 4.2)**. Tick performance parameters were reduced in association with the transmission-feeding phase of these trials. Interestingly, reductions in performance parameters were also observed for ticks fed on calves that were immunized with adjuvant alone; however, in both trials, ticks fed on these controls had the highest detached female and egg mass weights.

Table 4.2. Performance of adult *D. variabilis* fed on immunized calves.

Calf	L & N fed	Trt	Feeding period (days)		Female weight (mg)		Survival (%)				Egg cluster (mg)		Ovi-posited (%)		TF Result
							BF		TF						
			BF	TF	BF	TF	F	M	F	M	BF	TF	BF	TF	
Trial 1															
B10	No	SG	13±5	14±2	820±229	118±87	87	40	70	50	404±151	51±62	85	57	ND
B4	No	MG	10±1	13±1	937±94	100±92	100	80	20	40	494±67	135*	100	25	+
B3	No	Adj.	10±2	12±3	918±91	282±168	100	93	90	55	497±90	155±113	100	100	+
Trial 2															
3356	No	SG	10±1	15±2	965±127	120±112	93	93	85	50	477±87	86±57	100	53	ND
3255	No	MG	10±0	15±2	847±250	126±111	100	87	60	50	567±208	39±51	80	50	+
8F	No	Adj.	11±3	13±2	914±90	201±131	100	93	80	60	467±100	109±51	93	81	+

Abbreviations: BF, baseline feeding; L, larvae; N, nymphae; ND, not detected; TF, transmission feeding; Trt, treatment group.

Treatment groups: MG, midgut homogenate; SG, salivary gland homogenate; Adj., Adjuvant.

*Egg mass from a single female.

ELISA

To confirm seroconversion of immunized calves, ELISA-based antibody titers to MG and SG were measured for weekly samples collected from each host. Antibody titers measured for trials 1 and 2 are illustrated in **(Figure 4.4)**. For the immunized calves, antibody titers were detected up to 1 x 10^5 or higher against unfed tick MG and SG antigens. Among calves B4 and 3255, immunized with MG, antibody titers reached 1 x 10^5 to MG and 1 x $10^{4.33 \pm 0.33}$ and 1 x $10^{5.66 \pm 0.33}$ to SG by Day 25 ± 5. Among calves B10 and 3356, immunized with SG, antibody titers reached 1 x 10^5 and 1 x $10^{6.66 \pm 0.33}$ to MG and 1 x $10^{5.66 \pm 0.33}$ and 1 x $10^{4.33 \pm 0.33}$ to SG by Days 25 ± 5 and 27 ± 5, respectively. Increasing titers and cross-reactivity to both tick tissue types were observed among calves immunized with MG or SG (**Figure 4.4**), and these titers remained at peak levels until TF. Lower levels of reactivity were measured for sera collected from the adjuvant control calves, reaching a peak of 1 x $10^{1.66 \pm 0.44}$ to MG and 1 x 10^2 to SG in B3 and 1 x $10^{0.66}$ to SG in 8F.

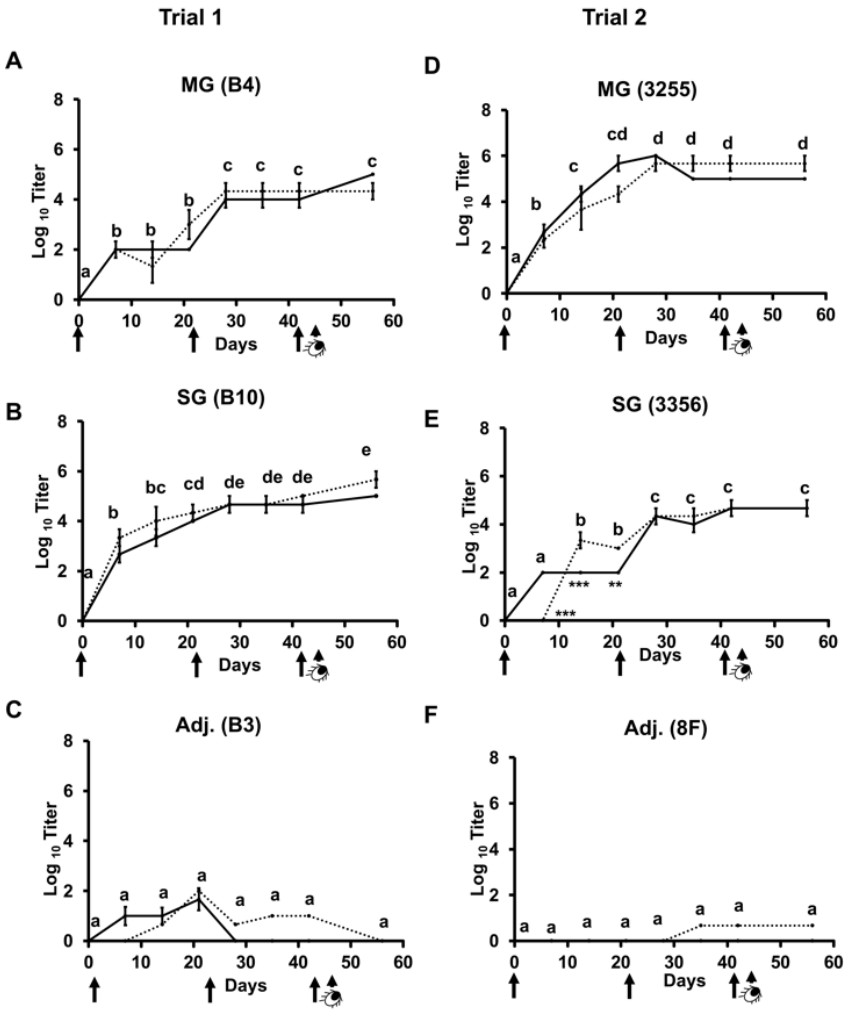

Figure 4.4. ELISA titers of calves immunized with tick tissues.

Calves were immunized with tick MG or SG. Antibody titers were monitored from immunization to transmission feeding challenge. Log_{10} titers are shown on the y-axis and duration in days is shown on the x-axis. Panels A, B and C represent the calves B4, B10 and B3 immunized with MG, SG, and adjuvant respectively (Trial 1). Similarly, 3255, 3356, and 8F immunized with MG, SG, and adjuvant respectively (Trial 2). Solid black lines represent titers represent MG antigen and dotted lines represent titers against SG. Immunizations are indicated by arrows and *A. marginale*-infected tick challenge infestations are indicated by an image of a tick. Results are shown as Least Square (LS) Means ± Standard Error; a two-way ANOVA was performed, and different superscripts in each panel indicate significantly different LS Means ($P < 0.05$) of different days, while asterisks show the difference in MG and SG at $P < 0.05$: *, < 0.01: ** and < 0.001: ***.

Transmission of *A. marginale*

Immunization with native tick MG or SG was associated with the inability of *D. andersoni* to biologically transmit *A. marginale* to susceptible cattle (Chapter 3). The *D. andersoni* used in aforementioned study did not perform as expected, and this colony was no longer available, so the purpose of this experiment was to adapt the closely related tick, *D. variabilis,* to the same immunization and transmission model system.

Measures of *A. marginale* infection levels and nonhemolytic anemia (PCVs) are illustrated in **Figure 4.5.** In both trials, 20 pairs of male and female *D. variabilis* infected with *A. marginale* as nymphs failed to transmit the pathogen to calves immunized with SG (calves B10 and 3356), while cohorts of these ticks transmitted the pathogen to calves immunized with MG (calves B4 and 3255) or adjuvant alone (calves B3 and 3356).

After the tick-transmission prepatent period had elapsed, all calves were challenged a second time by inoculation (IV) with *A. marginale*-infected blood to confirm their levels of susceptibility to infection with this pathogen. As expected, the SG-immune tick-transmission-negative calves, B10 and 3356, developed patent infections and clinical anaplasmosis, while no signs of infection or disease were observed among the previously tick-transmission-positive calves that had since become asymptomatic *A. marginale* carriers. Key parameters of infection and disease observed among all calves are summarized in **Table 4.3**.

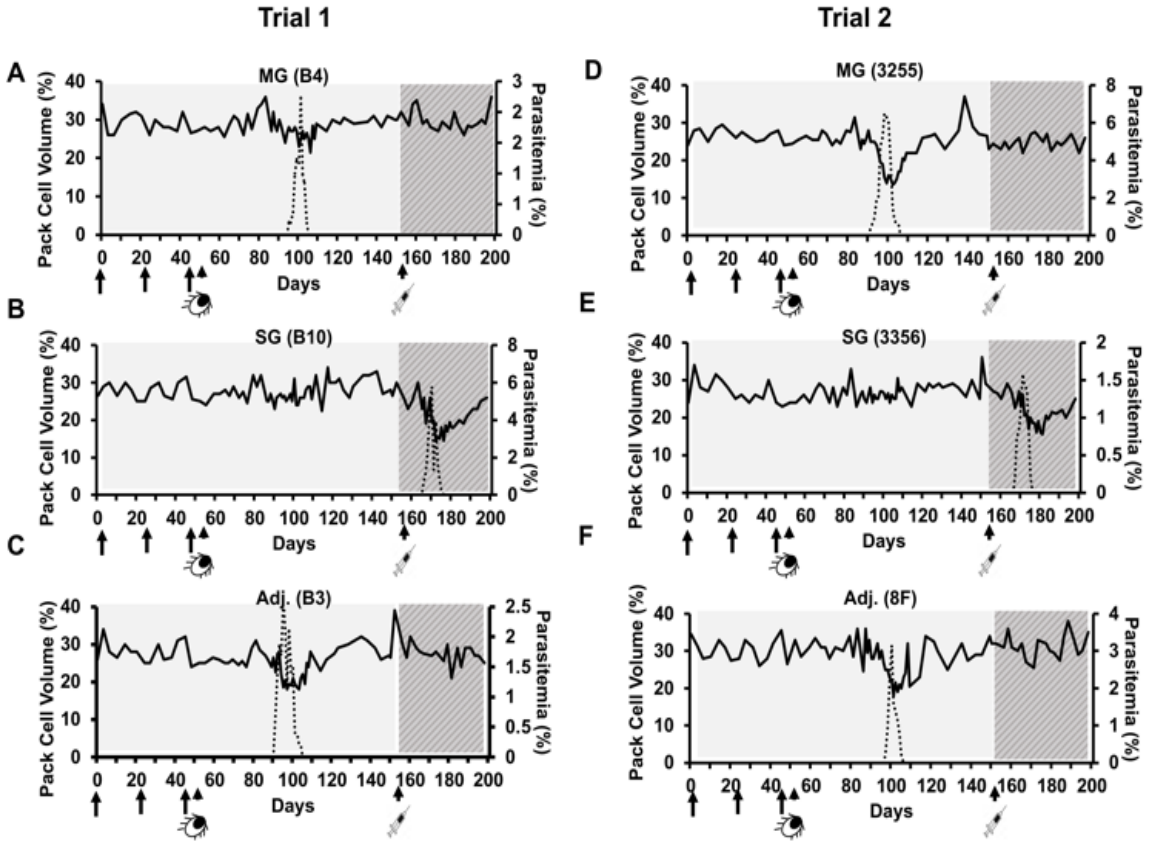

Figure 4.5. PCV and parasitemia of calves infected with *A. marginale*.

For each panel, solid and dotted lines illustrate PCV and parasitemia, respectively. Arrows indicate three immunizations administered to each host on days 0, 21 and 42. All calves were challenged with *A. marginale*-infected *D. variabilis* on day 49, which are indicated with an image resembling a female tick. All calves were inoculated with *A. marginale*-infected blood 100 days after initiation of TF challenge. Lightly shaded regions of each panel indicate immunization and TF-challenge phases for each host, and darkly shaded regions indicate IV-inoculation phases at the end of each experiment.

Table 4.3. Infection and disease parameters observed among cattle infected with *Anaplasma marginale*.

Calf	Treatment	*A. marginale* transmission		PCV (%)				Peak Parasitemia (%)	Prepatent period (days)
		Tick TF	Inoculation (IV)	Pre-infection $\bar{x} \pm SD$	Pre-infection N=	Nadir	Reduction		
B10	SG	**ND**	+	27.9±3	85	14.5	48.1	5.8	17
B4	MG	**+**	ND	29.6±3	36	21.5	27.1	2.2	45
B3	Adj.	**+**	ND	28.7±3	36	18.0	37.2	2.1	38
3356	SG	**ND**	+	28.5±3	83	15.5	45.6	1.6	20
3255	MG	**+**	ND	26.6±6	36	13.5	52.8	6.5	40
8F	Adj.	**+**	ND	30.0±2.8	35	18.0	40.0	3.2	44
3275F	Carrier	**NA**	+	41.1±3.7	83	18.0	56.2	2.5	48

*((Mean pre-infection PCV – post-infection PCV nadir) ÷ mean pre-infection PCV) x 100.
**Days from exposure to A. marginale-infected D. andersoni or bovine blood until observation of a parasitemia ≥1%.
Treatments: MG, midgut; SG, salivary glands; Adj., adjuvant.
Abbreviations: IV, intravenous; ND, not detected; NA, not applicable; TF, transmission feeding.

Discussion

The results of this study demonstrated humoral immune responses after immunization of cattle with homogenates of *D. variabilis* MG and SG. This study also compared protection elicited by these homogeneous to an adjuvant-only control. Overall transmission-feeding tick performance was reduced on immunized calves in comparison to baseline-feeding performance on pre-immune calves. Although the adjuvant-only control group also presented reduced performance of transmission-fed ticks, the overall performance of this control group was higher than the same parameters among calves immunized with MG or SG. Importantly, *D. variabilis* adults did not transmit *A. marginale* to the SG-immune calves, while cohorts from the same pools of ticks infected as nymphs transmitted the pathogen to the adjuvant-control and MG-immune calves. Both tick-transmission-negative calves were demonstrated to be susceptible to *A. marginale* after challenge through inoculation (IV) with *A. marginale*-infected carrier blood, while all tick-transmission-positive calves were demonstrated to be premune to homologous challenge infection with the same carrier blood. The findings of this work indicated that host immunization with *D. variabilis* SG homogenates can interfere with biologic transmission of *A. marginale* to susceptible cattle.

Other groups have reported interference with transmission of different vector-borne pathogens by immunization with molecules derived from tick salivary glands. Vaccination of mice with salivary protein 64TRP significantly diminished tick-borne encephalitis virus transmission by *Rhipicephalus appendiculatus* (Labuda *et al.*, 2006), Similarly, immunization of mice with Salp15 showed

significant protection against transmission of *B. burgdorferi* by *Ixodes scapularis* (Dai *et al*., 2009).

To the best of our knowledge, this is the first report of *D. variabilis* performance with this model system to date. The reason for reduction in performance of ticks transmission fed on adjuvant controls could be due to several factors, including (1) baseline-fed and transmission-fed *D. variabilis,* respectively, were reared with the original supplier colony and fed on an *A. marginale*-infected calf, (2) baseline and transmission feeding phases of this experiment were conducted several months apart, during different seasons and (3) the adjuvant alone could be responsible for reduced tick performance. Similar effects of adjuvant controls have been observed with *R. (B.) microplus* ticks fed on cattle or rabbits (Jittapalapong *et al.,* 2004a; Jittapalapong *et al.,* 2010).

In a recent study, immunization with similarly prepared native tick SG or MG homogenates both interfered with transmission of *A. marginale* by *D. andersoni* (Chapter 3). However, in the current study, *D. variabilis* transmitted *A. marginale* to native MG-immune calves while cohorts from the same tick pools failed to transmit this pathogen to native SG-immune calves. In the current study, the numbers of infected ticks transmission fed for challenge infestations were doubled in comparison to the work described in Chapter 3, to 20 female and male tick pairs per calf. This difference in transmission results could be due to (1) difference in midgut compositions between the two *Dermacentor* species, (2) other differences such as salivary components between these tick species, (3) better feeding performance of the *D. variabilis* specimens, (4) the aforementioned greater

numbers of *D. variabilis* used for transmission feeding or (5) perhaps in relation to the latter three points, higher pathogen levels delivered in saliva from the *D. variabilis* described in the current report. Notably, the four latter hypotheses suggest the possibility of breakthrough transmission of infections, which could be instructive for future investigations during the development phase of any defined subunit vaccine designed to interfere with pathogen transmission by targeting a tick vector.

In summary, in two trials, *D. variabilis* infected with *A. marginale* as nymphs did not transmit the pathogen to SG-immune susceptible cattle. Conversely, cohort ticks transmitted this pathogen to MG-immune and adjuvant control cattle. These results further validated this bovine-tick-pathogen model system, and revealed future directions with respect to model refinement, anti-tick vaccine candidate discovery and factors to be considered for vaccine candidate development.

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CHAPTER 5

Preliminary observations of *Anaplasma marginale* infection levels in previously fed male *Dermacentor andersoni* ticks

Abstract

Globally, ticks transmit a diverse range of disease-causing agents to vertebrate hosts. *Anaplasma marginale*, the primary etiologic agent of bovine anaplasmosis, is a tick-transmitted, gram-negative bacterial parasite classified in the rickettsial family *Anaplasmataceae*. Ixodid tribe Rhipicephalinae ticks biologically transmit *A. marginale* to cattle. Thus, disruption of the acquisition of *A. marginale* by the tick vector represents an effective strategy to prevent the spread of this pathogen from silent carriers or infected host to susceptible hosts. This study reports work on a well-established bovine-tick model for *A. marginale*, which can serve as a model for other tick-borne infections. Two groups of *Dermacentor andersoni* male ticks (previously mated and unmated or flat) were fed on an *A. marginale*-infected animal for the acquisition of the infection. Quantitative PCR determined a lower level of *A. marginale* infection in previously mated male *D. andersoni* as compared to flat male ticks that simultaneously fed on the same bovine host. The results were unexpected because the current paradigm implements that once the infection is established within tick vectors, *A. marginale* is believed to grow to the same level among all infected ticks.

Keywords: *Dermacentor andersoni;* infection; cattle; *Anaplasma marginale;* anaplasmosis

Introduction

Ticks impact human and animal health through infestation and transmission of a wide range of pathogens, including viral, bacterial, and protozoal disease-causing agents (Jongejan and Uilenberg, 2004). Ixodid ticks, transmit several etiologic agents of medically important diseases, including arboviral diseases, anaplasmosis, babesiosis, ehrlichiosis, Lyme disease, and Rocky Mountain spotted fever (de la Fuente *et al*., 2008; Ahantarig *et al*., 2013). Tick control is essential to control tick borne diseases, but it is a difficult task. Common approaches to tick control such as habitat modification, host exclusions, and depopulation may reduce the tick population for only a short period (Garris and Barré, 1991; Jongejan and Uilenberg, 1994). An alternative approach for control involves the use of chemicals such as organophosphates or pyrethroids. The use of chemicals causes environmental contamination and consequent toxicity in both animals and humans (Nolan, 1990).

A. marginale is an etiological agent of bovine anaplasmosis which leads to enormous economic loss to the cattle industry worldwide (Kocan *et al*., 2010). Bovine anaplasmosis is transmitted by ticks belonging to the family *Rhipicephalinae* and biting flies. In the United States *D. andersoni* and *D. variabilis* ticks are the major competent vectors of bovine anaplasmosis (Kocan *et al*., 2004). These vectors acquire the pathogen while feeding on an infected host. The

infection level within ticks remains the same as *A. marginale* divides within ticks after acquisition (Palmer *et al*., 2000).

In the present study, we observed that two groups of ticks (previously mated, and flat) acquire different levels of *A. marginale* infection after feeding simultaneously on the same bovine host.

Materials and Methods

Ticks

Dermacentor andersoni ticks were used for all the experiments in this study. Adult ticks were obtained from the Oklahoma State University Medical Entomology Laboratory. Ticks were stored at 25°C, >90% relative humidity, and provided a photoperiod of 12h:12h (L:D).

Calves

Two Holstein-Angus cross calves numbered 3250 and 635 were obtained from the Foremost Dairy Research Center, University of Missouri. Both calves were of 3 months to 1 year of age with no history of *A. marginale* infection. The animals tested negative for anaplasmosis by *msp5*-based PCR assay (Ueti *et al*., 2007). Calves were kept at the Middlebush Farm, University of Missouri containment facilities. The calves were housed under conditions of constant temperature (68°F) and provided a photoperiod of 12h:12h (L:D). Dedicated boots and coveralls remained in the dedicated room. The calves were observed, cleaned, fed, and given fresh water twice daily. The feed consisted of a pelleted ration (12% protein). Hay was provided *ad libitum*. The protocols for the

experiments and animal care were approved by the University of Missouri Animal Care and Use Committee (ACUC #8981).

Tick Feeding

Tick containment cells were made of a sleeve (10 cm in diameter) of a stockinette (Medline Industries, Northfield, IL). Tick feeding cells were adhered to the calf with Kamar adhesive (Kamar Products Inc., Zionsville, IN) (**Figure 5.1 A**) and ticks were fed on a localized area on both sides of the calf's vertebral column (**Figure 5.1 B**).

DNA isolation

The Roche High Pure Viral Kit (Roche, Basel, Switzerland) was used to isolate the tick DNA, as described by McClure *et al.*, 2009.

Tick acquisition of *A. marginale*

Primers as previously described by Ueti *et al*. 2007 were used for the detection of *A. marginale* targeting *msp5.* Briefly, primers chosen included a forward primer named StM194752-194775_S 5' (CTTATCGGCATGGTCGCCTAGTTT) and a reverse primer named StM194954-194931_A, 5' (CTTCCGAAGTTGTAAGTGAGGGCA). For conventional PCR, HotStart PCR Master Mix (Lamda Biotech, Ballwin, MO) was used, and the manufacturer's instructions were followed. Briefly, each reaction of 25 µL contained 1X HotStart PCR Master Mix, 0.9 µM forward and 0.9 µM reverse

primers, and HPLC H_2O. Thermocycler conditions for the reaction were 95°C for 3 min, followed by 45 cycles at 94°C for 30 sec, 65°C for 30 sec, and 72°C for 30 sec with a final extension time at 72°C for 5 min. Amplified products were detected on a 1.5% agarose gel containing ethidium bromide.

For quantitative PCR (qPCR), a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) was used, and Powerup SYBR Green (Thermo Fisher Scientific, Waltham, MA) master mix was used for reactions. Cycling conditions were UNG activation which allows previous PCR amplifications or mis-primed, nonspecific products to degrade, leaving native nucleic acid templates intended for amplification intact) at 50°C for 2 min, polymerase activation at 95°C for 2 min, 50 cycles of melting at 95°C for 15 seconds, annealing at 55°C for 30 sec, and extension at 72°C for 1 min, followed by a dissociation stage. For amplicon confirmations samples were run on a 1.5% agarose gel containing ethidium bromide.

A. marginale infection

Calf number 635 was intravenously inoculated with 2 ml of *A. marginale* infected blood. During ascending parasitemia, *D. andersoni* were fed on calf number 635 to obtain transstadially infected ticks. Blood samples were collected from the jugular vein into containers treated with ethylene diamine tetraacetic acid (EDTA). Dedicated sampling materials and gloves were used to prevent mechanical transmission between animals. Uncoagulated whole blood was used to determine packed cell volume (PCV) by micro-hematocrit centrifugation and

parasitemia (percent parasitized erythrocytes), was monitored by examination of stained blood smears. A commercial staining kit (Diff-Quick; Dade Behring, Newark, DE), was used to stain the blood smears. An oil immersion lens (100X magnification) of a compound microscope was used to determine the parasitemia. Estimates of parasitemia were based on counts of the erythrocytes in 5 different fields of view with more than 500 cells counted.

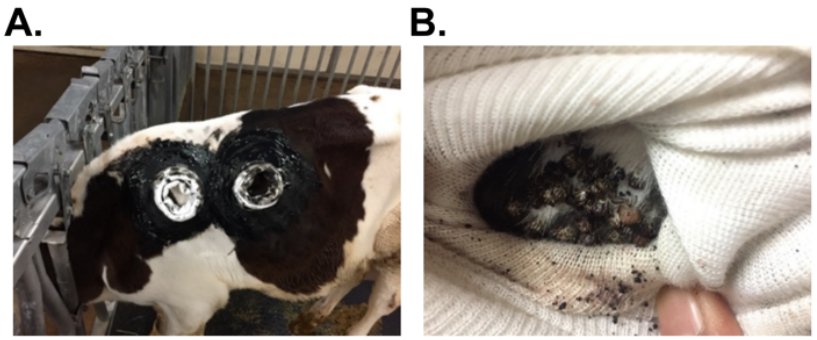

Figure 5.1. Tick feeding in stockinette

Results

Preliminary observation of *A. marginale* infection within ticks

Two groups of ticks (previously mated, and flat) were fed on the *A. marginale* infected calf 635. After 14 days, ticks were removed from the animal and stored at 25°C, >90% relative humidity, and provided a photoperiod of 12h:12h (L:D) in the chamber for 10 days to ensure the digestion of blood meal. Ticks were chopped in half and one half was used to test for PCR and PCR showed the qualitative difference between the two groups of ticks (**Figure 5.2**).

Quantitative PCR (qPCR)

D. andersoni male ticks (previously mated, and flat) were kept in a humidity chamber for 10 days. For quantification of *A. marginale* within ticks, DNA from the whole tick was isolated and qPCR was run to calculate the copy numbers. For every qPCR trial, a dissociation curve was generated which showed the target and plasmid peaks (**Figure 5.3**). It was noticed that there was a significant difference in the copy numbers of *A. marginale* between flat and mated *D. andersoni* male ticks (**Figure 5.4 C**).

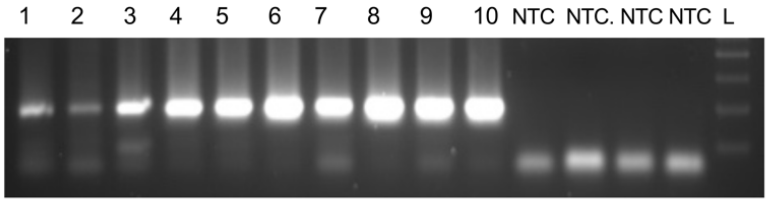

Figure 5.2. PCR of tick halves to detect *A. marginale.*

The *msp5*-based PCR assay was used to detect *A. marginale* within tick halves. 1-5 are previously mated male tick halves and 6-10 are flat male tick halves, NTC is non-template control and L is 100 bp Ladder (GoldBio, Olivette, MO). Expected size of amplicon was 200 bp.

