# EPIDEMIOLOGICAL ASSOCIATION OF *ANAPLASMA MARGINALE* THEILER WITH IMPORTANT CATTLE TICKS

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EPIDEMIOLOGICAL ASSOCIATION OF ANAPLASMA MARGINALE THEILER WITH IMPORTANT CATTLE TICKS

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# CHAPTER I

## INTRODUCTION

Anaplasmosis is an infectious, noncontagious blood disease which causes severe anemia in cattle. The causative organism, Anaplasma marginale Theiler was originally described in 1910 (1). Presently the organism is classified in the Order Rickettsiales, Family Anaplasmataceae (2). An erythrocytic cycle of development of A. marginale has been proposed by Ristic (3, 4) and consists of invasion of the erythrocyte by initial bodies which invaginate the cytoplasmic membrane to form a parasitic vacuole. Intraerythrocytic initial bodies multiply by binary fission and form inclusion bodies which mature and give rise to particles which may represent the actual infectious stage (3, 4, 5).

Recent studies of the disease by Kocan et al. (6, 7) have revealed a developmental sequence in midgut epithelial cells of infected ticks. Colonies of A. marginale, studied with light and electron microscopy in adult tick midgut epithelial cells, were observed to occur in five morphologic types which may contain four distinct forms of the parasite. Type one colonies were small, dense, and contained electron dense forms. Type two colonies were similar to type one, but the mass of organisms was separated from the limiting membrane forming an open area clearly visible by light microscopy. Type three colonies contained electron dense forms, larger reticulated forms and small particles,

often outside the limiting membrane. Type four colonies contained many reticulated forms which often had small particles within the cell membrane. Type five colonies contained reticulated forms and sometimes had large pleomorphic reticulated forms that often formed clusters. It was postulated that colonies sequentially develop from type one through type five in the adult tick gut cells (6). Similar studies conducted on recently fed replete nymphal ticks showed that two distinct morphological types were present and were categorized as nymphal type one and nymphal type two colonies, with light microscopy. Nymphal type one colonies contained small particles and reticulated forms which appeared to be dividing by binary fission. Nymphal type two colonies contained rodlike reticulated organisms with no evidence of binary fission present when examined with electron microscopy. Some colonies which had characteristics common to both nymphal colony types were designated transitional nymphal colonies (8).

Anaplasmosis is one of the major tick-borne hemotropic diseases which affect cattle. It occurs in most tropical and subtropical regions of the world, as well as in some temperate areas (9). The major endemic areas in the United States are along the central coastal area of California, areas in the northwestern section of the country, also from Maryland along the southern Atlantic coast to Florida and across the southeastern Gulf states to the Mexican border. Areas of medium incidence occur over the eastern portion of Oklahoma (10, 11).

Anaplasma marginale may be transmitted between animals either mechanically or biologically by many different arthropod vectors. Ticks are the only recognized biological vectors and many different species in several different genera are capable of maintaining and transmitting the

disease under laboratory conditions. Identification of specific ticks as natural vectors of anaplasmosis is complicated by the presence of certain Tabanidae which may serve as mechanical vectors (12, 13, 14). The Pacific coast tick, Dermacentor occidentalis Marx is an important vector in the Coast Range area of California (15, 16). Dermacentor albipictus (Packard) is known to transmit A. marginale transstadially (17) and was assumed to be the vector in several clinical cases in Texas (18). Dermacentor andersoni Stiles is capable of transmission and maintenance of the organism transstadially under laboratory conditions (19, 20). This tick appears to be a principal vector of anaplasmosis in the western U.S. based on results of controlled natural exposure studies (21) and upon a correlation between the seasonal occurrence of D. andersoni adults on cattle and the seasonal occurrence of the disease in areas of the geographic range of the tick. Because immature stages of D. andersoni are not known to feed on cattle or deer it has been postulated that disease transmission occurs when feeding males are dislodged from an infected host and reattach to a susceptible animal (13). The American dog tick, D. variabilis (Say) may be a natural vector of anaplasmosis in southern and eastern areas of the United States for much the same reason that D. andersoni is a probable vector is the western United States. Transstadial but not transovarial passage occurs (20) and this tick is a prominent species over the eastern half of the United States including Oklahoma. However, the immature stages prefer to engorge on small rodents and are not known to attack cattle until reaching the adult stage.

Ticks found in Oklahoma which attack cattle, other than D. variabilis and D. albipictus, include Amblyomma americanum (L.), A. maculatum Koch and Ixodes scapularis (Say). Adult stages of the Amblyomma ticks, A. americanum and A. maculatum coincide with areas of high anaplasmosis prevalence in Oklahoma but have not transmitted the disease under controlled conditions (20). The black-legged tick, I. scapularis, may be a possible vector (20) of Anaplasma in deer populations, but the tick's seasonal occurrence on cattle does not correlate with the seasonal occurrence of anaplasmosis (13).

The present study was undertaken in an effort to gain a better understanding of the relationship of Anaplasma marginale to its biological vectors, the Ixodoidea, and specifically to provide a more complete understanding of the relationship between A. marginale and possible natural vectors in Oklahoma.

Initially a serologic survey was performed to delineate the distribution and abundance of the disease in Oklahoma. This was followed by an effort to provide information about possible biological vectors associated with cattle in which midgut epithelial cells of important cattle ticks were examined for A. marginale colony development. Two experiments were designed to provide information about the biological relationship of A. marginale and D. variabilis. These included a study of overwintering and longevity capabilities of A. marginale in adult D. variabilis and evaluation of D. variabilis as an experimental vector of A. marginale from the larval through adult stages.

# CHAPTER II

# PREVALENCE AND DISTRIBUTION OF ANAPLASMOSIS IN OKLAHOMA AS INDICATED BY A SEROLOGIC SURVEY

## Introduction

Anaplasmosis is an infectious blood disease of cattle caused by Anaplasma marginale Theiler. A knowledge of the distribution of this disease in Oklahoma would lead to a better understanding of this economically important hematozoan. Serologic surveys have been performed in other states utilizing the complement fixation test (15, 22, 23, 24). In a questionnaire survey in 1972, over 13% of beef cattle producers in Oklahoma listed anaplasmosis as their primary cause of economic loss (25). This study provides information about the prevalence and distribution of anaplasmosis based on a serologic survey conducted on bovine sera collected in the 77 counties of Oklahoma in 1978-79. The serologic testing utilized a recently developed fluorescent antibody (FIAX®) test for anaplasmosis (26).

## Materials and Methods

Bovine serum samples representing animals from all 77 counties of Oklahoma were tested to establish an anaplasmosis prevalence profile for the state. Approximately 50 sera were collected in each county with no

more than 10 samples coming from any one ranch. Some background information about many of the samples in the survey was known: i.e. sex, age, breed and location. Only limited information was available for some of the sera; where missing the data were classified as unknowns in the tables.

## Serum samples

Blood samples used in the survey were collected from slaughterhouses throughout the state with the help of the Federal Brucellosis Laboratory (Oklahoma City). The sera tested in this survey were collected during 1978-79 and were frozen and stored at -20°C until serology was performed in 1982. For serodiagnosis 10 µl of serum was diluted in 750 µl of phosphate buffered saline solution with 0.15% Tween 20, pH 7.3. Samples in the survey were tested in groups ranging from 50 to 200 sera.

## Antigen

The antigen used in the FIAX test was the standard anaplasmosis CF antigen prepared by the National Veterinary Services Laboratory, Ames, Iowa. The stock CF antigen was diluted 1:8 in PBS buffer (pH 7.3), spotted on FIAX StiQ® samplers in 25 µl volumes and allowed to air dry.

## Conjugate

Fluorescein-conjugated rabbit-antibovine IgG antisera (Cooper Biomedical, Malvern, PA) was diluted 1:200 in PBS buffer (pH 7.3 and 0.15% Tween 20). It was used in tests in 0.5 ml amounts.

FIAX® Serology

The FIAX® instrumentation for serology was developed by Whittaker M.A. Bioproducts, Walkersville, MD. It is based on a microfluorometric antibody technique which uses a special fluorometer (FIAX 100®) that can measure minute amounts of fluorescence. This immunoassay has been used primarily as a serodiagnostic tool in human medicine. Fox et al. (26), adapted a FIAX method to detect serum antibodies (IgG class) specific for diagnosis of bovine anaplasmosis. The test is rapid, economical and several hundred samples can be processed with ease. The technique consisted of spotting 25 µl of titrated anaplasmosis CF antigen onto a cellulose acetate membrane (Millipore filter) attached to the StiQ® samples which was then allowed to air dry. The StiQ with CF antigen was shaken for 30 minutes in a serum sample diluted 1:76 in PBS buffer (pH 7.3 and 0.15% Tween 20) using an automated microdiluter. It was then transferred to 0.6 ml of the PBS buffer and washed for 10 minutes. Following washing the StiQ was placed in 500 µl of a 1:200 solution of fluorescein-conjugated rabbit antibovine IgG antisera for 15 minutes followed by a second 10-minute wash in the PBS buffer. The StiQ was then placed in the fluorometer to measure the fluorescent signal units. The amount of nonspecific binding of antibodies was determined by using a double-sided StiQ, whereby the fluorescent signal units from the back-side of the StiQ were subtracted from those on the reaction side. The resultant net fluorescent signal unit values were used as a measure of anaplasmosis antibody in the bovine serum.

