A STUDY OF SPOROGONY OF *THEILERIA CERVI* (BETTENCOURT) IN THE SALIVARY GLANDS OF *AMBLYOMMA AMERICANUM* (L.)

By

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I thank and dedicate this thesis to my husband, Jerry, and stepsons, Matt and Ben. Their patience, understanding, and unfailing support have made this crazy final semester bearable, and enabled me to reach my goal.

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CHAPTER I

INTRODUCTION

Literature Review

The first reported case of a Theileria sp. in the United States occurred in a calf that had been splenectomized as part of an anaplasmosis study (Splitter, 1950) and this blood piroplasm was identified as Theileria mutans. In 1961, a white-tailed deer (Odocoileus virginianus) native to Missouri, also splenectomized as part of an anaplasmosis experiment, developed a blood piroplasm that was identified as a Theileria sp. (Krier et.al., 1962). The parasitemia in the deer reached 50%; the animal became anemic and died approximately 12 weeks post surgery. Before the deer died, piroplasms were successfully transmitted to another deer by intravenous injection of infected whole blood. This same route of administration was used in an attempt to transmit piroplasms to a splenectomized calf and a sheep, but no piroplasms or clinical disease was noted. Because this Theileria could not be transmitted to cattle, it was not classified as T. mutans.

Studies dealing with the piroplasm from deer resulted in the designation of this organism as Theileria cervi (Schaffler, 1962). The taxonomic designation was based on a

morphologic comparison of the erythrocytic piroplasms observed in the white-tailed deer and those described in fallow deer (Dama dama) by Bettencourt, et.al., 1907. Schaffler described and classified the piroplasms according to their shape as commas, safety pins, bipolar, and signet rings. He also transmitted the parasite successfully to six other deer via intravenous and subcutaneous injections of infected whole blood. All deer developed severe anemias, a notable inflammatory response and died. Schaffler's attempts to transmit the organism to sheep and cattle failed. Using various histochemical techniques, he demonstrated the presence of DNA, RNA, basic proteins and lipids in the piroplasms. In a serologic evaluation using the fluorescent antibody test, gel-diffusion test, and capillary tube agglutination test to identify the various species of Theileria, including T. parva, T. annulata, T. lawrencei, T. recondita and T. cervi, neither the fluorescent antibody test or the gel-diffusion test could detect serum antibody levels in infected animals. The capillary tube agglutination test did provide more reliable results but cross-reactivity was observed between T. cervi antigen and sera from animals infected T. recondita, T. annulata, T. lawrencei, and T. parva, indicating a strong antigenic relationship between these species (Schaffler, 1963).

Robinson et.al. (1967) conducted a survey in Texas to determine if T. cervi infection contributed to the decline in the health of the white-tailed deer in that state. Field

necropsies of deer and blood film screenings indicated that Theileria sp. was a common parasite in most Texas deer herds. Infective blood (5 ml.) from an experimentally infected deer was inoculated into five intact and five splenectomized fawns. One of the splenectomized fawns died; the remaining four became anemic and eventually recovered. The average high parasitemia for the fawns was 30%. Of the intact deer, anemia was not observed and parasitemias were less than 4%. The results of these studies suggested that in immunocompromised or nutritionally deprived deer, clinically apparent T. cervi infections may occur and thus may play a role in the periodic die-offs that had been reported in Texas.

Field studies conducted in Texas revealed that areas with a high prevalence of the lone star tick, Amblyomma americanum, also had a high prevalence of Theileria sp. in deer (Kuttler, et.al., 1967A). In experimental transmission studies utilizing adult A. americanum that fed as nymphs on an infected deer, prepatent periods for T. cervi infections were 14 to 21 days.

Because of the pleomorphic nature and marked morphological similarities within members of the family Theileridae, recognition of individual species is difficult. Kuttler et,al., (1967B), attempted to develop a system of serologic classification using the complement fixation (CF) technique. The test results proved to be quite variable, and animals with chronic, low level parasitemias were not readily

identified by this test. Since the capillary tube agglutination (CA) test did not appear to correlate with the parasitemia level, (Schaffler, 1963), Kuttler et.al., (1967C), compared the efficiency of the two tests. In all aspects of the study, the CA test was found to be more sensitive, especially in detecting animals with low parasitemias (less than 2%) and more consistent in detecting infected animals regardless of the level of infection. The CA test was also found to be highly selective for Theileria sp. as it did not crossreact with any other known hemoparasites of deer.

Theileria sp. was first reported in white-tailed deer in Oklahoma in 1969 (Barker et.al., 1973) in a study of the interaction of white-tailed deer and lone star ticks. The Theileria sp.was experimentally transmitted from infected field collected ticks to susceptible fawns. Piroplasms appeared in the blood films of all tick infested fawns and did not appear in the controls. Theileria sp. did not appear to have any effect on the blood parameters measured in deer infested with low numbers of ticks; parasitemia levels in these fawns did not rise above 10%. In the deer that had greater numbers of ticks, parasitemias were as high as 27%.

Hoch (1973) evaluated the effects of Theileria sp. and A. americanum on fawn survival and hematology. In the study, a variety of blood parameters were compared between groups of fawns infested with uninfected A. americanum,

Theileria sp. infected A. americanum, and fawns infected with Theileria sp. from intravenous whole blood injection. The major factor in fawn mortality was attributed to the total number of ticks that fed on the fawn. The Theileria sp. infection did not appear to cause clinical disease.

Durham et.al. (1976) conducted the first studies describing T. cervi in ticks. A fluorescent antibody (FA) technique was used to determine the infectivity rates of ticks by examining sectioned salivary glands and oral secretion smears. Although the results were not consistent, parasites were demonstrated in both the sections and smears. The data suggested that A. americanum was a reservoir host (overwinter as well) for T. cervi in Oklahoma.

Extensive field studies have shown that Theileria sp. is a hemoprotozoon that commonly occurs in white-tailed deer throughout the southeastern U.S. (Davidson et.al., 1983). These studies also demonstrated that T. cervi was most often found in areas infested by A. americanum.

The life cycle of Theileria sp. occurring outside North America, especially T. parva, has been studied extensively in the mammalian (Hullinger et.al.,1964, Mugera et.al., 1972, Schein et.al., 1978B, Young et.al., 1978, Fawcett et.al., 1982) and arthropod hosts (Cowdry et.al.,1932, Martin et.al., 1964, Purnell et.al., 1968, 1975, MacMillan et.al., 1971, Schein, 1975, Schein et.al., 1977, 1978A, Mehlhorn et.al., 1977, 1979, Warnecke et.al., 1979, 1980, Irwin et.al., 1981). Sporogony in the tick

salivary gland has been described with light microscopy for Theileria parva in Rhipicephalus appendiculatus (Cowdry et.al., 1932, Martin et.al., 1964, Binnington et.al., 1983), T. annulata in Hyalomma anatolicum excavatum (Schein et.al., 1978), T. mutans in Amblyomma variegatum (Purnell et.al., 1975) and T. taurotragi in R. appendiculatus (Young et.al., 1980). Detailed ultrastructural observations of sporogony by T. parva in R. appendiculatus (MacMillan et.al., 1971, Fawcett et.al., 1982A), T. ovis in R. evertsi evertsi (Mehlhorn et.al., 1979), and T. taurotragi in R. appendiculatus (Fawcett et.al., 1985), have also been reported.

Research Objectives

Although a great deal of information about the life cycle of Theileria sp., especially T. parva has been generated, many aspects of the cycle are still not well understood. The life cycle of T. cervi has not been studied in its vector, except for the fluorescent antibody study on the salivary glands of infected A. americanum (Durham et.al., 1976). The purpose of this study was to demonstrate T. cervi in the female A. americanum and to describe sporogony with light and electron microscopies. The information obtained may help with classification of the Theileriidae, lead to field studies on both ticks and deer and provide a research model for other species of Theileria.

CHAPTER II

MATERIALS AND METHODS

Description of Facilities

Deer Facilities

A 15 acre deer research facility was used for these studies. The facility consists of two, four acre fenced pens, a barn with six 6x20 foot enclosed runs and four cement floored holding pens (each 20x20 foot).

Tick Laboratory

Ticks were reared at the OSU Department of Entomology Tick Laboratory. This laboratory is 10,000 square feet and provided facilities for rearing and holding ticks. Facilities were also available for feeding ticks on rabbits or sheep to obtain developmental stages desired. An acaridarium, with controlled humidity, was used for retention of ticks while molting (Patrick, et.al., 1975).

Electron Microscope Laboratory

The College of Veterinary Medicine has an Electron Microscope Laboratory with a JEOL 100CX II transmission-scanning electron microscope. Also available was a

photography laboratory for processing of film plates and printing of electron micrographs.

Veterinary Research Laboratory

The Veterinary Research Laboratory in the College of Veterinary Medicine provided facilities, instruments and supplies for the dissection and processing of tick tissues for light and electron microscopies. Equipment used included dissecting microscopes, chemical hood, vacuum oven, and Sorvall MT 5000 ultramicrotome.