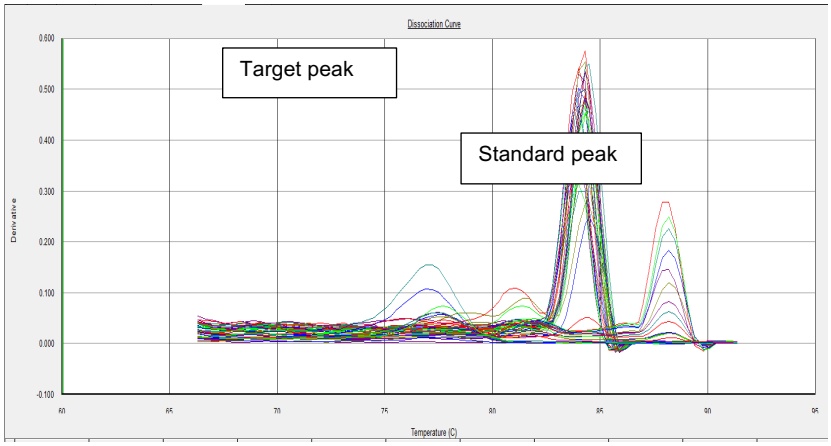

Figure 5.3. Dissociation curve for qPCR assays.
Amplicons from the *msp5*-based PCR assay reveal a single peak following melt curve analysis, while plasmid used as standard, and amplicon peak were separated from target amplicon after melting curve analysis.

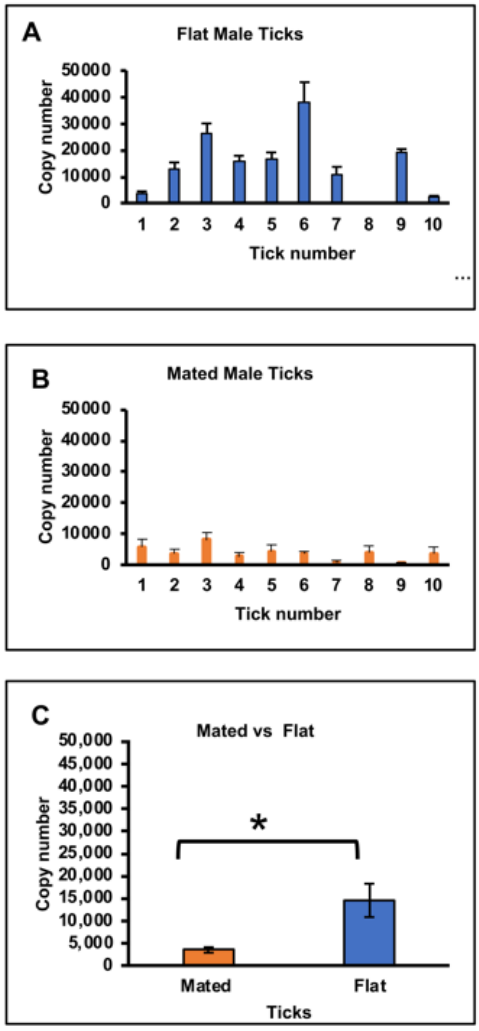

Figure 5.4. Quantitative PCR of *A. marginale* in male *D. andersoni.*

Copy number of *msp5*-based real-time PCR with SYBR green chemistry. PCR-positive samples were confirmed by amplicon T_m. **Panel A:** 9 out of 10 ticks were PCR positive. Mean + standard deviation of copy number for two separate assays with the independent standard curve are shown for PCR-positive flat *D. andersoni* ticks**. Panel B:** 10 out of 10 ticks were PCR positive**.** Mean + SD of copy number of previously mated male *D. andersoni* ticks simultaneously acquisition fed on the same calf are shown. **Panel C:** flat and previously mated male ticks were acquisition fed on the same calf. After removal ticks were held in a humidity chamber for ten days. The template was extracted, and qPCR was run on 10 ticks/group in two separate trials with an independent standard curve for calculation of copy number. The least-square means ± standard error indicates that the groups differ $P< 0.015$.

Discussion

The most accepted paradigm among researchers is that, although acquisition rates vary, *A. marginale* replicates to the same level among infected ticks, regardless of the rickettsemic load among the bovine source of tick infection. Thus, tick infection levels (*not infection rates*) are considered independent of the high or low points in persistent cyclic rickettsemia of cattle (Palmer *et al*., 2000). In contrast to this paradigm, in this study, different infection levels between two different groups of male *D. andersoni* ticks simultaneously fed on the same calf were observed. Quantitative PCR (qPCR) (Ueti *et al*., 2007) of individual male ticks from these groups (n=10 ticks per group) was used to demonstrate a statistically significant difference in average copy number between mated and flat *D. andersoni* male ticks. Briefly, an *msp5*-based qPCR was used to measure a lower mean infection level (copy number), among older male *D. andersoni* that had previously mated, compared to the mean copy number of normal, flat male *D. andersoni*. This result was unexpected in the context of the above paradigm. Previously it was documented that after the acquisition of *A. marginale,* infection level within ticks remains the same regardless of the rickettsemia of the host (Eriks *et al*., 1993; Palmer *et al*., 2000). In both previous studies, a nucleic acid probe was developed and used to detect the low level of *A. marginale* infection. The probe was specific for *A. marginale* and was able to detect 0.01 ng of genomic DNA and 500 to 1,000 infected erythrocytes in 0.5 ml of blood, which is equivalent to a parasitemia of 1 x 10^{-5} %. That made the probe method at least 4,000 times more sensitive than light microscopy. In this study quantitative PCR was used to calculate the copy number

of *A. marginale* which is more sensitive than the nucleic acid probe (Palmer *et al*., 2000).

Moreover, in this study, only male *D. andersoni* were used. Both male and female *D. andersoni* ticks can transmit infection, however, the intermittent feeding of male *D. andersoni* makes them particularly important for the transmission of the infection to other susceptible hosts (Eriks *et al*., 1993). Since male ticks and bovine hosts become persistently infected with *A. marginale*, they serve as a reservoir of infection (Kocan *et al*., 2010). Ticks can transmit *A. marginale* transstadialy (*i.e.,* pathogen passage between different developmental stages of the same tick) or intrastadially (*i.e*., within the same life stage, by males) (Stich *et al*., 2008). Thus, male *D. andersoni* ticks can continuously transmit *A. marginale* among hosts without the additional acquisition of infection or molting periods.

In summary, more experiments are needed to answer the question of why mated *D. andersoni* male ticks acquire less infection as compared to flat *D. andersoni* male ticks.

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CHAPTER 6

Preliminary observations on the effect of tetracycline on transmission of *Anaplasma marginale* by *Dermacentor andersoni*

Abstract

Anaplasmosis, caused by the tick-borne pathogen, *Anaplasma marginale,* is an economically important disease of cattle in the US as well as globally. Cattle become carriers of *A. marginale* after acute infection when low or microscopically undetectable infection persists. Currently, control options are limited, and FDA-approved antimicrobial control options do not have a defined duration of use. Feeding free-choice chlortetracycline (CTC)-medicated minerals to pastured cattle for several months is a practical and routinely used anaplasmosis control method. In the USA, tetracycline antimicrobials are used for the treatment of acute anaplasmosis and are in use for the elimination of persistent infections. In this study, calf 3149 was inoculated with *A. marginale* stabilate. During ascending parasitemia *Dermacentor andersoni* ticks were fed on the calf. During tick feeding, the calf was treated with a single dose of oxytetracycline administered at 22 mg/kg, intramuscular (IM). Ticks were PCR-positive for *A. marginale* after the acquisition but unable to transmit the infection to naïve bovine hosts. These observations suggested that tetracycline may affect the transmissibility of *A. marginale* to naïve bovine hosts by *D. andersoni* ticks.

Keywords: tetracycline, *Dermacentor andersoni;* infection; cattle; *Anaplasma marginale;* anaplasmosis

Introduction

Anaplasma marginale is a rickettsial heamoparasite of cattle and other ruminants (Kocan *et al.*, 1978; Fuente, Garcia *et al*., 2001). It is transmitted by ticks and biting flies, as well as by contaminated fomites, such as needles (Jones and Brock, 1966; Ristic and Carson, 1977; Aubry and Geale, 2011). Economically, anaplasmosis is a very important disease of cattle worldwide because it has a severe impact on meat and milk production in tropical and subtropical regions (McCallon, 1973; Ristic and Carson, 1977). In the US alone, the estimated annual loss is $100 million and includes 50,000 to 100,000 cattle deaths (Eriks *et al*., 1993). Moreover, due to the socio-economic importance and significance of anaplasmosis, it is currently classified in List B of the Office International des Epizooties (OIE) Terrestrial Animal Health (OIE 2021).

Cattle remain persistently infected with *A. marginale* even after recovery from the acute infection (Kocan and Fuente, 2003). Oxytetracycline is the drug of choice for the treatment of the acute phase of anaplasmosis in the US (Coetzee *et al*., 2005). Sequential rickettsemic cycles ranging from 10^2 to 10^6 - 10^7 are characteristic of persistent infection and occur at approximately 5-week intervals (Kieser *et al*., 1990; French *et al*., 1998; French *et al*., 1999; Barbet *et al*., 2001). During these cycles, infected erythrocytes are not always detectable in stained blood smears, but ticks are able to acquire *A. marginale* infection from carrier cattle

(Eriks *et al*., 1989). Thus, carrier animals serve as reservoirs of infection for mechanical transmission and infection of ticks which are biological vectors (Reeves and Swift, 1977; Eriks, Palmer *et al*., 1989; Ueti *et al*., 2003).

Tetracycline inhibits microbial replication by binding to the 30S subunit of the ribosome resulting in the inhibition of protein synthesis (Blouin *et al*., 2002). The Food and Drug Administration (FDA) approved only two compounds i.e., Chlortetracycline and Oxytetracycline for the control of bovine anaplasmosis in the pastured cattle in the US (Coetzee *et al*., 2005). However, the recommended therapeutic doses of these compounds do not always clear the *A. marginale* infection from cattle (Kuttler and Simpson, 1978; Stewart *et al*., 1979).

In this study, it was observed that tetracycline treatment of an *A. marginale* infected calf with tetracycline during acquisition feeding of *D. andersoni* ticks interferes with the transmissibility of *A. marginale* to uninfected calves by those ticks.

Materials and Methods

The protocols for the experiments and animal care were approved by the University of Missouri Animal Care and Use Committee (ACUC #8981).

Experimental cattle

A Holstein calf 3149 of 3 mo. was used. The calf was obtained from the Foremost Dairy Research Center, University of Missouri and had no history of anaplasmosis. This study was conducted in 2017 and the calf was housed at the Middlebush Farm, University of Missouri containment facilities under constant

temperature (68°F) providing a photoperiod of 12h:12h (L:D). Dedicated boots and coveralls remained in the dedicated room. The calf was observed, cleaned, fed, and given fresh water twice daily. The feed consisted of a pelleted ration (12% protein). Hay was provided *ad libitum*.

Ticks and ticks feeding

Dermacentor andersoni ticks were used in this study. Adult ticks were obtained from the Oklahoma State University Medical Entomology Laboratory. Ticks were stored at 25°C, >90% relative humidity, and provided a photoperiod of 12h:12h (L:D). Ticks were fed on a localized area on both sides of the calf vertebral column. Tick containment cells were made of a sleeve (10 cm in diameter) of a stockinette (Medline Industries, Northfield, IL). Tick feeding cells were adhered to the calf with Kamar adhesive (Kamar Products Inc., Zionsville, IN).

***A. marginale* infection**

Calf 3149 was inoculated with 2 ml of *A. marginale* (Virginia strain) stabilate. Blood samples were collected from the jugular vein into containers treated with ethylene diamine tetraacetic acid (EDTA). Dedicated sampling materials and gloves were used to prevent mechanical transmission between animals. Uncoagulated whole blood was used to determine packed cell volume (PCV) by micro-hematocrit centrifugation and parasitemia, percent parasitized erythrocytes, was monitored by examination of stained blood smears. A commercial staining kit (Diff-Quick; Dade Behring, Newark, DE), was used to stain the blood smears. An

oil immersion lens (100X magnification) of a compound microscope was used to determine the parasitemia. Estimates of parasitemia were based on counts of the erythrocytes in 5 different fields of view with more than 500 cells counted.

Treatment of an *A. marginale* infected calf with tetracycline (LA 200)

After needle inoculation of calf 3149 with infected *A. marginale* stabilate, the PCV% dropped below 11% and the animal became recumbent. The calf was treated with a single dose of oxytetracycline (Liquamycin LA-200, Zoetis, Parsippany-Troy Hills, NJ) administered at 22 mg/kg, intramuscular (IM).

Results

A. marginale infection

Calf 3149 was inoculated with 2 ml of *A. marginale* (Virginia strain) stabilate. Infection was monitored microscopically with percent infected erythrocyte (PPE) also called parasitemia percentage, packed cell volume percentage (PCV percentage), and prepatent period (PPP). On 18 days post-inoculation (dpi) PPE was 1% and after that, on 27 dpi PPE reached 26% and PCV% dropped to 11 % (**Figure 6.1**) and the calf was recumbent. The calf was treated with a single dose of oxytetracycline on 29 dpi.

Tick feeding

After needle inoculation of the calf 3149 during ascending parasitemia on 25 dpi *D. andersoni* were fed on the calf and another cohort of *D. andersoni* ticks

were fed on 40 dpi. A sample of 5 ticks per cohort were PCR tested and both cohorts of *D. andersoni* were PCR positive for *A. marginale* (**Figure 6.3 A and B**).

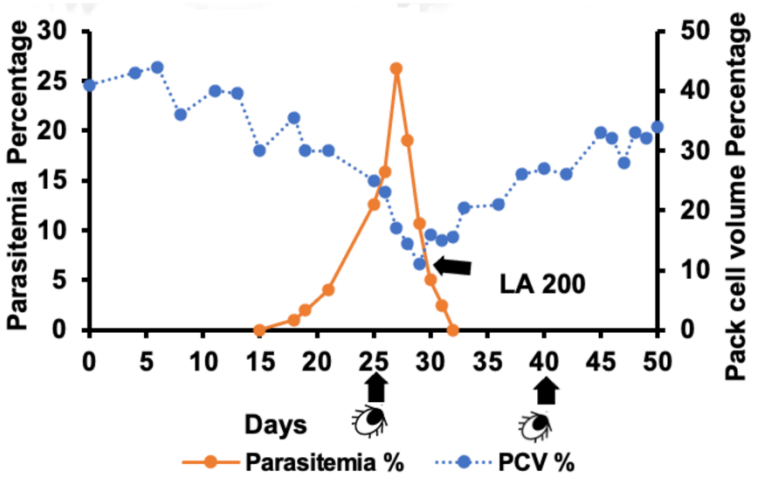

Figure 6.1. PCV and parasitemia of calf No. 3149.

Calf 3149 was inoculated with *A. marginale* infected blood. PCV and parasitemia are shown in blue and orange lines, respectively. Ticks were applied on day 25 and day 40 post-inoculation. The calf was treated with LA 200 at 11% PCV on day 29 dpi.

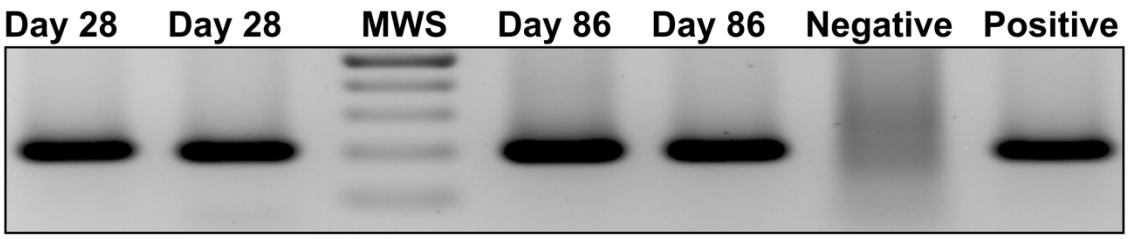

Figure 6.2. PCR of Calf # 3149 before and after treatment with LA 200

Calf 3149 was PCR tested on day 28 and day 86 post-inoculation with an *msp5* - based assay. 100 bp molecular mass marker (MWS) used, template from calf # 635 was used as a positive control and template from an uninfected calf was used as a negative control.

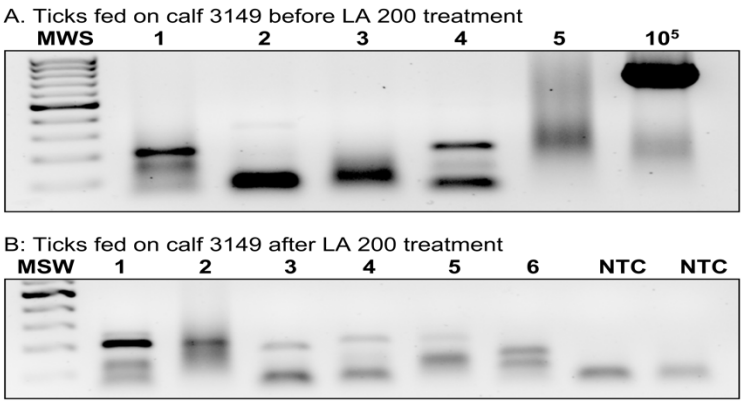

Figure 6.3. *D. andersoni* ticks fed on calf 3149 before and after LA 200.

Two groups of ticks i.e., before LA 200 (A) and after LA 200 (B) were fed on day 25 and day 40 post-inoculation, respectively. 2μl of 10^5 copy/μl plasmid was used as positive control and NTC (non-template control) were used as contamination controls. 100 bp molecular mass marker (MWS) was used and 200 bp was an expected size of *msp5* gene of *A. marginale* amplified by PCR. Tick # 1 and 4 were PCR positive in cohort fed before LA 200 treatment (A) and Tick # 1,2,3,4, and 5 were positive in cohort fed after LA 200 treatments (B).

Effect of tetracycline on the transmissibility of *A. marginale*

Two cohorts of D. andersoni ticks were fed on calf 3149, one before tetracycline treatment on 25 dpi and the other after tetracycline treatment on 40 dpi. Tick # 1 and 4 were PCR positive in cohort fed before LA 200 treatment (**Figure 6.3 A**) (A) and Tick # 1,2,3,4, and 5 were positive in cohort fed after LA 200 treatments (**Figure 6.3 B**), *i.e.,* ticks acquire infection but when naïve calves were challenged with these infected ticks. *D.andersoni* ticks were unable to transmit the infection to naïve calves (**Figure 6.4**).

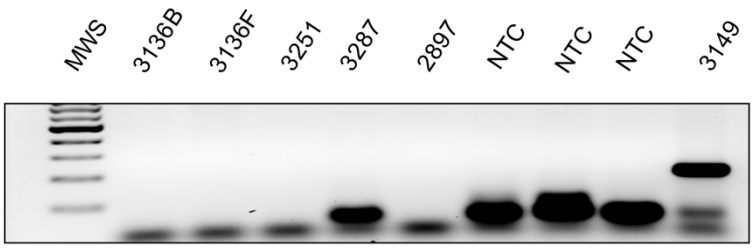

Figure 6.4. Infected ticks fed on normal calves.

Infected ticks were fed on five naïve calves,3136B, 3136F, 3251, 3287, 2897 for 12 days, calves were tested with PCR, *msp5* - based assay, DNA extracted from a carrier calf 3149 was used as a positive control, and non-template control (NTC) was used as a contamination control.100 bp molecular weight standard (MWS) was used. Expected positive amplicon size was 200 bp, only positive control was positive.

Discussion

Our study found that in bovine anaplasmosis, tetracycline treatment will not interfere with the acquisition of *A. marginale* by *D. andersoni* ticks, but it will impact the transmission of *A. marginale* to the naïve bovine host. Tetracycline is a broad-spectrum antibiotic that is bacteriostatic, acting at the ribosomal level, interfering with protein synthesis, and has immunomodulatory functions (Santos & Garcia, 2018). Ticks transmit pathogens like *Anaplasma* and *Ehrlichia* after feeding on infected animal hosts and transfer them to susceptible hosts during future feedings. Following the acquisition of bacterial pathogens, replication of the bacteria occurs in the midgut epithelium of the tick before migration to the salivary glands and the final transmission to the susceptible host (Ueti *et al*., 2007).

A similar observation was reported in a murine model with *B. burgdorferi*, where ticks fed on doxycycline-treated mice were able to acquire spirochetes but

unable to transmit them (Bockenstedt *et al.*, 2002). Previously, clearance of the *A. marginale* with oxytetracycline was reported is several studies (Magonigle and Newby, 1982; Swift and Thomas, 1983; Rogers and Dunster, 1984; Özlem, 1988), while in a couple of studies, *A. marginale* infection was not cleared successfully from the bovine host (Kuttler *et al*., 1980; Coetzee et al., 2005).

Tetracycline has an impact on the biological vector feeding on the host, for instance in *Litomosoides sigmodontis*, a filarial nematode of rodents, which carries intracellular bacteria. Tetracycline therapy, when initiated with *L. sigmodontis* infection, eliminated the bacteria, and resulted in filarial growth retardation and infertility (Hoerauf *et al*., 1999). Multiple studies are reporting the use of tetracyclines to cure the *Wolbachia* infection of the arthropods in class Arachnida, for example from the spider mites (Stouthamer and Mak, 2002; Wang *et al*., 2017; Wong *et al*., 2020). Tetracycline treatment also impacted *Coxiella sp*., in all stages of *Amblyomma americanum* (Zhong *et al*., 2007; Jasinskas *et al*., 2007).

Previous studies about the impact of tetracycline on the arthropod vector and our observation after treatment of an *A. marginale* infected calf with oxytetracycline suggested to us two hypotheses that tetracycline can disturbs the tick midgut and salivary gland, or tetracycline disturb the normal microbiota.

There is precedent for endurance of weakened pathogens after antimicrobial therapy for infection with *Mycobacterium tuberculosis*. Under treatment patients may continue to have positive results of sputum smear testing but are less infectious (Sultan *et al*.,1962: Riley *et al*., 1962). Moreover, sputum samples of undertreatment patients may remain positive for acid-fast bacilli but test negative

by culture, suggesting that the observed organisms either are nonviable or have reduced infectivity (Vidal *et al*.,1996). Results of this study shows that although it is possible to detect some *A. marginale* after antibiotic therapy, pathogen do not have the same transmissibility or pathogenicity as those of the original infectious pathogen (without exposure to tetracycline). The *D. andersoni* ticks are the natural reservoir for *A. marginale*, even if attenuated, may be particularly suited to persisting in specific microenvironments within this invertebrate host. Further studies needed if these antimicrobial treated *A. marginale* can multiply within the ticks or not. There is currently no evidence that infectious *A. marginale* survive antimicrobial therapy, and our results show that arguably attenuated *A. marginale* do not cause persistent disease i.e., bovine anaplasmosis to new naïve cattle. In summary, we provide xenodiagnostic evidence that *A. marginale* (exposed to tetracycline) can persist for after antibiotic treatment within invertebrate hosts but are not transmissible to uninfected cattle.

Acknowledgements

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CHAPTER 7

Attempted optimization of an artificial feeding apparatus for ixodid ticks

Abstract

Ticks are ectoparasites that can transmit several pathogens to animals and people. Our knowledge of pathogen transmission by ticks is limited because the use of many live ticks raised under controlled conditions is required for comprehensive studies on the transmission, maintenance, infectivity and virulence of tick-borne pathogens. The prolonged duration of tick feeding on the host makes tick rearing difficult. Tick feeding is a complex and long process and there is a need to better understand the parasite-host-vector-relationships and to investigate new control strategies for ticks and tick-borne pathogens. In this study, we optimized tick feeding conditions on an artificial feeding apparatus (AFA) over both static and continuous flow conditions for adults, nymphs and larvae of *Amblyomma americanum*, *D. andersoni,* and *D. variabilis*. We focused on the optimization of glucose and heparin in blood meals, and concentration of CO_2 of the incubator in which the feeding trial was conducted. It is hoped this study will contribute to the development of a reliable *in vitro* system and procedure to experimentally feed different stages of *A. americanum*, *D. andersoni* and *D. variabilis* ticks and confirmation of transmission of *A. marginale* infection by *D. andersoni* ticks.

Key Words: Artificial feeding apparatus, *Amblyomma americanum, Dermacentor andersoni,* blood meal, *in vitro* system.

Introduction

Ticks have complex feeding behavior and require time to attach and feed on the host (Tahir *et al*., 2020). Due to the complex feeding of ticks less is known about the competency of different species of ticks for different pathogens. Since the beginning of the 19th century, the concept of artificial feeding of hematophagous arthropods was in the discussion of scientific communities. In 1912, defibrinated ovine blood and rat skin was used to feed *Glossina palpalis* (Galun, 1967). Moreover, the importance of stimulants like temperature, relative humidity, and chemical factors to regulate the feeding behavior of hematophagous arthropods has been discussed (Davis *et al*., 1983). In the case of ticks, different methods were used to feed ticks and study transmission and acquisition of several pathogens by ticks on the laboratory bench which includes injections, capillary feeding, animal skin, or silicone membranes (Purnell and Joyner, 1967; Kocan *et al*., 1986; Kariu, *et al*., 2011). Silicone membrane was used as an interface to artificially feed *Ixodes holocyclus* (Stone *et al*., 1983). Among all the arthropods, hard ticks present the greatest difficulties in the development of an artificial feeding system capable of attending to their needs. The feeding strategy of two tick families *i.e.,* hard and soft, is different. Hard ticks use the mechanical reaction of their buccal appendages which effectively secretes chemical substances such as the prostaglandin E2 and antihistamines and forms a dermal pool where they feed (Sonenshine *et al*., 2013). Soft ticks also known as Argasid ticks have a completely

different strategy to feed on the host (Brown, 1989). Typically, they feed for a short period, although larvae of *Argas* species and some *Ornithodoros* feed for longer periods. Moreover, argasid ticks tend to be opportunistic feeders; when one blood-meal proves insufficient in quantity or quality the instar can readily feed again if another host is available (Balashov, 1972). Because of this, the artificial feeding of the ixodids is more difficult, due to the necessities of greater attachment time and the necessary combination of host reactions required to meet their feeding needs. The objective of this study was to determine the effectiveness of different phagostimulants collected from the hosts in initiating the attachment and feeding of different tick species native to the US in the laboratory utilizing silicone membrane or animal skin and heparinized bovine blood.

Materials and Methods

Ticks and tick feeding

Three different tick species which are native to the US, *A. americanum*, *D. andersoni* and *D. variabilis* were used in this study. Three different artificial feeding systems (one static and two continuous-flow systems) were used to optimize *in vitro* tick feeding (**Figure 7.1 and 7.2**). *A. americanum* (larvae, nymphs, and adults) were purchased from the Oklahoma State University, while *D. andersoni* and *D. variabilis ticks* were reared on the bovine host. Ticks were kept and maintained in the laboratory until use in experiments. Humidity chambers with 12-hour photoperiods were used. Supersaturated KNO_3 was used to maintain the humidity level > 95%. Blood was collected from experimental calves at Middlebush Farm from an ongoing project. A blood meal was prepared by

supplementing the heparinized blood (Militzer *et al*., 2021). All components of artificial feeding apparatus (AFA) in contact with blood were autoclaved or sterilized with 70% ethanol.