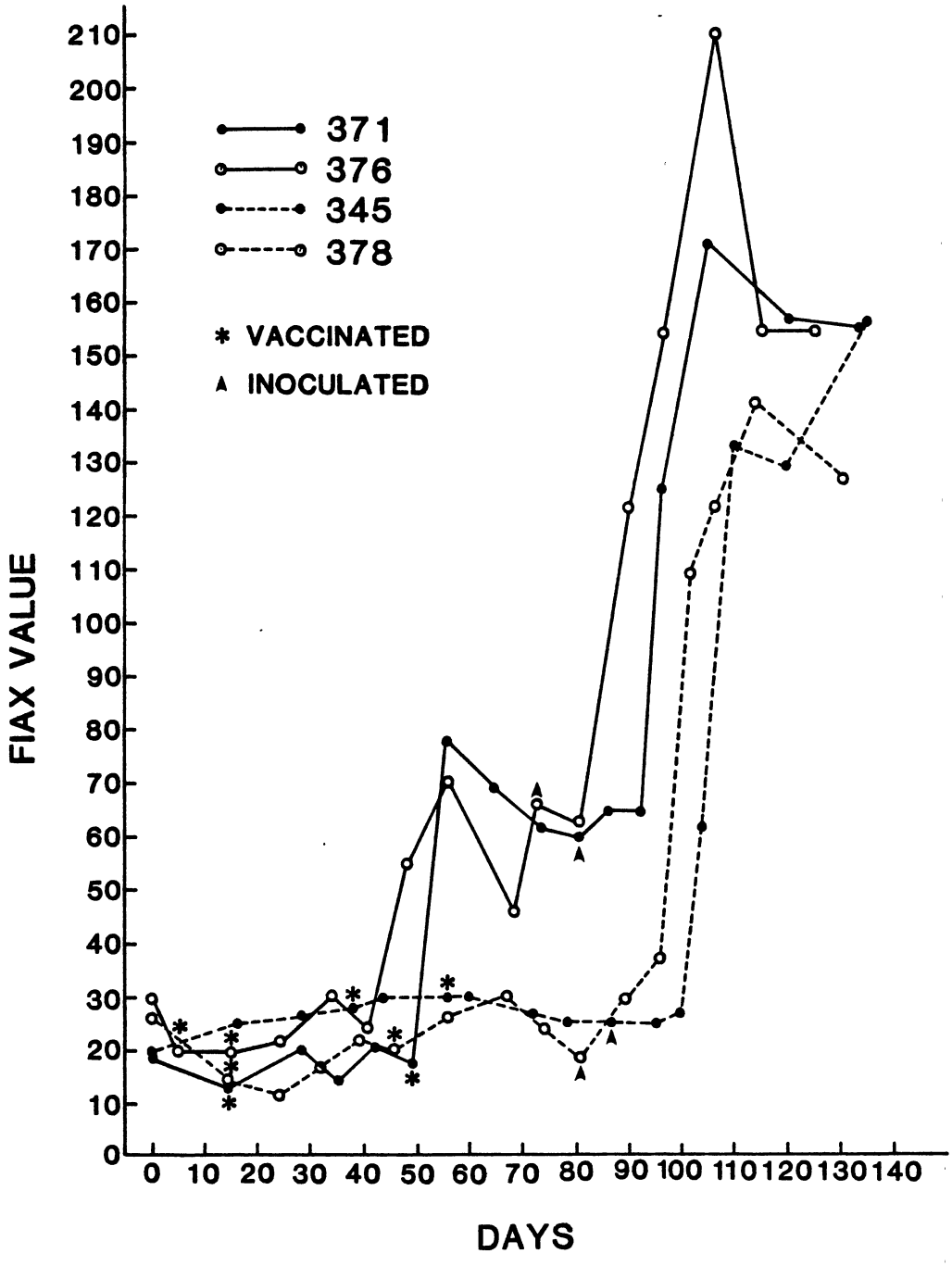
## Standardization of Control Sera

Serially collected sera from two experimentally infected cattle were titrated in order to set FIAX values for representative low and high titered control sera. The control with known FIAX values were run in duplicate with each group of serum samples and were used to determine the FIAX values for all other samples tested. The net fluorescent signal units for the control sera and their previously assigned FIAX values were used to compute a regression curve from which FIAX values were extrapolated for all other sera in each test.

## Interpretation of Antibody Levels

To determine the FIAX values which could be considered as negative, low-positive or high-positive, four splenectomized calves were inoculated with A. marginale and monitored by FIAX serology (Figure 1). Infections were produced in two of the calves by vaccination with an immunogen made from infected tick gut homogenates (27), and the remaining two calves were injected with adjuvant. The calves were monitored for serum antibody during the course of infection. The mean FIAX values for the uninfected calves and infected calves prior to inoculation (32 samples total) was added to 2.5 times the standard deviation of the mean to mark the maximum FIAX value which would be considered negative. The low-positive range of FIAX values was approximated from the rise in antibody levels after vaccination with an immunogen made from infected tick gut homogenates. The mean value for these samples (total of eight samples) was added to 2.5 times the

Figure 1. FIAX values (serum antibody levels) in calves experimentally inoculated with Anaplasma marginale. Solid lines indicate calves vaccinated with an immunogen made from infected tick gut homogenates. Broken lines indicate calves injected with adjuvant only.


371
376
345
378
* VACCINATED
▲ INOCULATED
FIAX VALUE
DAYS
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210

standard deviation of the mean to approximate the maximum FIAX value for low-positive sera. FIAX values above this level were considered high-positive.

## Results

The maximum FIAX value that was considered negative in the survey was 36. Serum samples with FIAX values between 37 and 89 were considered low-positive and FIAX values of 90 and above were considered high-positive.

A total of 3,367 bovine sera were subjected to the FIAX test for anaplasmosis. All 77 counties in the state were represented in the survey. There were 1,523 (45%) negative sera, 1,844 (55%) sera reacted positively in the FIAX tests; 533 (16%) sera had strong responses (Table I).

Female animals constituted the bulk of the survey (3,126) and 55% (1,708) were seropositives, 16% (495) were classified as high-positive reactors. Fifty-three male cattle were tested; 60% (32) were seronegative, whereas 40% (21) had low-positive titers (Table II).

Classifications of all animals by age (Table III) showed that the highest percentage of positive samples 58% of 554 was in the oldest age group, 5 years and older. Serum samples from animals classified as adults (over 2 yrs. of age) tested 57% of 1,946 seropositive. Serum samples from cattle less than two years of age revealed the lowest percentage of positive reactors, 36% of 348. Animals between two and four years of age tested 55% (122) negative, 34% (74) low-positive, and 11% (25) high-positive. The 3 serological classifications according to

TABLE I

RESULTS OF AN *ANAPLASMA MARGINALE* ANTIBODY SURVEY IN OKLAHOMA CATTLE, 1978-1979, USING A FIAX® MICROFLUOROMETRIC IMMUNOASSAY[1]

| | Total number sampled | Percent positive | | |
|---|---|---|---|---|
| | | Total | Low-positive[2] | High-positive[2] |
| Cattle | 3367 | 55 | 39 | 16 |
| Herds[3] | 604 | 77 | 77 | 17 |
| Counties[3] | 77 | 100 | 100 | 83 |

[1] FIAX® is a microfluorometric assay which uses equipment developed by Whittaker M.A. Bioproducts, Walkersville, MD. *Anaplasma marginale* CF antigen was obtained from the National Veterinary Sciences Laboratory and IgG class antibodies were detected using FITC-conjugated antisera from Cooper Biomedical, Malvern, PA.

[2] Responses determined using sera from noninfected and experimentally inoculated cattle: Low-positive FIAX values ranged from 37-89; High-positive values were 90 or greater.

[3] Percentage of herds and counties where low-positive and/or high-positive animals were found.

TABLE II

ANAPLASMA MARGINALE ANTIBODY[1] PREVALENCE
IN OKLAHOMA CATTLE, 1978-1979,
ACCORDING TO SEX

| Sex | Total tested | Percent positive | | |
|---|---|---|---|---|
| | | Total | Low-positive[2] | High-positive[2] |
| Unknown | 188 | 61 | 41 | 20 |
| Female | 3126 | 55 | 39 | 16 |
| Male | 53 | 40 | 40 | 0 |

[1] IgG class antibody response measured using a FIAX® microfluorometric immunoassay.

[2] Responses determined using sera from noninfected and experimentally inoculated cattle: Low-positive FIAX values ranged from 37-89; High-positive values were 90 or greater.

TABLE III

ANAPLASMA MARGINALE ANTIBODY[1] PREVALENCE IN OKLAHOMA CATTLE, 1978-1979, ACCORDING TO AGE

| Age | Total tested | Percent Positive | | |
|---|---|---|---|---|
| | | Total | Low-positive[2] | High-positive[2] |
| Adult unclassified | 1946 | 57 | 41 | 16 |
| Less than 2 | 348 | 36 | 29 | 7 |
| 2 - 4 | 221 | 45 | 34 | 11 |
| 5 and older | 554 | 58 | 39 | 19 |
| Unknown | 297 | 60 | 38 | 22 |

[1] IgG class antibody response measured using a FIAX® microfluorometric immunoassay.

[2] Responses determined using sera from noninfected and experimentally inoculated cattle: Low-positive FIAX values ranged from 37-89; High-positive values were 90 or greater.