Study Procedures

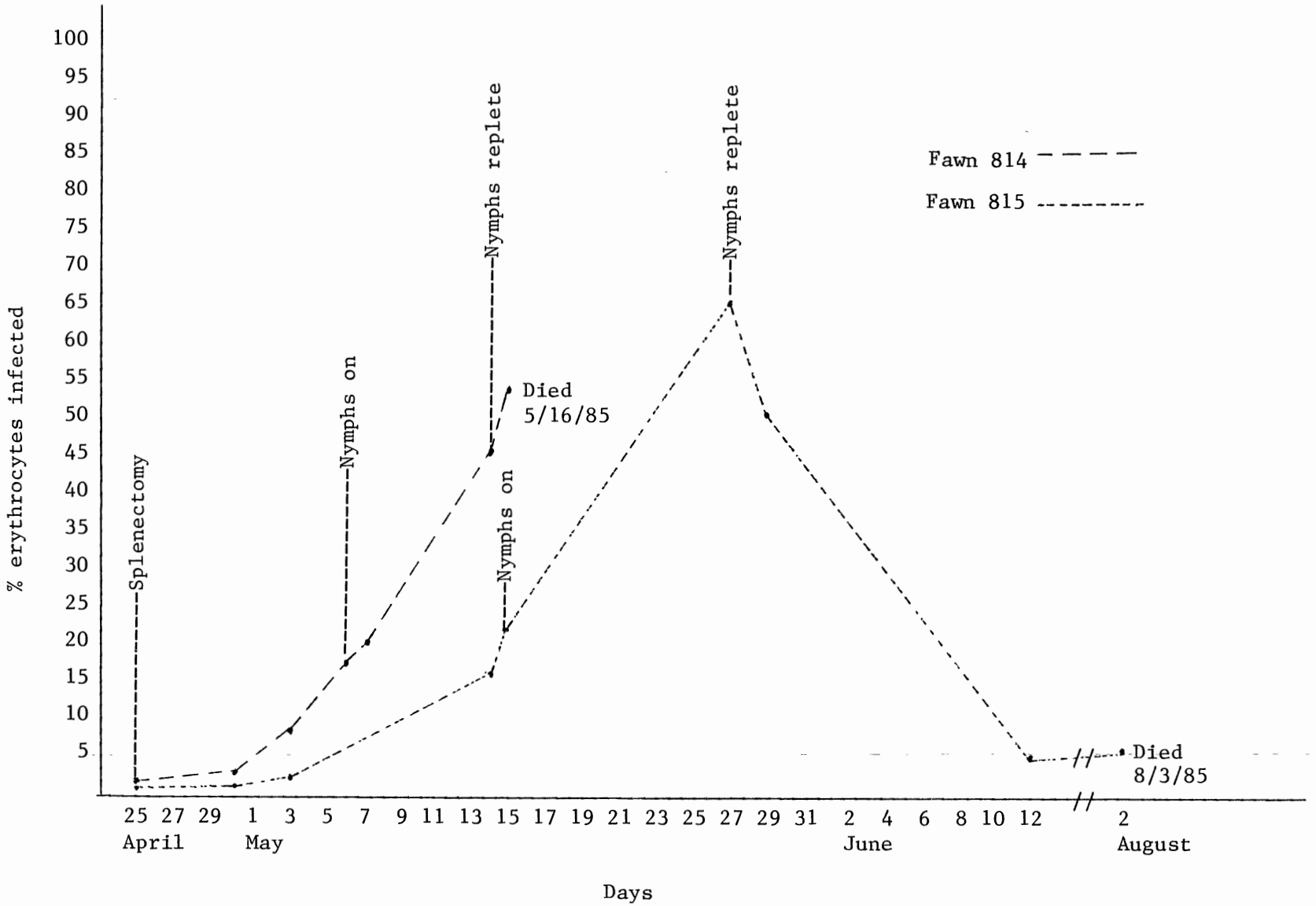
Infection of Ticks

Two white-tailed deer fawns (Odocoileus virginianus) obtained from eastern Oklahoma, No.s 814 and 815, naturally infected with Theileria sp. were bled to determine their present level of infection. Thin blood films were made, stained with Diff-Quik and studied under oil immersion. A parasitemia (percentage of piroplasm infected erythrocytes) was determined by counting the number of infected cells out of a total of 500 total erythrocytes and expressing it as a percentage. The fawns were then splenectomized under fluothane anesthesia. Blood was collected at the time of surgery and every few days there after to monitor the rate of parasite increase (Figure 1). When the parasitemia reached 10% (approximately 10-21 days post splenectomy), each deer was placed in a solid wooden box (24x44x39 inches)

with approximately 2,000 Amblyomma americanum nymphs which were reared and maintained at the Oklahoma State University Department of Entomology Tick Laboratory (Patrick et.al., 1975). The fawns remained in the box for 24 hours to allow the nymphs to attach. The infested fawns were then placed in a soldered wire cage (35x25x31 inches) suspended over stainless steel pans lined with newspaper to catch the replete nymphs as they detached. The edges of the pans were trimmed with masking tape, allowing 1/2 to 1" of the adhesive side to extend above the edge to prevent replete nymphs from leaving the pans. The nymphs were collected daily, counted and placed in paper cartons that were stored in an acaridarium (90-98% relative humidity, 25°c temperature and a 14 hour photophase). Approximately 50 days post repletion, the newly molted adult ticks were divided into two groups. One group was used for light and electron microscopy, the other was used for transmission studies.

The fawns remained in the cage for 7 days or until all of the nymphs became replete. Upon removal, the deer was dusted with an acaricide powder, bled to determine the parasitemia level, and returned to outside pens. Periodic blood samples were taken to monitor the fawns' parasitemias. (Figure 1)

Figure 1. Percentage of erythrocytes infected with *Theileria cervi* in white-tailed deer fawns after splenectomy and during feeding of *Amblyomma americanum* nymphs.


% erythrocytes infected
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
Splenectomy
Nymphs on
Nymphs replete
Died
5/16/85
Nymphs on
Nymphs replete
Fawn 814
Fawn 815
Died
8/3/85
25 27 29 1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 2 4 6 8 10 12 2
April
May
June
August
Days

Collection of Tick Salivary Glands

After molting to the adult stage, ticks were used for histologic studies. One hundred and fifty pairs of *Theileria* sp. infected *A. americanum* from fawn 814, 150 pairs from fawn 815 and 100 pairs of laboratory reared control *A. americanum* adult ticks were placed in separate stockinette cells attached to a closely shorn sheep. Twenty infected pairs from each trial and 10 control pairs of ticks were removed from the sheep, beginning with unfed ticks and continuing every day of feeding until repletion. The ticks were dissected by removing the dorsal surface of the tick's exoskeleton with a razor blade and salivary glands were removed with finely-sharpened forceps.

Fixation and Processing of Tick Salivary Glands for Light and Electron Microscopy

Dissected salivary glands were placed immediately in cold 2% glutaraldehyde (pH 7.4) in 0.2M sodium cacodylate and allowed to fix at 4°c for 24-48 hours. After washing in sucrose buffered 0.2M sodium cacodylate, the tissues were post-fixed in 2% osmium tetroxide (in the same), dehydrated through graded alcohols, and held for 48 hours in capped vials with a mixture of 1:1 propylene oxide/DER 732. The vials were uncapped for approximately 12 hours and embedded in DER in flat embedding molds, and allowed to harden in a vacuum oven at 60°c at 8 mm. of Hg for a minimum of two days.

Thick sections (1µm) of tissues were prepared using glass knives and a Sorvall MT 5000 ultramicrotome. The sections were stained at 60°c with Mallory's stain and examined with a light microscope.

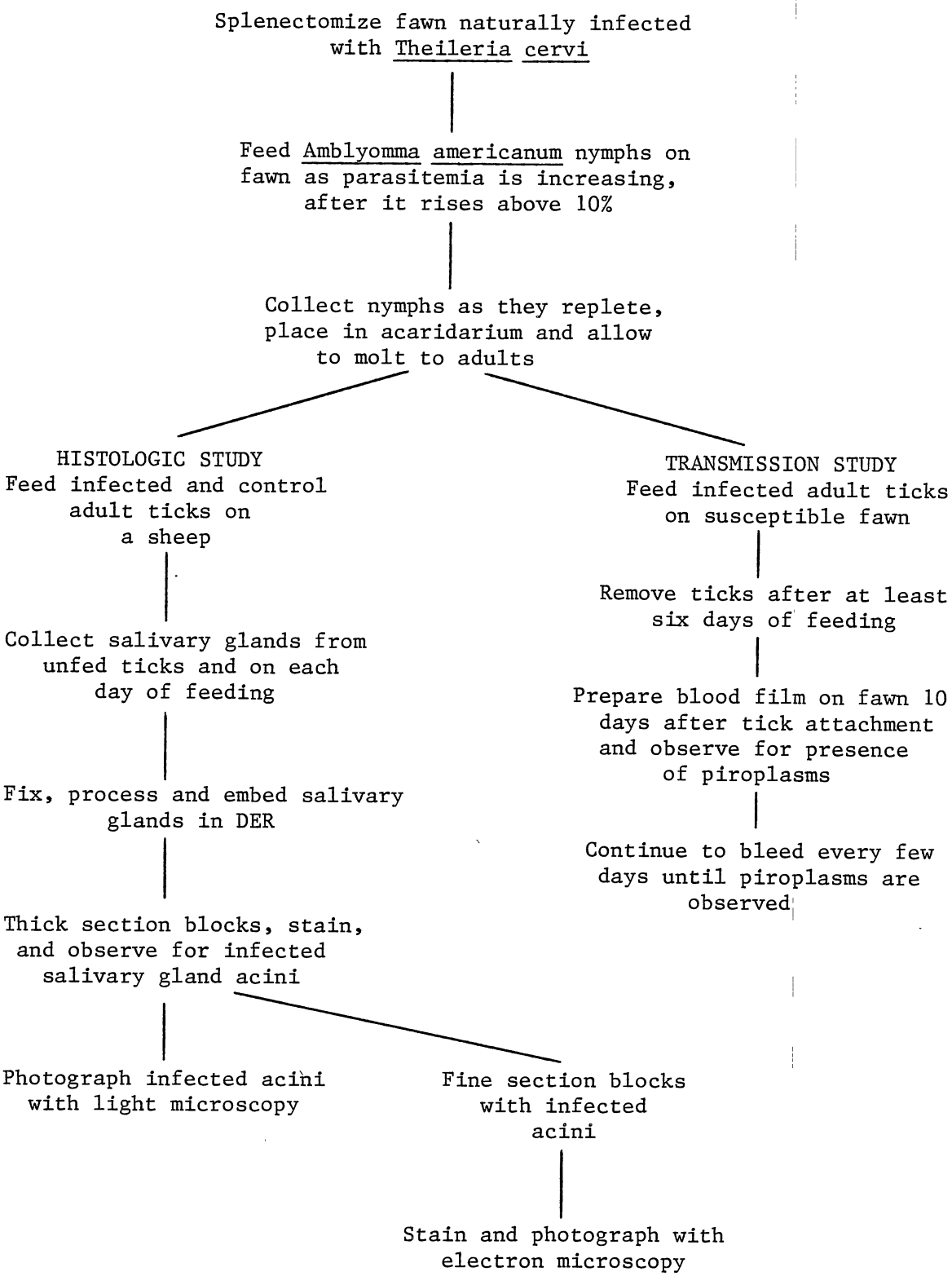
Blocks with infected salivary gland acini were fine sectioned with a Dupont diamond knife and a Sorvall MT 5000 ultramicrotome at 75-85 nm. (gold to dark gold interference color). The sections were treated with chloroform, collected on 200 mesh copper grids and stained with 5% uranyl acetate for 30 minutes and lead citrate for 1-1 1/2 minutes (Venable et.al., 1965). Sections were examined and photographed on a JEOL 100CX II transmission-scanning electron microscope at 100 KV.

Confirmation of Infectivity of Ticks

Stockinette ear bags were attached to two susceptible intact white-tailed deer fawns, No.s 904 and 905. Twenty five pairs of infected A. americanum adults (12 pairs to one ear bag, 13 pairs to the other) were placed on each fawn. Deer 904 received ticks infected on deer 814 and deer 905 received ticks infected on deer 815. The ticks were allowed to attach and feed a minimum of six days to allow for maturation and transmission of the parasite. Blood films were prepared and stained beginning 10 days post tick attachment and every few days there after until piroplasms were demonstrated in the fawn's blood. After piroplasms

were observed, the fawns were bled periodically to monitor parasitemia levels.

Figure 2. Experimental Design for collection, preparation and examination of samples.