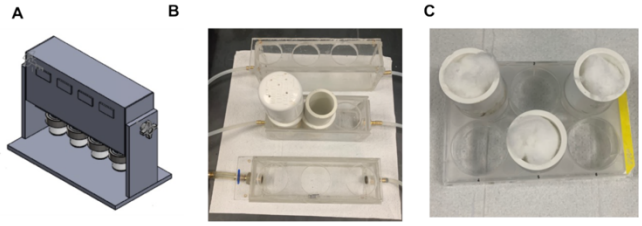


Figure 7.1. Artificial feeding apparatuses for ixodid ticks. Continuous-flow apparatuses (A and B), static artificial feeding apparatus (C).

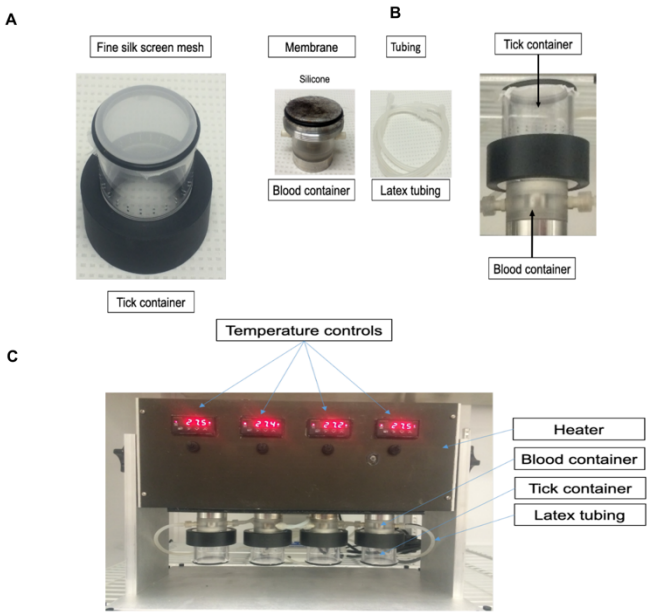


Figure 7.2. Assembly of a continuous-flow artificial tick feeding system**.**

Tick container covered with cloth (A), blood container with a membrane interface (B), four tick chambers attached to a digital heater to maintain the temperature (C)

Membranes used

Three types of membrane interfaces were used: a) goldbeater's skin, b) Lens paper, c) bovine/mouse skin. In the case of Goldbeater's skin or lens paper interface, siliconized membranes were prepared using commercially available Goldbeater's skin (Brooklyn, NY) or lens paper. Five (5) ml of each Ecoflex Super soft 00–50 silicone components A and B (Smooth-On, Easton, PA) was mixed and thinned with 0.75 ml of hexane (Sigma-Aldrich, St. Louis, MO). Goldbeater's membranes were taped to a smooth surface and saturated with the silicone mixture to yield an approximately 100 μm thick membrane. Membranes were air-dried overnight at room temperature (**Figure 7.3**).

In the case of the animal skin interface, bovine skin was obtained from the sacrificed animals from our ongoing study, and mouse skin was obtained from the sacrificed mice (uninfected) from the mouse room **(Figure 7.4)**. In some of the experiments, to avoid deterioration, skin was treated with commercially available gentamicin (Fresenius, Lake Zurich, IL) and Gibco™ Antibiotic-Antimycotic (Thermo Fisher Scientific, Waltham, MA) preparations prior to use in the AFA.

Blood meal

Normal bovine blood was supplemented with 20 IU/ml heparin, 0.2% [w/v] glucose, 51 mg/ml ATP and 5 μg/ml gentamicin. Blood meals were refreshed every 12 h.

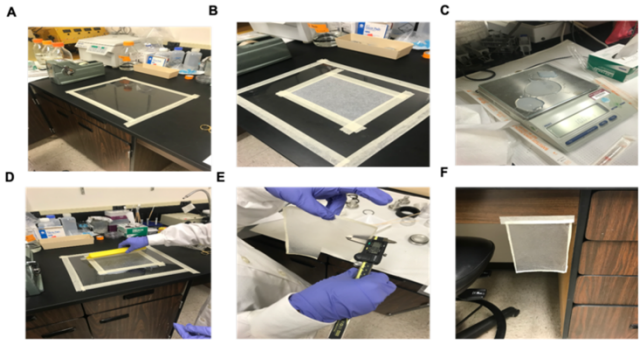


Figure 7.3. Siliconized membrane preparation

Plastic sheet taped on the bench top (A), paste lens paper/goldbeater's membrane (B) weighing silicone solution A and B (C), spreading silicone mixture (D), measuring thickness of membrane (E), drying membrane for 24 hr.

Figure 7.4. Different membrane interfaces used in continuous-flow AFA

Results

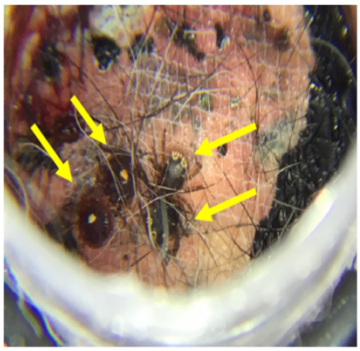
A. americanum feeding on artificial feeding apparatus

A. americanum adults, larvae and nymphs were fed on different membrane interfaces, multiple compositions of a bloodmeal and under different conditions to find the optimum conditions for *in vitro* feeding.

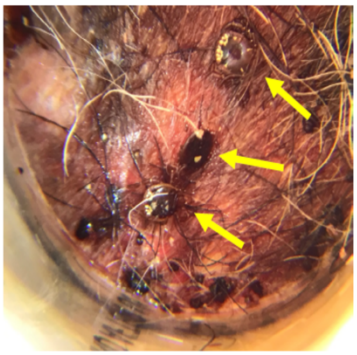
1) *A. americanum adults* feeding on siliconized membrane and bovine skin

It was observed that *A. americanum* larvae, nymphs, and adults feed for 3-5, 5-7, and 10-14 days, respectively followed by ~30 days to molt or to oviposit after feeding *in vivo* (Doube and Kemp, 1979). *A. americanum* adults were fed on two different membranes, a) siliconized goldbeater's membrane and b) bovine skin. Tick attachment started after 24 h of the experiment and the maximum number of ticks were attached after 48 h (**Figure 7.5A and 7.5B**). However, tick attachment varied with thickness of the interface (**Figure 7.5C**). Most ticks attached on thin siliconized membrane and bovine skin but there was a slight difference in attachment between bovine and siliconized interfaces.

A. Silicone



B. Bovine Skin



C. Adult tick attachment rates (N=10 ticks/group)
Attachment of *A. americanum* adults to silicone or bovine skin interface (static bloodmeals, 48 hr, 37°C, 5% CO_2). 5 male + 5 female ticks per group.

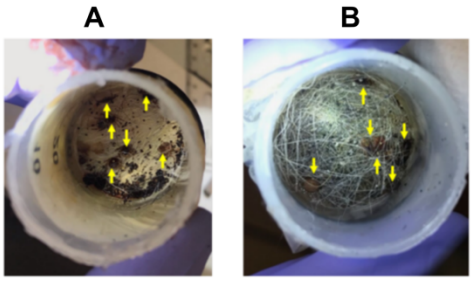
	Number Attached (out of 10)			
Trial	**Silicone (thick)**	**Silicone (thin)**	**Bovine (thick)**	**Bovine (thin)**
I	2	6	1	6
II	3	6	2	6
III	3	6	2	8
$\bar{X}$ (SD)	2.7 (0.6)	6 (0)	1.7 (0.6)	6.7 (1.2)

Figure 7.5. *A. americanum* feeding on siliconized membrane and bovine skin.

Panels A and B show *A. americanum* adults (arrows) attached to silicone membrane or bovine skin, respectively. Panel C summarizes tick attachment to different *in vitro* membrane preparations.

A. americanum adults feeding on siliconized membrane and bovine skin in static artificial feeding apparatus

A. americanum adult ticks were fed on the static artificial feeding apparatus which included a six-well cell culture plate and polypropylene feeding chambers. Blood meal was changed twice a day. Again, ticks were fed on bovine skin and siliconized membrane and phagostimulants *i.e.,* tick feces were added to both feeding chambers, but cattle hairs were added only to the siliconized chamber (**Figure 7.6A and 7.6B**). In two trials, a greater number of ticks were attached to bovine skin as compared to the siliconized interface (**Figure 7.6C**). One partially engorged tick that had detached on day 6 from the siliconized membrane, was sized and compared to the unfed *A. americanum* and tick weight was documented (**Figure 7.6D, 7.6E, 7.6F and 7.6G**).



C

Attachment of adult *A. americanum* on bovine and siliconized membrane and bovine skin after 48 hr at 37ºC,5% CO_2 10 pairs per group

Number of ticks attached out of 10		
Trial	Silicone membrane	Calf skin thin
I	6	6
II	5	7
x̄ (SD)	5.5(0.7)	6.5(0.7)

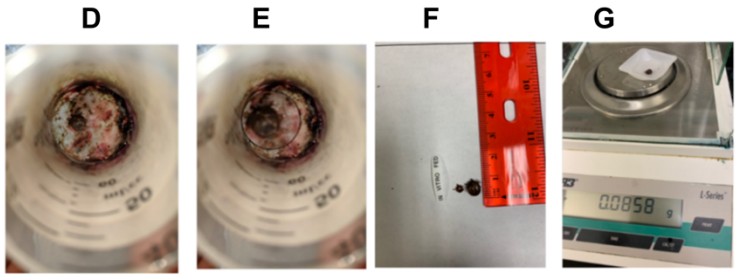


Figure 7.6. Comparison of *A. americanum* (adult feeding) on bovine skin and siliconized membrane on static AFA**.**

Bovine skin with tick feces (A), the siliconized membrane with tick feces and cattle hair as phagostimulant (B). In both chambers the same blood meal was used. Female *A. americanum* engorged on siliconized membrane on day 6 (D, E), Size comparison of the engorged and flat tick (F), weighing the engorged female tick (G).

A. americanum nymphs feeding on mouse skin

A. americanum nymphs were fed on the continuous-flow AFA shown in **Figure 7.7**. Mouse skin was used as interface and 50 nymphs were applied to each of three chambers and ~ 80% attachment was observed in all three chambers after 36 h (**Figure 7.7A**) but due to deterioration of the skin, nymphs started dying on the skin (**Figure 7.7B**) and after 72 h about 90 % nymphs were found dead (**Figure 7.7C**).

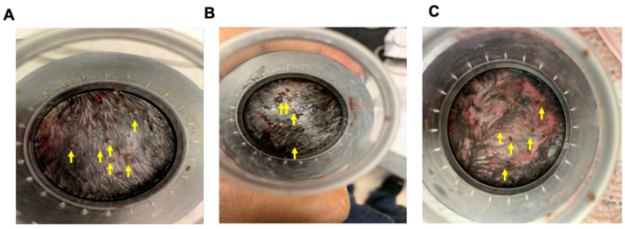


Figure 7.7. *A. americanum* nymphs feeding on mouse skin on a continuous-flow AFA

Attachment of *A. americanum* nymphs feeding on mouse skin in continuous-flow AFA after 36 h, 48 h and 72 h (A, B and C).

A. americanum larvae feeding on mouse skin

Lone star larvae were fed on mouse skin in the continuous-flow AFA shown in **Figure 7.8**. Interface thickness was reduced as compared to the AFA with nymphs. Larvae attachment was observed daily under the dissection scope and ~20% attachment was noticed on day 2 (**Figure 7.8B**) but there was complete deterioration of mouse skin observed on day 4 of the experiment (**Figure 7.8C**).

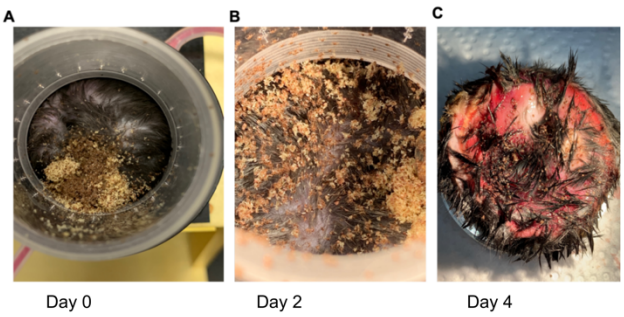


Figure 7.8. *A. americanum* larvae feeding on mouse skin in continuous-flow AFA. *A. americanum* larvae feeding on mouse skin (A), attachment on mouse skin on day two (B), deterioration of skin on day four of the experiment (C).

D. andersoni feeding on the artificial feeding apparatus

In these experiments, the objective was to determine the optimized condition for the *D. andersoni* feeding on the AFA. Three different feeding apparatuses were used in all these experiments (**Figure 7.1**).

In the continuous-flow AFA, a silicone membrane was used and eight pairs of *D. andersoni* were fed at 37ºC (**Figure 7.9A, 7.9B and 7.9C**). After 24 h, attachment of *D. andersoni* had started but the membrane leaked and was fixed with glue to continue the experiment to observe an average 11 ticks attached on the siliconized membrane (**Figure 7.9B**).

In the second experiment the continuous-flow AFA with silicone membrane as the interface was used to feed five pairs of *D. andersoni* adults. 70% of ticks attached after 48 h (**Figure 7.9D, 7.9 E and 7.9F**). The temperature of the blood meal was maintained by keeping the whole system in a water bath.

In the third experiment, the same apparatus and interface were used but the whole system was kept in an incubator and 5% CO_2 was continuously provided

which substantially improved the attachment rate of the ticks (**Figure 7.9G**, **7.9H and Table 7.1)**. Further, one of the *D. andersoni* females was observed partially engorged (**Figure 7.9I**).

In the last experiment, the bovine skin interface was compared with the siliconized membrane by using the static apparatus (six-well cell culture plate) and it was found that there was slightly more attachment to bovine skin than siliconized membrane (**Figure 7.9J, 7.9K, and 7.9L**) and **Table 7.1**.

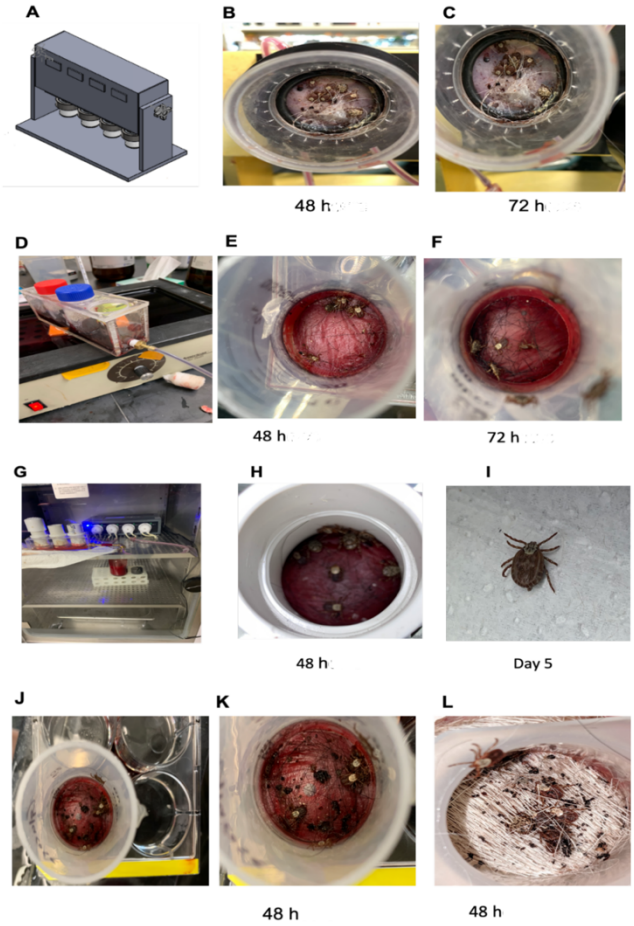


Figure 7.9. *D. andersoni* adult male and female ticks feeding on a siliconized membrane and a bovine skin on different artificial feeding systems.

D.andersoni feeding on continuous-flow AFA on a siliconized membrane (A, B, C). *D. andersoni* feeding on continuous-flow AFA placed on a water bath on a siliconized membrane (D, E, F). *D. andersoni* feeding on silicone membrane interface on continuous-flow AFA placed in a CO_2 incubator (G, H,) partially engorged female detached from the membrane (I). *D. andersoni* feeding on a silicone membrane and bovine skin interface on static AFA placed on a water bath.

Table 7.1. Summary of the number of *D. andersoni* adults attached to a different interface on different AFA.

Trial	Continuous flow AFA silicone membrane at 37ºC	Continuous flow AFA silicone membrane on water bath at 37ºC	Continuous flow AFA silicone membrane on 5% CO_2 at 37ºC	Static AFA with silicone membrane on water bath at 37ºC	Static AFA with bovine skin on water bath at 37ºC
	8 pairs applied	5 pairs applied	5 pairs applied	5 pairs applied	5 pairs applied
I	11	3	8	5	5
II	12	4	7	3	4
$\bar{x}$ (SD)	11.5 (0.7)	3.5 (0.7)	7.5 (0.7)	4 (1.41)	4.5 (0.7)

***D. andersoni* larvae feeding on a continuous flow artificial feeding apparatus**

D. andersoni larvae were fed on the siliconized membrane and after 24 h 80% of the larvae attached and after 36 h larvae detached and after 48 h only 20% larvae remained attached.

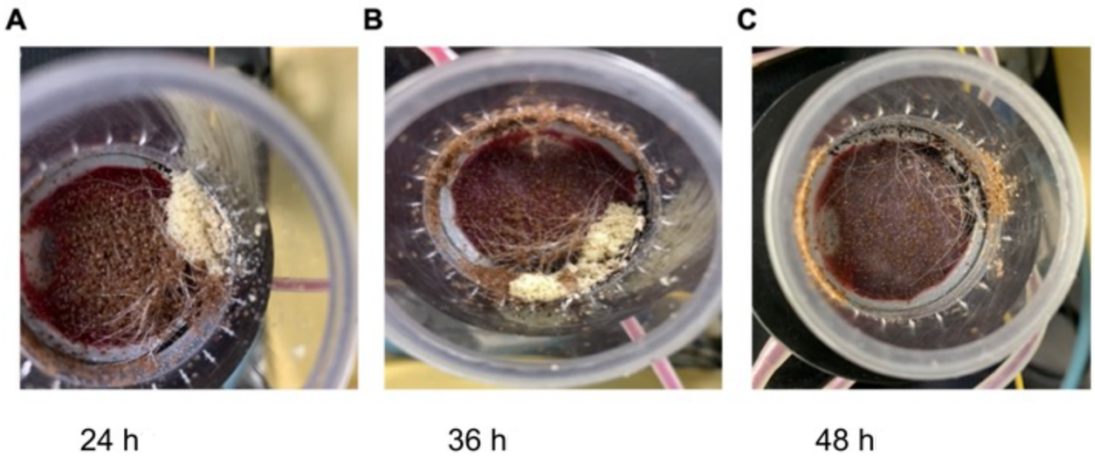


Figure 7.10. Feeding of *D. andersoni* larvae on a continuous-flow apparatus**.**

One cluster of *D. andersoni* larvae was applied to a continuous-flow apparatus. Attachment was observed after 24 h, 36 h and 48 h (A, B, C)

***D. andersoni* feeding on *A. marginale* infected blood on a static artificial feeding apparatus**

It has been documented previously that *D. andersoni* can acquire *Leptospira pomona* via capillary feeding (Burgdorfer, 1957) and *A. marginale* via a continuous-flow AFA (Vimonish *et al*., 2020). In this study heparinized bovine blood from an *A. marginale* carrier animal was used and five pairs of *D. andersoni* ticks were fed on siliconized membrane and after 24 h about 60% ticks were attached. A partially engorged tick was detached on day 7 which was kept in a

humidity chamber for 5 days. The tick was tested for *A. marginale* by *msp5*-based PCR assay and *A. marginale* carrier calf blood was used as a positive control. It was found that ticks acquire *A. marginale* during feeding on infected blood.

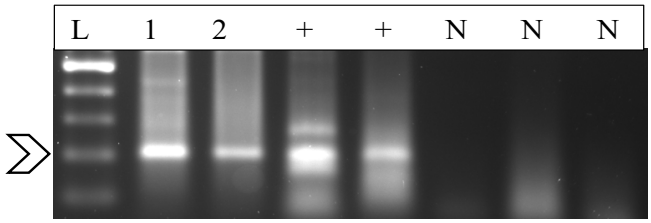


Figure 7.11. Detection of *A. marginale* in *D. andersoni* adult tick after *in vitro* feeding.

msp5-based assay was used to detect *A. marginale* in the tick. Template was run in duplicate (1 and 2) and blood from carrier calf was used as positive control (+, (N) was non-template control. 100 bp ladder (L) was used. The expected size of target (200 bp) is denoted by arrow.

Discussion

Vector-borne diseases are a major cause of death and illness around the world; every year there are more than 700 000 deaths from diseases such as malaria, dengue, African trypanosomiasis, leishmaniasis, Chagas disease, yellow fever, Japanese encephalitis, and onchocerciasis (Nigusie, *et al*., 2021). Trends of tick and mosquito-borne diseases have increased two-fold from 2004 -2016 in the US (Rosenberg *et al*., 2018). Ticks can transmit a wide variety of pathogens including fungi, viruses, bacteria, and protozoa but there is limited knowledge on transmission, maintenance, and acquisition of the pathogens transmitted by these arachnids (Ackerman *et al*., 1980; Abbassy *et al*., 1994; Waladde *et al*., 1996; Krull *et al*., 2017). One of the major reasons that puts a hold on this sort of research is that maintenance of the tick colony under laboratory conditions is a tough task and tick feeding behavior is also not supported as it takes several days to feed on the host (Kröber and Guerin, 2007).

In these experiments, different stages of *A. americanum* and *D. andersoni* were fed on synthetic and biological interfaces by using heparinized bovine blood. There was more attachment of *A. americanum* adults on bovine skin as compared to the siliconized membrane, but bovine skin deteriorated on day 4 of the experiment. Antifungal and antibiotic treatment of the skin only added 2-3 more days to the life of the skin. However, one partially engorged *A. americanum* female was documented on siliconized membrane. Immature stages of *A. americanum* were fed on mouse skin and attachment was observed after 36 h in the case of nymphs, while larvae attached after 24 h. In both cases mouse skin rotted on day

4. In another set of experiments *D. andersoni* adults were fed on the different interfaces by using continuous-flow AFA and static AFA under different experimental conditions. 80% attachment of adult *D. andersoni* was observed in the continuous-flow apparatus at 37°C while in the presence of CO_2 the attachment rate increased, and a partially engorged female was also documented when ticks were fed in the presence of CO_2.

In a previous study, 75 *D. andersoni* and 25 *A. maculatum* were fed artificially with a capillary tube and were induced to ingest a suspension of *Leptospira pomona* in Verwoort' s medium. The ticks fed readily at RT for 4 to 6 h and ingested ~ 0.01 to 0.03 ml of the suspension. After keeping the ticks in a humidity chamber for 14 days, the ticks were allowed to feed on guinea pigs and were shown to transmit *Leptospira* (Burgdorfer, 1957). Recently Vimonish and colleagues described the acquisition of the *A. marginale* by *D. andersoni* males by using an *in vitro* feeding apparatus (Vimonish *et al*., 2020). It was a quite similar experiment to the one described herein except defibrinated blood was used while in this experiment heparinized blood was used. For the confirmation of *A. marginale* presence a TaqMan probe was used while in this study the *msp5*-based PCR assay was used.

In summary, this work provided the optimum conditions for the attachment of different stages of *A. americanum*, and *D. andersoni* but there were problems including membrane thickness, leakage, and deterioration. Further experiments are needed to answer the questions regarding appropriate thickness of membrane and to avoid the deterioration of the animal skin.

Acknowledgements

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CHAPTER 8

Summary and Conclusion

As indicated throughout this dissertation, the goal of anti-tick vaccine research is to develop sustainable interventions to decrease the incidence of tick-borne diseases. Paradoxically, direct interference with the tick-pathogen interface is frequently overlooked as the *primary* criterion when screening for protective antigens. Some investigations led to identification of antigenic targets for interference with the tick life cycle, but interference with transmission of tick-borne pathogens has often been a secondary parameter for such work. The overall objective of this dissertation was to determine the feasibility of direct interference with an infectious disease agent that is transmitted by ticks.

The first step toward our overall objective was the merging or co-adaptation of two established model systems used (1) to investigate the effects of host immunization with tick tissues and (2) to study the biologic transmission of *Anaplasma marginale.* This work involved ticks indigenous to the USA, *Dermacentor andersoni* and *D. variabilis*, which feed on cattle in nature and are also incriminated as competent vectors of *A. marginale.*

In the pilot study, we hypothesized that immunization with *D. andersoni* salivary gland (SG) or midgut (MG) tissue would induce immune responses which would impact the tick performance parameters. Immunization of cattle with SG

resulted in a greater reduction in tick performance as compared to the baseline feeding ticks. Two-dimensional SDS-polyacrylamide gel electrophoresis (2D-PAGE) combined with Western blots developed with sera from SG-immune animals reacted with 44 candidate protein spots associated with reduced tick performance, which were divided into regions A-E. Protein spots were plucked and pooled according to the region and identified with trapped ion mobility spectrometry time-of-flight (timsTOF) mass spectrometry, and resultantly 258 proteins were identified. As a proof-of-concept, bioinformatics analysis was performed on 46 proteins from regions E and F. Forty-six proteins were searched for in the *D. andersoni* MG and SG transcriptome database, which resulted in five candidates recognized by anti-SG sera. Open reading frames encoding these proteins were cloned by Twist Biosciences in the pET-28a(+) expression vector (**Table 2.1**). IPTG induction conditions were optimized, and all five clones were expressed and tested for seroreactivity using anti-SG and anti-MG sera. This pilot study suggested that tick SG has potential candidates for an anti-tick vaccine to interfere with tick performance, but we were more interested in the effect this approach would have with transmission of a tick-borne pathogen.

To test the long-standing hypothesis that immunization with SG will interfere with the transmission of *A. marginale* to susceptible cattle, calves were immunized with denatured (d) or native (n) homogenates of SG or MG collected from the same ticks. After immunizations, calves were challenged with infected *D. andersoni* ticks. All but one calf, 3241J, immunized with dMG or dSG became infected with *A. marginale* after tick challenge feeding. However, all calves immunized with nMG

or nSG did not become infected after tick challenge. The susceptibilities of these calves to infection with *A. marginale* infection was confirmed after inoculation with infected blood. In a separate group, it was hypothesized that denatured infected tick tissues will interfere with the transmission of the *A. marginale* to the susceptible bovine host. Those results corroborated those observed for other denatured immunogens, as both animals which were immunized with dIT became infected upon infected tick challenge. Overall, these findings suggested that denaturation of tick tissues will not protect against transmission of the *A. marginale* to the host.