TABLE IV

ANAPLASMA MARGINALE ANTIBODY[1] PREVALENCE IN OKLAHOMA CATTLE, 1978-1979, ACCORDING TO BREED

| Breed | Total tested | Percent positive | | |
|---|---|---|---|---|
| | | Total | Low-positive[2] | High-positive[2] |
| Unknown | 2017 | 57 | 40 | 17 |
| Hereford | 483 | 52 | 38 | 14 |
| Holstein | 310 | 41 | 31 | 10 |
| Angus | 305 | 63 | 39 | 24 |
| Charolais | 138 | 52 | 41 | 11 |
| Limousin | 25 | 28 | 24 | 4 |
| Jersey | 36 | 45 | 40 | 5 |
| Simmental | 30 | 50 | 43 | 7 |
| Maine Angouis | 3 | 67 | 67 | 0 |
| Guernsey | 20 | 35 | 30 | 5 |

[1] IgG class antibody response measured using a FIAX® microfluorometric immunoassay.

[2] Responses determined using sera from noninfected and experimentally inoculated cattle: Low-positive FIAX values ranged from 37-89; High-positive values were 90 or greater.

breeds are given in Table IV. Angus cattle exhibited higher percentages of positives than other breeds, showing 63% (192) positive. Hereford cattle tested 52% (251) seropositive. Holstein cattle reflected 59% (182) of the samples negative and Charlois showed 48% (66) negative samples. Several other breeds were represented in the survey, however, not in numbers large enough to be meaningful. Distribution of prevalence rates of positive sera by county are depicted in Figure 2. Distribution of prevalence rates by county of high-positive sera are shown in Figure 3.

## Discussion

A questionnaire survey in 1972 rated anaplasmosis as a priority disease (25) but more substantial evidence was needed to determine the effect of the disease. Therefore, the serologic survey was undertaken to provide direct evidence of disease prevalence and distribution.

The statewide prevalence rate for cattle exhibiting titers against Anaplasma marginale was 55%. High-positive responses were observed in 16% of the samples. The prevalence rate for Anaplasma in Oklahoma is considerably higher than rates previously reported by researchers in other states. A questionnaire survey in Texas indicated (18) only 0.254% incidence of clinical anaplasmosis in 1980. The prevalence of complement fixing antibodies in cattle in northern California was reported (15) to be 40% in 1969-70. The prevalence rate reported (22) from northern Idaho and southeastern Washington in 1972 was 8% as tested by the complement fixation or anaplasmosis card test. A survey in

Figure 2. Prevalence of Anaplasma marginale in cattle in Oklahoma according to a serological survey conducted in 1978-1979. Data represents percentages of cattle exhibiting titers using a microfluorometric immunoassay.

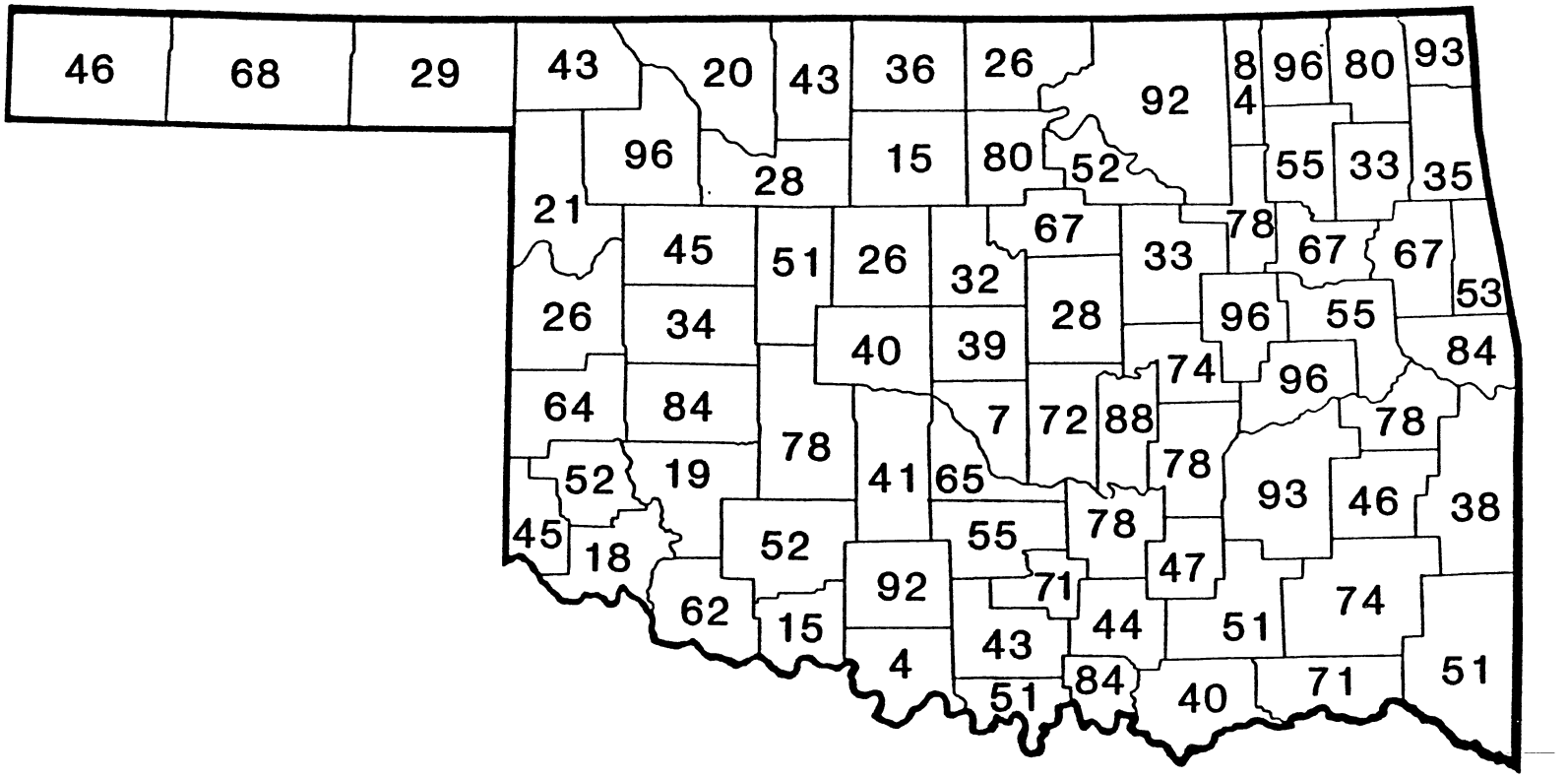
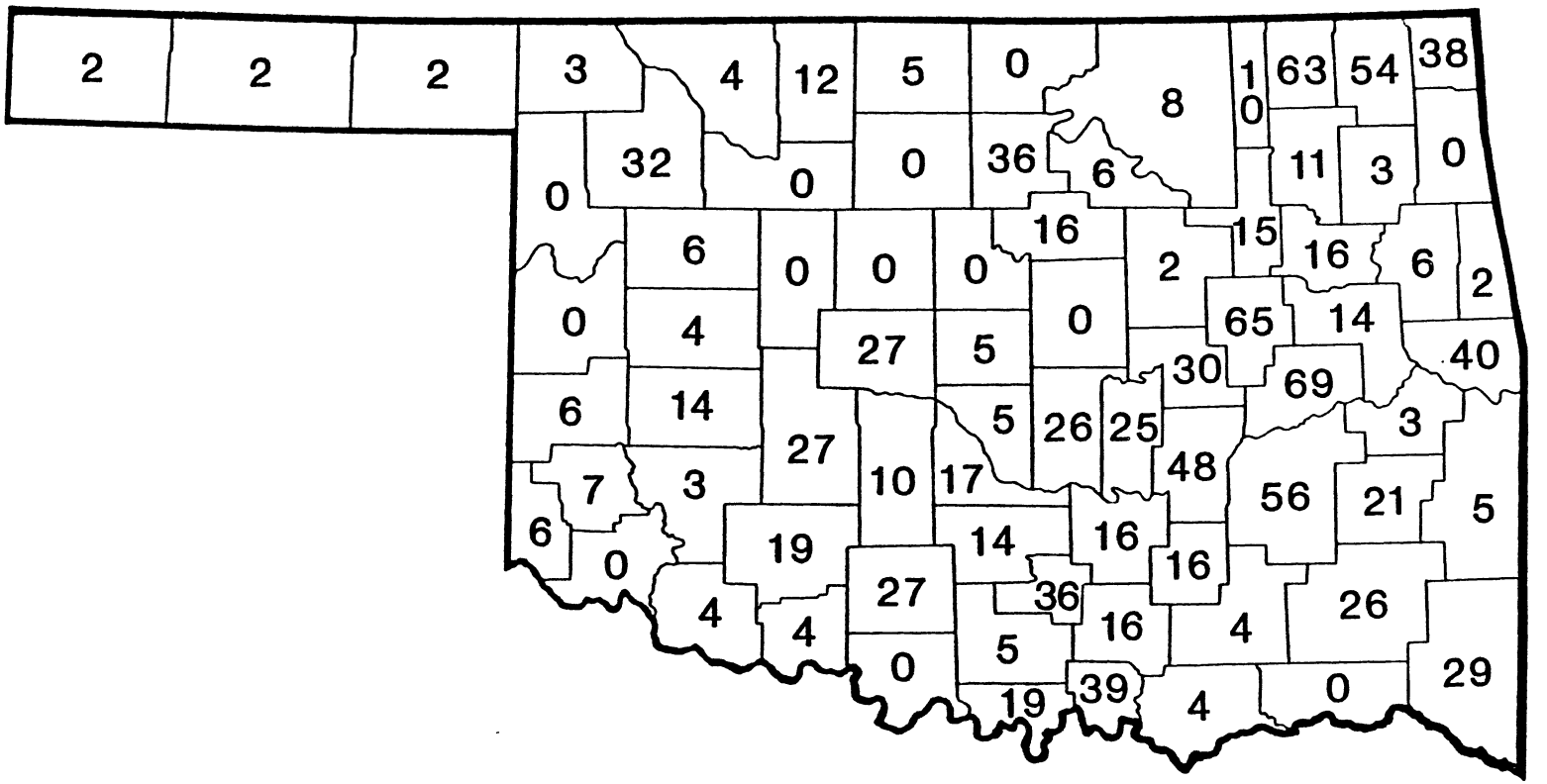