Splenectomize fawn naturally infected with Theileria cervi
Feed Amblyomma americanum nymphs on fawn as parasitemia is increasing, after it rises above 10%
Collect nymphs as they replete, place in acaridarium and allow to molt to adults
HISTOLOGIC STUDY
Feed infected and control adult ticks on a sheep
Collect salivary glands from unfed ticks and on each day of feeding
Fix, process and embed salivary glands in DER
Thick section blocks, stain, and observe for infected salivary gland acini
Photograph infected acini with light microscopy
Fine section blocks with infected acini
Stain and photograph with electron microscopy
TRANSMISSION STUDY
Feed infected adult ticks on susceptible fawn
Remove ticks after at least six days of feeding
Prepare blood film on fawn 10 days after tick attachment and observe for presence of piroplasms
Continue to bleed every few days until piroplasms are observed

CHAPTER III

RESULTS

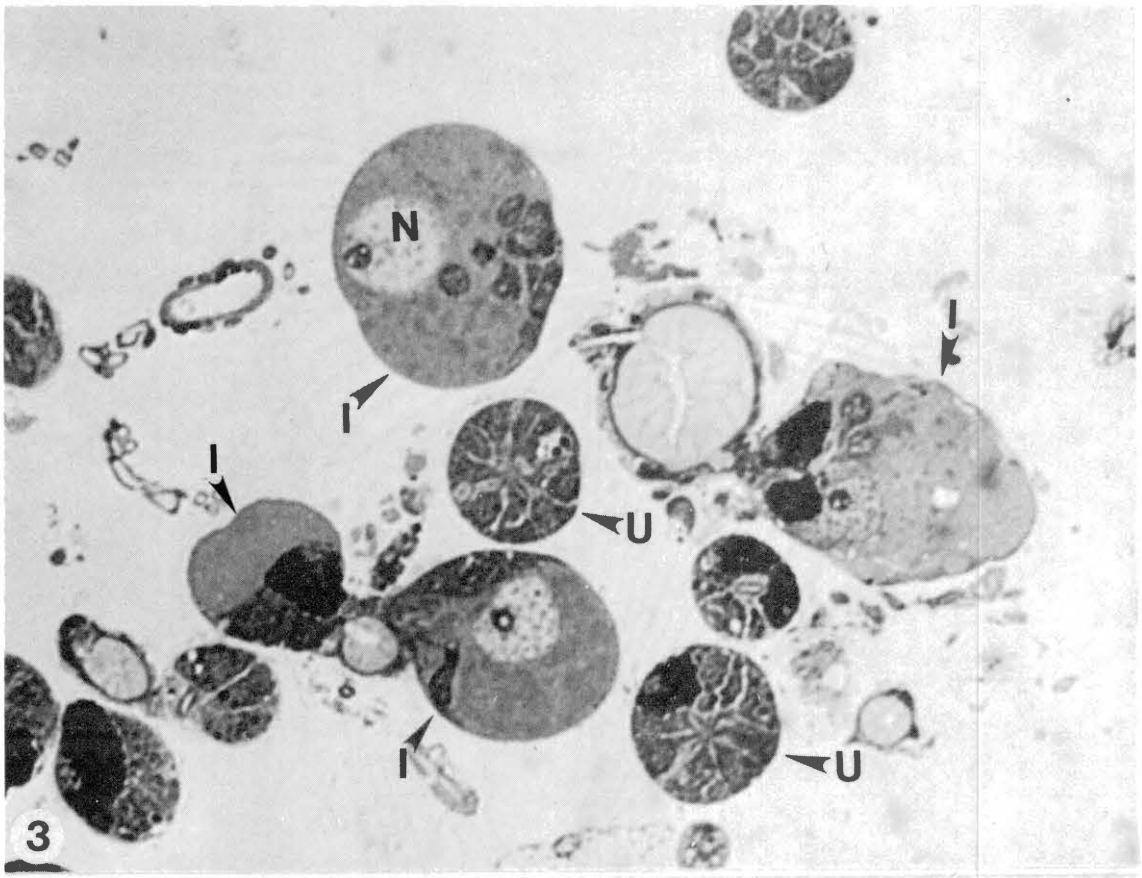
Light Microscopy

Theileria cervi was observed in the salivary gland acini of adult female Amblyomma americanum that were exposed as nymphs on parasitemic white-tailed deer. Infected acini were identified by comparison with associated control tissues. In unfed ticks, the infected host cells in the salivary glands were greatly enlarged. The nucleus was also enlarged. The one or two nucleoli that were present were intensely stained while the small clumps of chromatin were less evident and located towards the periphery. The host cell and nucleus remained unchanged throughout the course of parasite development (Figure 3). Uninfected cells were normal in size and heterochromatic. It was not possible to differentiate host and parasite material. The cell cytoplasm was moderately stained and had numerous randomly scattered vacuoles. Occasionally, slightly darker areas of the cytoplasm were noted.

After 24 hours of tick feeding, parasite development could be observed with light microscopy. Within the infected acini, the rapidly developing, darker staining areas appeared to be of parasitic origin while the lighter

Figure 3. Photomicrograph of a section of salivary gland from an unfed female Amblyomma americanum infected with Theileria cervi. Infected acini(I) are enlarged compared to uninfected acini(U). Infected cells contain hypertrophied nuclei (N) and a homogeneously staining cytoplasm. Plastic embedment, Mallory's stain. x 8,944.

Figure 4. Photomicrograph of a section of salivary gland from a feeding female Amblyomma americanum infected with an early developmental stage of Theileria cervi. The parasite (P) has developed into a large interconnected mass. The parasite (P) stains more darkly than the surrounding host cell cytoplasm (H). Plastic embedment, Mallory's stain. x 8,944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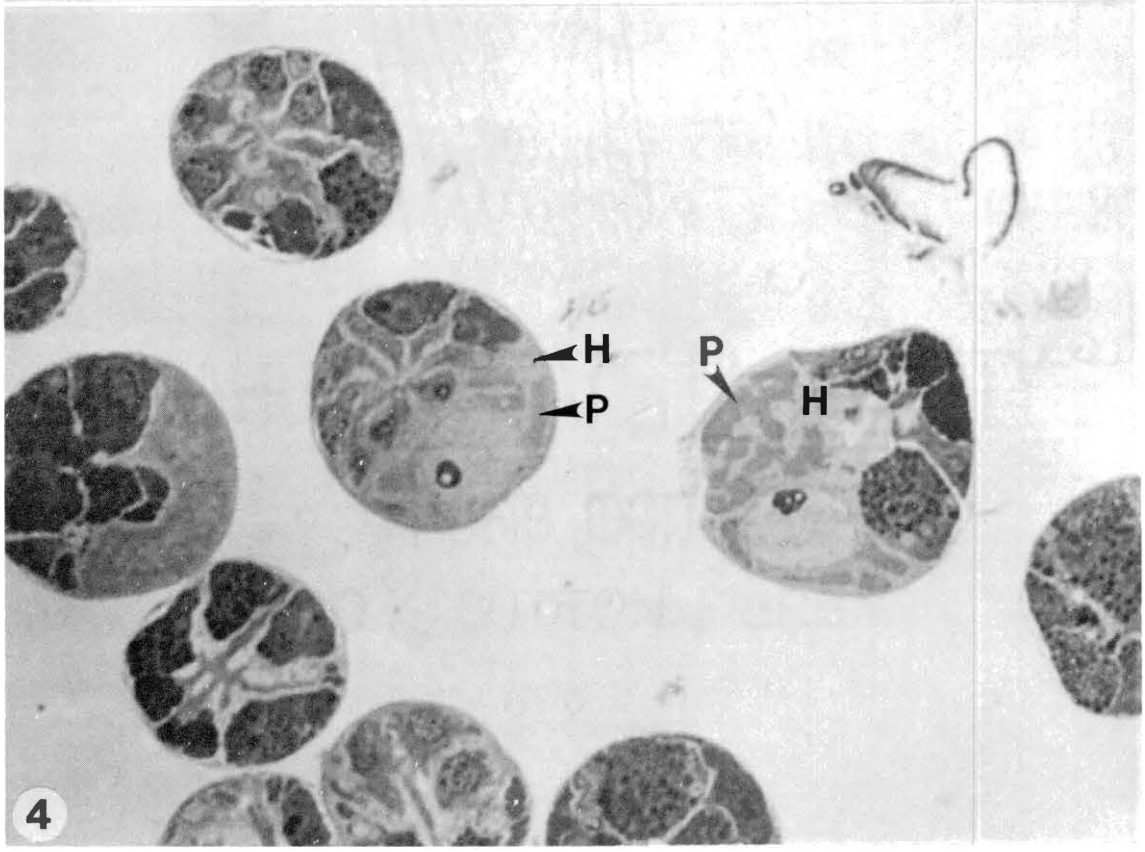
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areas were host. The parasite cytoplasm was homogeneous and appeared as one large, irregularly shaped mass.

As feeding continued into the second and third days, the parasite began involuting and formed into large irregularly shaped, connecting masses (Figure 4). Differentiation between host and parasite was obvious as the parasite continued to become more darkly stained and the host lighter. The parasite continued to involute, becoming progressively thinner. The slender processes of the parasite formed a complex network with the host cell cytoplasm (Figure 5). By the third day, parasite nuclei could be distinguished, primarily in the wider areas of the protoplasm.

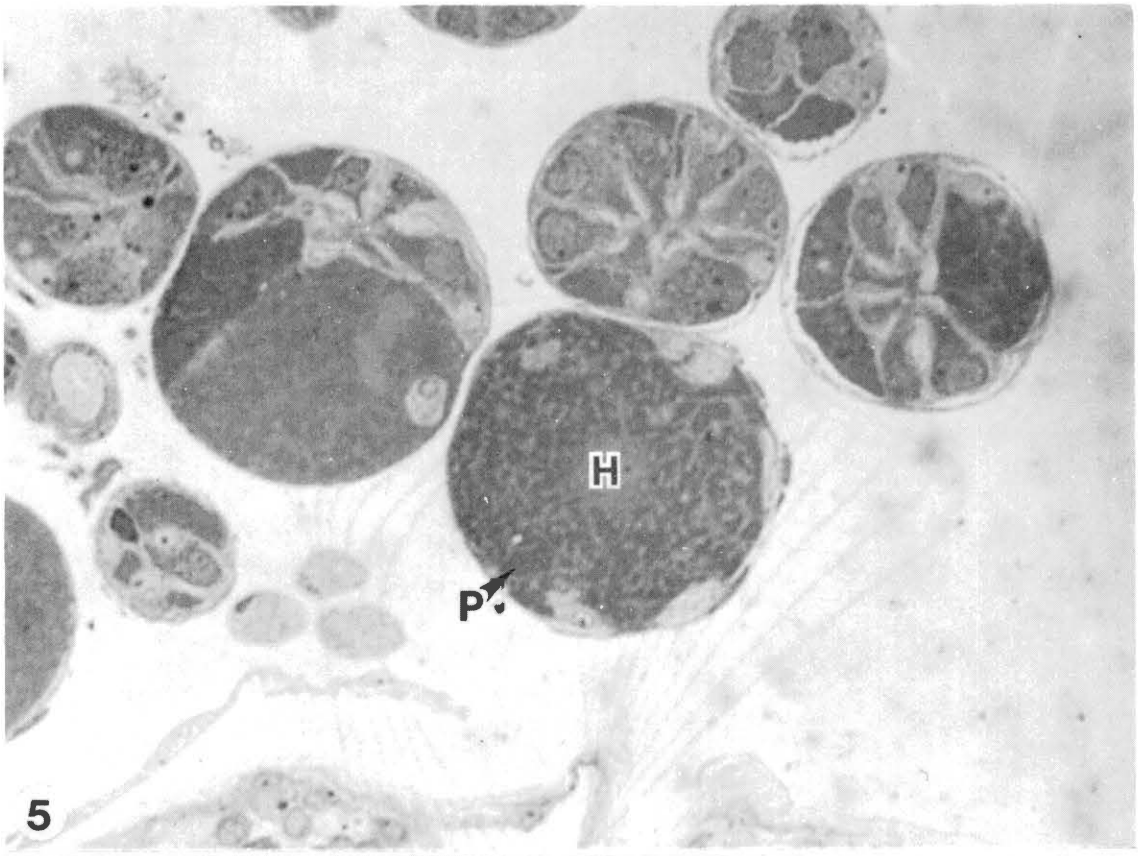
By the fourth and fifth days of tick feeding, the infected cells were filled with round, darkly staining sporozoites. Larger, irregularly shaped residual bodies, which stained less intensely than the sporozoites, were also seen. (Figure 6). Some residual bodies appeared to contain several nuclei and other organelles.