Although these results suggested that conformational epitopes are involved in protection against tick transmission of *A. marginale,* it is important to remember that this idea should be treated as a newly justified hypothesis rather than a speculative conclusion.

In our hands, the *D. andersoni* ticks used in the second study (Chapter 3) did not feed or perform as expected, and the Oklahoma State University Tick Rearing Facility decided to stop selling *D. andersoni.* These limitations to the decision to use *D. variabilis.*

In the next study, calves were immunized with nSG, nMG tissue or adjuvant-only as a control, and the numbers of infected ticks used for challenge infestations were doubled to 20 female and male tick pairs. Interestingly, these *D. variabilis* transmitted *A. marginale* to all but the nSG-immune calves (Chapter 4). This difference in transmission results could be due to (1) difference in midgut compositions of the two species, (2) other differences such as salivary components between these tick species, (3) feeding performance of the *D. variabilis* specimens,

(4) higher tick numbers used for challenge infestation or (5) perhaps in relation to the latter three points, higher pathogen levels delivered in saliva from the *D. variabilis* described in Chapter 4. Notably, the four latter hypotheses would suggest the possibility of breakthrough infections, which could be instructive in identification of protective tick target molecule candidates as well as future investigations during the development phase of a defined subunit vaccine.

Immunization with denatured tissue failed to interfere with tick transmission of *A. marginale,* suggesting the need for more innovative approaches to identification of tick molecules or epitopes that are uniquely reactive with protective antisera. This change in strategy is because denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE), which separates denatured proteins by size, is now expected to make protective epitopes less reactive with protective immune sera. Immunoprecipitation, native PAGE and phage display are methods that may lead to identification of protective tick antigens, but all these methods have limitations.

Immunoprecipitation, in theory, is a direct approach for identification of antigens. Protein A- or Protein G-coated magnetic beads can be incubated with antisera followed by antigen. After elution, antigen specific to the bead-attached antibody could be identified and quantified by Bruker Mass Spec. A limitation with this approach will be the contamination of the eluted antigen with antibodies. A second limitation could be the amount of antigen isolated from immunoprecipitation. This could result in higher background and the protective antigens may remain unidentifiable due to technical limitations.

Assuming the premise that protection from *A. marginale* transmission was due to conformational protein epitopes is correct, Western blotting after native (*i.e.,* non-denaturing) PAGE is another possibility for identification of protective protein antigens. Limitations of native PAGE include (1) proteins are not separated based solely on size and (2) less information is available about the use of native gels for the second dimension of 2D-PAGE (Willadsen, 1987; Arndt *et al*., 2012; Lasserre and Ménard, 2012; Hou *et al*., 2014). Furthermore, it is not certain that 2D-PAGE would not render protective antigens non-reactive with immune sera.

Phage display involves genetically modified bacteriophages, which express small random peptides on one of their coat proteins (Wu, Liu, Lu, and Wu, 2016). These bacteriophages could be used for bio panning to isolate specific epitopes that are bound by immune sera. Positive and negative selection could be used to distinguish epitopes and mimotopes reactive to immunoglobulins derived from cattle immunized with native versus denatured MG and/or SG. Selection will eliminate the cross-reactive epitopes that are presumably less protective.

The phage display approach has a few limitations. First, the identified amino acid sequences may have no known match to any currently available protein in a *Dermacentor* sp.-derived data set. Second, bias with over incorporation of guanine (G) into the nucleotides encoding the displayed peptides reduces the randomness of randomly generated phage peptide libraries, which can limit sequence coverage (Rodi, Soares, and Makowski, 2002; Krumpe and Mori, 2006; Wu *et al*., 2016; Ryvkin *et al*., 2018).

An old-fashioned approach used method is to identify potentially protective antigens is through differential screening of a cDNA expression library, which could identify full-length as well as truncated recombinant fusion proteins. The expression library can be screened to identify proteins that react with sera from immunized calves that were not infected by *A. marginale* during infected tick challenge. However, bacterial expression libraries do not incorporate the post-translational modifications performed in eukaryotic cells, which means this approach is not expected to detect epitopes of protective carbohydrate or lipid moieties. Expression libraries utilizing eukaryotic expression vectors and host cells would be expected to post-translationally modify overexpressed recombinant proteins, increasing the likelihood of detecting non-proteinaceous antigens. However, the latter approach would be relatively complex and expected to increase the likelihood of detecting epitopes that are cross-reactive among different eukaryotic species.

Taken together, identification of putatively protective tick antigens is expected to require a combination of the approaches described above. Perhaps the most direct approach, initially, would involve identification of immunogen components that are uniquely reactive to protective immune sera. Arguably, the most direct molecular biological approach could be to use phage display to identify randomized peptide sequences uniquely reactive to protective immune sera, which could then require more in-depth analyses to identify specific tick molecules presenting such epitopes.

In Chapters 5 and 6 two interesting observations were discussed. The first relates to *A. marginale* infection levels in ticks. We observed that two groups of ticks, flat (unfed) and previously mated male ticks acquire different levels of infection after feeding on the same host simultaneously. It has been reported that rickettsemia levels in infected cattle fluctuate between $<10^4$ to 10^7 infected erythrocytes per ml of blood, and during long-term persistent infections, cattle maintain very low rickettsemia levels (*i.e.*, $<10^3$ infected erythrocytes per ml of blood) (Eriks *et al*., 1993). The most accepted paradigm among researchers is that, although acquisition rates vary, *A. marginale* replicates to the same level among infected ticks, regardless of the rickettsemic load among the bovine source of tick infection. Thus, tick infection levels (*not* infection rates) are considered independent of the high or low points in persistent cyclic rickettsemia of cattle (Palmer *et al*, 2000). Thus, this observation from Chapter 5 needs to be further investigated with proper controls and larger sample sizes.

In Chapter 6, a second observation is discussed which was made during acute anaplasmosis, when ticks were feeding on a patent, recumbent carrier calf with a packed cell volume (PCV) of 10.5%) and so this calf was treated with one dose of tetracycline (LA 200). After recovery, the calf was still PCR-positive for *A. marginale.* A second cohort of ticks were fed on the same calf, and both cohorts of ticks were PCR-positive for *A. marginale*. However, when these ticks were transmission fed on susceptible cattle, none of these cattle became infected with *A. marginale.* These results suggested that treatment of the donor host with tetracycline did not prevent tick acquisition of the *A. marginale*, yet these ticks

somehow did not transmit the infection. It is not clear if this is a repeatable observation, underscoring the importance of distinguishing hypotheses from conclusions, and, if repeatable, it would need to be determine if such effects of tetracycline are on the pathogen, tick, microbiome, vertebrate host or some other component of this interaction. Interestingly, a similar observation was reported in a murine model with *B. burgdorferi*, where ticks fed on doxycycline-treated mice were able to acquire spirochetes but unable to transmit them (Bockenstedt *et al*., 2002). This observation in chapter 6 led to a couple of testable hypotheses, (1) tetracycline disturbs the SG or MG transmission barrier(s) of ticks, which led to failure of transmission of *A. marginale* infection to the susceptible host, (2) tetracycline impacts the microbiota of ticks which hinders the transmission of the *A. marginale* to the bovine host, (3) tetracycline affects *A. marginale* in a way that interferes with it's adaptation to biologic transmission by ticks and (4) tetracycline affects the vertebrate host through an undescribed mechanism (to our knowledge) that impacts subsequent transmission of *A. marginale*.

In Chapter 7, efforts toward optimization of different parameters for artificial feeding of metastriate ticks were described. Ticks require an vertebrate host to feed on, and during this feeding process ticks acquire and transmit pathogens including viruses, bacteria and protozoa. To study the biology of ticks or their interactions with associated pathogens, it is invaluable to be able to maintain tick colonies under laboratory conditions and to have efficient techniques to artificially infect them.

During natural transmission, the consensus paradigm is that tick-borne pathogens are injected into the vertebrate host at the same time as tick saliva, which favors infection by tick adaptations to evade immunologic responses of vertebrate hosts (Nuttall and Labuda, 2004). This emphasizes the need of tick rearing, and infecting ticks under laboratory conditions, which is a difficult task because of the complex feeding behavior of ticks. Maintenance of productive tick colonies doubtlessly explains a significant proportion of the existing gaps in our knowledge of tick vector competence and transmission pathways.

In Chapter 7, a continuous-flow artificial feeding apparatus (AFA) and some static AFAs were used to optimize different feeding parameters for different stages of *Amblyomma americanum* and *D. andersoni.* Siliconized membranes and animal skins (bovine and mouse) were used. Tick attachment and semi-engorged ticks were collected. Further, it was shown that AFA can be used to infect ticks (**Figure 7.11**). There were certain problems encountered including leakage of the membrane interface, deterioration of the biological membrane after a certain time, clotting of blood and detachment of larvae after certain times. Therefore, more experiments are required to make AFAs reliable for maintaining tick colonies and infecting ticks in laboratory settings.

In conclusion, this dissertation discussed different facets of tick, host and pathogen interaction. The long-term goal of this research was mitigating ticks and tick-borne diseases. Three major findings of this research were (1) the *D. andersoni*, *A. marginale* and bovine model system can be adopted in the USA; (2) the host immune system can be used to target tick molecules involved in

reduced tick performance; (3) proteomics combined with bioinformatic analyses can be used to find proteins potentially involved in host protection against ticks; (4) immunization with crude homogenates of *D. andersoni* or *D. variabilis* tissues can interfere with the transmission of *A. marginale* infections; (5) artificial feeding apparatus can be used to infect ticks under laboratory conditions; (6) several novel observations may become instrumental in the formulation of testable hypotheses for future work. It is the sincere hope of this PhD candidate, and his mentor, that data generated from these studies will add useful information to the body of knowledge of tick, pathogen and host interaction.

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APPENDICES

Appendix 1.1

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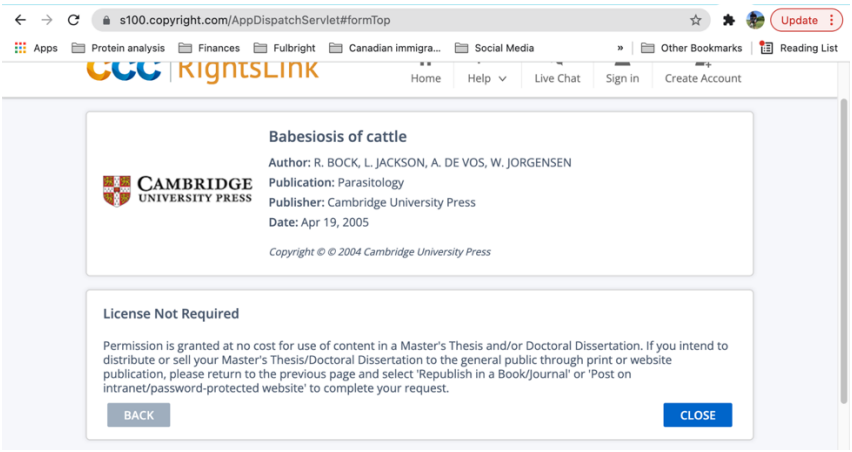

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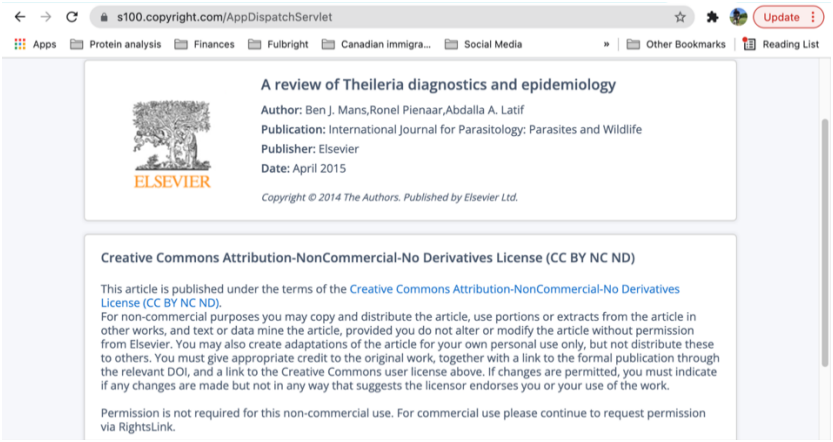

Appendix 2.1

Mass spectrometry (MS)

Gel regions expected to contain proteins of interest were excised from stained gels, destained, trypsin digested (Promega, Madison, WI) and subjected to C18 tip purification according to manufacturer instructions (Pierce, Appleton, WI). Samples containing peptides were lyophilized, resuspended in 10 µl of 5% (v/v) acetonitrile, 0.1% (v/v) formic acid and transferred to an autosampler vial.

MS was performed at the University of Missouri Gehrke Proteomics Center by injection (1 µL) directly onto a 20 cm long x 75 µm inner diameter pulled-needle analytical column packed with ethylene bridged hybrid-C18 (BEH-C18) (Waters, Milford, MA), 1.7 µm, reversed phase resin. Peptides were separated and eluted from the analytical column with a gradient of acetonitrile at 300 nl/min. The Bruker nano Elute system (Billerica, MA) was attached to a Bruker timsTOF-PRO mass spectrometer via a Bruker CaptiveSpray source. Liquid chromatography (LC) gradient conditions Initially were 3%B (A: 0.1% formic acid in water, B: 99.9% acetonitrile, 0.1% formic acid), followed by a 7.5 min gradient to 17%B,7.5 min to 17-25%B, 5 min to 25-37%B, 2 min to 37-80%B, a 5 min hold at 80%B, for a 1 min and ramp back and 2 min hold at initial conditions, for a total run time of 30 min. MS data were collected in a positive-ion data-dependent parallel accumulation-serial fragmentation (PASEF) mode (1) over an m/z range of 100 to 1700, with samples run five days after the most recent calibration date. PASEF and trapped ion mobility spectrometry (TIMS) were set to “on.” One MS and ten PASEF frames were acquired per cycle of 1.27

sec (~1 MS and 120 MS/MS). Target MS intensity for MS was set at 20,000 counts/sec with a minimum threshold of 250 counts/s. A charge-state-based rolling collision energy table was used from 76-123% of 42.0 eV. An active exclusion/reconsider precursor method was used with release after 0.4 min. If the precursor (within mass width error of 0.015 m/z) was >4X signal intensity in subsequent scans, a second MS/MS spectrum was collected. Isolation width was set to 2 (<700 m/z) or 3 m/z (800-1500 m/z).

Expression of recombinant proteins

Proteins identified by bioinformatic analysis were expressed by following the strategy described in Current Protocols in Protein Science (Francis and Page, 2010). Schematic flow chart is as follows. Sequences encoding proteins of interest were synthesized and inserted into pET-28 c (+) expression vectors by Twist Biosciences (San Francisco, CA).

Preparation of competent *Escherichia coli* (*E. coli)*

E. coli BL21 (DE3) were used for the preparation of competent cells. 1M $CaCl_2$, 0.1M $CaCl_2$ and 0.1M $CaCl_2$ with 15% glycerol were prepared. 1 mL of Luria Broth (LB) broth was inoculated with *E. coli* and kept in a shaking incubator at 37 °C and 200 rpm, overnight. The next day, the overnight culture was diluted 1:100 into fresh LB broth and placed in a shaking incubator at 37 °C and 200 rpm for 3-4 hours until the optical density (OD) at 600 nm reached 0.5. Then these cells were harvested and washed with ice-cold 0.1M $CaCl_2$. *E. coli* culture was equally divided in multiple Oakridge tubes and kept on ice for 20 min. Cells were centrifuged at 4 °C at 700 X g for 10 min. Supernatants were discarded and pellets were resuspended in 20 ml ice-cold 0.1M $CaCl_2$ and kept on ice for 30 min. Again, cells were centrifuged at 4 °C at 700 X g for 10 min. Supernatants were discarded and pellets were resuspended with 20 ml in ice-cold 0.1M $CaCl_2$ and kept on ice for 30 min. Again, cells were centrifuged at 4 °C at 700 X g for 10 min, supernatants were discarded, and combined pellets resuspended in 5 ml ice-cold 0.1M $CaCl_2$ with 15% glycerol and stored at -80°C for downstream transformation.

Transformation of clones

Competent cells were thawed on wet ice. The required number of 1.5-ml polypropylene microcentrifuge tubes were placed on wet ice. Cells were mixed gently, then 50 µl aliquots of competent cells were placed in the chilled 1.5-ml microcentrifuge tubes. Then, 1−5 µl of sample DNA was directly added into a

tube of competent cells. DNA was mixed with cells by gently flicking the tube several times. Cells were incubated on ice for 30 min. Cells were heat-shocked for exactly 30 sec in a 42 °C water bath. Tubes were not mixed or shaken. Cells were incubated on ice for 2 min. 250 µl of super optimal broth (SOC) with catabolite suppression (SOC+ Glucose) medium at RT was added to cells and then placed in a shaking incubator at 225 rpm for 1 h at 37 °C. At least two different volumes (20 or 200 µL) of cells were spread from each transformation reaction on separate LB plates containing kanamycin (30 µg/ml). The plates were labeled with the plating volume so that the amount providing the best colony density could be identified. Plates were inverted and incubated overnight at 37 °C. The next day, individual colonies were picked and grown overnight in 5 ml LB broth with 15 µl of 10 mg/ml kanamycin at 37 °C on a shaker at 250 rpm. Overnight broth cultures were diluted (1:100) into fresh LB/ kanamycin medium and grown for several hours until OD_{600} > 0.3.

Optimization of Isopropyl β- D-1-thiogalactopyranoside (IPTG) induction

IPTG is an analog of allolactose, a lactose metabolite that triggers transcription of the *lac* operon, and it is therefore used to induce protein expression where the gene is under the control of the *lac* operator. In this study, IPTG was used to induce the gene for T7 RNA polymerase and to optimize different parameters to get the maximum expression of recombinant clones. Before induction, an overnight culture was diluted and grown at 37 °C with

shaking until they reached saturation (OD_{600} ≥3). Expression conditions were optimized following the strategy shown below (**Figure S2.3**).

Appendix 2.2

ELISA analysis

Two Way Analysis of Variance Saturday, October 10, 2020, 12:42:10 PM

Data source: 2249 Two Way ANOVA Data in Titer ANOVAs w. transformed data Graphs w. stdev.JNB

Balanced Design

Dependent Variable: Data

Normality Test (Shapiro-Wilk): Failed (P < 0.050)

Equal Variance Test (Brown-Forsythe): Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
Day	8	180.338	22.542	47.521	<0.001
Antigen	1	6.325	6.325	13.333	<0.001
Day x Antigen	8	2.938	0.367	0.774	0.628
Residual	36	17.077	0.474		
Total	53	206.678	3.900		

The difference in the mean values among the different levels of Day is greater than would be expected by chance after allowing for effects of differences in Antigen. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Antigen is greater than would be expected by chance after allowing for effects of differences in Day. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of Day does not depend on what level of Antigen is present. There is not a statistically significant interaction between Day and Antigen. (P = 0.628)

Power of performed test with alpha = 0.0500: for Day : 1.000
Power of performed test with alpha = 0.0500: for Antigen : 0.927
Power of performed test with alpha = 0.0500: for Day x Antigen : 0.0500

Least square means for Day :

Group	Mean
1.000	0.000
9.000	4.290
16.000	4.457
23.000	5.167
30.000	5.167
34.000	5.333
42.000	6.167
48.000	6.333
55.000	6.167

Std Err of LS Mean = 0.281

Least square means for Antigen :

Group	Mean
MG	5.129
SG	4.444

Std Err of LS Mean = 0.133

Least square means for Day x Antigen :

Group	Mean

1.000 x MG	0.000
1.000 x SG	0.000
9.000 x MG	4.580
9.000 x SG	4.000
16.000 x MG	4.580
16.000 x SG	4.333
23.000 x MG	5.667
23.000 x SG	4.667
30.000 x MG	5.667
30.000 x SG	4.667
34.000 x MG	5.667
34.000 x SG	5.000
42.000 x MG	6.333
42.000 x SG	6.000
48.000 x MG	6.667
48.000 x SG	6.000
55.000 x MG	7.000
55.000 x SG	5.333

Std Err of LS Mean = 0.398

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **Day**

Comparison	**Diff of Means**		**t**	**P**	**P<0.050**
48.000 vs. 1.000	6.333	15.927	<0.001	Yes	
55.000 vs. 1.000	6.167	15.508	<0.001	Yes	
42.000 vs. 1.000	6.167	15.508	<0.001	Yes	
34.000 vs. 1.000	5.333	13.412	<0.001	Yes	
30.000 vs. 1.000	5.167	12.993	<0.001	Yes	
23.000 vs. 1.000	5.167	12.993	<0.001	Yes	
16.000 vs. 1.000	4.457	11.208	<0.001	Yes	
9.000 vs. 1.000	4.290	10.789	<0.001	Yes	
48.000 vs. 9.000	2.043	5.138	<0.001	Yes	
48.000 vs. 16.000	1.877	4.719	<0.001	Yes	
42.000 vs. 9.000	1.877	4.719	<0.001	Yes	
55.000 vs. 9.000	1.877	4.719	<0.001	Yes	
55.000 vs. 16.000	1.710	4.300	0.003	Yes	
42.000 vs. 16.000	1.710	4.300	0.003	Yes	
48.000 vs. 30.000	1.167	2.934	0.120	No	
48.000 vs. 23.000	1.167	2.934	0.115	No	
34.000 vs. 9.000	1.043	2.624	0.225	No	
48.000 vs. 34.000	1.000	2.515	0.271	No	
42.000 vs. 23.000	1.000	2.515	0.259	No	
42.000 vs. 30.000	1.000	2.515	0.247	No	
55.000 vs. 23.000	1.000	2.515	0.234	No	
55.000 vs. 30.000	1.000	2.515	0.221	No	
34.000 vs. 16.000	0.877	2.205	0.384	No	
30.000 vs. 9.000	0.877	2.205	0.362	No	
23.000 vs. 9.000	0.877	2.205	0.339	No	
42.000 vs. 34.000	0.833	2.096	0.385	No	
55.000 vs. 34.000	0.833	2.096	0.357	No	
23.000 vs. 16.000	0.710	1.785	0.540	No	
30.000 vs. 16.000	0.710	1.785	0.498	No	
34.000 vs. 23.000	0.167	0.419	1.000	No	
16.000 vs. 9.000	0.167	0.419	0.999	No	
34.000 vs. 30.000	0.167	0.419	0.997	No	
48.000 vs. 55.000	0.167	0.419	0.989	No	
48.000 vs. 42.000	0.167	0.419	0.966	No	
23.000 vs. 30.000	0.000	0.000	1.000	No	
42.000 vs. 55.000	0.000	0.000	1.000	No	

Comparisons for factor: **Antigen**

Comparison	**Diff of Means**		**t**	**P**	**P<0.050**
MG vs. SG	0.684	3.651	<0.001	Yes	

Two Way Analysis of Variance Friday, May 15, 2020, 12:50:28 PM

Data source: 2335 Ttests in Titer ANOVAs w. transformed data Graphs w. stdev

Balanced Design

Dependent Variable: Data

Normality Test (Shapiro-Wilk): Failed (P < 0.050)

Equal Variance Test (Brown-Forsythe): Passed (P = 1.000)

Source of Variation		DF	SS	MS	F	P
Day	8	155.593	19.449	38.898	<0.001	
Antigen	1	2.667	2.667	5.333	0.027	
Day x Antigen		8	3.667	0.458	0.917	0.514
Residual	36	18.000	0.500			
Total	53	179.926	3.395			

The difference in the mean values among the different levels of Day is greater than would be expected by chance after allowing for effects of differences in Antigen. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Antigen is greater than would be expected by chance after allowing for effects of differences in Day. There is a statistically significant difference (P = 0.027). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of Day does not depend on what level of Antigen is present. There is not a statistically significant interaction between Day and Antigen. (P = 0.514)

Power of performed test with alpha = 0.0500: for Day : 1.000
Power of performed test with alpha = 0.0500: for Antigen : 0.526
Power of performed test with alpha = 0.0500: for Day x Antigen : 0.0500

Least square means for Day :

Group	Mean
1.000	0.000
9.000	3.000
16.000	3.333
23.000	3.333
30.000	4.500
34.000	5.667
42.000	5.500
48.000	5.000
55.000	5.333

Std Err of LS Mean = 0.289

Least square means for Antigen :

Group	Mean
MG	3.741
SG	4.185

Std Err of LS Mean = 0.136

Least square means for Day x Antigen :

Group	Mean
1.000 x MG	0.000
1.000 x SG	0.000
9.000 x MG	2.667
9.000 x SG	3.333
16.000 x MG	2.667
16.000 x SG	4.000
23.000 x MG	2.667
23.000 x SG	4.000
30.000 x MG	4.333
30.000 x SG	4.667
34.000 x MG	5.667
34.000 x SG	5.667

42.000 x MG	5.333
42.000 x SG	5.667
48.000 x MG	5.000
48.000 x SG	5.000
55.000 x MG	5.333
55.000 x SG	5.333

Std Err of LS Mean = 0.408

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **Day**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
34.000 vs. 1.000	5.667	13.880	<0.001	Yes
42.000 vs. 1.000	5.500	13.472	<0.001	Yes
55.000 vs. 1.000	5.333	13.064	<0.001	Yes
48.000 vs. 1.000	5.000	12.247	<0.001	Yes
30.000 vs. 1.000	4.500	11.023	<0.001	Yes
16.000 vs. 1.000	3.333	8.165	<0.001	Yes
23.000 vs. 1.000	3.333	8.165	<0.001	Yes
9.000 vs. 1.000	3.000	7.348	<0.001	Yes
34.000 vs. 9.000	2.667	6.532	<0.001	Yes
42.000 vs. 9.000	2.500	6.124	<0.001	Yes
34.000 vs. 23.000	2.333	5.715	<0.001	Yes
34.000 vs. 16.000	2.333	5.715	<0.001	Yes
55.000 vs. 9.000	2.333	5.715	<0.001	Yes
42.000 vs. 23.000	2.167	5.307	<0.001	Yes
42.000 vs. 16.000	2.167	5.307	<0.001	Yes
48.000 vs. 9.000	2.000	4.899	<0.001	Yes
55.000 vs. 23.000	2.000	4.899	<0.001	Yes
55.000 vs. 16.000	2.000	4.899	<0.001	Yes
48.000 vs. 23.000	1.667	4.082	0.004	Yes
48.000 vs. 16.000	1.667	4.082	0.004	Yes
30.000 vs. 9.000	1.500	3.674	0.012	Yes
30.000 vs. 23.000	1.167	2.858	0.101	No
30.000 vs. 16.000	1.167	2.858	0.094	No
34.000 vs. 30.000	1.167	2.858	0.088	No
42.000 vs. 30.000	1.000	2.449	0.209	No
55.000 vs. 30.000	0.833	2.041	0.422	No
34.000 vs. 48.000	0.667	1.633	0.692	No
48.000 vs. 30.000	0.500	1.225	0.903	No
42.000 vs. 48.000	0.500	1.225	0.875	No
34.000 vs. 55.000	0.333	0.816	0.978	No
55.000 vs. 48.000	0.333	0.816	0.962	No
23.000 vs. 9.000	0.333	0.816	0.934	No
16.000 vs. 9.000	0.333	0.816	0.887	No
34.000 vs. 42.000	0.167	0.408	0.969	No
42.000 vs. 55.000	0.167	0.408	0.901	No
23.000 vs. 16.000	0.000	0.000	1.000	No