46
68
29
43
20
43
36
26
92
8
4
96
80
93
96
28
15
80
52
55
33
35
21
67
78
45
51
26
32
33
67
67
53
26
34
40
39
28
96
55
84
74
64
84
7
72
88
96
78
78
52
19
78
41
65
78
93
46
38
45
18
52
55
47
92
71
74
62
15
44
51
4
43
84
40
71
51

Figure 3. Distribution of cattle in Oklahoma with antibody levels indicative of recent Anaplasma marginale infections. Data represents percentages of animals with high antibody titers as estimated from responses in experimentally infected calves.


2
2
2
3
4
12
5
0
8
1
0
63
54
38
32
0
0
36
6
11
3
0
0
16
15
6
6
0
0
0
2
16
2
0
4
27
5
0
65
14
40
30
69
6
14
5
26
25
3
27
48
7
3
10
17
56
21
5
6
0
19
14
16
16
27
36
4
4
16
4
26
0
5
39
4
0
29
19

Wyoming (23) in 1958 reported 13% reactors to the complement fixation test.

The 3 serological classifications used in the survey: negative, low-positive and high-positive were based upon FIAX test results obtained by monitoring 4 animals during the course of an experimental infection. The low-positive category may be indicative of vaccinated animals or animals previously exposed to the disease. The number of cattle vaccinated for anaplasmosis in Oklahoma is probably small, however the extent to which the vaccine is used is not presently known. Vaccinated cattle would react as false positives in this survey. The sera placed in the high-positive category were considered to have antibody levels high enough to suggest recent exposures to infection. The possibility exists that if a larger number of calves were monitored during the course of an experimental infection then the FIAX values used to partition the samples into serological groupings may be changed, thereby altering the corresponding prevalence rates to some degree. However, that would not change the overall trends presented in this survey.

The prevalence rates for anaplasmosis varied greatly within the state, but there was a marked trend toward higher prevalence in the eastern part of Oklahoma (Figures 2 and 3). This may be due in part to a larger number of vectors in that area. Species of Amblyomma and Dermacentor ticks are more commonly found to attack cattle in eastern Oklahoma than in western Oklahoma as are many species of horseflies (Tabanus sp), some of which are mechanical vectors of the disease (13).

The youngest age group less than 2 years, had the lowest percentage of positive animals, whereas the oldest age group had the highest (Table III). This may be a reflection of the longer time period that older animals have to become exposed to the disease.

The serologic survey identified areas of high disease prevalence and could provide direction for field studies of vector transmission. Based on these results field studies could be conducted to determine how the disease is transmitted under natural conditions.

# CHAPTER III

# EXAMINATION OF IMPORTANT CATTLE TICKS FOR *ANAPLASMA MARGINALE* THEILER COLONY FORMATION IN TICK MIDGUT EPITHELIAL CELLS

## Introduction

*Anaplasma marginale* Theiler colonies were first described in ticks by Kocan et al. (6). Studies conducted with the light microscope on midgut epithelial cells of *D. andersoni* Stiles unfed adults, experimentally infected as nymphs, revealed an apparent developmental sequence of *A. marginale*, which was categorized into five morphologic types based on staining characteristics of colonies. Similar studies conducted on ticks of a different genus, *Rhipicephalus simus* Koch, corraborated the pattern of development detailed in *D. andersoni* (28). Midgut epithelial tissues of unfed adult *R. simus*, experimentally infected as nymphs, exhibited similar types of colonies as those seen in *D. andersoni*. Colonies of *A. marginale* are also known to occur in midgut epithelial tissues of infected *D. variabilis* (Say) (7).

The precise mode of transmission of the disease organism from ticks to cattle is not presently known, although it is apparent that a phase of development occurs in the midgut epithelial cells of some ticks which

are capable of biologically transmitting the disease. The present experiment was designed to determine if Anaplasma colonies also form in the midgut epithelial cells of Amblyomma ticks, a genus previously reported to be incapable of biological transmission of anaplasmosis (20).

Amblyomma americanum (L.), A. maculatum (Koch), and A. cajennense (F.), are cattle associated ticks that occur in large numbers on cattle during the summer in areas where anaplasmosis is prevalent. Amblyomma americanum and A. maculatum are prevalent in the southern United States, including eastern Oklahoma, while A. cajennense is limited to southern Texas (29). Attempts at experimental transmission with these Amblyomma species have been unsuccessful (20, 30), but because of an association with cattle in endemic areas where frequent exposure to Anaplasma can occur in the natural environment, it may be possible that a biological relationship exists between these organisms and Amblyomma ticks.

## Materials and Methods

One splenectomized dairy calf (three months old), found to be negative by the anaplasmosis complement fixation test, was infected with a Virginia isolate[1] of A. marginale by feeding 25 pair of infected D. andersoni. When the donor calf developed a parasitemia of two percent, unfed nymphs of three Amblyomma species, A. americanum, A. maculatum and A. cajennense as well as Dermacentor

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[1] United States Department of Agriculture, Animal Parasitology Institute, Beltsville, Maryland.

variabilis, which was used as a control, were placed in orthopedic stockinette cells attached to the side of the calf and allowed to engorge. The rapid phase of tick engorgement occurred during the period of highest parasitemia. Engorged nymphs were removed daily after detachment and held in a humidity chamber (90-98 percent relative humidity) at 25C with a 14:10 light:dark photophase until one month postmolt. Ten pairs of each tick species were then selected at random and examined for Anaplasma colony formation. All ticks used in this study were obtained from colonies at the Oklahoma State University Medical Entomology Laboratory (31).

Collection of Gut Tissues and Light Microscopy

Dissected gut tissues were processed individually by immediate immersion in cold two percent glutaraldehyde in a 0.27M sodium cacodylate buffer for two hours, then washed three times in the same buffer with sucrose added. The tissues were postfixed with two percent osmium tetroxide in 0.27M sodium cacodylate buffer for one hour followed by three sucrose-buffer washes. Tissues were dehydrated through a graded series (50-100 percent) of ethanol and infiltrated with Dow Epoxy resin 731[1] using propylene oxide as the intermediate solvent. Thick sections (1 μM) of the epoxy embedded tissue were stained with Mallory's stain (32) at 60C for two minutes. Cross sections of each tick gut were examined for A. marginale colonies using light microscopy (x1000). Gut area was measured with a calibrated ocular grid

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[1] Dow Epoxy resin (732) Polysciences Inc. Paul Valley Industrial Park, Warrington, PA.

and colony density (No. colonies per 0.1 $mm^2$ gut tissue examined) was calculated.

## Results

The experimentally infected dairy calf, which served as the source of infection for ticks in this experiment, developed a parasitemia of 28 percent during the period of tick engorgement. Low levels of A. marginale colonies were present, 0.29 colonies per 0.1$mm^2$ gut tissue, in D. variabilis ticks which were used as a control to which tissue from Amblyomma was compared. No evidence of colony formation was seen in gut tissues from the Amblyomma species (Table V).

## Discussion

Amblyomma americanum and A. maculatum were chosen for this study because they are most frequently found to feed on cattle in eastern Oklahoma, A. cajennense was included because it is a cattle associated tick in southern Texas which, like A. americanum, attacks cattle in the immature as well as adult stages. These species would be good candidates for transstadial transmission if they became infected with A. marginale. Dermacentor variabilis was chosen as a control species because it has been shown to become infected with Anaplasma marginale (7) and adult ticks attack cattle during the spring and summer in Oklahoma.

This study suggested that the three species of Amblyomma ticks were not vectors of A. marginale because there was no evidence that they had become infected as colonies were not found in the gut cells.

TABLE V

COLONY DENSITIES[1] IN MIDGUT EPITHELIAL CELLS OF *AMBLYOMMA* AND *DERMACENTOR* TICKS INFECTED WITH *ANAPLASMA MARGINALE*[2]

| Tick species | Female | | | Male | | |
|---|---|---|---|---|---|---|
| | N | Colony density | Range | N | Colony density | Range |
| *D. variabilis* | 10 | 0.29 | 0-3 | 10 | 0.29 | 0-2 |
| *A. americanum* | 10 | 0 | 0 | 10 | 0 | 0 |
| *A. maculatum* | 10 | 0 | 0 | 10 | 0 | 0 |
| *A. cajennense* | 10 | 0 | 0 | 10 | 0 | 0 |

[1] Number of colonies per $0.1/mm^2$ gut tissue examined.

[2] Donor calf had a prepatent period of 37 days and peak parasitemia of 25.9%.