By the sixth day of feeding, the residual bodies had become smaller and more rounded. The sporozoites were also more rounded and condensed. (Figure 7). Sporogony appeared to be complete.

Throughout the course of the six days of tick feeding that were studied, the non-infected acini in the salivary glands appeared normal. The cells adjacent to the infected cell in the acinus also appeared normal, even though they

Figure 5. Photomicrograph of a section of salivary gland acini with a later developmental stage of Theileria cervi. The parasite (P) has formed a complex labyrinth with the lighter staining host cell cytoplasm (H). Plastic embedment, Mallory's stain. x 8,944.

Figure 6. Photomicrograph of an infected salivary gland acini containing numerous sporozoites. Uninfected cells (U) in the acinus are displaced by the infected cell (I). Plastic embedment, Mallory's stain. x 8,944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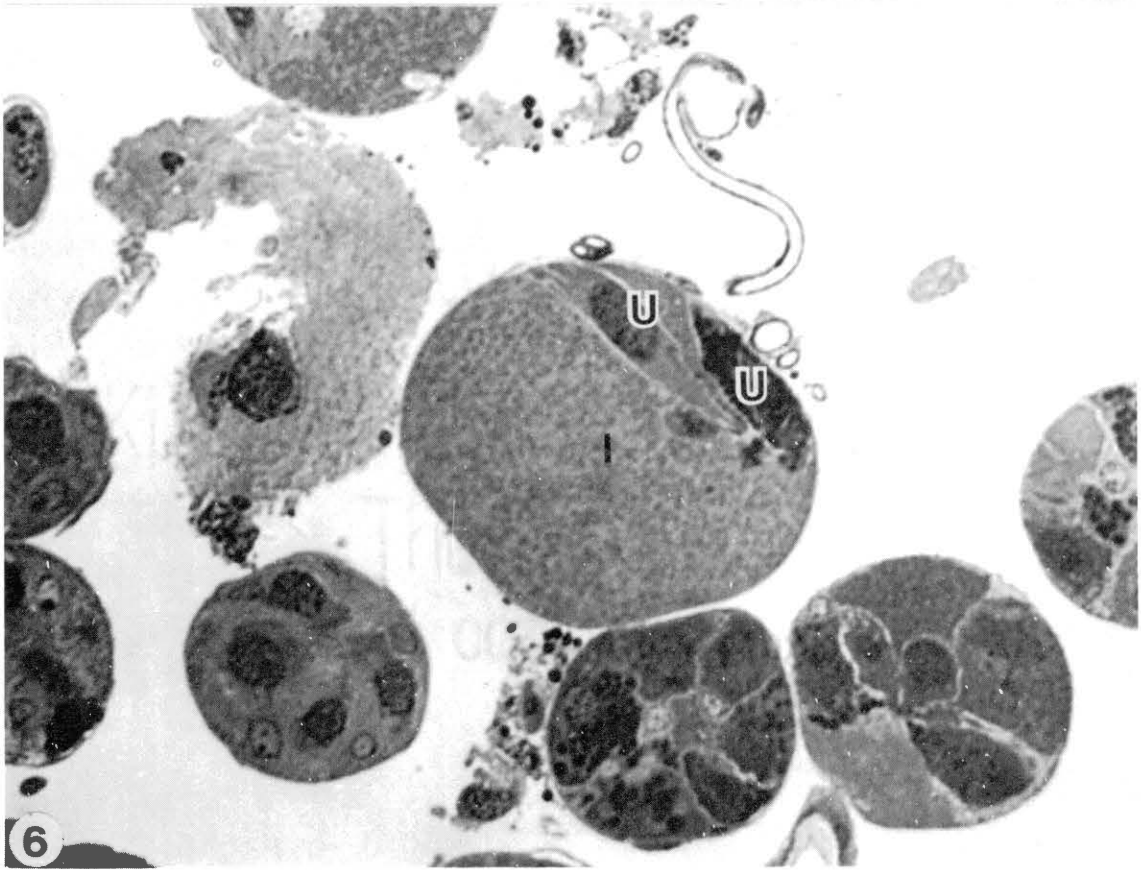
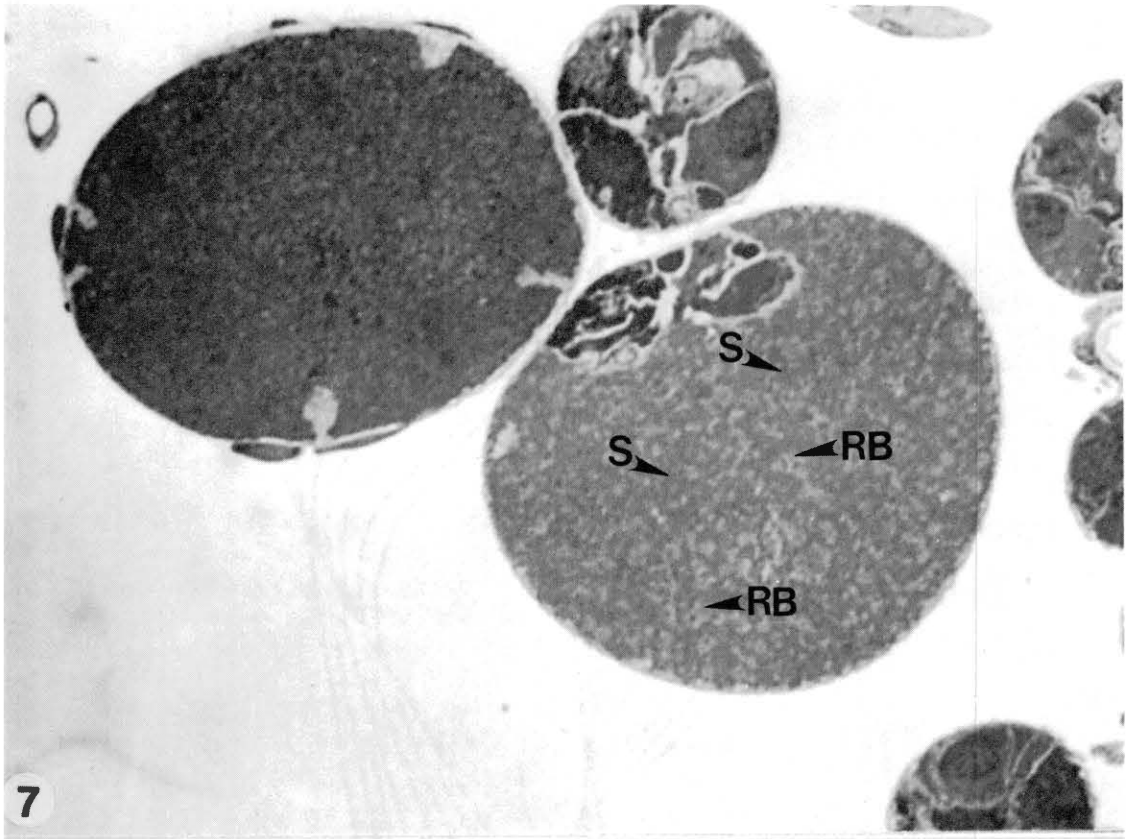

U
U
I
6

Figure 7. Photomicrograph of a granular acinus cell filled with sporozoites and residual bodies. Sporozoites (S) are darkly stained, residual bodies (RB) are lightly stained. Plastic embedment, Mallory's stain. x 8,944.

Figure 8. Photomicrograph of a lower magnification of a cluster of salivary gland acini from a female lone star tick infected with Theileria cervi. Infected acini (I) are enlarged when compared to the uninfected acini (U). Plastic embedment, Mallory's stain. x 2,236.



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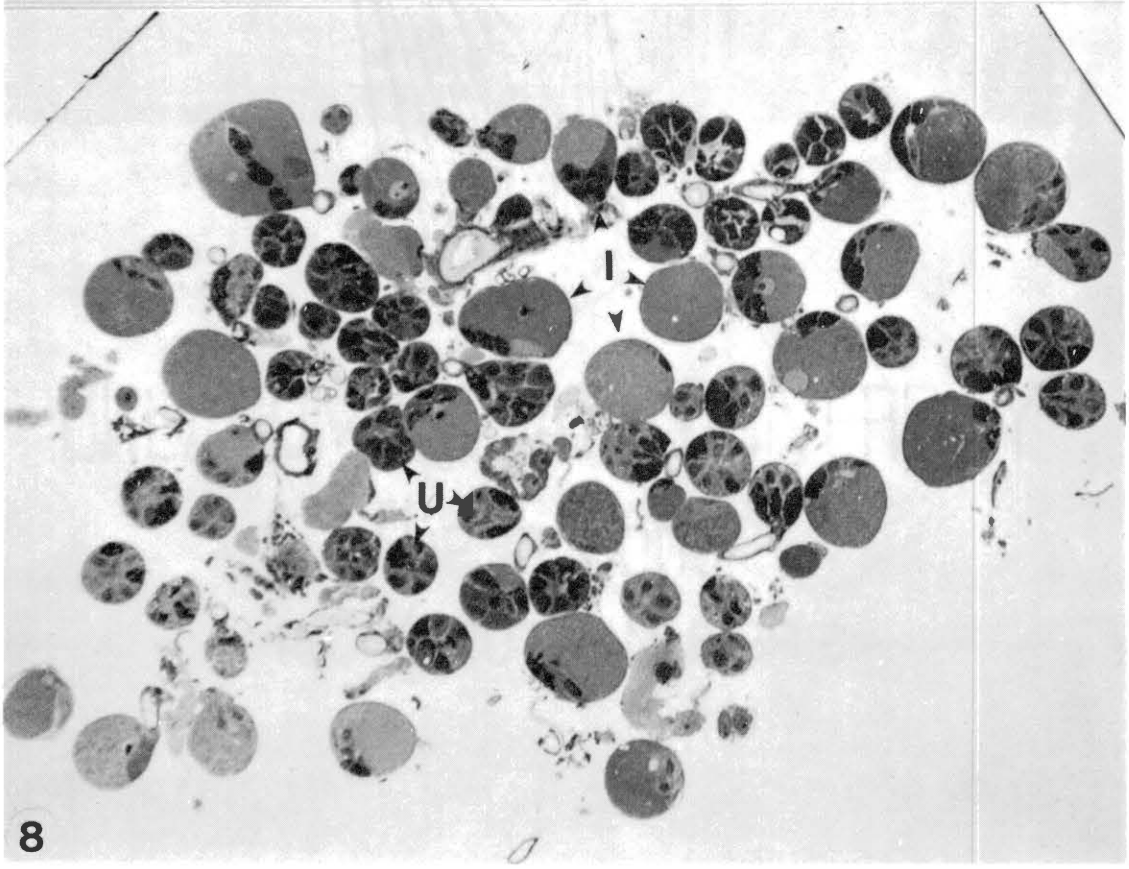