Comparisons for factor: **Antigen**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
SG vs. MG	0.444	2.309	0.027	Yes

Two Way Analysis of Variance Friday, May 15, 2020, 12:43:36 PM

Data source: 2324 Ttests in Titer ANOVAs w. transformed data Graphs w. stdev

Balanced Design

Dependent Variable: Data

Normality Test (Shapiro-Wilk): Failed (P < 0.050)

Equal Variance Test (Brown-Forsythe): Failed (P < 0.050)

Source of Variation		DF	SS	MS	F	P
Day	8	129.481	16.185	33.615	<0.001	
Antigen	1	2.667	2.667	5.538	0.024	
Day x Antigen		8	4.000	0.500	1.038	0.426
Residual	36	17.333	0.481			
Total	53	153.481	2.896			

The difference in the mean values among the different levels of Day is greater than would be expected by chance after allowing for effects of differences in Antigen. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Antigen is greater than would be expected by chance after allowing for effects of differences in Day. There is a statistically significant difference (P = 0.024). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of Day does not depend on what level of Antigen is present. There is not a statistically significant interaction between Day and Antigen. (P = 0.426)

Power of performed test with alpha = 0.0500: for Day : 1.000
Power of performed test with alpha = 0.0500: for Antigen : 0.545
Power of performed test with alpha = 0.0500: for Day x Antigen : 0.0585

Least square means for Day :

Group	Mean
1.000	0.333
9.000	4.000
16.000	4.333
23.000	4.833
30.000	5.333
34.000	5.167
42.000	5.500
48.000	5.500
55.000	5.333

Std Err of LS Mean = 0.283

Least square means for Antigen :

Group	Mean
MG	4.259
SG	4.704

Std Err of LS Mean = 0.134

Least square means for Day x Antigen :

Group	Mean
1.000 x MG	0.667
1.000 x SG	0.000
9.000 x MG	3.667
9.000 x SG	4.333
16.000 x MG	4.000
16.000 x SG	4.667
23.000 x MG	4.333
23.000 x SG	5.333
30.000 x MG	4.667
30.000 x SG	6.000
34.000 x MG	5.000
34.000 x SG	5.333
42.000 x MG	5.333
42.000 x SG	5.667
48.000 x MG	5.333
48.000 x SG	5.667
55.000 x MG	5.333
55.000 x SG	5.333

Std Err of LS Mean = 0.401

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **Day**

Comparison	Diff of Means	t	P	P<0.050

48.000 vs. 1.000	5.167	12.897	<0.001	Yes	
42.000 vs. 1.000	5.167	12.897	<0.001	Yes	
55.000 vs. 1.000	5.000	12.481	<0.001	Yes	
30.000 vs. 1.000	5.000	12.481	<0.001	Yes	
34.000 vs. 1.000	4.833	12.065	<0.001	Yes	
23.000 vs. 1.000	4.500	11.233	<0.001	Yes	
16.000 vs. 1.000	4.000	9.985	<0.001	Yes	
9.000 vs. 1.000	3.667	9.153	<0.001	Yes	
48.000 vs. 9.000	1.500	3.744	0.018	Yes	
42.000 vs. 9.000	1.500	3.744	0.017	Yes	
30.000 vs. 9.000	1.333	3.328	0.051	No	
55.000 vs. 9.000	1.333	3.328	0.049	Yes	
42.000 vs. 16.000	1.167	2.91 2	0.137	No	
48.000 vs. 16.000	1.167	2.912	0.132	No	
34.000 vs. 9.000	1.167	2.912	0.126	No	
30.000 vs. 16.000	1.000	2.496	0.306	No	
55.000 vs. 16.000	1.000	2.496	0.294	No	
23.000 vs. 9.000	0.833	2.080	0.581	No	
34.000 vs. 16.000	0.833	2.080	0.561	No	
42.000 vs. 23.000	0.667	1.664	0.848	No	
48.000 vs. 23.000	0.667	1.664	0.830	No	
30.000 vs. 23.000	0.500	1.248	0.976	No	
55.000 vs. 23.000	0.500	1.248	0.969	No	
23.000 vs. 16.000	0.500	1.248	0.960	No	
16.000 vs. 9.000	0.333	0.832	0.998	No	
42.000 vs. 34.000	0.333	0.832	0.997	No	
48.000 vs. 34.000	0.333	0.832	0.995	No	
34.000 vs. 23.000	0.333	0.832	0.991	No	
48.000 vs. 55.000	0.167	0.416	1.000	No	
30.000 vs. 34.000	0.167	0.416	1.000	No	
42.000 vs. 55.000	0.167	0.416	0.999	No	
55.000 vs. 34.000	0.167	0.416	0.997	No	
42.000 vs. 30.000	0.167	0.416	0.989	No	
48.000 vs. 30.000	0.167	0.416	0.967	No	
30.000 vs. 55.000	8.882E-016		2.217E-015		1.000 No
48.000 vs. 42.000	0.000	0.000	1.000	No	

Comparisons for factor: **Antigen**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
SG vs. MG	0.444	2.353	0.024	Yes

Two Way Analysis of Variance Friday, May 15, 2020, 12:53:29 PM

Data source: 2468 Ttests in Titer ANOVAs w. transformed data Graphs w. stdev

Balanced Design

Dependent Variable: Data

Normality Test (Shapiro-Wilk): Failed (P < 0.050)

Equal Variance Test (Brown-Forsythe): Passed (P = 1.000)

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Day	8	158.333	19.792	48.580	<0.001
Antigen	1	1.500	1.500	3.682	0.063
Day x Antigen	8	2.333	0.292	0.716	0.676
Residual	36	14.667	0.407		
Total	53	176.833	3.336		

The difference in the mean values among the different levels of Day is greater than would be expected by chance after allowing for effects of differences in Antigen. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Antigen is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Day. There is not a statistically significant difference (P = 0.063).

The effect of different levels of Day does not depend on what level of Antigen is present. There is not a statistically significant interaction between Day and Antigen. (P = 0.676)

Power of performed test with alpha = 0.0500: for Day : 1.000
Power of performed test with alpha = 0.0500: for Antigen : 0.357
Power of performed test with alpha = 0.0500: for Day x Antigen : 0.0500

Least square means for Day :

Group	**Mean**
1.000	0.000
9.000	3.667
16.000	3.667
23.000	5.000
30.000	5.167
34.000	5.167
42.000	5.667
48.000	5.500
55.000	5.667

Std Err of LS Mean = 0.261

Least square means for Antigen :

Group	**Mean**
MG	4.222
SG	4.556

Std Err of LS Mean = 0.123

Least square means for Day x Antigen :

Group	**Mean**
1.000 x MG	0.000
1.000 x SG	0.000
9.000 x MG	3.667
9.000 x SG	3.667
16.000 x MG	3.667
16.000 x SG	3.667
23.000 x MG	4.667
23.000 x SG	5.333
30.000 x MG	4.667
30.000 x SG	5.667
34.000 x MG	4.667
34.000 x SG	5.667
42.000 x MG	5.667
42.000 x SG	5.667
48.000 x MG	5.333
48.000 x SG	5.667
55.000 x MG	5.667
55.000 x SG	5.667

Std Err of LS Mean = 0.369

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **Day**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
42.000 vs. 1.000	5.667	15.377	<0.001	Yes
55.000 vs. 1.000	5.667	15.377	<0.001	Yes
48.000 vs. 1.000	5.500	14.925	<0.001	Yes
30.000 vs. 1.000	5.167	14.020	<0.001	Yes
34.000 vs. 1.000	5.167	14.020	<0.001	Yes
23.000 vs. 1.000	5.000	13.568	<0.001	Yes
16.000 vs. 1.000	3.667	9.950	<0.001	Yes
9.000 vs. 1.000	3.667	9.950	<0.001	Yes
42.000 vs. 9.000	2.000	5.427	<0.001	Yes
55.000 vs. 9.000	2.000	5.427	<0.001	Yes
55.000 vs. 16.000	2.000	5.427	<0.001	Yes
42.000 vs. 16.000	2.000	5.427	<0.001	Yes
48.000 vs. 9.000	1.833	4.975	<0.001	Yes
48.000 vs. 16.000	1.833	4.975	<0.001	Yes
30.000 vs. 16.000	1.500	4.070	0.005	Yes
34.000 vs. 16.000	1.500	4.070	0.005	Yes

30.000 vs. 9.000	1.500	4.070	0.005	Yes
34.000 vs. 9.000	1.500	4.070	0.005	Yes
23.000 vs. 9.000	1.333	3.618	0.016	Yes
23.000 vs. 16.000	1.333	3.618	0.015	Yes
55.000 vs. 23.000	0.667	1.809	0.731	No
42.000 vs. 23.000	0.667	1.809	0.708	No
55.000 vs. 30.000	0.500	1.357	0.941	No
42.000 vs. 30.000	0.500	1.357	0.928	No
55.000 vs. 34.000	0.500	1.357	0.912	No
42.000 vs. 34.000	0.500	1.357	0.892	No
48.000 vs. 23.000	0.500	1.357	0.868	No
48.000 vs. 34.000	0.333	0.905	0.985	No
48.000 vs. 30.000	0.333	0.905	0.976	No
30.000 vs. 23.000	0.167	0.452	0.999	No
42.000 vs. 48.000	0.167	0.452	0.998	No
34.000 vs. 23.000	0.167	0.452	0.995	No
55.000 vs. 48.000	0.167	0.452	0.986	No
42.000 vs. 55.000	0.000	0.000	1.000	No
16.000 vs. 9.000	0.000	0.000	1.000	No
34.000 vs. 30.000	0.000	0.000	1.000	No

Appendix 3.1

ELISA ANALYSIS

TRIAL 1

Two Way Analysis of Variance 3342 DSG Friday, January 28, 2022, 2:00:35 AM

Data source: Data 1 in Notebook1

Balanced Design

Dependent Variable: DATA

Normality Test (Shapiro-Wilk): Failed (P < 0.050)

Equal Variance Test (Brown-Forsythe): Passed (P = 1.000)

Source of Variation	DF	SS	MS	F	P
DAYS	8	154.000	19.250	51.975	<0.001
ANTIGEN	1	6.000	6.000	16.200	<0.001
DAYS x ANTIGEN	8	8.000	1.000	2.700	0.019
Residual	36	13.333	0.370		
Total	53	181.333	3.421		

Main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor.

The effect of different levels of DAYS depends on what level of ANTIGEN is present. There is a statistically significant interaction between DAYS and ANTIGEN. (P = 0.019)

Power of performed test with alpha = 0.0500: for DAYS : 1.000
Power of performed test with alpha = 0.0500: for ANTIGEN : 0.967
Power of performed test with alpha = 0.0500: for DAYS x ANTIGEN : 0.643

Least square means for DAYS :

Group	Mean
0.000	0.000
7.000	4.500
14.000	4.667
21.000	4.333
28.000	5.333
35.000	5.333
42.000	5.167
49.000	5.833
69.000	5.833

Std Err of LS Mean = 0.248

Least square means for ANTIGEN :

Group	Mean
SG	4.889
MG	4.222

Std Err of LS Mean = 0.117

Least square means for DAYS x ANTIGEN :

Group	Mean
0.000 x SG	0.000
0.000 x MG	0.000
7.000 x SG	4.000
7.000 x MG	5.000
14.000 x SG	5.000
14.000 x MG	4.333
21.000 x SG	4.667

21.000 x MG	4.000
28.000 x SG	5.667
28.000 x MG	5.000
35.000 x SG	5.667
35.000 x MG	5.000
42.000 x SG	5.667
42.000 x MG	4.667
49.000 x SG	6.667
49.000 x MG	5.000
69.000 x SG	6.667
69.000 x MG	5.000

Std Err of LS Mean = 0.351

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **DAYS**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
49.000 vs. 0.000	5.833	16.602	<0.001	Yes
69.000 vs. 0.000	5.833	16.602	<0.001	Yes
28.000 vs. 0.000	5.333	15.179	<0.001	Yes
35.000 vs. 0.000	5.333	15.179	<0.001	Yes
42.000 vs. 0.000	5.167	14.705	<0.001	Yes
14.000 vs. 0.000	4.667	13.282	<0.001	Yes
7.000 vs. 0.000	4.500	12.807	<0.001	Yes
21.000 vs. 0.000	4.333	12.333	<0.001	Yes
49.000 vs. 21.000	1.500	4.269	0.004	Yes
69.000 vs. 21.000	1.500	4.269	0.004	Yes
49.000 vs. 7.000	1.333	3.795	0.014	Yes
69.000 vs. 7.000	1.333	3.795	0.014	Yes
69.000 vs. 14.000	1.167	3.320	0.048	Yes
49.000 vs. 14.000	1.167	3.320	0.046	Yes
28.000 vs. 21.000	1.000	2.846	0.148	No
35.000 vs. 21.000	1.000	2.846	0.142	No
28.000 vs. 7.000	0.833	2.372	0.374	No
35.000 vs. 7.000	0.833	2.372	0.359	No
42.000 vs. 21.000	0.833	2.372	0.344	No
35.000 vs. 14.000	0.667	1.897	0.686	No
28.000 vs. 14.000	0.667	1.897	0.664	No
69.000 vs. 42.000	0.667	1.897	0.640	No
42.000 vs. 7.000	0.667	1.897	0.615	No
49.000 vs. 42.000	0.667	1.897	0.587	No
42.000 vs. 14.000	0.500	1.423	0.882	No
69.000 vs. 28.000	0.500	1.423	0.859	No
49.000 vs. 35.000	0.500	1.423	0.832	No
69.000 vs. 35.000	0.500	1.423	0.799	No
49.000 vs. 28.000	0.500	1.423	0.760	No
14.000 vs. 21.000	0.333	0.949	0.951	No
28.000 vs. 42.000	0.167	0.474	0.998	No
14.000 vs. 7.000	0.167	0.474	0.994	No
35.000 vs. 42.000	0.167	0.474	0.983	No
7.000 vs. 21.000	0.167	0.474	0.953	No
49.000 vs. 69.000	0.000	0.000	1.000	No
35.000 vs. 28.000	0.000	0.000	1.000	No

Comparisons for factor: **ANTIGEN**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
SG vs. MG	0.667	4.025	<0.001	Yes

Comparisons for factor: **ANTIGEN within 0**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
SG vs. MG	0.000	0.000	1.000	No

Comparisons for factor: **ANTIGEN within 7**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
MG vs. SG	1.000	2.012	0.052	No

Comparisons for factor: **ANTIGEN within 14**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
SG vs. MG	0.667	1.342	0.188	No

Comparisons for factor: **ANTIGEN within 21**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
SG vs. MG	0.667	1.342	0.188	No

Comparisons for factor: **ANTIGEN within 28**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
SG vs. MG	0.667	1.342	0.188	No

Comparisons for factor: **ANTIGEN within 35**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
SG vs. MG	0.667	1.342	0.188	No

Comparisons for factor: **ANTIGEN within 42**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
SG vs. MG	1.000	2.012	0.052	No

Comparisons for factor: **ANTIGEN within 49**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
SG vs. MG	1.667	3.354	0.002	Yes

Comparisons for factor: **ANTIGEN within 69**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
SG vs. MG	1.667	3.354	0.002	Yes

Comparisons for factor: **DAYS within SG**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
49.000 vs. 0.000	6.667	13.416	<0.001	Yes
69.000 vs. 0.000	6.667	13.416	<0.001	Yes
28.000 vs. 0.000	5.667	11.404	<0.001	Yes
42.000 vs. 0.000	5.667	11.404	<0.001	Yes
35.000 vs. 0.000	5.667	11.404	<0.001	Yes
14.000 vs. 0.000	5.000	10.062	<0.001	Yes
21.000 vs. 0.000	4.667	9.391	<0.001	Yes
7.000 vs. 0.000	4.000	8.050	<0.001	Yes
49.000 vs. 7.000	2.667	5.367	<0.001	Yes
69.000 vs. 7.000	2.667	5.367	<0.001	Yes
49.000 vs. 21.000	2.000	4.025	0.007	Yes
69.000 vs. 21.000	2.000	4.025	0.007	Yes
49.000 vs. 14.000	1.667	3.354	0.044	Yes
28.000 vs. 7.000	1.667	3.354	0.042	Yes
35.000 vs. 7.000	1.667	3.354	0.041	Yes
42.000 vs. 7.000	1.667	3.354	0.039	Yes
69.000 vs. 14.000	1.667	3.354	0.037	Yes
49.000 vs. 28.000	1.000	2.012	0.635	No
14.000 vs. 7.000	1.000	2.012	0.615	No
42.000 vs. 21.000	1.000	2.012	0.594	No
28.000 vs. 21.000	1.000	2.012	0.572	No
35.000 vs. 21.000	1.000	2.012	0.549	No
49.000 vs. 42.000	1.000	2.012	0.524	No
69.000 vs. 42.000	1.000	2.012	0.498	No
69.000 vs. 35.000	1.000	2.012	0.471	No
49.000 vs. 35.000	1.000	2.012	0.442	No
69.000 vs. 28.000	1.000	2.012	0.412	No
21.000 vs. 7.000	0.667	1.342	0.847	No
28.000 vs. 14.000	0.667	1.342	0.811	No
42.000 vs. 14.000	0.667	1.342	0.767	No
35.000 vs. 14.000	0.667	1.342	0.714	No

14.000 vs. 21.000	0.333	0.671	0.971	No
42.000 vs. 28.000	0.000	0.000	1.000	No
35.000 vs. 42.000	0.000	0.000	1.000	No
35.000 vs. 28.000	0.000	0.000	1.000	No
49.000 vs. 69.000	0.000	0.000	1.000	No

Comparisons for factor: **DAYS within MG**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
7.000 vs. 0.000	5.000	10.062	<0.001	Yes
69.000 vs. 0.000	5.000	10.062	<0.001	Yes
28.000 vs. 0.000	5.000	10.062	<0.001	Yes
35.000 vs. 0.000	5.000	10.062	<0.001	Yes
49.000 vs. 0.000	5.000	10.062	<0.001	Yes
42.000 vs. 0.000	4.667	9.391	<0.001	Yes
14.000 vs. 0.000	4.333	8.721	<0.001	Yes
21.000 vs. 0.000	4.000	8.050	<0.001	Yes
69.000 vs. 21.000	1.000	2.012	0.774	No
7.000 vs. 21.000	1.000	2.012	0.761	No
28.000 vs. 21.000	1.000	2.012	0.748	No
35.000 vs. 21.000	1.000	2.012	0.735	No
49.000 vs. 21.000	1.000	2.012	0.720	No
28.000 vs. 14.000	0.667	1.342	0.992	No
42.000 vs. 21.000	0.667	1.342	0.990	No
69.000 vs. 14.000	0.667	1.342	0.987	No
7.000 vs. 14.000	0.667	1.342	0.985	No
35.000 vs. 14.000	0.667	1.342	0.981	No
49.000 vs. 14.000	0.667	1.342	0.977	No
14.000 vs. 21.000	0.333	0.671	1.000	No
42.000 vs. 14.000	0.333	0.671	1.000	No
49.000 vs. 42.000	0.333	0.671	1.000	No
7.000 vs. 42.000	0.333	0.671	1.000	No
35.000 vs. 42.000	0.333	0.671	1.000	No
28.000 vs. 42.000	0.333	0.671	1.000	No
69.000 vs. 42.000	0.333	0.671	1.000	No
49.000 vs. 7.000	0.000	0.000	1.000	No
35.000 vs. 7.000	0.000	0.000	1.000	No
35.000 vs. 69.000	0.000	0.000	1.000	No
28.000 vs. 49.000	0.000	0.000	1.000	No
28.000 vs. 69.000	0.000	0.000	1.000	No
28.000 vs. 7.000	0.000	0.000	1.000	No
69.000 vs. 7.000	0.000	0.000	1.000	No
28.000 vs. 35.000	0.000	0.000	1.000	No
49.000 vs. 69.000	0.000	0.000	1.000	No
49.000 vs. 35.000	0.000	0.000	1.000	No

Two Way Analysis of Variance 3248 NSG Friday, January 28, 2022, 2:18:26 AM

Data source: Data 2 in Notebook1

Balanced Design

Dependent Variable: DATA

Normality Test (Shapiro-Wilk): Failed (P < 0.050)

Equal Variance Test (Brown-Forsythe): Passed (P = 1.000)

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
DAYS	8	94.815	11.852	40.000	<0.001
ANTIGEN	1	0.167	0.167	0.562	0.458
DAYS x ANTIGEN	8	2.000	0.250	0.844	0.571
Residual	36	10.667	0.296		
Total	53	107.648	2.031		

The difference in the mean values among the different levels of DAYS is greater than would be expected by chance after allowing for effects of differences in ANTIGEN. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of ANTIGEN is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in DAYS. There is not a statistically significant difference (P = 0.458).

The effect of different levels of DAYS does not depend on what level of ANTIGEN is present. There is not a statistically significant interaction between DAYS and ANTIGEN. (P = 0.571)

Power of performed test with alpha = 0.0500: for DAYS : 1.000
Power of performed test with alpha = 0.0500: for ANTIGEN : 0.0500
Power of performed test with alpha = 0.0500: for DAYS x ANTIGEN : 0.0500

Least square means for DAYS :

Group	Mean
0.000	0.000
7.000	2.667
14.000	2.667
21.000	4.333
28.000	4.167
35.000	3.833
42.000	3.833
49.000	4.500
69.000	3.833

Std Err of LS Mean = 0.222

Least square means for ANTIGEN :

Group	Mean
SG	3.259
MG	3.370

Std Err of LS Mean = 0.105

Least square means for DAYS x ANTIGEN :

Group	Mean
0.000 x SG	0.000
0.000 x MG	0.000
7.000 x SG	2.333
7.000 x MG	3.000
14.000 x SG	2.667
14.000 x MG	2.667
21.000 x SG	4.000
21.000 x MG	4.667
28.000 x SG	4.333
28.000 x MG	4.000
35.000 x SG	4.000
35.000 x MG	3.667
42.000 x SG	3.667
42.000 x MG	4.000
49.000 x SG	4.333
49.000 x MG	4.667
69.000 x SG	4.000
69.000 x MG	3.667

Std Err of LS Mean = 0.314

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **DAYS**

Comparison	Diff of Means	t	P	P<0.050
49.000 vs. 0.000	4.500	14.319	<0.001	Yes
21.000 vs. 0.000	4.333	13.789	<0.001	Yes
28.000 vs. 0.000	4.167	13.258	<0.001	Yes
69.000 vs. 0.000	3.833	12.198	<0.001	Yes
42.000 vs. 0.000	3.833	12.198	<0.001	Yes
35.000 vs. 0.000	3.833	12.198	<0.001	Yes
7.000 vs. 0.000	2.667	8.485	<0.001	Yes
14.000 vs. 0.000	2.667	8.485	<0.001	Yes
49.000 vs. 14.000	1.833	5.834	<0.001	Yes
49.000 vs. 7.000	1.833	5.834	<0.001	Yes
21.000 vs. 14.000	1.667	5.303	<0.001	Yes

21.000 vs. 7.000	1.667	5.303	<0.001	Yes
28.000 vs. 14.000	1.500	4.773	<0.001	Yes
28.000 vs. 7.000	1.500	4.773	<0.001	Yes
69.000 vs. 14.000	1.167	3.712	0.015	Yes
42.000 vs. 14.000	1.167	3.712	0.014	Yes
35.000 vs. 14.000	1.167	3.712	0.014	Yes
69.000 vs. 7.000	1.167	3.712	0.013	Yes
42.000 vs. 7.000	1.167	3.712	0.012	Yes
35.000 vs. 7.000	1.167	3.712	0.012	Yes
49.000 vs. 69.000	0.667	2.121	0.487	No
49.000 vs. 42.000	0.667	2.121	0.465	No
49.000 vs. 35.000	0.667	2.121	0.442	No
21.000 vs. 35.000	0.500	1.591	0.811	No
21.000 vs. 42.000	0.500	1.591	0.785	No
21.000 vs. 69.000	0.500	1.591	0.756	No
49.000 vs. 28.000	0.333	1.061	0.970	No
28.000 vs. 42.000	0.333	1.061	0.957	No
28.000 vs. 35.000	0.333	1.061	0.940	No
28.000 vs. 69.000	0.333	1.061	0.914	No
21.000 vs. 28.000	0.167	0.530	0.996	No
49.000 vs. 21.000	0.167	0.530	0.990	No
42.000 vs. 69.000	0.000	0.000	1.000	No
7.000 vs. 14.000	4.441E-016	1.413E-015	1.000	No
35.000 vs. 69.000	0.000	0.000	1.000	No
35.000 vs. 42.000	0.000	0.000	1.000	No

Comparisons for factor: **ANTIGEN**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
MG vs. SG	0.111	0.750	0.458	No

Two Way Analysis of Variance Friday, January 28, 2022, 2:29:40 AM

Data source: 3371 DMG DATA in Notebook1

Balanced Design

Dependent Variable: DATA

Normality Test (Shapiro-Wilk): Failed (P < 0.050)

Equal Variance Test (Brown-Forsythe): Passed (P = 1.000)

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
DAYS	8	475.815	59.477	458.821	<0.001
ANTIGEN	1	0.0185	0.0185	0.143	0.708
DAYS x ANTIGEN	8	1.148	0.144	1.107	0.381
Residual	36	4.667	0.130		
Total	53	481.648	9.088		

The difference in the mean values among the different levels of DAYS is greater than would be expected by chance after allowing for effects of differences in ANTIGEN. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of ANTIGEN is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in DAYS. There is not a statistically significant difference (P = 0.708).