All species of ticks were fed on the same donor calf during the period of ascending parasitemia in a controlled laboratory experiment. Animal transmission studies would have been helpful in confirming these data, they were planned and conducted as part of this experiment. However, cattle feed was found to be contaminated with tetracyclines and caused these studies to be terminated. Previous studies (27, 33) have shown that infected ticks that transmit the disease have colonies in gut cells. Colonies were demonstrated in D. variabilis and this species was therefore chosen for the following studies.

Rickettsial organisms other than Anaplasma are known to occur in Amblyomma ticks. Rickettsia rickettsi occurs in A. americanum and A. cajennense (34) and A. maculatum can be infected with R. parkeri (35) and Cowdria ruminantium (36). Little is known about factors that influence the ability of ticks to become infected with disease agents. Some ticks have a peritrophic membrane which may act as a barrier to the passage of pathogens between the midgut contents and the epithelial cells of the gut (37) but such a membrane has not been seen in either Dermacentor or Amblyomma ticks. Dermacentor variabilis concentrates the ingested host blood meal to a greater extent than does A. cajennense or A. maculatum which could lead to a higher exposure level to pathogens for D. variabilis (38).

# CHAPTER IV

# EVALUATION OF *DERMACENTOR VARIABILIS* (SAY) AS AN EXPERIMENTAL VECTOR OF *ANAPLASMA MARGINALE* THEILER FROM THE LARVAL THROUGH ADULT STAGES

## Introduction

The ability of *Dermacentor variabilis* (Say) ticks to transmit anaplasmosis under laboratory conditions has been demonstrated (19, 20, 39). Ticks experimentally infected as larvae or nymphs transmit the disease as nymphs or adults. Several attempts to demonstrate transovarial passage of *Anaplasma marginale* Theiler in *D. variabilis* were unsuccessful (40, 30). Anthony and Roby (30) reported the results of a transmission trial in which adult *D. variabilis*, infected as larvae, did not transmit although the intervening nymphs were infective to a susceptible calf.

The role of *D. variabilis* as a natural vector for anaplasmosis remains unproven. However, because the presence of adult stages of these ticks on cattle in nature correlates with the seasonal occurrence of anaplasmosis in Oklahoma, it should be considered as a potential vector. Dikmans (13) postulated that transmission may occur when feeding males reattach to a susceptible animal after being dislodged from an infected host. Another possible route of transmission could be

through infection of immature tick stages, if ticks in these stages were to engorge on an infected animal and feed on susceptible animals in subsequent stages. It is not presently known whether immature D. variabilis attack cattle in the natural environment. The following experiment was designed to evaluate, under laboratory conditions, the ability of D. variabilis infected with A. marginale as larvae to transmit the disease in the subsequent nymphal and adult stages. Anthony and Roby (30) reported no transmission in the adult stage in a similar experiment but it seems likely that both nymphs and adults would transmit the disease thereby increasing the vector potential of D. variabilis, if immature stages engorge on infected animals in the natural environment.

## Materials and Methods

### Animals

Six splenectomized dairy calves (two to three months old), found to be negative by the anaplasmosis complement fixation test, were used in this study. A susceptible animal was inoculated intravenously with a Virginia isolate[1] of A. marginale. After developing the disease, large numbers of laboratory reared ticks were allowed to engorge on this animal. Gut homogenate prepared from infected ticks was made with gut tissues dissected from 25 pairs of ticks in RPMI medium[2] and

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[1] United States Department of Agriculture, Animal Parasitology Institute, Beltsville, Maryland.

[2] GIBCO Inc., Grand Island, NY.

homogenized in ground glass grinders. Gut homogenate was injected intravenously into a splenectomized dairy calf (No. 520) which after developing anaplasmosis, served as the source of infection for ticks used in this study. Following exposure to ticks, the calves were monitored biweekly for infection by microscopic examination of Wright-stained blood smears for the presence of organisms. After detection of marginal bodies in the peripheral blood the calves were monitored daily. Calves were housed in a temperature controlled building and confined to individual stalls with head stanchions.

## Ticks

Dermacentor variabilis ticks were maintained in a colony at the Oklahoma State University Medical Entomology Laboratory (31). Colony ticks were held in humidity chambers (90-98% relative humidity) at 25C with a 14:10 light:dark photophase. Larval and nymphal stages were routinely fed on rabbits, whereas adult ticks were fed on sheep.

Approximately 1000 to 2000 unfed larvae and 500 unfed nymphs were placed in orthopedic stockinette cells attached to the donor calf (No. 520) when the parasitemia level reached two percent. Thus, the rapid phase of tick feeding (four to eight days post attachment) coincided with the days of highest parasitemias. Engorged ticks were collected daily after engorgement and held in a humidity chamber until one month postmolt.

Adult ticks (25 pairs) infected as nymphs on donor calf No. 520 were placed in a cell attached to a susceptible calf (No. B-2) to

determine infectivity. Gut tissues from ten pairs of unfed adults from the same group were also collected at this time.

Nymphal ticks, infected as larvae on calf No. 520, were placed in a cell attached to a susceptible calf (No. B-1) to determine infectivity. Gut tissues of several unfed infected nymphs from the same group were collected at this time. Engorged nymphs were held in a humidity chamber until one month postmolt at which time 25 pairs of the adult ticks were allowed to feed on a susceptible calf (No. B-25). Gut tissues of ten pairs of unfed adult ticks were collected at this time.

Collection of Gut Tissues and Light Microscopy

Gut tissues were collected from ticks in each group after 2.5 days incubation at 37C (38). Dissected gut tissues were processed individually by immediate immersion in cold two percent glutaraldehyde in a 0.27M sodium cacodylate buffer for two hours, then washed three times in the same buffer with sucrose added. The tissues were postfixed with two percent osmium tetroxide in 0.27M sodium cacodylate buffer for one hour followed by three sucrose-buffer washes. Tissues were dehydrated through a graded series (50-100 percent) of ethanol and infiltrated with Dow Epoxy resin 732[1] using propylene oxide as the intermediate solvent. Thick sections (1 μM) of the epoxy embedded tissue were stained with Mallory's stain (32) at 60C for two minutes. Cross sections of each tick gut were examined for A. marginale

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[1] Dow Epoxy resin (732), Polysciences Inc. Paul Valley Industrial Park, Warrington, PA.

colonies using light microscopy (X1000). Gut area was measured with a calibrated ocular grid and colony density (No. colonies per 0.1 $mm^2$ gut tissue examined) was calculated.

## Results

### Tick Transmission of A. marginale

When D. variabilis were infected as larvae and fed in the subsequent nymph and adult stages on susceptible calves, it was determined that the organism was transmitted by nymphs infected as larvae and again as adults without reinfection in the nymphal stage (Figure 4). Ticks infected as larvae on calf No. 520 transmitted the disease in the nymphal stage to calf No. B-1 which had a prepatent period of 23 days and peak parasitemia of 35.4 percent (Table VI). The subsequent adult ticks transmitted the disease to calf No. B-25 which had a prepatent period of 32 days and peak parasitemia of 31.2 percent. The ticks infected as nymphs on calf No. 520 transmitted the disease to calf No. B-2 in the adult stage. The prepatent period was 30 days and the parasitemia reached 31.8 percent.

### Examination of Gut Tissues

Gut tissues from incubated unfed nymphs, infected as larvae on calf No. 520, contained A. marginale colonies (8). No colonies were observed in gut tissues of 10 pairs of the subsequent adult ticks which were incubated even though ticks of the same group transmitted the disease to calf No. B-25 (Table VI).

Gut tissues of 10 pairs of incubated unfed adult ticks, infected as

Figure 4. Schematic diagram of the experimental design to test transstadial transmission of Anaplasma marginale in Dermacentor variabilis.