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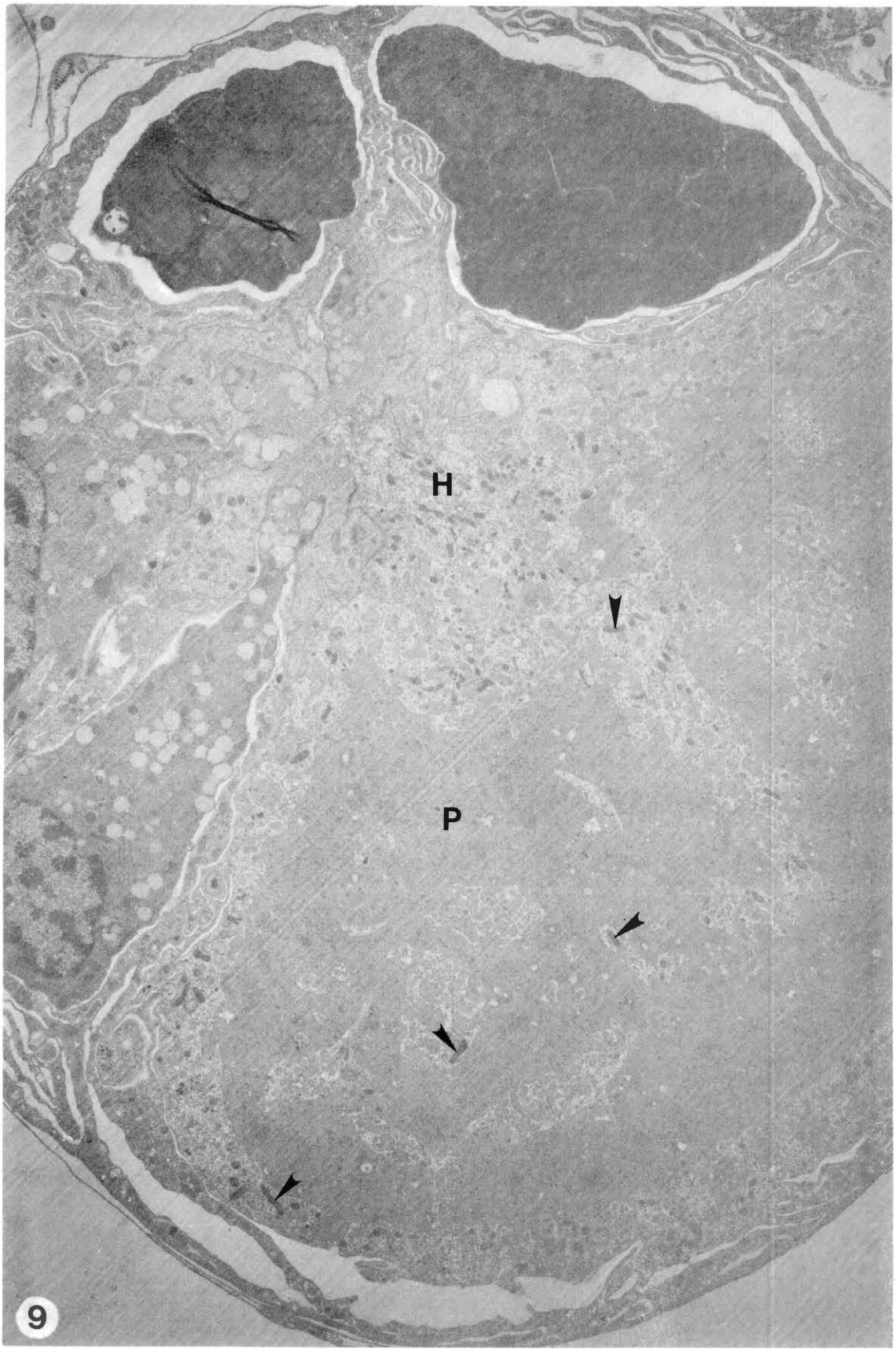
were displaced by the enlarged cell. In many of the thick sections of the salivary glands that were examined, several infected acini were apparent. In these sections, a range of parasite development in the infected acini were observed. Thus, it was possible to observe in a single section of salivary gland from an individual tick, acini with very early parasite development as well as acini filled with mature sporozoites. (Figure 8)

Electron Microscopy

Organelles of the Theileriidae are morphologically unlike tick organelles. Mitochondria of Theileria sp. are double membraned, without cristae and are frequently found in pairs, while those of tick origin are larger and contain foliate cristae. The endoplasmic reticulum of Theileria sp. consists of short anastomosing segments with few attached ribosomes. Tick endoplasmic reticulum occurs in long strands, richly studded with ribosomes. Cytostomes, mouth-like specializations of the protozoa cell membrane, are not seen in tick tissue. By using these organelle distinctions as landmarks, distinction between tick cell cytoplasm and developing parasite was simplified. Transmission electron microscopy verified and enhanced what was observed at the light microscope level. (Figure 9)

In unfed, infected female ticks it was possible to distinguish parasite from host cytoplasm within the infected cells of the acini. Parasite mitochondria were present and

Figure 9. Electron micrograph of an early developmental stage of Theileria cervi in a granular acinus of the salivary gland of a feeding female Amblyomma americanum. Parasite cytoplasm (P) is homogeneous in texture. Host cell cytoplasm (H) is less densely stained and filled with organelles, most notably mitochondria (arrows). x 11,358.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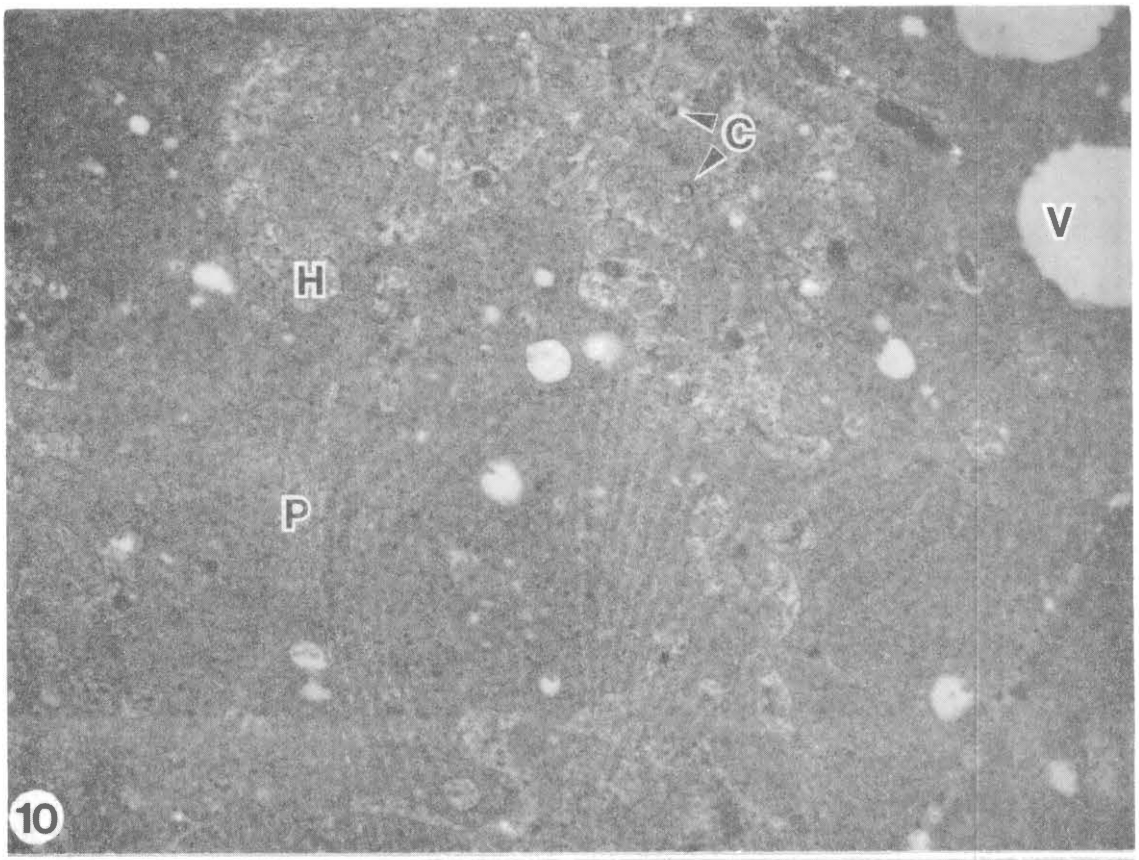
dispersed throughout the parasite cytoplasm. Cytostomes were observed on the parasite plasmalemma. The electron densities of the parasite and the host cell cytoplasm were very similar. Single membrane bound vacuoles were electron lucent and located primarily in the parasite cytoplasm. (Figure 10) Irregular and various sized parasite nuclei were noted within the parasite cytoplasm and could be differentiated only by the presence of nuclear envelopes, as the densities of the nuclei were nearly identical to the surrounding cytoplasm. (Figure 11) The developing parasite configuration was difficult to determine because the parasite was amorphous and interspersed with the host cytoplasm. The majority of the host organelles, mostly mitochondria and short branches of endoplasmic reticulum lined with ribosomes, were located towards the periphery of the cell and close to the host cell nucleus.

Once tick feeding had begun, the parasite became more condensed and formed a network of large, interconnected, irregularly shaped masses. (Figure 12) Cytostomes were abundant on the border of the parasitic membrane, and most notably on the smaller projections of the parasite. Small sections of smooth endoplasmic reticulum could be seen as well as numerous mitochondria in the parasite cytoplasm. The host cytoplasm became more electron lucent, leaving host mitochondria and strands of rough endoplasmic reticulum clearly visible. Numerous small projections of the parasite were also observed more clearly in this area. Parasite

Figure 10. Electron micrograph of a higher magnification of a Theileria cervi infected cell in the salivary gland of an unfed female Amblyomma americanum. Parasite cytoplasm (P), containing vacuoles (V) and cytostomes (C), is ınterspersed with host cell cytoplasm (H). x 18,792.

Figure 11. Electron micrograph of a higher magnification of an infected acinus cell of an unfed female Amblyomma americanum. Parasite cytoplasm (P) stains more densely than host cytoplasm and contains vacuoles (V) and irregularly shaped nuclei (N). x 46,980.