The effect of different levels of DAYS does not depend on what level of ANTIGEN is present. There is not a statistically significant interaction between DAYS and ANTIGEN. (P = 0.381)

Power of performed test with alpha = 0.0500: for DAYS : 1.000
Power of performed test with alpha = 0.0500: for ANTIGEN : 0.0500
Power of performed test with alpha = 0.0500: for DAYS x ANTIGEN : 0.0749

Least square means for DAYS :

Group	**Mean**
0.000	0.000
7.000	0.000

14.000	0.000
21.000	0.000
28.000	5.833
35.000	5.833
42.000	5.833
49.000	6.000
69.000	6.333

Std Err of LS Mean = 0.147

Least square means for ANTIGEN :

Group	**Mean**
SG	3.333
MG	3.296

Std Err of LS Mean = 0.0693

Least square means for DAYS x ANTIGEN :

Group	**Mean**
0.000 x SG	0.000
0.000 x MG	0.000
7.000 x SG	0.000
7.000 x MG	0.000
14.000 x SG	0.000
14.000 x MG	0.000
21.000 x SG	0.000
21.000 x MG	0.000
28.000 x SG	6.000
28.000 x MG	5.667
35.000 x SG	6.000
35.000 x MG	5.667
42.000 x SG	6.000
42.000 x MG	5.667
49.000 x SG	6.000
49.000 x MG	6.000
69.000 x SG	6.000
69.000 x MG	6.667

Std Err of LS Mean = 0.208

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **DAYS**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
69.000 vs. 7.000	6.333	30.468	<0.001	Yes
69.000 vs. 21.000	6.333	30.468	<0.001	Yes
69.000 vs. 0.000	6.333	30.468	<0.001	Yes
69.000 vs. 14.000	6.333	30.468	<0.001	Yes
49.000 vs. 14.000	6.000	28.864	<0.001	Yes
49.000 vs. 7.000	6.000	28.864	<0.001	Yes
49.000 vs. 21.000	6.000	28.864	<0.001	Yes
49.000 vs. 0.000	6.000	28.864	<0.001	Yes
35.000 vs. 21.000	5.833	28.062	<0.001	Yes
42.000 vs. 7.000	5.833	28.062	<0.001	Yes
35.000 vs. 0.000	5.833	28.062	<0.001	Yes
35.000 vs. 14.000	5.833	28.062	<0.001	Yes
28.000 vs. 21.000	5.833	28.062	<0.001	Yes
28.000 vs. 7.000	5.833	28.062	<0.001	Yes
28.000 vs. 14.000	5.833	28.062	<0.001	Yes
28.000 vs. 0.000	5.833	28.062	<0.001	Yes
35.000 vs. 7.000	5.833	28.062	<0.001	Yes
42.000 vs. 21.000	5.833	28.062	<0.001	Yes
42.000 vs. 0.000	5.833	28.062	<0.001	Yes
42.000 vs. 14.000	5.833	28.062	<0.001	Yes
69.000 vs. 28.000	0.500	2.405	0.293	No
69.000 vs. 35.000	0.500	2.405	0.277	No
69.000 vs. 42.000	0.500	2.405	0.262	No
69.000 vs. 49.000	0.333	1.604	0.803	No
49.000 vs. 35.000	0.167	0.802	0.999	No
49.000 vs. 42.000	0.167	0.802	0.998	No
49.000 vs. 28.000	0.167	0.802	0.996	No

42.000 vs. 28.000	0.000	0.000	1.000	No
21.000 vs. 14.000	0.000	0.000	1.000	No
21.000 vs. 0.000	0.000	0.000	1.000	No
7.000 vs. 21.000	0.000	0.000	1.000	No
14.000 vs. 0.000	0.000	0.000	1.000	No
7.000 vs. 14.000	0.000	0.000	1.000	No
7.000 vs. 0.000	0.000	0.000	1.000	No
42.000 vs. 35.000	0.000	0.000	1.000	No
35.000 vs. 28.000	0.000	0.000	1.000	No

Comparisons for factor: **ANTIGEN**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
SG vs. MG	0.0370	0.378	0.708	No

Two Way Analysis of Variance Friday, January 28, 2022, 2:36:27 AM

Data source: 3241J DMG DATA in Notebook1

Balanced Design

Dependent Variable: DATA

Normality Test (Shapiro-Wilk): Failed (P < 0.050)

Equal Variance Test (Brown-Forsythe): Passed (P = 1.000)

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
DAYS	8	130.333	16.292	97.750	<0.001
ANTIGEN	1	0.0741	0.0741	0.444	0.509
DAYS x ANTIGEN	8	2.926	0.366	2.194	0.051
Residual	36	6.000	0.167		
Total	53	139.333	2.629		

The difference in the mean values among the different levels of DAYS is greater than would be expected by chance after allowing for effects of differences in ANTIGEN. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of ANTIGEN is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in DAYS. There is not a statistically significant difference (P = 0.509).

The effect of different levels of DAYS does not depend on what level of ANTIGEN is present. There is not a statistically significant interaction between DAYS and ANTIGEN. (P = 0.051)

Power of performed test with alpha = 0.0500: for DAYS : 1.000
Power of performed test with alpha = 0.0500: for ANTIGEN : 0.0500
Power of performed test with alpha = 0.0500: for DAYS x ANTIGEN : 0.465

Least square means for DAYS :

Group	**Mean**
0.000	0.000
7.000	2.500
14.000	2.833
21.000	3.000
28.000	4.167
35.000	4.167
42.000	3.833
49.000	5.833
69.000	4.667

Std Err of LS Mean = 0.167

Least square means for ANTIGEN :

Group	**Mean**
SG	3.481
MG	3.407

Std Err of LS Mean = 0.0786

Least square means for DAYS x ANTIGEN :

Group	Mean
0.000 x SG	0.000
0.000 x MG	0.000
7.000 x SG	2.000
7.000 x MG	3.000
14.000 x SG	2.667
14.000 x MG	3.000
21.000 x SG	3.000
21.000 x MG	3.000
28.000 x SG	4.333
28.000 x MG	4.000
35.000 x SG	4.333
35.000 x MG	4.000
42.000 x SG	4.000
42.000 x MG	3.667
49.000 x SG	6.000
49.000 x MG	5.667
69.000 x SG	5.000
69.000 x MG	4.333

Std Err of LS Mean = 0.236

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **DAYS**

Comparison	Diff of Means	t	P	P<0.050
49.000 vs. 0.000	5.833	24.749	<0.001	Yes
69.000 vs. 0.000	4.667	19.799	<0.001	Yes
28.000 vs. 0.000	4.167	17.678	<0.001	Yes
35.000 vs. 0.000	4.167	17.678	<0.001	Yes
42.000 vs. 0.000	3.833	16.263	<0.001	Yes
49.000 vs. 7.000	3.333	14.142	<0.001	Yes
49.000 vs. 14.000	3.000	12.728	<0.001	Yes
21.000 vs. 0.000	3.000	12.728	<0.001	Yes
49.000 vs. 21.000	2.833	12.021	<0.001	Yes
14.000 vs. 0.000	2.833	12.021	<0.001	Yes
7.000 vs. 0.000	2.500	10.607	<0.001	Yes
69.000 vs. 7.000	2.167	9.192	<0.001	Yes
49.000 vs. 42.000	2.000	8.485	<0.001	Yes
69.000 vs. 14.000	1.833	7.778	<0.001	Yes
49.000 vs. 28.000	1.667	7.071	<0.001	Yes
49.000 vs. 35.000	1.667	7.071	<0.001	Yes
35.000 vs. 7.000	1.667	7.071	<0.001	Yes
69.000 vs. 21.000	1.667	7.071	<0.001	Yes
28.000 vs. 7.000	1.667	7.071	<0.001	Yes
42.000 vs. 7.000	1.333	5.657	<0.001	Yes
28.000 vs. 14.000	1.333	5.657	<0.001	Yes
35.000 vs. 14.000	1.333	5.657	<0.001	Yes
49.000 vs. 69.000	1.167	4.950	<0.001	Yes
35.000 vs. 21.000	1.167	4.950	<0.001	Yes
28.000 vs. 21.000	1.167	4.950	<0.001	Yes
42.000 vs. 14.000	1.000	4.243	0.002	Yes
42.000 vs. 21.000	0.833	3.536	0.011	Yes
69.000 vs. 42.000	0.833	3.536	0.010	Yes
21.000 vs. 7.000	0.500	2.121	0.284	No
69.000 vs. 28.000	0.500	2.121	0.253	No
69.000 vs. 35.000	0.500	2.121	0.221	No
35.000 vs. 42.000	0.333	1.414	0.596	No
14.000 vs. 7.000	0.333	1.414	0.516	No
28.000 vs. 42.000	0.333	1.414	0.420	No
21.000 vs. 14.000	0.167	0.707	0.734	No
35.000 vs. 28.000	0.000	0.000	1.000	No

Comparisons for factor: **ANTIGEN**

Comparison	Diff of Means	t	P	P<0.050
SG vs. MG	0.0741	0.667	0.509	No

Two Way Analysis of Variance Friday, January 28, 2022, 2:42:57 AM

Data source: 3275 NMG DATA in Notebook1

Balanced Design

Dependent Variable: DATA

Normality Test (Shapiro-Wilk): Failed (P < 0.050)

Equal Variance Test (Brown-Forsythe): Passed (P = 1.000)

Source of Variation	DF	SS	MS	F	P
DAYS	8	127.259	15.907	214.750	<0.001
ANTIGEN	1	2.667	2.667	36.000	<0.001
DAYS x ANTIGEN	8	2.000	0.250	3.375	0.005
Residual	36	2.667	0.0741		
Total	53	134.593	2.539		

Main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor.

The effect of different levels of DAYS depends on what level of ANTIGEN is present. There is a statistically significant interaction between DAYS and ANTIGEN. (P = 0.005)

Power of performed test with alpha = 0.0500: for DAYS : 1.000
Power of performed test with alpha = 0.0500: for ANTIGEN : 1.000
Power of performed test with alpha = 0.0500: for DAYS x ANTIGEN : 0.814

Least square means for DAYS :

Group	Mean
1.000	0.000
7.000	2.833
14.000	3.500
21.000	3.333
28.000	5.000
35.000	3.667
42.000	3.833
49.000	5.500
69.000	5.000

Std Err of LS Mean = 0.111

Least square means for ANTIGEN :

Group	Mean
SG	3.852
MG	3.407

Std Err of LS Mean = 0.0524

Least square means for DAYS x ANTIGEN :

Group	Mean
1.000 x SG	0.000
1.000 x MG	0.000
7.000 x SG	3.000
7.000 x MG	2.667
14.000 x SG	4.000
14.000 x MG	3.000
21.000 x SG	3.667
21.000 x MG	3.000
28.000 x SG	5.000
28.000 x MG	5.000
35.000 x SG	4.000
35.000 x MG	3.333
42.000 x SG	4.000
42.000 x MG	3.667
49.000 x SG	6.000
49.000 x MG	5.000

69.000 x SG 5.000
69.000 x MG 5.000
Std Err of LS Mean = 0.157

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **DAYS**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
49.000 vs. 1.000	5.500	35.002	<0.001	Yes
69.000 vs. 1.000	5.000	31.820	<0.001	Yes
28.000 vs. 1.000	5.000	31.820	<0.001	Yes
42.000 vs. 1.000	3.833	24.395	<0.001	Yes
35.000 vs. 1.000	3.667	23.335	<0.001	Yes
14.000 vs. 1.000	3.500	22.274	<0.001	Yes
21.000 vs. 1.000	3.333	21.213	<0.001	Yes
7.000 vs. 1.000	2.833	18.031	<0.001	Yes
49.000 vs. 7.000	2.667	16.971	<0.001	Yes
28.000 vs. 7.000	2.167	13.789	<0.001	Yes
49.000 vs. 21.000	2.167	13.789	<0.001	Yes
69.000 vs. 7.000	2.167	13.789	<0.001	Yes
49.000 vs. 14.000	2.000	12.728	<0.001	Yes
49.000 vs. 35.000	1.833	11.667	<0.001	Yes
28.000 vs. 21.000	1.667	10.607	<0.001	Yes
49.000 vs. 42.000	1.667	10.607	<0.001	Yes
69.000 vs. 21.000	1.667	10.607	<0.001	Yes
28.000 vs. 14.000	1.500	9.546	<0.001	Yes
69.000 vs. 14.000	1.500	9.546	<0.001	Yes
28.000 vs. 35.000	1.333	8.485	<0.001	Yes
69.000 vs. 35.000	1.333	8.485	<0.001	Yes
69.000 vs. 42.000	1.167	7.425	<0.001	Yes
28.000 vs. 42.000	1.167	7.425	<0.001	Yes
42.000 vs. 7.000	1.000	6.364	<0.001	Yes
35.000 vs. 7.000	0.833	5.303	<0.001	Yes
14.000 vs. 7.000	0.667	4.243	0.002	Yes
21.000 vs. 7.000	0.500	3.182	0.030	Yes
49.000 vs. 69.000	0.500	3.182	0.027	Yes
49.000 vs. 28.000	0.500	3.182	0.024	Yes
42.000 vs. 21.000	0.500	3.182	0.021	Yes
35.000 vs. 21.000	0.333	2.121	0.221	No
42.000 vs. 14.000	0.333	2.121	0.188	No
35.000 vs. 14.000	0.167	1.061	0.754	No
14.000 vs. 21.000	0.167	1.061	0.651	No
42.000 vs. 35.000	0.167	1.061	0.504	No
28.000 vs. 69.000	0.000	0.000	1.000	No

Comparisons for factor: **ANTIGEN**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
SG vs. MG	0.444	6.000	<0.001	Yes

Comparisons for factor: **ANTIGEN within 1**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
SG vs. MG	0.000	0.000	1.000	No

Comparisons for factor: **ANTIGEN within 7**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
SG vs. MG	0.333	1.500	0.142	No

Comparisons for factor: **ANTIGEN within 14**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
SG vs. MG	1.000	4.500	<0.001	Yes

Comparisons for factor: **ANTIGEN within 21**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**

SG vs. MG	0.667	3.000	0.005	Yes

Comparisons for factor: **ANTIGEN within 28**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
SG vs. MG	0.000	0.000	1.000	No

Comparisons for factor: **ANTIGEN within 35**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
SG vs. MG	0.667	3.000	0.005	Yes

Comparisons for factor: **ANTIGEN within 42**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
SG vs. MG	0.333	1.500	0.142	No

Comparisons for factor: **ANTIGEN within 49**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
SG vs. MG	1.000	4.500	<0.001	Yes

Comparisons for factor: **ANTIGEN within 69**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
SG vs. MG	0.000	0.000	1.000	No

Comparisons for factor: **DAYS within SG**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
49.000 vs. 1.000	6.000	27.000	<0.001	Yes
69.000 vs. 1.000	5.000	22.500	<0.001	Yes
28.000 vs. 1.000	5.000	22.500	<0.001	Yes
14.000 vs. 1.000	4.000	18.000	<0.001	Yes
42.000 vs. 1.000	4.000	18.000	<0.001	Yes
35.000 vs. 1.000	4.000	18.000	<0.001	Yes
21.000 vs. 1.000	3.667	16.500	<0.001	Yes
7.000 vs. 1.000	3.000	13.500	<0.001	Yes
49.000 vs. 7.000	3.000	13.500	<0.001	Yes
49.000 vs. 21.000	2.333	10.500	<0.001	Yes
49.000 vs. 14.000	2.000	9.000	<0.001	Yes
49.000 vs. 35.000	2.000	9.000	<0.001	Yes
69.000 vs. 7.000	2.000	9.000	<0.001	Yes
49.000 vs. 42.000	2.000	9.000	<0.001	Yes
28.000 vs. 7.000	2.000	9.000	<0.001	Yes
28.000 vs. 21.000	1.333	6.000	<0.001	Yes
69.000 vs. 21.000	1.333	6.000	<0.001	Yes
49.000 vs. 69.000	1.000	4.500	0.001	Yes
14.000 vs. 7.000	1.000	4.500	0.001	Yes
28.000 vs. 35.000	1.000	4.500	0.001	Yes
42.000 vs. 7.000	1.000	4.500	0.001	Yes
28.000 vs. 42.000	1.000	4.500	0.001	Yes
28.000 vs. 14.000	1.000	4.500	<0.001	Yes
69.000 vs. 42.000	1.000	4.500	<0.001	Yes
69.000 vs. 35.000	1.000	4.500	<0.001	Yes
35.000 vs. 7.000	1.000	4.500	<0.001	Yes
49.000 vs. 28.000	1.000	4.500	<0.001	Yes
69.000 vs. 14.000	1.000	4.500	<0.001	Yes
21.000 vs. 7.000	0.667	3.000	0.038	Yes
42.000 vs. 21.000	0.333	1.500	0.659	No
35.000 vs. 21.000	0.333	1.500	0.602	No
14.000 vs. 21.000	0.333	1.500	0.536	No
42.000 vs. 14.000	0.000	0.000	1.000	No
35.000 vs. 14.000	0.000	0.000	1.000	No
35.000 vs. 42.000	0.000	0.000	1.000	No
28.000 vs. 69.000	0.000	0.000	1.000	No

Comparisons for factor: **DAYS within MG**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**

28.000 vs. 1.000	5.000	22.500	<0.001	Yes
69.000 vs. 1.000	5.000	22.500	<0.001	Yes
49.000 vs. 1.000	5.000	22.500	<0.001	Yes
42.000 vs. 1.000	3.667	16.500	<0.001	Yes
35.000 vs. 1.000	3.333	15.000	<0.001	Yes
14.000 vs. 1.000	3.000	13.500	<0.001	Yes
21.000 vs. 1.000	3.000	13.500	<0.001	Yes
7.000 vs. 1.000	2.667	12.000	<0.001	Yes
28.000 vs. 7.000	2.333	10.500	<0.001	Yes
49.000 vs. 7.000	2.333	10.500	<0.001	Yes
69.000 vs. 7.000	2.333	10.500	<0.001	Yes
28.000 vs. 14.000	2.000	9.000	<0.001	Yes
28.000 vs. 21.000	2.000	9.000	<0.001	Yes
69.000 vs. 21.000	2.000	9.000	<0.001	Yes
69.000 vs. 14.000	2.000	9.000	<0.001	Yes
49.000 vs. 14.000	2.000	9.000	<0.001	Yes
49.000 vs. 21.000	2.000	9.000	<0.001	Yes
49.000 vs. 35.000	1.667	7.500	<0.001	Yes
69.000 vs. 35.000	1.667	7.500	<0.001	Yes
28.000 vs. 35.000	1.667	7.500	<0.001	Yes
28.000 vs. 42.000	1.333	6.000	<0.001	Yes
69.000 vs. 42.000	1.333	6.000	<0.001	Yes
49.000 vs. 42.000	1.333	6.000	<0.001	Yes
42.000 vs. 7.000	1.000	4.500	<0.001	Yes
35.000 vs. 7.000	0.667	3.000	0.057	No
42.000 vs. 21.000	0.667	3.000	0.052	No
42.000 vs. 14.000	0.667	3.000	0.048	Yes
14.000 vs. 7.000	0.333	1.500	0.749	No
35.000 vs. 14.000	0.333	1.500	0.707	No
35.000 vs. 21.000	0.333	1.500	0.659	No
21.000 vs. 7.000	0.333	1.500	0.602	No
42.000 vs. 35.000	0.333	1.500	0.536	No
21.000 vs. 14.000	0.000	0.000	1.000	No
49.000 vs. 69.000	0.000	0.000	1.000	No
28.000 vs. 69.000	0.000	0.000	1.000	No
28.000 vs. 49.000	0.000	0.000	1.000	No

Two Way Analysis of Variance Friday, January 28, 2022, 2:55:12 AM

Data source: 3280 DIT DATA in Notebook1

Balanced Design

Dependent Variable: DATA

Normality Test (Shapiro-Wilk): Failed (P < 0.050)

Equal Variance Test (Brown-Forsythe): Passed (P = 1.000)

Source of Variation	DF	SS	MS	F	P
DAYS	7	23.979	3.426	9.135	<0.001
ANTIGEN	1	0.0208	0.0208	0.0556	0.815
DAYS x ANTIGEN	7	3.813	0.545	1.452	0.220
Residual	32	12.000	0.375		
Total	47	39.813	0.847		

The difference in the mean values among the different levels of DAYS is greater than would be expected by chance after allowing for effects of differences in ANTIGEN. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of ANTIGEN is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in DAYS. There is not a statistically significant difference (P = 0.815).

The effect of different levels of DAYS does not depend on what level of ANTIGEN is present. There is not a statistically significant interaction between DAYS and ANTIGEN. (P = 0.220)

Power of performed test with alpha = 0.0500: for DAYS : 1.000
Power of performed test with alpha = 0.0500: for ANTIGEN : 0.0500
Power of performed test with alpha = 0.0500: for DAYS x ANTIGEN : 0.169

Least square means for DAYS :

Group	**Mean**
0.000	2.000
14.000	3.000
21.000	3.500
33.000	3.833
40.000	4.333
49.000	4.000
56.000	4.167
61.000	3.667

Std Err of LS Mean = 0.250

Least square means for ANTIGEN :

Group	**Mean**
SG	3.542
MG	3.583

Std Err of LS Mean = 0.125

Least square means for DAYS x ANTIGEN :

Group	**Mean**
0.000 x SG	2.000
0.000 x MG	2.000
14.000 x SG	2.667
14.000 x MG	3.333
21.000 x SG	3.000
21.000 x MG	4.000
33.000 x SG	3.667
33.000 x MG	4.000
40.000 x SG	4.333
40.000 x MG	4.333
49.000 x SG	4.333
49.000 x MG	3.667
56.000 x SG	4.333
56.000 x MG	4.000
61.000 x SG	4.000
61.000 x MG	3.333

Std Err of LS Mean = 0.354

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **DAYS**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
40.000 vs. 0.000	2.333	6.600	<0.001	Yes
56.000 vs. 0.000	2.167	6.128	<0.001	Yes
49.000 vs. 0.000	2.000	5.657	<0.001	Yes
33.000 vs. 0.000	1.833	5.185	<0.001	Yes
61.000 vs. 0.000	1.667	4.714	0.001	Yes
21.000 vs. 0.000	1.500	4.243	0.004	Yes
40.000 vs. 14.000	1.333	3.771	0.014	Yes
56.000 vs. 14.000	1.167	3.300	0.049	Yes
49.000 vs. 14.000	1.000	2.828	0.149	No
14.000 vs. 0.000	1.000	2.828	0.142	No
40.000 vs. 21.000	0.833	2.357	0.363	No
33.000 vs. 14.000	0.833	2.357	0.346	No
61.000 vs. 14.000	0.667	1.886	0.678	No
56.000 vs. 21.000	0.667	1.886	0.655	No
40.000 vs. 61.000	0.667	1.886	0.629	No
21.000 vs. 14.000	0.500	1.414	0.907	No
40.000 vs. 33.000	0.500	1.414	0.888	No
49.000 vs. 21.000	0.500	1.414	0.866	No
56.000 vs. 61.000	0.500	1.414	0.839	No

56.000 vs. 33.000	0.333	0.943	0.980	No
49.000 vs. 61.000	0.333	0.943	0.969	No
40.000 vs. 49.000	0.333	0.943	0.952	No
33.000 vs. 21.000	0.333	0.943	0.927	No
33.000 vs. 61.000	0.167	0.471	0.994	No
61.000 vs. 21.000	0.167	0.471	0.983	No
56.000 vs. 49.000	0.167	0.471	0.954	No
49.000 vs. 33.000	0.167	0.471	0.871	No
40.000 vs. 56.000	0.167	0.471	0.641	No

Comparisons for factor: **ANTIGEN**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
MG vs. SG	0.0417	0.236	0.815	No

Two Way Analysis of Variance Friday, January 28, 2022, 2:58:56 AM

Data source: 3124 DIT DATA in Notebook1

Balanced Design

Dependent Variable: DATA

Normality Test (Shapiro-Wilk): Failed (P < 0.050)

Equal Variance Test (Brown-Forsythe): Passed (P = 1.000)

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
DAYS	7	18.813	2.688	3.308	0.009
ANTIGEN	1	1.688	1.688	2.077	0.159
DAYS x ANTIGEN	7	3.479	0.497	0.612	0.742
Residual	32	26.000	0.813		
Total	47	49.979	1.063		

The difference in the mean values among the different levels of DAYS is greater than would be expected by chance after allowing for effects of differences in ANTIGEN. There is a statistically significant difference (P = 0.009). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of ANTIGEN is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in DAYS. There is not a statistically significant difference (P = 0.159).

The effect of different levels of DAYS does not depend on what level of ANTIGEN is present. There is not a statistically significant interaction between DAYS and ANTIGEN. (P = 0.742)

Power of performed test with alpha = 0.0500: for DAYS : 0.755
Power of performed test with alpha = 0.0500: for ANTIGEN : 0.172
Power of performed test with alpha = 0.0500: for DAYS x ANTIGEN : 0.0500

Least square means for DAYS :

Group	**Mean**
0.000	1.000
14.000	2.833
21.000	2.333
33.000	3.000
40.000	3.000
49.000	2.500
56.000	3.000
61.000	2.500

Std Err of LS Mean = 0.368

Least square means for ANTIGEN :

Group	**Mean**
SG	2.708
MG	2.333

Std Err of LS Mean = 0.184

Least square means for DAYS x ANTIGEN :

Group	Mean
0.000 x SG	1.333
0.000 x MG	0.667
14.000 x SG	3.000
14.000 x MG	2.667
21.000 x SG	2.000
21.000 x MG	2.667
33.000 x SG	3.333
33.000 x MG	2.667
40.000 x SG	3.000
40.000 x MG	3.000
49.000 x SG	3.000
49.000 x MG	2.000
56.000 x SG	3.000
56.000 x MG	3.000
61.000 x SG	3.000
61.000 x MG	2.000

Std Err of LS Mean = 0.520

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **DAYS**

Comparison	Diff of Means	t	P	P<0.050
56.000 vs. 0.000	2.000	3.843	0.015	Yes
40.000 vs. 0.000	2.000	3.843	0.015	Yes
33.000 vs. 0.000	2.000	3.843	0.014	Yes
14.000 vs. 0.000	1.833	3.523	0.032	Yes
61.000 vs. 0.000	1.500	2.882	0.155	No
49.000 vs. 0.000	1.500	2.882	0.149	No
21.000 vs. 0.000	1.333	2.562	0.288	No
40.000 vs. 21.000	0.667	1.281	0.993	No
33.000 vs. 21.000	0.667	1.281	0.991	No
56.000 vs. 21.000	0.667	1.281	0.988	No
14.000 vs. 21.000	0.500	0.961	0.999	No
56.000 vs. 61.000	0.500	0.961	0.999	No
33.000 vs. 49.000	0.500	0.961	0.999	No
56.000 vs. 49.000	0.500	0.961	0.998	No
33.000 vs. 61.000	0.500	0.961	0.997	No
40.000 vs. 61.000	0.500	0.961	0.996	No
40.000 vs. 49.000	0.500	0.961	0.994	No
14.000 vs. 61.000	0.333	0.641	1.000	No
14.000 vs. 49.000	0.333	0.641	0.999	No
49.000 vs. 21.000	0.167	0.320	1.000	No
61.000 vs. 21.000	0.167	0.320	1.000	No
56.000 vs. 14.000	0.167	0.320	1.000	No
33.000 vs. 14.000	0.167	0.320	1.000	No
40.000 vs. 14.000	0.167	0.320	0.999	No
56.000 vs. 40.000	0.000	0.000	1.000	No
49.000 vs. 61.000	0.000	0.000	1.000	No
56.000 vs. 33.000	0.000	0.000	1.000	No
40.000 vs. 33.000	0.000	0.000	1.000	No

Comparisons for factor: **ANTIGEN**

Comparison	Diff of Means	t	P	P<0.050
SG vs. MG	0.375	1.441	0.159	No

Balanced Design

Dependent Variable: DATA

Normality Test (Shapiro-Wilk): Failed (P < 0.050)

Equal Variance Test (Brown-Forsythe): Passed (P = 1.000)

Source of Variation	DF	SS	MS	F	P
DAYS	7	1.250	0.179	0.429	0.877
ANTIGEN	1	2.083	2.083	5.000	0.032
DAYS x ANTIGEN	7	1.250	0.179	0.429	0.877
Residual	32	13.333	0.417		
Total	47	17.917	0.381		

The difference in the mean values among the different levels of DAYS is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in ANTIGEN. There is not a statistically significant difference (P = 0.877).