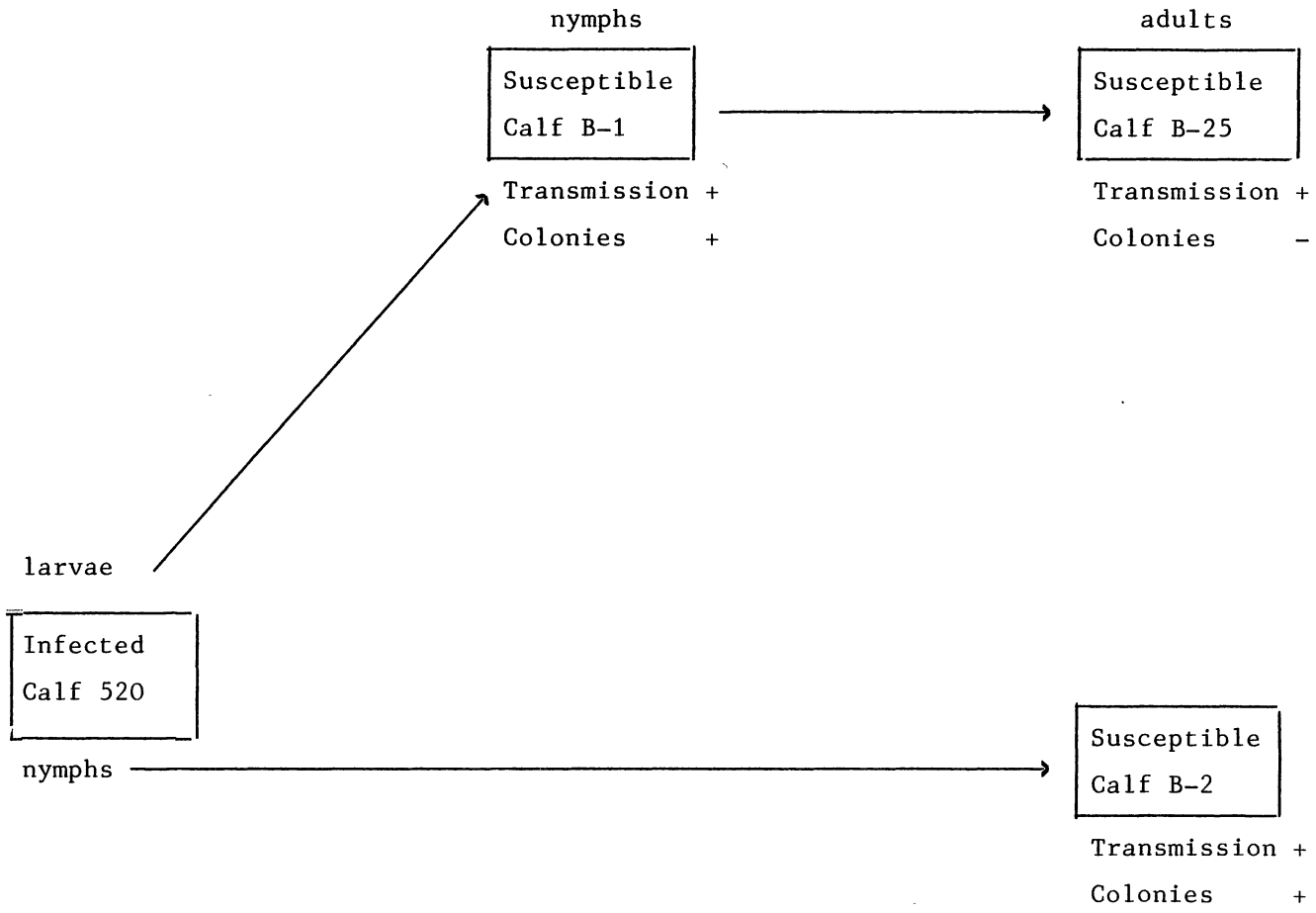
nymphs
adults
Susceptible
Calf B-1
Susceptible
Calf B-25
Transmission +
Colonies +
Transmission +
Colonies -
larvae
Infected
Calf 520
nymphs
Susceptible
Calf B-2
Transmission +
Colonies +

TABLE VI

RESULTS OF TRANSSTADIAL TRANSMISSIONS OF *ANAPLASMA MARGINALE* IN *DERMACENTOR VARIABILIS*

| Tick Stage | Calf No. | Prepatent[c] period(days) | Peak parasitemia(%) | Colony[e] density |
|---|---|---|---|---|
| Nymph[a] | B-1 | 23 | 35.4[d] | +[f] |
| Adult[b] | B-25 | 32 | 31.2[d] | 0 |
| Adult[a] | B-2 | 30 | 31.8[d] | 0.8 |

[a] Preceding stage infected on calf No. 520 with a peak parasitemia of 38.6%

[b] Preceding stage fed on calf No. B-1

[c] Prepatent period is the time between tick attachment and the appearance of marginal bodies in the peripheral blood smear.

[d] Calves found to undergo seroconversion and became CF-positive.

[e] Number of colonies per 0.1 $mm^2$ gut tissue examined.

[f] Colonies present but not quantified.

nymphs on calf No. 520, had A. marginale colonies present in 14 of the 20 ticks examined. The mean average colony density in this group of ticks (total number of colonies/total cross section area of gut tissue examined) was 0.8 colonies per 0.1 $mm^2$ of tissue examined.

## Discussion

This study confirmed transstadial transmission of A. marginale in D. variabilis reported previously (19, 20, 39). However, transmission from larvae to adult without reexposure as nymphs had not been previously confirmed (30). This is in contrast to tick infections with Theileria and Babesia, in which ticks infected as larvae transmit and lose the infection as nymphs and are therefore noninfective as adults unless the nymphs are reinfected by feeding (41).

The exact mechanism of transmission of A. marginale by ticks has not been determined. Although the gut is a site of extensive development, the organism is believed to be transmitted through the salivary glands. These studies support this concept because adult D. variabilis, infected as larvae, transmitted the disease but no colonies were found in the gut which does not support the hypothesis of tick transmission by infected gut regurgitation.

Gut tissues taken from ticks in this study were incubated 2.5 days in an effort to increase colony densities (33). However, adult ticks infected as nymphs on No. 520 held low colony densities in comparison to colony densities of D. andersoni infected on donor calves with low parasitemias (27). Since colony densities were low in adult ticks infected as nymphs it seems possible that colony densities of adult

ticks infected as larvae would also be low or possibly lower due to the intervening nymph stage, thus decreasing the chances of locating gut colonies of A. marginale if they were present.

Previous studies have documented ticks with gut colonies that did not transmit the organism (42) but this report of transmission with no gut colonies present is unique. Further studies are needed to investigate transmitting adults without gut colonies. This may be a method of possibly increasing Anaplasma levels in salivary glands thereby providing a basis for understanding transmission through salivary glands.

# CHAPTER V

## OVERWINTERING AND LONGEVITY OF *ANAPLASMA MARGINALE* THEILER IN MIDGUT EPITHELIAL CELLS OF *DERMACENTOR VARIABILIS* (SAY) IN OKLAHOMA

### Introduction

To be an effective biological vector of anaplasmosis in temperate climates *Dermacentor variabilis* (Say) must harbor *Anaplasma marginale* Theiler transstadially and from one vector season to the next. The ability of *A. marginale* to pass the winter in *D. variabilis* and the persistence of infection in the tick are factors that may be of epizootilogical importance for control of tick transmitted anaplasmosis in endemic areas.

Kocan et al. (42) determined under laboratory conditions that colonies of *A. marginale* in midgut epithelial cells of *Dermacentor andersoni* Stiles unfed adults decreased over time, markedly so between three to six months of age. A relationship between increased *A. marginale* colony density and tick infectivity was observed in *D. andersoni* ticks when incubated 2.5 days at 37°C (43). Inoculation of gut homogenates made from incubated ticks into susceptible calves resulted in shortened prepatent periods and a greater potential to produce infection (43, 33).

The objective of the present study was to monitor colony densities in midgut epithelial cells of experimentally infected D. variabilis unfed adult ticks under laboratory conditions, cold storage conditions and natural conditions for a period of one year.

## Materials and Methods

A splenectomized dairy calf (two months old), anaplasmosis complement fixation test negative, was infected with a Virginia isolate of A. marginale by feeding 30 pairs of artificially infected D. andersoni ticks on it. The animal developed a patent infection of anaplasmosis 37 days later and was used as the source of infection for unfed D. variabilis nymphs used in this study. Ticks were reared and maintained in colony at the Oklahoma State University Medical Entomology Laboratory (31).

Approximately 4000 unfed D. variabilis nymphs were placed in orthopedic stockinette cells attached to the infected calf when the parasitemia reached two percent. Elevated parasitemia levels occurred during the rapid phase of tick engorgement. Replete nymphs were collected after detachment and held in a humidity chamber (90-98 percent relative humidity) at 25C with a 14:10 light:dark photophase until two weeks postmolt. The unfed adult ticks were then separated into three groups and each group placed under a different set of environmental conditions, either laboratory conditions, cold storage conditions or natural conditions as described below.

Laboratory Conditions: groups of 100 unfed adult D.

variabilis were placed in 237 ml paper containers fitted with plastic lids and held in a humidity chamber as earlier described.

Cold Storage Conditions: groups of 100 unfed adult D. variabilis were placed in 237 ml paper containers fitted with plastic lids and held in a cold chamber (94-98 percent relative humidity) at 4.5C with a 14:10 light:dark photophase.

Outdoor Conditions: groups of 100 unfed adult D. variabilis were placed outdoors on 1 December 1983. Each group was held in a length of four inch white PVC pipe covered with hardware cloth to prevent tick escape and provide protection from predators.

A sample of infected ticks was taken at two weeks postmolt before the adults were separated into different environments. Samples were collected at ca two month intervals from each group for the following year. The initial sample collected at two weeks postmolt consisted of collecting gut tissues from 10 pairs of randomly selected ticks. Subsequent samples collected at two month intervals consisted of collecting gut tissues from 10 pairs of ticks randomly retrieved from each of the three environments.

## Collection of Gut Tissues and Light Microscopy

For each sample period indicated gut tissues from 10 pairs of unfed adult ticks from each environmental condition were dissected and processed individually by immediate immersion in cold two percent glutaraldehyde in a 0.27M sodium cacodylate buffer for two hours,

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[1] Dow Epoxy resin (732), Polysciences Inc. Paul Valley Industrial Park, Warrington, PA.

then washed three times in the same buffer with sucrose added. The tissues were postfixed with two percent osmium tetroxide in 0.27M sodium-cacodylate buffer for one hour followed by three sucrose-buffer washes. Tissues were dehydrated through a graded series (50-100 percent) of ethanol and infiltrated with Dow Epoxy resin 732[1] using propylene oxide as the intermediate solvent. Thick sections (1 µm) of the epoxy embedded tissue were stained with Mallory's stain (32) at 60C for two minutes. Cross sections of each tick gut were examined for A. marginale colonies using light microscopy (x1000). When colonies were present each was assigned a type number (1-5) based on the morphologic characteristics as described by Kocan et al. (6). Each colony was measured with a calibrated ocular micrometer and the area of the gut cross section was determined with a calibrated ocular grid. A colony density was calculated per $0.1mm^2$ gut tissue examined.