C
V
H
P
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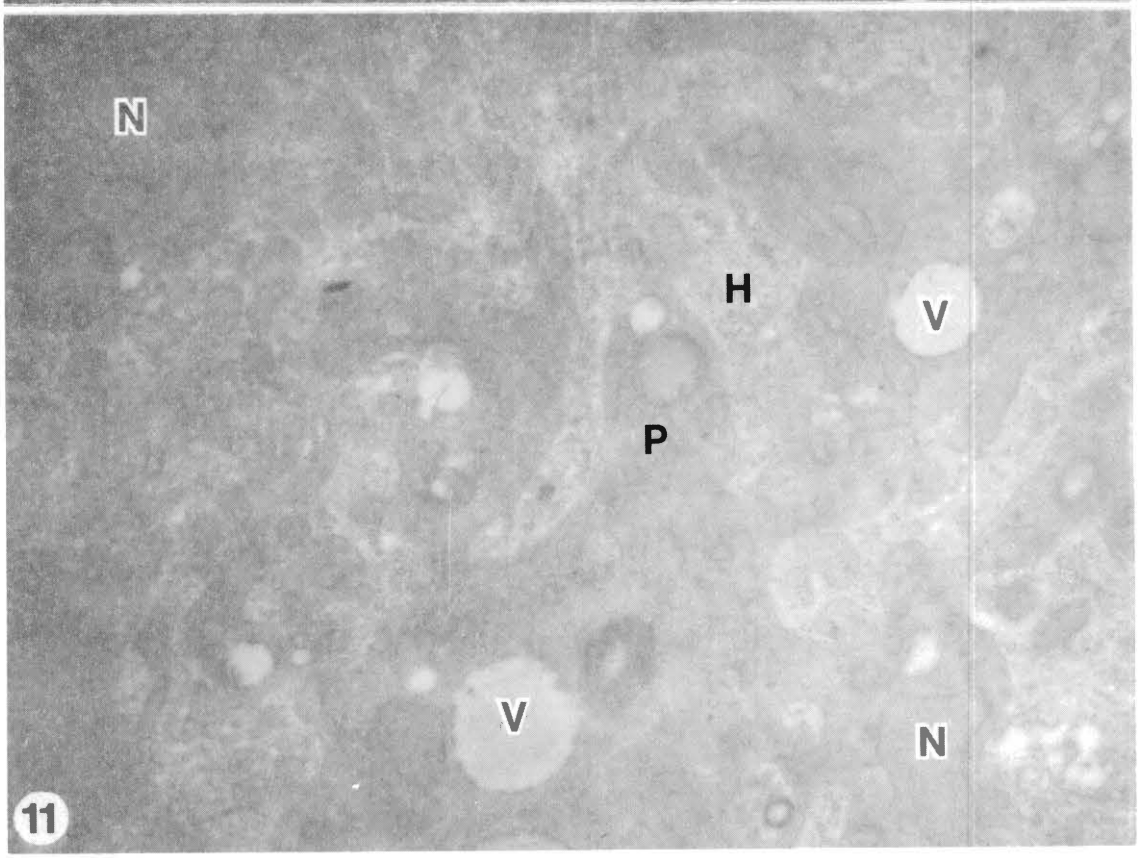
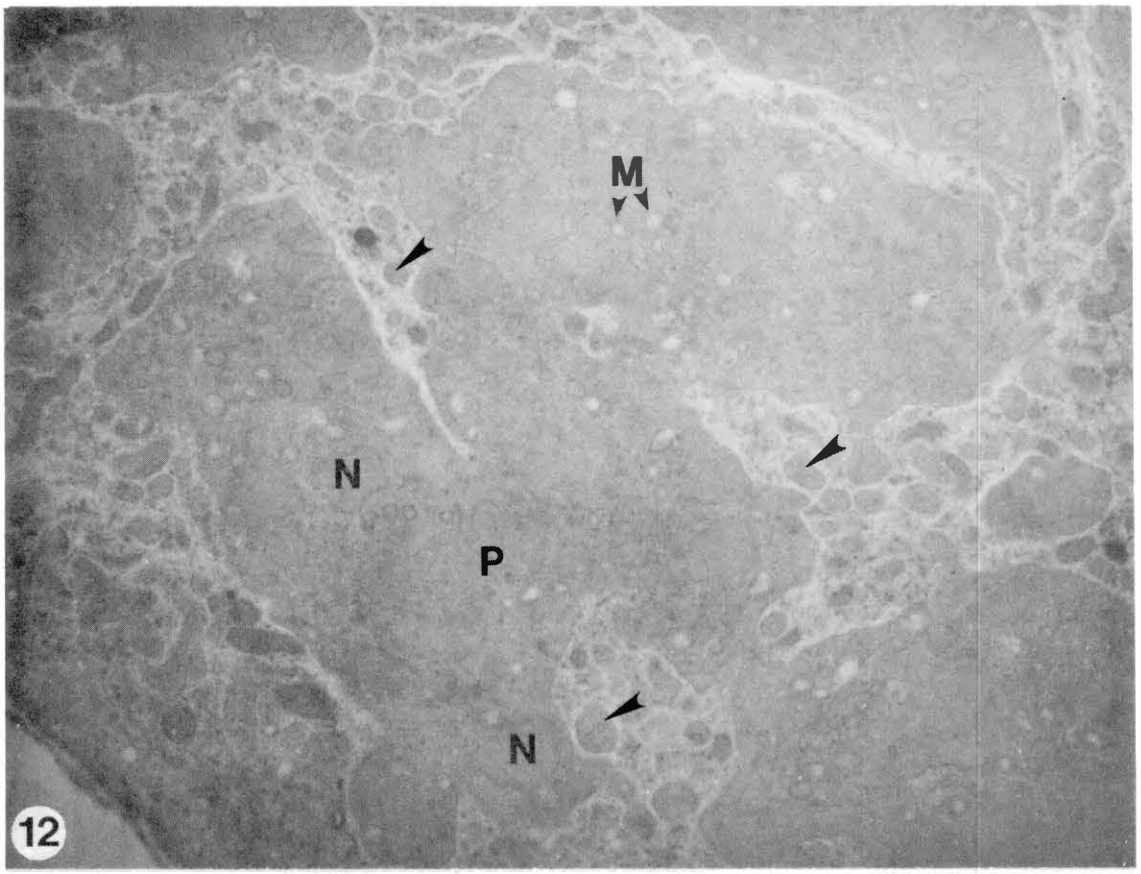
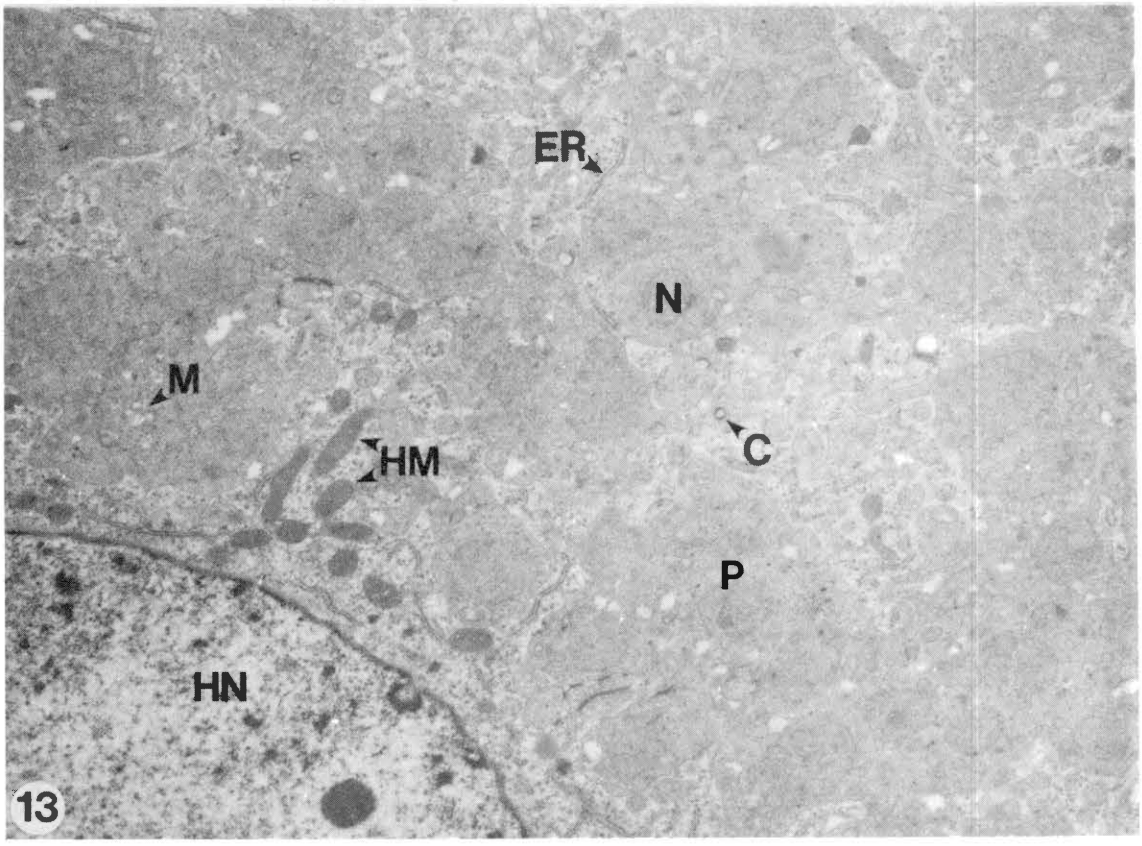

N
H
V
P
V
N
11

Figure 12. Electron micrograph of developing Theileria cervi in the salivary gland of a feeding female Amblyomma americanum. Parasite cytoplasm (P) contains numerous nuclei (N) and mitochondria (M) and stains more densely than host cytoplasm (H). Small, thin branches of the parasite (arrows) are dispersed throughout the host cell cytoplasm (H). x 12,528.

Figure 13. Electron micrograph of a later developmental stage of Theileria cervi in the salivary gland of a feeding female Amblyomma americanum. Host cytoplasm adjacent to the nucleus (HN) contains mitochondria with transverse cristae (HM) and rough endoplasmic reticulum (ER). Parasite cytoplasm (P) contains nuclei (N) and circular mitochondria (M) that lack cristae. Cytostomes (C) are present on the parasite plasma membrane. x 12,528.


M
N
P
N
12


ER
N
M
HM
C
P
HN
13

nuclei gradually became more electron dense and were readily visible at low magnification. The parasite continued to involute, become narrower and formed what appeared to be a three dimensional labyrinth within the host cytoplasm. (Figure 13) As the parasite developed, increasing numbers of cytostomes were observed as well as thickened plaque-like areas on the parasite cell membrane.

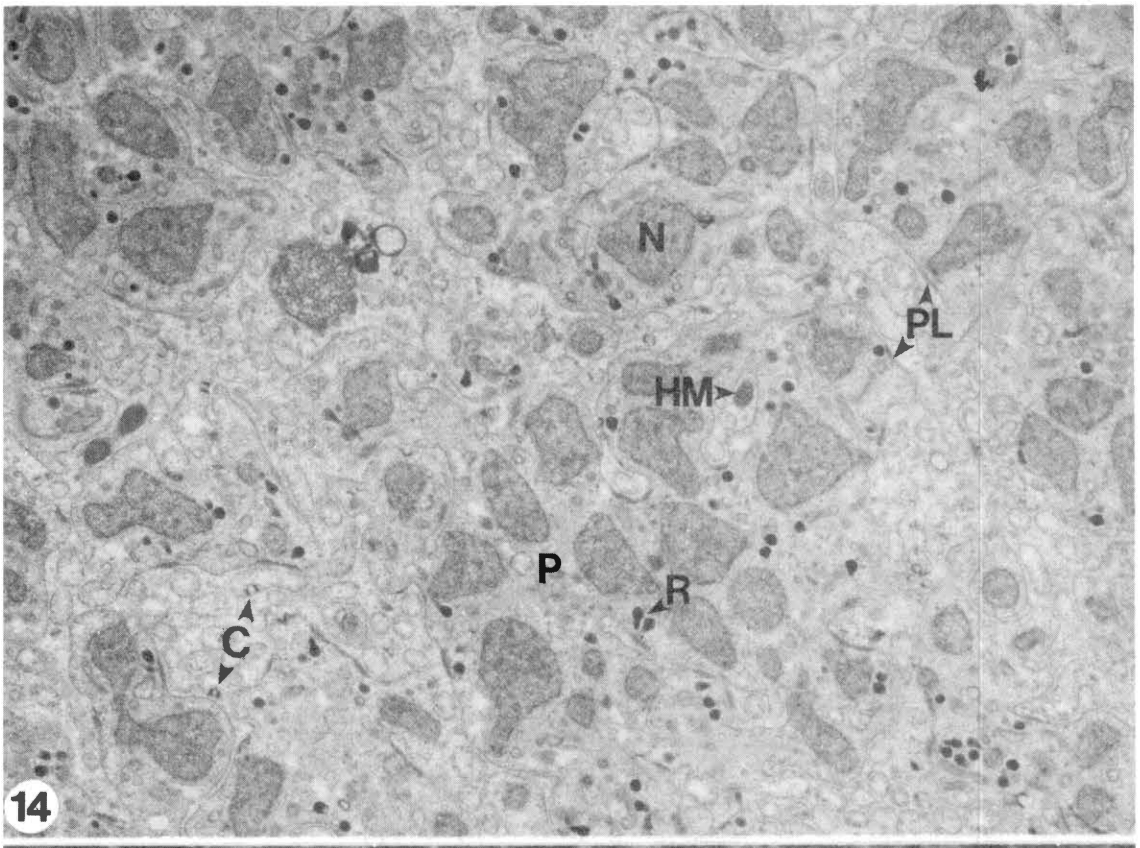
By the fourth day of feeding, the host cell cytoplasm was diminished, leaving an intricate maze of interweaving processes of the parasite. The numerous parasite nuclei were irregular in shape and much more electron dense than the surrounding cytoplasm. Rhoptries appeared and oriented around the plaque-like areas of the cytoplasmic membrane. Occasionally, some host mitochondria were observed between the lobules of the parasite. (Figure 14)