The difference in the mean values among the different levels of ANTIGEN is greater than would be expected by chance after allowing for effects of differences in DAYS. There is a statistically significant difference (P = 0.032). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of DAYS does not depend on what level of ANTIGEN is present. There is not a statistically significant interaction between DAYS and ANTIGEN. (P = 0.877)

Power of performed test with alpha = 0.0500: for DAYS : 0.0500
Power of performed test with alpha = 0.0500: for ANTIGEN : 0.492
Power of performed test with alpha = 0.0500: for DAYS x ANTIGEN : 0.0500

Least square means for DAYS :

Group	Mean
0.000	0.000
14.000	0.333
21.000	0.000
33.000	0.333
40.000	0.333
49.000	0.333
56.000	0.333
61.000	0.000

Std Err of LS Mean = 0.264

Least square means for ANTIGEN :

Group	Mean
SG	0.000
MG	0.417

Std Err of LS Mean = 0.132

Least square means for DAYS x ANTIGEN :

Group	Mean
0.000 x SG	0.000
0.000 x MG	0.000
14.000 x SG	0.000
14.000 x MG	0.667
21.000 x SG	0.000
21.000 x MG	0.000
33.000 x SG	0.000
33.000 x MG	0.667
40.000 x SG	0.000
40.000 x MG	0.667
49.000 x SG	0.000
49.000 x MG	0.667
56.000 x SG	0.000
56.000 x MG	0.667
61.000 x SG	0.000
61.000 x MG	0.000

Std Err of LS Mean = 0.373

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **DAYS**

Comparison	Diff of Means	t	P	P<0.050
33.000 vs. 21.000	0.333	0.894	1.000	No
40.000 vs. 21.000	0.333	0.894	1.000	No
40.000 vs. 0.000	0.333	0.894	1.000	No
33.000 vs. 61.000	0.333	0.894	1.000	No
56.000 vs. 61.000	0.333	0.894	1.000	No
14.000 vs. 0.000	0.333	0.894	1.000	No
33.000 vs. 0.000	0.333	0.894	1.000	No
14.000 vs. 61.000	0.333	0.894	1.000	No
14.000 vs. 21.000	0.333	0.894	1.000	No
40.000 vs. 61.000	0.333	0.894	1.000	No
56.000 vs. 0.000	0.333	0.894	1.000	No
56.000 vs. 21.000	0.333	0.894	1.000	No
49.000 vs. 61.000	0.333	0.894	0.999	No
49.000 vs. 21.000	0.333	0.894	0.999	No
49.000 vs. 0.000	0.333	0.894	0.999	No
56.000 vs. 49.000	0.000	0.000	1.000	No
0.000 vs. 61.000	0.000	0.000	1.000	No
0.000 vs. 21.000	0.000	0.000	1.000	No
56.000 vs. 14.000	0.000	0.000	1.000	No
33.000 vs. 14.000	0.000	0.000	1.000	No
21.000 vs. 61.000	0.000	0.000	1.000	No
56.000 vs. 33.000	0.000	0.000	1.000	No
56.000 vs. 40.000	0.000	0.000	1.000	No
49.000 vs. 14.000	0.000	0.000	1.000	No
40.000 vs. 33.000	0.000	0.000	1.000	No
40.000 vs. 14.000	0.000	0.000	1.000	No
49.000 vs. 40.000	0.000	0.000	1.000	No
49.000 vs. 33.000	0.000	0.000	1.000	No

Comparisons for factor: **ANTIGEN**

Comparison	Diff of Means	t	P	P<0.050
MG vs. SG	0.417	2.236	0.032	Yes

Trail 2
Two Way Analysis of Variance Friday, January 28, 2022, 3:18:48 AM

Data source: 648 DMG Data in Notebook2

Balanced Design

Dependent Variable: DATA

Normality Test (Shapiro-Wilk): Failed (P < 0.050)

Equal Variance Test (Brown-Forsythe): Passed (P = 1.000)

Source of Variation	DF	SS	MS	F	P
DAYS	7	129.979	18.568	111.411	<0.001
ANTIGEN	1	1.021	1.021	6.125	0.019
DAYS x ANTIGEN	7	1.146	0.164	0.982	0.461
Residual	32	5.333	0.167		
Total	47	137.479	2.925		

The difference in the mean values among the different levels of DAYS is greater than would be expected by chance after allowing for effects of differences in ANTIGEN. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of ANTIGEN is greater than would be expected by chance after allowing for effects of differences in DAYS. There is a statistically significant difference (P = 0.019). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of DAYS does not depend on what level of ANTIGEN is present. There is not a statistically significant interaction between DAYS and ANTIGEN. (P = 0.461)

Power of performed test with alpha = 0.0500: for DAYS : 1.000
Power of performed test with alpha = 0.0500: for ANTIGEN : 0.593
Power of performed test with alpha = 0.0500: for DAYS x ANTIGEN : 0.0500

Least square means for DAYS :

Group	**Mean**
0.000	0.000
7.000	4.667
14.000	4.667
17.000	4.833
28.000	5.000
35.000	5.333
42.000	5.333
52.000	4.333

Std Err of LS Mean = 0.167

Least square means for ANTIGEN :

Group	**Mean**
SG	4.125
MG	4.417

Std Err of LS Mean = 0.0833

Least square means for DAYS x ANTIGEN :

Group	**Mean**
0.000 x SG	0.000
0.000 x MG	0.000
7.000 x SG	4.667
7.000 x MG	4.667
14.000 x SG	4.333
14.000 x MG	5.000
17.000 x SG	4.667
17.000 x MG	5.000
28.000 x SG	5.000
28.000 x MG	5.000
35.000 x SG	5.000
35.000 x MG	5.667
42.000 x SG	5.000
42.000 x MG	5.667
52.000 x SG	4.333
52.000 x MG	4.333

Std Err of LS Mean = 0.236

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **DAYS**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
42.000 vs. 0.000	5.333	22.627	<0.001	Yes
35.000 vs. 0.000	5.333	22.627	<0.001	Yes
28.000 vs. 0.000	5.000	21.213	<0.001	Yes
17.000 vs. 0.000	4.833	20.506	<0.001	Yes
7.000 vs. 0.000	4.667	19.799	<0.001	Yes
14.000 vs. 0.000	4.667	19.799	<0.001	Yes
52.000 vs. 0.000	4.333	18.385	<0.001	Yes
35.000 vs. 52.000	1.000	4.243	0.004	Yes
42.000 vs. 52.000	1.000	4.243	0.004	Yes
42.000 vs. 14.000	0.667	2.828	0.142	No
35.000 vs. 14.000	0.667	2.828	0.135	No
35.000 vs. 7.000	0.667	2.828	0.128	No
42.000 vs. 7.000	0.667	2.828	0.121	No
28.000 vs. 52.000	0.667	2.828	0.114	No
17.000 vs. 52.000	0.500	2.121	0.449	No
42.000 vs. 17.000	0.500	2.121	0.425	No
35.000 vs. 17.000	0.500	2.121	0.400	No
42.000 vs. 28.000	0.333	1.414	0.866	No
7.000 vs. 52.000	0.333	1.414	0.839	No

28.000 vs. 14.000	0.333	1.414	0.807	No
35.000 vs. 28.000	0.333	1.414	0.768	No
14.000 vs. 52.000	0.333	1.414	0.722	No
28.000 vs. 7.000	0.333	1.414	0.666	No
17.000 vs. 14.000	0.167	0.707	0.964	No
17.000 vs. 7.000	0.167	0.707	0.929	No
28.000 vs. 17.000	0.167	0.707	0.863	No
7.000 vs. 14.000	8.882E-016	3.768E-015	1.000	No
42.000 vs. 35.000	0.000	0.000	1.000	No

Comparisons for factor: **ANTIGEN**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
MG vs. SG	0.292	2.475	0.019	Yes

Two Way Analysis of Variance Friday, January 28, 2022, 3:29:12 AM

Data source: 3250 NSG DATA in Notebook2

Balanced Design

Dependent Variable: DATA

Normality Test (Shapiro-Wilk): Failed (P < 0.050)

Equal Variance Test (Brown-Forsythe): Passed (P = 1.000)

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
DAYS	7	96.333	13.762	44.038	<0.001
ANTIGEN	1	8.333	8.333	26.667	<0.001
DAYS x ANTIGEN	7	3.000	0.429	1.371	0.251
Residual	32	10.000	0.313		
Total	47	117.667	2.504		

The difference in the mean values among the different levels of DAYS is greater than would be expected by chance after allowing for effects of differences in ANTIGEN. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of ANTIGEN is greater than would be expected by chance after allowing for effects of differences in DAYS. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of DAYS does not depend on what level of ANTIGEN is present. There is not a statistically significant interaction between DAYS and ANTIGEN. (P = 0.251)

Power of performed test with alpha = 0.0500: for DAYS : 1.000
Power of performed test with alpha = 0.0500: for ANTIGEN : 0.998
Power of performed test with alpha = 0.0500: for DAYS x ANTIGEN : 0.144

Least square means for DAYS :

Group	**Mean**
0.000	2.000
7.000	4.167
14.000	4.833
17.000	5.333
28.000	5.833
35.000	5.667
42.000	5.667
52.000	7.167

Std Err of LS Mean = 0.228

Least square means for ANTIGEN :

Group	**Mean**
SG	5.500
MG	4.667

Std Err of LS Mean = 0.114

Least square means for DAYS x ANTIGEN :

Group	Mean
0.000 x SG	2.000
0.000 x MG	2.000
7.000 x SG	4.333
7.000 x MG	4.000
14.000 x SG	5.333
14.000 x MG	4.333
17.000 x SG	6.000
17.000 x MG	4.667
28.000 x SG	6.333
28.000 x MG	5.333
35.000 x SG	6.333
35.000 x MG	5.000
42.000 x SG	6.333
42.000 x MG	5.000
52.000 x SG	7.333
52.000 x MG	7.000

Std Err of LS Mean = 0.323

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **DAYS**

Comparison	Diff of Means	t	P	P<0.050
52.000 vs. 0.000	5.167	16.008	<0.001	Yes
28.000 vs. 0.000	3.833	11.877	<0.001	Yes
42.000 vs. 0.000	3.667	11.361	<0.001	Yes
35.000 vs. 0.000	3.667	11.361	<0.001	Yes
17.000 vs. 0.000	3.333	10.328	<0.001	Yes
52.000 vs. 7.000	3.000	9.295	<0.001	Yes
14.000 vs. 0.000	2.833	8.779	<0.001	Yes
52.000 vs. 14.000	2.333	7.230	<0.001	Yes
7.000 vs. 0.000	2.167	6.713	<0.001	Yes
52.000 vs. 17.000	1.833	5.680	<0.001	Yes
28.000 vs. 7.000	1.667	5.164	<0.001	Yes
52.000 vs. 35.000	1.500	4.648	<0.001	Yes
35.000 vs. 7.000	1.500	4.648	<0.001	Yes
52.000 vs. 42.000	1.500	4.648	<0.001	Yes
42.000 vs. 7.000	1.500	4.648	<0.001	Yes
52.000 vs. 28.000	1.333	4.131	0.003	Yes
17.000 vs. 7.000	1.167	3.615	0.012	Yes
28.000 vs. 14.000	1.000	3.098	0.043	Yes
42.000 vs. 14.000	0.833	2.582	0.137	No
35.000 vs. 14.000	0.833	2.582	0.124	No
14.000 vs. 7.000	0.667	2.066	0.320	No
17.000 vs. 14.000	0.500	1.549	0.626	No
28.000 vs. 17.000	0.500	1.549	0.570	No
35.000 vs. 17.000	0.333	1.033	0.843	No
42.000 vs. 17.000	0.333	1.033	0.773	No
28.000 vs. 42.000	0.167	0.516	0.940	No
28.000 vs. 35.000	0.167	0.516	0.847	No
35.000 vs. 42.000	0.000	0.000	1.000	No

Comparisons for factor: **ANTIGEN**

Comparison	Diff of Means	t	P	P<0.050
SG vs. MG	0.833	5.164	<0.001	Yes

Two Way Analysis of Variance Friday, January 28, 2022, 3:33:40 AM

Data source: 3178 DMG DATA in Notebook2

Balanced Design

Dependent Variable: DATA

Normality Test (Shapiro-Wilk): Failed (P < 0.050)

Equal Variance Test (Brown-Forsythe): Passed (P = 1.000)

Source of Variation	DF	SS	MS	F	P
DAYS	7	225.917	32.274	129.095	<0.001
ANTIGEN	1	14.083	14.083	56.333	<0.001
DAYS x ANTIGEN	7	13.917	1.988	7.952	<0.001
Residual	32	8.000	0.250		
Total	47	261.917	5.573		

Main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor.

The effect of different levels of DAYS depends on what level of ANTIGEN is present. There is a statistically significant interaction between DAYS and ANTIGEN. (P = <0.001)

Power of performed test with alpha = 0.0500: for DAYS : 1.000
Power of performed test with alpha = 0.0500: for ANTIGEN : 1.000
Power of performed test with alpha = 0.0500: for DAYS x ANTIGEN : 0.999

Least square means for DAYS :

Group	Mean
0.000	0.667
7.000	2.833
14.000	4.333
17.000	5.667
28.000	6.000
35.000	6.167
42.000	7.500
52.000	7.167

Std Err of LS Mean = 0.204

Least square means for ANTIGEN :

Group	Mean
SG	5.583
MG	4.500

Std Err of LS Mean = 0.102

Least square means for DAYS x ANTIGEN :

Group	Mean
0.000 x SG	0.000
0.000 x MG	1.333
7.000 x SG	3.000
7.000 x MG	2.667
14.000 x SG	5.000
14.000 x MG	3.667
17.000 x SG	6.333
17.000 x MG	5.000
28.000 x SG	7.000
28.000 x MG	5.000
35.000 x SG	7.333
35.000 x MG	5.000
42.000 x SG	8.000
42.000 x MG	7.000
52.000 x SG	8.000
52.000 x MG	6.333

Std Err of LS Mean = 0.289

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **ANTIGEN**

Comparison	Diff of Means	t	P	P<0.050
SG vs. MG	1.083	7.506	<0.001	Yes

Comparisons for factor: **ANTIGEN within 0**

Comparison	Diff of Means	t	P	P<0.050
MG vs. SG	1.333	3.266	0.003	Yes

Comparisons for factor: **ANTIGEN within 7**

Comparison	Diff of Means	t	P	P<0.050
SG vs. MG	0.333	0.816	0.420	No

Comparisons for factor: **ANTIGEN within 14**

Comparison	Diff of Means	t	P	P<0.050
SG vs. MG	1.333	3.266	0.003	Yes

Comparisons for factor: **ANTIGEN within 17**

Comparison	Diff of Means	t	P	P<0.050
SG vs. MG	1.333	3.266	0.003	Yes

Comparisons for factor: **ANTIGEN within 28**

Comparison	Diff of Means	t	P	P<0.050
SG vs. MG	2.000	4.899	<0.001	Yes

Comparisons for factor: **ANTIGEN within 35**

Comparison	Diff of Means	t	P	P<0.050
SG vs. MG	2.333	5.715	<0.001	Yes

Comparisons for factor: **ANTIGEN within 42**

Comparison	Diff of Means	t	P	P<0.050
SG vs. MG	1.000	2.449	0.020	Yes

Comparisons for factor: **ANTIGEN within 52**

Comparison	Diff of Means	t	P	P<0.050
SG vs. MG	1.667	4.082	<0.001	Yes

Comparisons for factor: **DAYS within SG**

Comparison	Diff of Means	t	P	P<0.050
42.000 vs. 0.000	8.000	19.596	<0.001	Yes
52.000 vs. 0.000	8.000	19.596	<0.001	Yes
35.000 vs. 0.000	7.333	17.963	<0.001	Yes
28.000 vs. 0.000	7.000	17.146	<0.001	Yes
17.000 vs. 0.000	6.333	15.513	<0.001	Yes
52.000 vs. 7.000	5.000	12.247	<0.001	Yes
42.000 vs. 7.000	5.000	12.247	<0.001	Yes
14.000 vs. 0.000	5.000	12.247	<0.001	Yes
35.000 vs. 7.000	4.333	10.614	<0.001	Yes
28.000 vs. 7.000	4.000	9.798	<0.001	Yes
17.000 vs. 7.000	3.333	8.165	<0.001	Yes
7.000 vs. 0.000	3.000	7.348	<0.001	Yes
52.000 vs. 14.000	3.000	7.348	<0.001	Yes
42.000 vs. 14.000	3.000	7.348	<0.001	Yes
35.000 vs. 14.000	2.333	5.715	<0.001	Yes
14.000 vs. 7.000	2.000	4.899	<0.001	Yes
28.000 vs. 14.000	2.000	4.899	<0.001	Yes
52.000 vs. 17.000	1.667	4.082	0.003	Yes
42.000 vs. 17.000	1.667	4.082	0.003	Yes
17.000 vs. 14.000	1.333	3.266	0.023	Yes
35.000 vs. 17.000	1.000	2.449	0.149	No
52.000 vs. 28.000	1.000	2.449	0.132	No
42.000 vs. 28.000	1.000	2.449	0.114	No
28.000 vs. 17.000	0.667	1.633	0.449	No
42.000 vs. 35.000	0.667	1.633	0.379	No
52.000 vs. 35.000	0.667	1.633	0.300	No
35.000 vs. 28.000	0.333	0.816	0.664	No
42.000 vs. 52.000	0.000	0.000	1.000	No

Comparisons for factor: **DAYS within MG**

Comparison	Diff of Means	t	P	P<0.050
42.000 vs. 0.000	5.667	13.880	<0.001	Yes
52.000 vs. 0.000	5.000	12.247	<0.001	Yes
42.000 vs. 7.000	4.333	10.614	<0.001	Yes
17.000 vs. 0.000	3.667	8.981	<0.001	Yes
35.000 vs. 0.000	3.667	8.981	<0.001	Yes
28.000 vs. 0.000	3.667	8.981	<0.001	Yes
52.000 vs. 7.000	3.667	8.981	<0.001	Yes
42.000 vs. 14.000	3.333	8.165	<0.001	Yes
52.000 vs. 14.000	2.667	6.532	<0.001	Yes
35.000 vs. 7.000	2.333	5.715	<0.001	Yes
17.000 vs. 7.000	2.333	5.715	<0.001	Yes
28.000 vs. 7.000	2.333	5.715	<0.001	Yes
14.000 vs. 0.000	2.333	5.715	<0.001	Yes
42.000 vs. 35.000	2.000	4.899	<0.001	Yes
42.000 vs. 17.000	2.000	4.899	<0.001	Yes
42.000 vs. 28.000	2.000	4.899	<0.001	Yes
28.000 vs. 14.000	1.333	3.266	0.031	Yes
35.000 vs. 14.000	1.333	3.266	0.028	Yes
17.000 vs. 14.000	1.333	3.266	0.026	Yes
7.000 vs. 0.000	1.333	3.266	0.023	Yes
52.000 vs. 17.000	1.333	3.266	0.021	Yes
52.000 vs. 35.000	1.333	3.266	0.018	Yes
52.000 vs. 28.000	1.333	3.266	0.016	Yes
14.000 vs. 7.000	1.000	2.449	0.096	No
42.000 vs. 52.000	0.667	1.633	0.379	No
28.000 vs. 35.000	0.000	0.000	1.000	No
35.000 vs. 17.000	0.000	0.000	1.000	No
28.000 vs. 17.000	0.000	0.000	1.000	No

Two Way Analysis of Variance Friday, January 28, 2022, 3:25:16 AM

Data source: 3239 DSG DATA in Notebook2

Balanced Design

Dependent Variable: DATA

Normality Test (Shapiro-Wilk): Failed (P < 0.050)

Equal Variance Test (Brown-Forsythe): Passed (P = 1.000)

Source of Variation	DF	SS	MS	F	P
DAYS	7	135.646	19.378	62.010	<0.001
ANTIGEN	1	6.021	6.021	19.267	<0.001
DAYS x ANTIGEN	7	4.813	0.688	2.200	0.061
Residual	32	10.000	0.313		
Total	47	156.479	3.329		

The difference in the mean values among the different levels of DAYS is greater than would be expected by chance after allowing for effects of differences in ANTIGEN. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of ANTIGEN is greater than would be expected by chance after allowing for effects of differences in DAYS. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of DAYS does not depend on what level of ANTIGEN is present. There is not a statistically significant interaction between DAYS and ANTIGEN. (P = 0.061)

Power of performed test with alpha = 0.0500: for DAYS : 1.000
Power of performed test with alpha = 0.0500: for ANTIGEN : 0.985
Power of performed test with alpha = 0.0500: for DAYS x ANTIGEN : 0.430

Least square means for DAYS :

Group	Mean
0.000	0.000
7.000	4.000
14.000	4.667
17.000	4.667
28.000	5.000
35.000	6.000
42.000	4.667
52.000	4.833

Std Err of LS Mean = 0.228

Least square means for ANTIGEN :

Group	Mean
SG	4.583
MG	3.875

Std Err of LS Mean = 0.114

Least square means for DAYS x ANTIGEN :

Group	Mean
0.000 x SG	0.000
0.000 x MG	0.000
7.000 x SG	4.667
7.000 x MG	3.333
14.000 x SG	5.333
14.000 x MG	4.000
17.000 x SG	4.667
17.000 x MG	4.667
28.000 x SG	5.000
28.000 x MG	5.000
35.000 x SG	6.667
35.000 x MG	5.333
42.000 x SG	5.333
42.000 x MG	4.000
52.000 x SG	5.000
52.000 x MG	4.667

Std Err of LS Mean = 0.323

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **DAYS**

Comparison	Diff of Means	t	P	P<0.050
35.000 vs. 0.000	6.000	18.590	<0.001	Yes
28.000 vs. 0.000	5.000	15.492	<0.001	Yes
52.000 vs. 0.000	4.833	14.976	<0.001	Yes
17.000 vs. 0.000	4.667	14.459	<0.001	Yes
42.000 vs. 0.000	4.667	14.459	<0.001	Yes
14.000 vs. 0.000	4.667	14.459	<0.001	Yes
7.000 vs. 0.000	4.000	12.394	<0.001	Yes
35.000 vs. 7.000	2.000	6.197	<0.001	Yes
35.000 vs. 42.000	1.333	4.131	0.005	Yes
35.000 vs. 14.000	1.333	4.131	0.005	Yes
35.000 vs. 17.000	1.333	4.131	0.004	Yes
35.000 vs. 52.000	1.167	3.615	0.017	Yes
35.000 vs. 28.000	1.000	3.098	0.063	No
28.000 vs. 7.000	1.000	3.098	0.059	No
52.000 vs. 7.000	0.833	2.582	0.186	No
17.000 vs. 7.000	0.667	2.066	0.466	No
42.000 vs. 7.000	0.667	2.066	0.439	No
14.000 vs. 7.000	0.667	2.066	0.411	No
28.000 vs. 14.000	0.333	1.033	0.975	No
28.000 vs. 42.000	0.333	1.033	0.964	No
28.000 vs. 17.000	0.333	1.033	0.948	No
52.000 vs. 42.000	0.167	0.516	0.999	No
52.000 vs. 14.000	0.167	0.516	0.996	No
28.000 vs. 52.000	0.167	0.516	0.991	No
52.000 vs. 17.000	0.167	0.516	0.977	No
17.000 vs. 14.000	8.882E-016	2.752E-015	1.000	No

17.000 vs. 42.000	8.882E-016	2.752E-015	1.000	No
14.000 vs. 42.000	0.000	0.000	1.000	No

Comparisons for factor: **ANTIGEN**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
SG vs. MG	0.708	4.389	<0.001	Yes

Appendix 4.1

Two Way Analysis of Variance Tuesday, February 15, 2022, 2:37:18 AM

Data source: Data 1 in Notebook1

Balanced Design

Dependent Variable: Data

Normality Test (Shapiro-Wilk): Failed (P < 0.050)

Equal Variance Test (Brown-Forsythe): Passed (P = 1.000)

Source of Variation	DF	SS	MS	F	P
Days	7	123.146	17.592	70.369	<0.001
Antigen	1	1.021	1.021	4.083	0.052
Days x Antigen	7	1.146	0.164	0.655	0.708
Residual	32	8.000	0.250		
Total	47	133.313	2.836		

The difference in the mean values among the different levels of Days is greater than would be expected by chance after allowing for effects of differences in Antigen. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Antigen is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Days. There is not a statistically significant difference (P = 0.052).