## Results

The infected dairy calf which served as the source of infection for ticks used in the study developed a peak parasitemia of 25.9 percent during the period of tick engorgement. The colony density data obtained from collections made during the study are displayed in Figures 5 and 6. The average colony density reflects data from several gut cross sections of each tick pooled for each sample group.

Laboratory conditions: An increase in colony density was indicated in ticks from two weeks to three months of age followed by a decrease at five months of age. Colony density again increased in ticks

Figure 5. Anaplasma marginale colony density (mean No. of colonies/0.1 $mm^2$ of gut tissue examined) from infected adult Dermacentor variabilis held in different environments. [a] Cold storage temperature, 4.5°C. [b] Outdoor mean temperature between sample periods, respectively; -0.5°, 8.9°, 19.7°, 27.2°, 18.9°C. [c] Laboratory chamber temperature, 25°C.

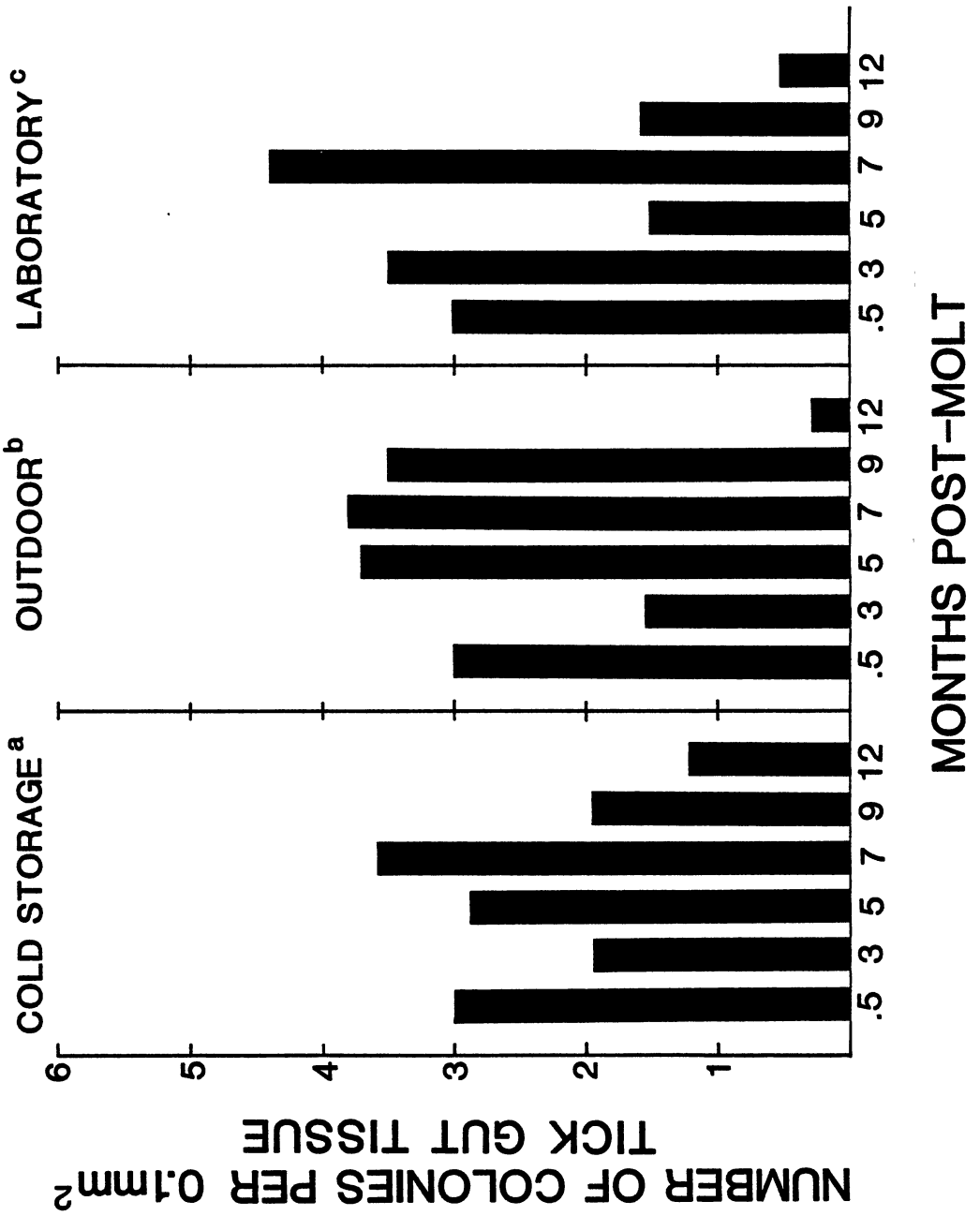
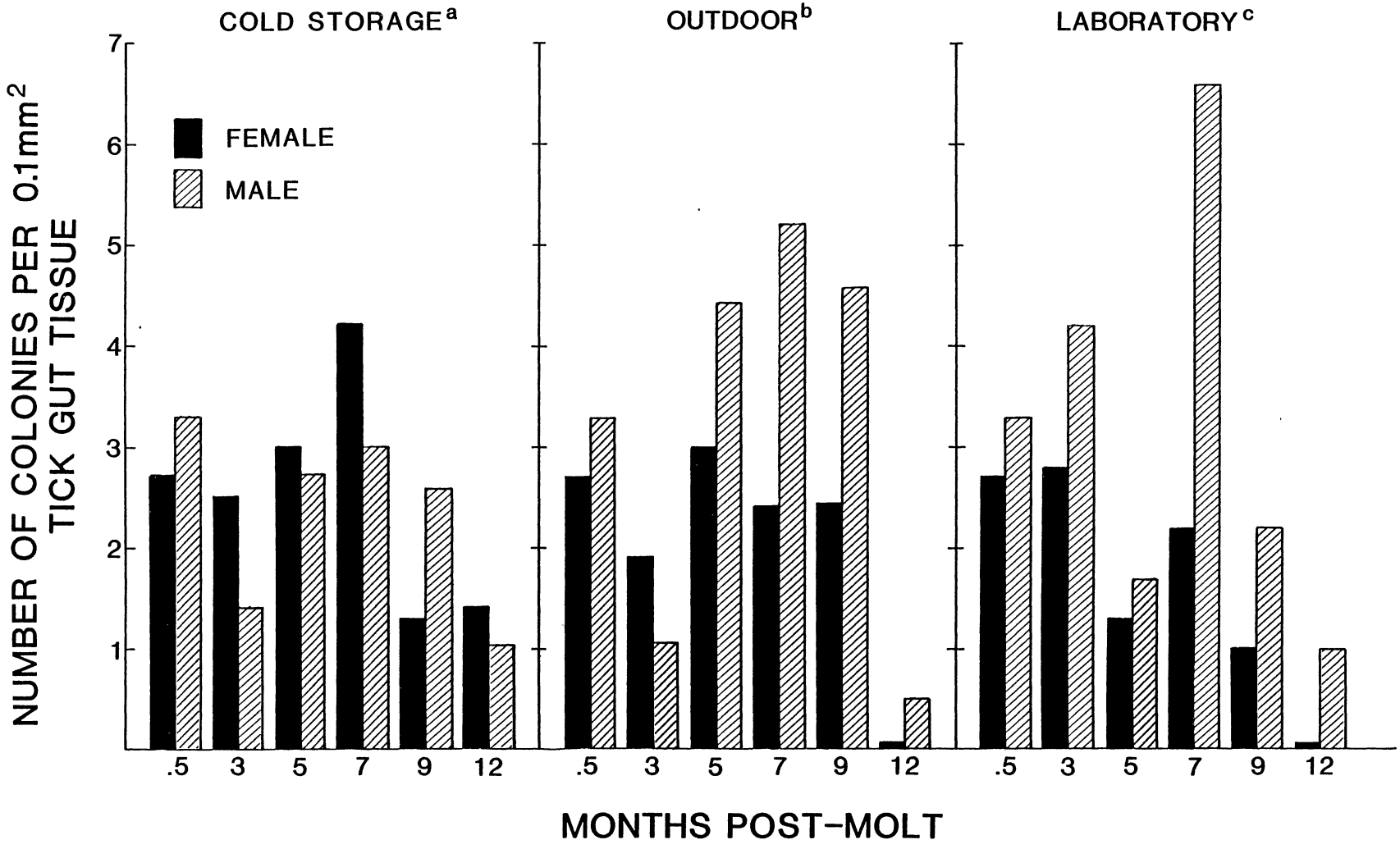

NUMBER OF COLONIES PER 0.1mm²
TICK GUT TISSUE
6
5
4
3
2
1
COLD STORAGE[a]
OUTDOOR[b]
LABORATORY[c]
.5 3 5 7 9 12
MONTHS POST-MOLT

Figure 6. *Anaplasma marginale* colony density (mean No. of colonies/0.1 $mm^2$ of gut tissue examined) from infected adult female and male *Dermacentor variabilis* held in different environments. [a] Cold storage temperature, 4.5°C. [b] Outdoor mean temperature between sample periods, respectively; -0.5°, 8.9°, 19.7°, 27.2°, 18.9°C. [c] Laboratory chamber temperature, 25°C.