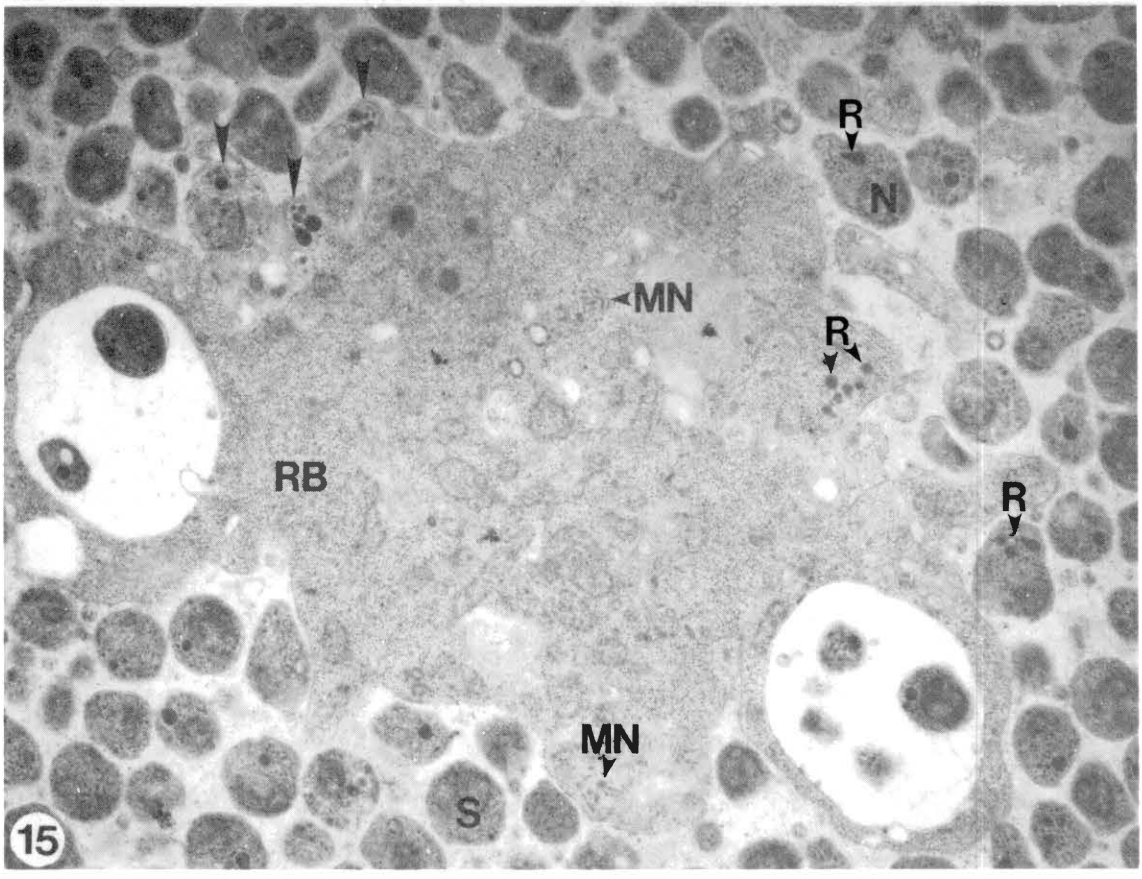
Between the fourth and fifth day of feeding, a rapid cytoplasmic fission occurred as sporozoites were now evident as were large irregularly shaped residual bodies. A few sporozoites were seen forming at the periphery of some of the residual bodies. The residual bodies also contained parasite organelles (mitochondria, cytostomes, micronemes, etc.) and vacuoles. Once formed, each sporozoite contained a single nucleus oriented to one end of the cell and a set of rhoptries and bar-shaped micronemes at the other. (Figure 15)

By the sixth day of tick feeding, sporozoites were less granular in texture, more electron dense and appeared

Figure 14. Electron micrograph of Theileria cervi prior to sporozoite formation in an acinus cell of a feeding female Amblyomma americanum. Parasite (P) organelles, nuclei (N) and rhoptries (R) are located adjacent to plaque-like areas (PL) on the plasmalemma. Cytostomes (C) are present in the plasma membrane of the parasite. Host mitochondria (HM) are occasionally observed between extensions of the parasite cytoplasm. x 15,138.

Figure 15. Electron micrograph of a residual body and sporozoites in the salivary gland of a feeding female Amblyomma americanum. Large, irregularly shaped residual body (RB) contains rhoptries (R), micronemes (MN) and other parasite organelles. Individual sporozoites (S) containing a nucleus (N) and rhoptries (R) are located around the residual body. Three sporozoites appear to be forming at the periphery of the residual body (arrows). x 18,792.


N
PL
HM
P
R
C
14


R
N
MN
R
RB
R
MN
S
15

uniform in development. The sporozoite nuclei were well defined. Mitochondria, usually located near the nucleus could be seen in some sporozoites. The residual bodies were smaller and more rounded in shape than previously observed and no sporozoites were seen forming from them. (Figure 16)

Confirmation of Tick Infectivity

Both susceptible fawns exposed to T. cervi infected ticks became infected. Fourteen days post attachment of ticks, fawn 904 showed piroplasms in its erythrocytes (less than 1% parasitemia). On the 20th day post tick attachment, the parasitemia had risen to 21.5%. No piroplasms were observed in fawn 905 on the 14th day post tick attachment, but on day 20, the piroplasm parasitemia was 25.3%. (Figure 17)

Figure 16. Electron micrograph of developed sporozoites and associated residual bodies in the salivary gland of a feeding female Amblyomma americanum. Sporozoites (S) are well formed containing parasite organelles, including mitochondria (M), rhoptries (R), nucleus (N), and micronemes (MN). Residual bodies (RB) are smaller and more rounded in shape than observed in Figure 15. x 26,100.

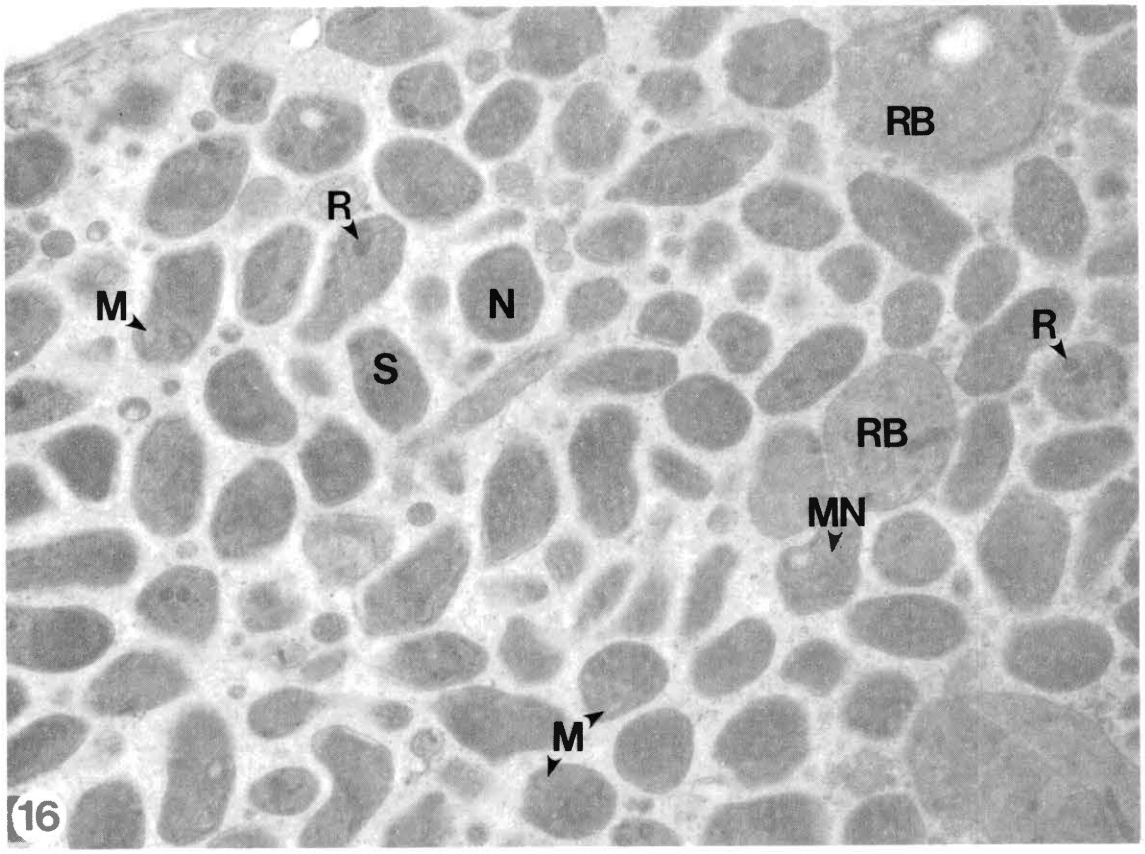
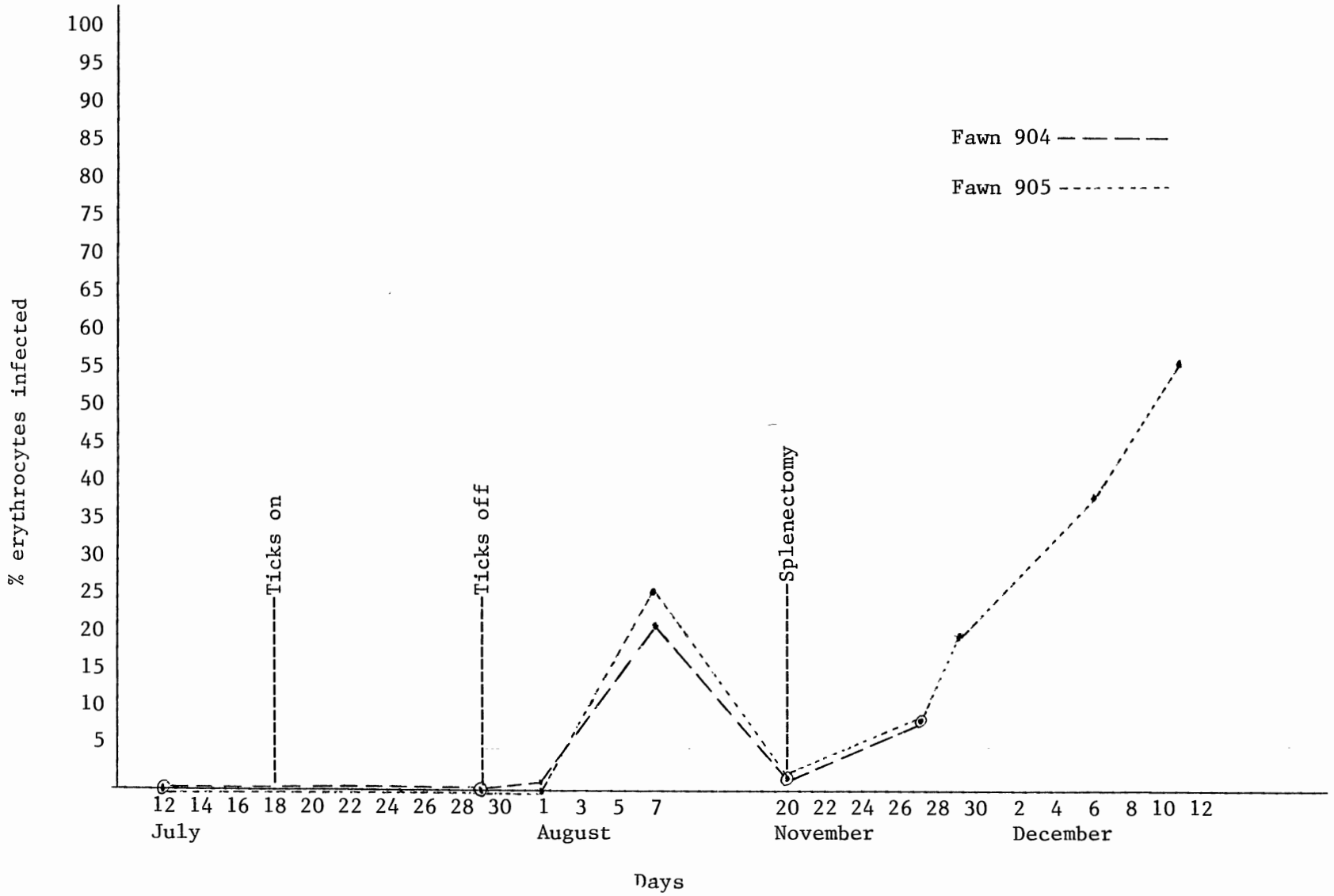