The effect of different levels of Days does not depend on what level of Antigen is present. There is not a statistically significant interaction between Days and Antigen. (P = 0.708)

Power of performed test with alpha = 0.0500: for Days : 1.000
Power of performed test with alpha = 0.0500: for Antigen : 0.399
Power of performed test with alpha = 0.0500: for Days x Antigen : 0.0500

Least square means for Days :

Group	Mean
0.000	0.000
7.000	3.000
14.000	3.667
21.000	4.167
28.000	4.667
35.000	4.667
42.000	5.000
56.000	5.333

Std Err of LS Mean = 0.204

Least square means for Antigen :

Group	Mean
SG	3.958
MG	3.667

Std Err of LS Mean = 0.102

Least square means for Days x Antigen :

Group	Mean
0.000 x SG	0.000
0.000 x MG	0.000
7.000 x SG	3.333
7.000 x MG	2.667
14.000 x SG	4.000
14.000 x MG	3.333

21.000 x SG	4.333
21.000 x MG	4.000
28.000 x SG	4.667
28.000 x MG	4.667
35.000 x SG	4.667
35.000 x MG	4.667
42.000 x SG	5.000
42.000 x MG	5.000
56.000 x SG	5.667
56.000 x MG	5.000

Std Err of LS Mean = 0.289

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Days**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
56.000 vs. 0.000	5.333	8	26.128	<0.001	Yes
56.000 vs. 7.000	2.333	8	11.431	<0.001	Yes
56.000 vs. 14.000	1.667	8	8.165	<0.001	Yes
56.000 vs. 21.000	1.167	8	5.715	0.007	Yes
56.000 vs. 35.000	0.667	8	3.266	0.320	No
56.000 vs. 28.000	0.667	8	3.266	0.320	Do Not Test
56.000 vs. 42.000	0.333	8	1.633	0.939	Do Not Test
42.000 vs. 0.000	5.000	8	24.495	<0.001	Yes
42.000 vs. 7.000	2.000	8	9.798	<0.001	Yes
42.000 vs. 14.000	1.333	8	6.532	0.001	Yes
42.000 vs. 21.000	0.833	8	4.082	0.109	No
42.000 vs. 35.000	0.333	8	1.633	0.939	Do Not Test
42.000 vs. 28.000	0.333	8	1.633	0.939	Do Not Test
28.000 vs. 0.000	4.667	8	22.862	<0.001	Yes
28.000 vs. 7.000	1.667	8	8.165	<0.001	Yes
28.000 vs. 14.000	1.000	8	4.899	0.029	Yes
28.000 vs. 21.000	0.500	8	2.449	0.667	Do Not Test
28.000 vs. 35.000	0.000	8	0.000	1.000	Do Not Test
35.000 vs. 0.000	4.667	8	22.862	<0.001	Yes
35.000 vs. 7.000	1.667	8	8.165	<0.001	Yes
35.000 vs. 14.000	1.000	8	4.899	0.029	Yes
35.000 vs. 21.000	0.500	8	2.449	0.667	Do Not Test
21.000 vs. 0.000	4.167	8	20.412	<0.001	Yes
21.000 vs. 7.000	1.167	8	5.715	0.007	Yes
21.000 vs. 14.000	0.500	8	2.449	0.667	No
14.000 vs. 0.000	3.667	8	17.963	<0.001	Yes
14.000 vs. 7.000	0.667	8	3.266	0.320	No
7.000 vs. 0.000	3.000	8	14.697	<0.001	Yes

Comparisons for factor: **Antigen**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
SG vs. MG	0.292	2	2.858	0.052	No

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

Two Way Analysis of Variance Tuesday, February 15, 2022, 2:36:10 AM

Data source: Data 2 in Notebook1

Balanced Design

Dependent Variable: DATA

Normality Test (Shapiro-Wilk): Failed (P < 0.050)

Equal Variance Test (Brown-Forsythe): Passed (P = 1.000)

Source of Variation	DF	SS	MS	F	P
Days	7	132.333	18.905	113.429	<0.001
Antigen	1	0.0833	0.0833	0.500	0.485
Days x Antigen	7	10.250	1.464	8.786	<0.001
Residual	32	5.333	0.167		
Total	47	148.000	3.149		

Main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor.

The effect of different levels of Days depends on what level of Antigen is present. There is a statistically significant interaction between Days and Antigen. (P = <0.001)

Power of performed test with alpha = 0.0500: for Days : 1.000
Power of performed test with alpha = 0.0500: for Antigen : 0.0500
Power of performed test with alpha = 0.0500: for Days x Antigen : 1.000

Least square means for Days :

Group	Mean
0.000	0.000
7.000	1.000
14.000	2.667
21.000	2.500
28.000	4.333
35.000	4.167
42.000	4.667
56.000	4.667

Std Err of LS Mean = 0.167

Least square means for Antigen :

Group	Mean
SG	3.042
MG	2.958

Std Err of LS Mean = 0.0833

Least square means for Days x Antigen :

Group	Mean
0.000 x SG	0.000
0.000 x MG	0.000
7.000 x SG	0.000
7.000 x MG	2.000
14.000 x SG	3.333
14.000 x MG	2.000
21.000 x SG	3.000
21.000 x MG	2.000
28.000 x SG	4.333
28.000 x MG	4.333
35.000 x SG	4.333
35.000 x MG	4.000
42.000 x SG	4.667
42.000 x MG	4.667
56.000 x SG	4.667
56.000 x MG	4.667

Std Err of LS Mean = 0.236

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Days**

Comparison	Diff of Means	p	q	P	P<0.050
42.000 vs. 0.000	4.667	8	28.000	<0.001	Yes
42.000 vs. 7.000	3.667	8	22.000	<0.001	Yes
42.000 vs. 21.000	2.167	8	13.000	<0.001	Yes
42.000 vs. 14.000	2.000	8	12.000	<0.001	Yes
42.000 vs. 35.000	0.500	8	3.000	0.424	No
42.000 vs. 28.000	0.333	8	2.000	0.844	Do Not Test

42.000 vs. 56.000	0.000	8	0.000	1.000	Do Not Test
56.000 vs. 0.000	4.667	8	28.000	<0.001	Yes
56.000 vs. 7.000	3.667	8	22.000	<0.001	Yes
56.000 vs. 21.000	2.167	8	13.000	<0.001	Yes
56.000 vs. 14.000	2.000	8	12.000	<0.001	Yes
56.000 vs. 35.000	0.500	8	3.000	0.424	Do Not Test
56.000 vs. 28.000	0.333	8	2.000	0.844	Do Not Test
28.000 vs. 0.000	4.333	8	26.000	<0.001	Yes
28.000 vs. 7.000	3.333	8	20.000	<0.001	Yes
28.000 vs. 21.000	1.833	8	11.000	<0.001	Yes
28.000 vs. 14.000	1.667	8	10.000	<0.001	Yes
28.000 vs. 35.000	0.167	8	1.000	0.996	Do Not Test
35.000 vs. 0.000	4.167	8	25.000	<0.001	Yes
35.000 vs. 7.000	3.167	8	19.000	<0.001	Yes
35.000 vs. 21.000	1.667	8	10.000	<0.001	Yes
35.000 vs. 14.000	1.500	8	9.000	<0.001	Yes
14.000 vs. 0.000	2.667	8	16.000	<0.001	Yes
14.000 vs. 7.000	1.667	8	10.000	<0.001	Yes
14.000 vs. 21.000	0.167	8	1.000	0.996	No
21.000 vs. 0.000	2.500	8	15.000	<0.001	Yes
21.000 vs. 7.000	1.500	8	9.000	<0.001	Yes
7.000 vs. 0.000	1.000	8	6.000	0.004	Yes

Comparisons for factor: **Antigen**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
SG vs. MG	0.0833	2	1.000	0.485	No

Comparisons for factor: **Antigen within 0**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
SG vs. MG	0.000	2	0.000	1.000	No

Comparisons for factor: **Antigen within 7**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
MG vs. SG	2.000	2	8.485	<0.001	Yes

Comparisons for factor: **Antigen within 14**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
SG vs. MG	1.333	2	5.657	<0.001	Yes

Comparisons for factor: **Antigen within 21**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
SG vs. MG	1.000	2	4.243	0.005	Yes

Comparisons for factor: **Antigen within 28**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
SG vs. MG	0.000	2	0.000	1.000	No

Comparisons for factor: **Antigen within 35**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
SG vs. MG	0.333	2	1.414	0.325	No

Comparisons for factor: **Antigen within 42**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
SG vs. MG	0.000	2	0.000	1.000	No

Comparisons for factor: **Antigen within 56**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
SG vs. MG	0.000	2	0.000	1.000	No

Comparisons for factor: **Days within SG**

Comparison	Diff of Means	p	q	P	P<0.050
42.000 vs. 7.000	4.667	8	19.799	<0.001	Yes
42.000 vs. 0.000	4.667	8	19.799	<0.001	Yes
42.000 vs. 21.000	1.667	8	7.071	<0.001	Yes
42.000 vs. 14.000	1.333	8	5.657	0.008	Yes
42.000 vs. 35.000	0.333	8	1.414	0.971	No
42.000 vs. 28.000	0.333	8	1.414	0.971	Do Not Test
42.000 vs. 56.000	0.000	8	0.000	1.000	Do Not Test
56.000 vs. 7.000	4.667	8	19.799	<0.001	Yes
56.000 vs. 0.000	4.667	8	19.799	<0.001	Yes
56.000 vs. 21.000	1.667	8	7.071	<0.001	Yes
56.000 vs. 14.000	1.333	8	5.657	0.008	Yes
56.000 vs. 35.000	0.333	8	1.414	0.971	Do Not Test
56.000 vs. 28.000	0.333	8	1.414	0.971	Do Not Test
28.000 vs. 7.000	4.333	8	18.385	<0.001	Yes
28.000 vs. 0.000	4.333	8	18.385	<0.001	Yes
28.000 vs. 21.000	1.333	8	5.657	0.008	Yes
28.000 vs. 14.000	1.000	8	4.243	0.086	No
28.000 vs. 35.000	0.000	8	0.000	1.000	Do Not Test
35.000 vs. 7.000	4.333	8	18.385	<0.001	Yes
35.000 vs. 0.000	4.333	8	18.385	<0.001	Yes
35.000 vs. 21.000	1.333	8	5.657	0.008	Yes
35.000 vs. 14.000	1.000	8	4.243	0.086	Do Not Test
14.000 vs. 7.000	3.333	8	14.142	<0.001	Yes
14.000 vs. 0.000	3.333	8	14.142	<0.001	Yes
14.000 vs. 21.000	0.333	8	1.414	0.971	No
21.000 vs. 7.000	3.000	8	12.728	<0.001	Yes
21.000 vs. 0.000	3.000	8	12.728	<0.001	Yes
0.000 vs. 7.000	0.000	8	0.000	1.000	No

Comparisons for factor: **Days within MG**

Comparison	Diff of Means	p	q	P	P<0.050
42.000 vs. 0.000	4.667	8	19.799	<0.001	Yes
42.000 vs. 21.000	2.667	8	11.314	<0.001	Yes
42.000 vs. 7.000	2.667	8	11.314	<0.001	Yes
42.000 vs. 14.000	2.667	8	11.314	<0.001	Yes
42.000 vs. 35.000	0.667	8	2.828	0.498	No
42.000 vs. 28.000	0.333	8	1.414	0.971	Do Not Test
42.000 vs. 56.000	0.000	8	0.000	1.000	Do Not Test
56.000 vs. 0.000	4.667	8	19.799	<0.001	Yes
56.000 vs. 21.000	2.667	8	11.314	<0.001	Yes
56.000 vs. 7.000	2.667	8	11.314	<0.001	Yes
56.000 vs. 14.000	2.667	8	11.314	<0.001	Yes
56.000 vs. 35.000	0.667	8	2.828	0.498	Do Not Test
56.000 vs. 28.000	0.333	8	1.414	0.971	Do Not Test
28.000 vs. 0.000	4.333	8	18.385	<0.001	Yes
28.000 vs. 21.000	2.333	8	9.899	<0.001	Yes
28.000 vs. 7.000	2.333	8	9.899	<0.001	Yes
28.000 vs. 14.000	2.333	8	9.899	<0.001	Yes
28.000 vs. 35.000	0.333	8	1.414	0.971	Do Not Test
35.000 vs. 0.000	4.000	8	16.971	<0.001	Yes
35.000 vs. 21.000	2.000	8	8.485	<0.001	Yes
35.000 vs. 7.000	2.000	8	8.485	<0.001	Yes
35.000 vs. 14.000	2.000	8	8.485	<0.001	Yes
14.000 vs. 0.000	2.000	8	8.485	<0.001	Yes
14.000 vs. 21.000	0.000	8	0.000	1.000	No
14.000 vs. 7.000	0.000	8	0.000	1.000	Do Not Test
7.000 vs. 0.000	2.000	8	8.485	<0.001	Yes
7.000 vs. 21.000	0.000	8	0.000	1.000	Do Not Test
21.000 vs. 0.000	2.000	8	8.485	<0.001	Yes

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

Two Way Analysis of Variance Tuesday, February 15, 2022, 2:44:06 AM

Data source: Data 3 in Notebook1

Balanced Design

Dependent Variable: Data

Normality Test (Shapiro-Wilk): Failed (P < 0.050)

Equal Variance Test (Brown-Forsythe): Passed (P = 1.000)

Source of Variation	DF	SS	MS	F	P
Days	7	115.646	16.521	66.083	<0.001
Antigen	1	0.0208	0.0208	0.0833	0.775
Days x Antigen	7	3.146	0.449	1.798	0.122
Residual	32	8.000	0.250		
Total	47	126.813	2.698		

The difference in the mean values among the different levels of Days is greater than would be expected by chance after allowing for effects of differences in Antigen. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Antigen is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Days. There is not a statistically significant difference (P = 0.775).

The effect of different levels of Days does not depend on what level of Antigen is present. There is not a statistically significant interaction between Days and Antigen. (P = 0.122)

Power of performed test with alpha = 0.0500: for Days : 1.000
Power of performed test with alpha = 0.0500: for Antigen : 0.0500
Power of performed test with alpha = 0.0500: for Days x Antigen : 0.286

Least square means for Days :

Group	Mean
0.000	0.000
7.000	2.000
14.000	1.667
21.000	2.500
28.000	4.167
35.000	4.167
42.000	4.333
56.000	4.667

Std Err of LS Mean = 0.204

Least square means for Antigen :

Group	Mean
SG	2.958
MG	2.917

Std Err of LS Mean = 0.102

Least square means for Days x Antigen :

Group	Mean
0.000 x SG	0.000
0.000 x MG	0.000
7.000 x SG	2.000
7.000 x MG	2.000
14.000 x SG	1.333
14.000 x MG	2.000
21.000 x SG	3.000
21.000 x MG	2.000
28.000 x SG	4.333
28.000 x MG	4.000
35.000 x SG	4.333
35.000 x MG	4.000
42.000 x SG	4.333
42.000 x MG	4.333
56.000 x SG	4.333

56.000 x MG 5.000
Std Err of LS Mean = 0.289

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Days**

Comparison	Diff of Means	p	q	P	P<0.050
56.000 vs. 0.000	4.667	8	22.862	<0.001	Yes
56.000 vs. 14.000	3.000	8	14.697	<0.001	Yes
56.000 vs. 7.000	2.667	8	13.064	<0.001	Yes
56.000 vs. 21.000	2.167	8	10.614	<0.001	Yes
56.000 vs. 35.000	0.500	8	2.449	0.667	No
56.000 vs. 28.000	0.500	8	2.449	0.667	Do Not Test
56.000 vs. 42.000	0.333	8	1.633	0.939	Do Not Test
42.000 vs. 0.000	4.333	8	21.229	<0.001	Yes
42.000 vs. 14.000	2.667	8	13.064	<0.001	Yes
42.000 vs. 7.000	2.333	8	11.431	<0.001	Yes
42.000 vs. 21.000	1.833	8	8.981	<0.001	Yes
42.000 vs. 35.000	0.167	8	0.816	0.999	Do Not Test
42.000 vs. 28.000	0.167	8	0.816	0.999	Do Not Test
28.000 vs. 0.000	4.167	8	20.412	<0.001	Yes
28.000 vs. 14.000	2.500	8	12.247	<0.001	Yes
28.000 vs. 7.000	2.167	8	10.614	<0.001	Yes
28.000 vs. 21.000	1.667	8	8.165	<0.001	Yes
28.000 vs. 35.000	0.000	8	0.000	1.000	Do Not Test
35.000 vs. 0.000	4.167	8	20.412	<0.001	Yes
35.000 vs. 14.000	2.500	8	12.247	<0.001	Yes
35.000 vs. 7.000	2.167	8	10.614	<0.001	Yes
35.000 vs. 21.000	1.667	8	8.165	<0.001	Yes
21.000 vs. 0.000	2.500	8	12.247	<0.001	Yes
21.000 vs. 14.000	0.833	8	4.082	0.109	No
21.000 vs. 7.000	0.500	8	2.449	0.667	Do Not Test
7.000 vs. 0.000	2.000	8	9.798	<0.001	Yes
7.000 vs. 14.000	0.333	8	1.633	0.939	Do Not Test
14.000 vs. 0.000	1.667	8	8.165	<0.001	Yes

Comparisons for factor: **Antigen**

Comparison	Diff of Means	p	q	P	P<0.050
SG vs. MG	0.0417	2	0.408	0.775	No

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

Two Way Analysis of Variance Tuesday, February 15, 2022, 2:46:51 AM

Data source: Data 4 in Notebook1

Balanced Design

Dependent Variable: Data

Normality Test (Shapiro-Wilk): Failed (P < 0.050)

Equal Variance Test (Brown-Forsythe): Passed (P = 1.000)

Source of Variation	DF	SS	MS	F	P
Days	7	171.583	24.512	69.210	<0.001
Antigen	1	0.333	0.333	0.941	0.339
Days x Antigen	7	4.667	0.667	1.882	0.105
Residual	32	11.333	0.354		
Total	47	187.917	3.998		

The difference in the mean values among the different levels of Days is greater than would be expected by chance after allowing for effects of differences in Antigen. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Antigen is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Days. There is not a statistically significant difference (P = 0.339).

The effect of different levels of Days does not depend on what level of Antigen is present. There is not a statistically significant interaction between Days and Antigen. (P = 0.105)

Power of performed test with alpha = 0.0500: for Days : 1.000
Power of performed test with alpha = 0.0500: for Antigen : 0.0500
Power of performed test with alpha = 0.0500: for Days x Antigen : 0.316

Least square means for Days :

Group	**Mean**
0.000	0.000
7.000	2.500
14.000	4.000
21.000	5.000
28.000	5.833
35.000	5.333
42.000	5.667
56.000	5.333

Std Err of LS Mean = 0.243

Least square means for Antigen :

Group	**Mean**
SG	4.125
MG	4.292

Std Err of LS Mean = 0.121

Least square means for Days x Antigen :

Group	**Mean**
0.000 x SG	0.000
0.000 x MG	0.000
7.000 x SG	2.333
7.000 x MG	2.667
14.000 x SG	3.667
14.000 x MG	4.333
21.000 x SG	4.333
21.000 x MG	5.667
28.000 x SG	5.667
28.000 x MG	6.000
35.000 x SG	5.667
35.000 x MG	5.000
42.000 x SG	5.667
42.000 x MG	5.667
56.000 x SG	5.667
56.000 x MG	5.000

Std Err of LS Mean = 0.344

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Days**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
28.000 vs. 0.000	5.833	8	24.010	<0.001	Yes
28.000 vs. 7.000	3.333	8	13.720	<0.001	Yes
28.000 vs. 14.000	1.833	8	7.546	<0.001	Yes
28.000 vs. 21.000	0.833	8	3.430	0.264	No
28.000 vs. 56.000	0.500	8	2.058	0.824	Do Not Test
28.000 vs. 35.000	0.500	8	2.058	0.824	Do Not Test
28.000 vs. 42.000	0.167	8	0.686	1.000	Do Not Test
42.000 vs. 0.000	5.667	8	23.324	<0.001	Yes
42.000 vs. 7.000	3.167	8	13.034	<0.001	Yes
42.000 vs. 14.000	1.667	8	6.860	<0.001	Yes
42.000 vs. 21.000	0.667	8	2.744	0.535	Do Not Test
42.000 vs. 56.000	0.333	8	1.372	0.976	Do Not Test

42.000 vs. 35.000	0.333	8	1.372	0.976	Do Not Test
35.000 vs. 0.000	5.333	8	21.952	<0.001	Yes
35.000 vs. 7.000	2.833	8	11.662	<0.001	Yes
35.000 vs. 14.000	1.333	8	5.488	0.010	Yes
35.000 vs. 21.000	0.333	8	1.372	0.976	Do Not Test
35.000 vs. 56.000	0.000	8	0.000	1.000	Do Not Test
56.000 vs. 0.000	5.333	8	21.952	<0.001	Yes
56.000 vs. 7.000	2.833	8	11.662	<0.001	Yes
56.000 vs. 14.000	1.333	8	5.488	0.010	Yes
56.000 vs. 21.000	0.333	8	1.372	0.976	Do Not Test
21.000 vs. 0.000	5.000	8	20.580	<0.001	Yes
21.000 vs. 7.000	2.500	8	10.290	<0.001	Yes
21.000 vs. 14.000	1.000	8	4.116	0.104	No
14.000 vs. 0.000	4.000	8	16.464	<0.001	Yes
14.000 vs. 7.000	1.500	8	6.174	0.003	Yes
7.000 vs. 0.000	2.500	8	10.290	<0.001	Yes

Comparisons for factor: **Antigen**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
MG vs. SG	0.167	2	1.372	0.339	No

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

Two Way Analysis of Variance Tuesday, February 15, 2022, 2:58:47 AM

Data source: Data 1 in Notebook1

Balanced Design

Dependent Variable: Data

Normality Test (Shapiro-Wilk): Failed (P < 0.050)

Equal Variance Test (Brown-Forsythe): Passed (P = 1.000)

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Days	7	2.667	0.381	1.143	0.362
Antigen	1	0.333	0.333	1.000	0.325
Days x Antigen	7	1.000	0.143	0.429	0.877
Residual	32	10.667	0.333		
Total	47	14.667	0.312		

The difference in the mean values among the different levels of Days is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Antigen. There is not a statistically significant difference (P = 0.362).

The difference in the mean values among the different levels of Antigen is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Days. There is not a statistically significant difference (P = 0.325).

The effect of different levels of Days does not depend on what level of Antigen is present. There is not a statistically significant interaction between Days and Antigen. (P = 0.877)

Power of performed test with alpha = 0.0500: for Days : 0.0818
Power of performed test with alpha = 0.0500: for Antigen : 0.0500
Power of performed test with alpha = 0.0500: for Days x Antigen : 0.0500

Least square means for Days :

Group	**Mean**
0.000	0.000
7.000	0.000
14.000	0.000
21.000	0.000

28.000 0.000
35.000 0.333
42.000 0.667
56.000 0.333
Std Err of LS Mean = 0.236

Least square means for Antigen :

Group	Mean
SG	0.250
MG	0.0833

Std Err of LS Mean = 0.118

Least square means for Days x Antigen :

Group	Mean
0.000 x SG	0.000
0.000 x MG	0.000
7.000 x SG	0.000
7.000 x MG	0.000
14.000 x SG	0.000
14.000 x MG	0.000
21.000 x SG	0.000
21.000 x MG	0.000
28.000 x SG	0.000
28.000 x MG	0.000
35.000 x SG	0.667
35.000 x MG	0.000
42.000 x SG	0.667
42.000 x MG	0.667
56.000 x SG	0.667
56.000 x MG	0.000

Std Err of LS Mean = 0.333

Two Way Analysis of Variance Tuesday, February 15, 2022, 3:00:53 AM

Data source: Data 2 in Notebook1

Balanced Design

Dependent Variable: Data

Normality Test (Shapiro-Wilk): Failed (P < 0.050)

Equal Variance Test (Brown-Forsythe): Passed (P = 1.000)

Source of Variation	DF	SS	MS	F	P
Days	7	15.250	2.179	1.743	0.134
Antigen	1	0.0833	0.0833	0.0667	0.798
Days x Antigen	7	3.917	0.560	0.448	0.864
Residual	32	40.000	1.250		
Total	47	59.250	1.261		

The difference in the mean values among the different levels of Days is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Antigen. There is not a statistically significant difference (P = 0.134).

The difference in the mean values among the different levels of Antigen is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Days. There is not a statistically significant difference (P = 0.798).

The effect of different levels of Days does not depend on what level of Antigen is present. There is not a statistically significant interaction between Days and Antigen. (P = 0.864)

Power of performed test with alpha = 0.0500: for Days : 0.267
Power of performed test with alpha = 0.0500: for Antigen : 0.0500
Power of performed test with alpha = 0.0500: for Days x Antigen : 0.0500

Least square means for Days :

Group	Mean
0.000	0.000
7.000	0.500
14.000	0.833
21.000	1.833
28.000	0.333
35.000	0.500
42.000	1.000
56.000	0.000

Std Err of LS Mean = 0.456

Least square means for Antigen :

Group	Mean
SG	0.667
MG	0.583

Std Err of LS Mean = 0.228

Least square means for Days x Antigen :

Group	Mean
0.000 x SG	0.000
0.000 x MG	0.000
7.000 x SG	0.000
7.000 x MG	1.000
14.000 x SG	0.667
14.000 x MG	1.000
21.000 x SG	2.000
21.000 x MG	1.667
28.000 x SG	0.667
28.000 x MG	0.000
35.000 x SG	1.000
35.000 x MG	0.000
42.000 x SG	1.000
42.000 x MG	1.000
56.000 x SG	0.000
56.000 x MG	0.000

Std Err of LS Mean = 0.645

Appendix 4.2

1. Tick Feeding

- Equal numbers of male and female *D. variabilis* ticks were fed on uninfected cattle

2. Tissue extraction

- Equal number of male and female ticks were removed from cattle from days 0-6
- Ticks were chopped and midgut (MG) and salivary gland (SG) tissues were extracted.
- MG and SG were kept separately in 0.1M PBS and 3X proteinase

3. Homogenization

- MG and SG were homogenized with Dunce homogenizer and store at -80℃.

4. Protein concentration

- Pool the SG tubes from 0-6 days into one tube.
- Pool the MG from 0-6 days into one tube.
- Protein concentration estimated by Bradford Assay for both SG and MG and stored at -80℃.

5. Immunogen preparation

- Just before immunization antigen and adjuvant were added to 5-cc syringes attached b ♀-♀ luer-lock adapter in ratio 2:3. Mix with 20 slow (~4 seconds/cycle) cycles and mix with 60 fast (~<1 second/cycle) cycles. Place (1 ml) of the emulsion into 1-cc syringe

6. Immunization

- Calves were shaved on both sides of vertebral column, just before immunization, disinfect shaved area with 70% ethanol, Immunize calves intradermally in 10 sites (0.1 cc/ site) along their backs

VITA

Sammuel Shahzad was born to Hameeda Bibi and Siddique Shahzad on March 8th, 1986, in Lahore, Pakistan. He was raised in Sahiwal, Pakistan and is the middle brother of Tanzeela, Emmanuel, Shakeela, Yousaf and Aneela Shahzad. He got married in 2012 to Shelly Saima Yaqub and was blessed with a son Zion Sammuel in 2013 and a daughter Zofia Sammuel in 2015. Sammuel graduated from Government High school Sahiwal in 2002 and received his DVM degree in 2010 from The University of Veterinary and Animal Sciences, Lahore, Pakistan. Sammuel worked as a veterinarian at the Lahore Zoo from 2012-2016. He completed his Master's in Veterinary Pathology in 2014 and was accepted to the University of Missouri in the Veterinary Pathobiology program in 2016 with a fully funded Fulbright scholarship. His PhD work was supervised by Dr. Roger William Stich, and he received his PhD degree in Veterinary Pathobiology in July 2021.