COLD STORAGE[a]
OUTDOOR[b]
LABORATORY[c]
FEMALE
MALE
NUMBER OF COLONIES PER 0.1mm²
TICK GUT TISSUE
1
2
3
4
5
6
7
.5
3
5
7
9
12
MONTHS POST-MOLT

seven months of age followed by decreased densities in ticks sampled at nine and twelve months of age (Figure 5). When colony densities of each sex were observed over time (Figure 6), the same pattern of colony density was shown for each sex, however, densities were greater in male ticks during each sampling period, particularly so in ticks seven months of age. Female ticks exhibited low colony densities at twelve months of age.

Cold Storage: Colony densities declined from two weeks to three months of age. Increased densities were observed in ticks at five months and colony densities peaked at seven months of age, followed by a decline at nine months and again at twelve months of age (Figure 5). Female and male ticks exhibited similar patterns of colony densities whether compared separately or combined. When the baseline data (two weeks of age) was excluded, colony densities were observed to increase from three months of age and peak at seven months of age followed by a decline at nine and twelve months of age. A slight rise in colony densities was observed in female ticks from nine to twelve months of age (Figure 6).

Outdoor Conditions: Colony densities declined from two weeks to three months of age. Increased densities were observed in ticks at five months and colony densities peaked at seven months of age, followed by a decline at nine months and again at twelve months of age (Figure 5); this was a similar trend to that observed in ticks under cold storage. Female tick colony densities, excluding baseline data, reflected increased densities from three to five months of age followed by a decline from five to nine months of age, and another decline at twelve months of age. Male ticks exhibited increased densities from three to

five and five to seven months of age followed by declining densities in samples collected at nine and twelve months of age.

A statistical analysis was conducted on these data and it was determined that the three treatments could be fitted to a quadratic equation. Further analysis revealed that the same equation could be used for data obtained from ticks under cold storage and outdoor conditions whereas data obtained from ticks held under laboratory conditions was marginally different ($P = .077$).

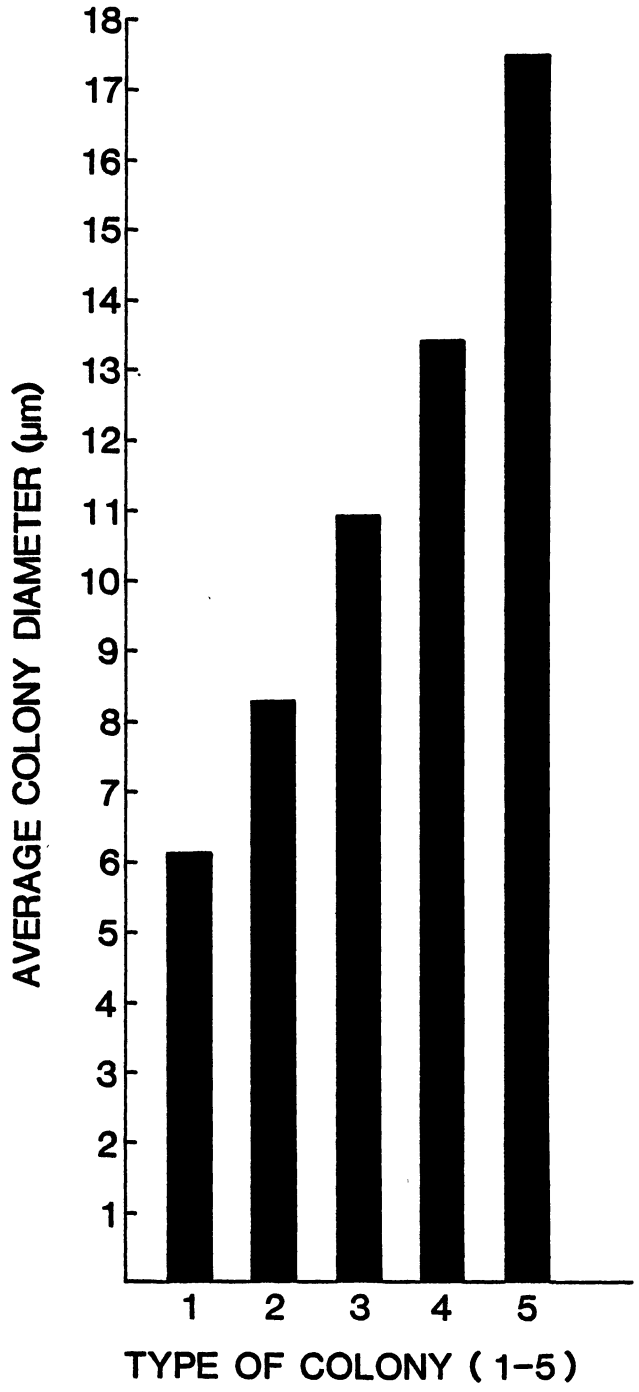
Data from female ticks analyzed separately showed that the same quadratic equation could be used in all three environmental conditions. This was also true for data from male ticks. However, data from the combined sexes was enough different to result in marginal significance.

Anaplasma Colony Diameters: When diameters of A. marginale colonies were recorded for each sex under the different environmental conditions during the study period, it was determined that colony diameter increased correspondingly in size for each colony type one through five, with colony type one being the smallest and type five the largest. These data were consistent for each observation period so the data was combined and is depicted in Figure 7.

## Discussion

In evaluating the role of D. variabilis as a vector of A. marginale it is important to determine experimentally if the parasite has the capability to overwinter in ticks under optimum conditions in the laboratory. Previous studies of A. marginale in D. andersoni (42) showed that A. marginale colony density decreased

Figure 7. Comparison of the mean diameters of the five morphologic types of colonies of Anaplasma marginale observed in adult Dermacentor variabilis from December 1983 through November 1984. Ticks were held under laboratory, cold storage and natural conditions.


AVERAGE COLONY DIAMETER (µm)
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
1
2
3
4
5
TYPE OF COLONY (1-5)

rapidly in adults after three months of age and did not appear to persist long enough to overwinter. However, these studies were done at room temperature and not under natural conditions in which the metabolism of the tick may be slowed by exposure to lower temperatures during the winter. Therefore, these studies were undertaken to study and compare survival of A. marginale in D. variabilis under laboratory conditions, cold storage conditions and natural conditions.

Infection of ticks was evaluated by colony density levels. This has been shown to be an indication of infectivity in ticks (43, 33). Transmission studies were planned concurrently but were discontinued when cattle feed was found to be contaminated with tetracyclines, these could not be repeated due to time constraints of the study and costs of experimental calves.

The pattern of colony density levels within each group were similar but more so in cold treated and outdoor groups. An increase was seen at seven months, followed by a decrease, especially noticable at twelve months of age. In contrast to previous studies of A. marginale in D. andersoni (42) in which colony levels dropped after three months of age, high colony levels were seen in the room temperature group at seven months of age. Dermacentor variabilis used in these studies survived much longer than D. andersoni of previous longevity studies (42), even though D. variabilis of the outdoor group were exposed to harsh overwintering temperatures (lowest temp. -24°C).

These studies suggest that D. variabilis could remain infected with A. marginale long enough to pass the winter and possibly be transmitted the following season. Dermacentor variabilis overwinter in adult and larvae stages in Virginia (44), thus it is likely that this

could occur in Oklahoma. This information provides further support for consideration of D. variabilis as potentially an effective vector of A. marginale. Further studies are needed to confirm that the overwintered ticks can transmit the disease. Also field studies are needed to demonstrate natural transmission as well as the specific mode of transmission. Immature stages of D. variabilis feed mainly on small mammals and are not known to attack cattle until the adult stage. Adult males may be important vectors if they reattach to susceptible animals after dislodgement from an infected animal. It is possible that adult males as well as immature stages could be involved in disease transmission under natural conditions.

Colony diameter data for the entire study was depicted in a combined graph because the pattern was consistent throughout all groups. The results were consistent with previous studies of A. marginale in D. andersoni (6, 33) which established increased diameter with colony type, type one being smallest and type five largest.

# CHAPTER VI

## SUMMARY

Anaplasmosis is one of the major tick-borne hemotropic diseases of cattle in the world. It is also of economic importance to cattle producers in Oklahoma. The primary goal of the present studies was to provide epidemiologic links to ticks which may serve as natural biological vectors of anaplasmosis in Oklahoma cattle. Initially the distribution and abundance of the disease in the state was delineated through a serological survey of cattle sera for Anaplasma antibodies. It was determined from the survey that the disease was most abundant in the eastern portion of Oklahoma. Because the disease was more prevalent in eastern Oklahoma, the three dominant cattle associated tick species in that region were examined for Anaplasma colonies in a controlled laboratory experiment. The results indicated that Amblyomma americanum (L.) and A. maculatum Koch did not exhibit Anaplasma colonies but Dermacentor variabilis (Say) exhibited colonies.

Based on the determination that D. variabilis was the only species of the three important cattle tick species which occur in the adult stage on cattle during the disease season in Oklahoma to exhibit Anaplasma colonies, this species was chosen for study as a probable disease vector.

Further experiments determined that under laboratory conditions, D. variabilis infected as larvae can transmit the disease in the nymphal and adult stages without reinfection. Overwintering of A. marginale in adult D. variabilis is likely to occur in Oklahoma based on results of the final portion of this study. Both of these biological relationships may become important in the control of the disease in endemic areas should D. variabilis be shown to be a major biological vector for anaplasmosis in Oklahoma.

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VITA 2

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