RB
R
M
N
S
R
RB
MN
M
16

Figure 17. Percentage of erythrocytes infected with *Theileria cervi* piroplasms in intact white-tailed deer fawns prior to and after natural feeding of infected *Amblyomma americanum* adults.


% erythrocytes infected
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
Ticks on
Ticks off
Splenectomy
Fawn 904
Fawn 905
12 14 16 18 20 22 24 26 28 30 1 3 5 7 20 22 24 26 28 30 2 4 6 8 10 12
July
August
November
December
Days

CHAPTER IV

DISCUSSION

Introduction

Although Theilerial parasites have been studied in several vectors, the development is not well understood and appears to vary with individual species. Theileria cervi morphology in the salivary glands of Amblyomma americanum has not previously been described. Sporogony has, however been investigated in a number of related species within the genus Theileria. It is apparent from the results of the present study and the descriptions of sporogony in T. parva, T. taurotragi, T. ovis, T. parva lawrencei, that the process of sporogony in T. cervi in the salivary glands of A. americanum has both common and dissimilar characteristics with those previously described.

Light Microscopy

Classification of tick salivary gland acini and cells has been an area of controversy. Binnington et.al. (1983), using Rhipicephalus appendiculatus females as a model, developed a scheme of classification based on histochemical stains. Krolak et.al. (1982), in studying female A. americanum, used ultrastructure morphology as their criteria

for classification. Both schemes do agree on the presence of three distinct acini types. Type I acini are agranular, while the Type II and Type III acini contain granular and agranular cells of different types and numbers. Because most acini observed in section are not cut in an exact crossection, demonstrating all the cell types, it is usually not possible to differentiate between the two types of granular acini. Also, because the infection of an acinus with Theileria sp. causes the acini to enlarge, the probability of obtaining an exact crossection is decreased. In the present study, sporogony of T. cervi, when an acinus type could be determined, was observed only in granular acini Types II or III. Sporogony in other species of Theileria, has been proposed to occur in only the Type II and Type III acini (Martin, et.al., 1964, Young et.al., 1980), only the Type III acinus (Purnell, et.al., 1968), or being restricted to only a single cell type of the Type III acinus (Binnington, et.al., 1983).

The process of sporogony has been described in a variety of developmental sequences. Most descriptions are relatively similar however, varying mostly in the terms used to describe the stages observed (Cowdry et.al., 1932, Martin et.al., 1964, Purnell et.al., 1968, Schein et.al., 1978, Young et.al., 1980). Early descriptions suggested that the parasite undergoes 2-3 series of multiple nuclear divisions followed by a cytokinesis, eventually resulting in uninuclear sporozoites. T. cervi does not appear to follow

this pattern. In the developmental sequence observed for T. cervi, the parasite cytoplasm forms a progressively more complex labyrinth of interconnected bodies and branches. This multinucleate syncitium then appears to undergo a synchronized multiple fission forming sporozoites and residual bodies.

Similarities in parasite development during sporogony in tick salivary glands between T. cervi and previously studied Theileria sp. were observed. A consistent finding is a great enlargement of the infected host acini cell, that causes compression of the non-infected cells in the acinus. The host cell nucleus also hypertrophies. In salivary glands with more than one infected acinus, a range of developmental stages were present in all species. One section of a gland infected with T. cervi contained developmental stages ranging from early developmental forms to cells with mature sporozoites.

The number of infected acini per salivary gland has been discussed in numerous studies and was reported to vary, depending on the species of tick and piroplasm parasitemia in the infected animal at the time of tick feeding (Martin et.al., 1964, Purnell et.al., 1968, 1975, Young et.al., 1980). The rate of infection for T. cervi in A. americanum in the present study appeared to be quite high. Although an exact rate was not determined, in one thick section of salivary glands from a tick which had fed for four days, 33% of the acini present were infected. This high percentage of

infected acini is probably related to the high piroplasm parasitemias of the fawns during nymphal feeding (beginning parasitemia 17.7%, end of feeding parasitemia 45.1% for fawn 814; beginning parasitemia 21.1%, end of feeding parasitemia 65% for fawn 815). Schein et.al. (1978A) reported that feeding on animals with parasitemias in excess of 40% resulted in tick mortality and disease for Hyalomma anatolicum excavatum ticks. The high parasitemias in the present study appeared to have no ill effects on the A. americanum as nearly all the replete nymphs appeared to molt to adults, attach and feed normally.

Electron Microscopy

The process of sporogony in T. parva has been described at the electron microscopic level by Fawcett et.al. (1982A). In that study, it was proposed that the parasite sporont developed into ramifying, multinucleate syncitium which increased in size and complexity until a terminal episode of cytoplasmic fission resulted in sporozoite and residual body formation. While an occasional sporozoite was observed forming from a residual body, this was not thought to be the principle form of sporozoite production. The description conflicted with previous studies which relied primarily on light microscope observations and reported the formation of primary, secondary and tertiary sporoblasts via cytokineses before sporozoites were formed (Cowdry et.al., 1932, Martin et.al. 1964, Purnell et.al., 1968, Schein et.al., 1978,

Mehlhorn et.al., 1979, Young, et.al. 1980). In a comparative study of sporogony in T. parva parva, T. parva lawrencei, and T. taurotragi, Fawcett et.al., (1985) confirmed their previous findings for T. parva parva and reported a similar process of sporogony for the other two Theileria species studied. T. cervi sporogony as studied in the salivary glands of A. americanum also appears to follow a similar but not identical sequence as that described by Fawcett et.al., (1982A, 1985).

Fawcett et.al., (1985) reported that a regional differentiation of the parasite occurred during development. The investigators observed that the thicker, nucleated portions of the parasite were located in one area of the host cell, while the slender, non-nucleated anastomosing processes of the parasite were located in another area of the cell. The non-nucleated area was further observed to form a close meshed network with the host cell cytoplasm which was termed the labyrinth. This regional differentiation was not observed in T. cervi. In the present study, the slender branches of the parasite were interspersed with the thicker, nucleated areas in an even mixture throughout the host cell. No concentrated areas of labyrinth were observed.

Accumulations of glycogen by Theileria infected host cells was reported as a frequent occurrence in T. parva parva, less frequent in T. parva lawrencei and rare in T.

taurotragi (Fawcett et.al., 1985). In T. cervi infected cells, no glycogen accumulations were observed.

Cytostomes on the parasite cell membrane of T. cervi appeared early in the developmental sequence and increased in number as development of the parasite progressed. By the fourth day of tick feeding, there were numerous, most notably on the smaller branches of the parasite. Fawcett et.al., (1982A, 1985) also reported the presence of cytostomes on the Theileria sp. studied, but they do not appear to be as abundant as seen in T. cervi.

The morphology of mature T. cervi sporozoites was different from that reported in other Theileria sp. Sporozoites of T. cervi were unique in that they were oval to oblong in shape and had a denser cytoplasm and nucleus than seen in the other Theileria. The micronemes of T. cervi, unlike the round dense ones previously described, were smaller, less dense, bar-shaped and primarily dispersed among the rhoptries. The rhoptries were situated in the smaller pole of the sporozoite and the nucleus was in the larger. Occasionally, an electron lucent mitochondria was observed, usually close to the nucleus which is similar to observations for the other Theileria species. Sporozoites of T. parva parva were mostly round or spheroid, had round dense micronemes which were located primarily towards the periphery of the cell, dense rhoptries and a fairly electron lucent cytoplasm. Those of T. parva lawrencei were similar, to T. parva parva but more oval in shape and more electron

dense. Sporozoites of T. taurotragi resembled those of T. parva but were highly variable in shape, some even possessed tail-like appendages giving them a racquet-like appearance.

CHAPTER V

SUMMARY AND CONCLUSIONS

Sporogony of Theileria cervi, was studied morphologically in the salivary glands of its arthropod vector Amblyomma americanum with light and electron microscopies. While T. cervi is not considered pathogenic under natural conditions in white-tailed deer, experimental information from this study, will help improve the level of understanding of the other pathogenic and nonpathogenic species of Theileria.

The process appeared to occur in only the granular acini. The developing parasite appeared to follow a sequence of becoming an increasingly complex multinucleate syncitium of long interconnecting processes, culminating in a rapid multiple cytoplasmic fission forming sporozoites and residual bodies. This description contradicts the majority of the previously reported descriptions of Theileria sporogony, which propose formation via cytokinesis of separate primary, secondary and tertiary sporoblasts. The present study is consistent, however, with the more recent findings of Fawcett et.al., (1982A, 1985), which are based on both light and ultrastructural observations.

Although T. cervi sporogony has the same sequence of events as those described by Fawcett et.al. (1985) for three

species of African Theileria, some unique morphological features were observed. The labyrinth the parasite forms with the host cell cytoplasm was spread throughout the acinus cell, and not restricted to one area of the cell. No glycogen accumulations by the infected host salivary gland acinus cell were observed. Cytostomes were abundant, and increased in number as parasite development progressed. The mature sporozoites were oval to oblong in shape and contained rhoptries, small bar-shaped micronemes, and an electron lucent, non-cristate mitochondria, usually located close to the dense well defined nucleus.

The ability of the protozoan, T. cervi, to develop to sporozoites in the A. americanum salivary glands and be successfully transmitted to a susceptible deer via natural feeding of an infected tick, indicates that this tick is most probably the natural intermediate host. This is supported by the fact that having large numbers of infected salivary gland acini in a single tick did not appear to impair the tick's ability to feed normally to repletion.

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