EXPERIMENTAL *HEPATOZOON AMERICANUM* INFECTION IN COYOTES (*CANIS LATRANS*)

by

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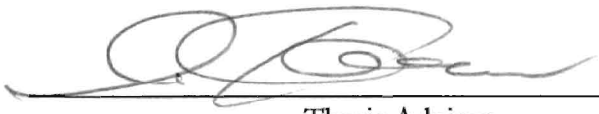
INFECTION IN COYOTES (*CANIS LATRANS*)

Thesis Approved



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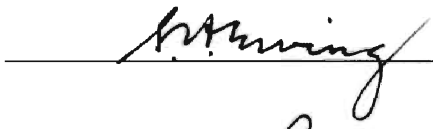

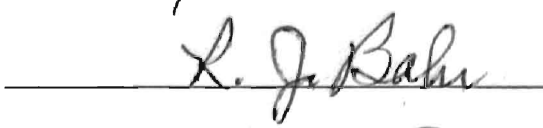

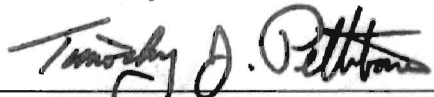

Dean of the Graduate College

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I. INTRODUCTION

Background

American canine hepatozoonosis (ACH) is an emerging vector-borne disease of dogs (Vincent-Johnson *et al.*, 1997) recognized with increasing frequency throughout the southcentral and southeastern United States (Ewing *et al.*, 2002a). The causative agent of ACH is the protozoan parasite *Hepatozoon americanum* (Apicomplexa: Adeleorina) (Vincent-Johnson *et al.*, 1997). Infections are characterized by fever, weakness, myalgia, mature neutrophilic leukocytosis, wasting, poor response to treatment and periosteal bone proliferation (Vincent-Johnson *et al.*, 1997; Macintire *et al.*, 2001; Panciera *et al.*, 1997). *Ambylomma maculatum* (Acari: Ixodidae), the Gulf Coast tick (GCT), is a suitable host and efficient vector for *H. americanum* (Mathew *et al.*, 1998; Ewing *et al.*, 2002b) with transstadial transmission occurring under experimental conditions between larval and nymphal stages (Ewing *et al.*, 2002a) and nymphal and adult stages (Mathew *et al.*, 1998). Field studies in Oklahoma and Texas have implicated coyotes (*Canis latrans*) as a possible natural reservoir for *H. americanum* (Davis *et al.*, 1978; Mercer *et al.*, 1988; Kocan *et al*, 1999). Approximately 56% of adult coyotes sampled in Oklahoma were naturally infected (Kocan *et al.*, 1999 and 2000). Transmission to both wild and domestic canids is presumed from ingestion of infected ticks during grooming activities or the consumption of tick-infested prey (Mathew *et al.*, 1998; Ewing *et al.*, 2000).

Geographic Distribution

American canine hepatozoonosis has been reported from Texas, Florida, Georgia, Alabama, Mississippi, Louisiana, and Oklahoma (Mathew *et al.*, 2000; Macintire *et al.*, 2001; Baneth, 2001) in geographic correlation with the known distribution (Semtner *et al.*, 1973) of its tick vector, *Amblyomma maculatum* (Ewing *et al.*, 2002b). However, the distribution of the Gulf Coast tick has expanded in recent years to include southern Kansas and Kentucky

(Williams *et al.*, 2002) although there have been no reported cases of ACH in these new areas (Ewing *et al.*, 2000).

Epidemiology

The primary vertebrate host for *H. americanum* is currently unknown (Ewing *et al.*, 2002a and 2002b). Infection in carnivores with *Hepatozoon* species, now believed to be *H. americanum*, has been reported from a variety of wild carnivores (Craig, 1978; Davis *et al.*, 1978; Lane and Kocan, 1983; Mercer *et al.*, 1988), including coyotes (*Canis latrans*) from Texas and Oklahoma (Davis *et al.*, 1978; Mercer *et al.*, 1988; Kocan *et al.*, 1999). Nine of seventeen free-ranging adult coyotes from Oklahoma were naturally infected with *H. americanum*, or an organism indistinguishable from *H. americanum*, as confirmed by the presence of muscle lesions (Kocan *et al.*, 1999). The characteristic bone lesions that occur in domestic dogs were not observed (Davis *et al.*, 1978; Kocan *et al.*, 1999; Kocan *et al.*, 2000). On the other hand, experimentally infected juvenile coyotes developed clinical disease and proliferative bone lesions similar in nature and severity to that exhibited by domestic dogs (Kocan *et al.*, 2000; Panciera *et al.*, 2000). It remains to be determined whether *H. americanum* infection in coyotes and domestic dogs result from accidental insertion of these animals into an existing enzootic cycle of Gulf Coast ticks and an unknown vertebrate host or whether *H. americanum* is a newly emerged species and canine infection a recent event (Ewing *et al.*, 2000).

Life Cycle

Amblyomma maculatum acquire gamonts of *H. americanum*, contained inside a leukocyte, in a blood meal from an infected canine host (Craig, 2001; Ewing *et al.*, 2002b). Inside the gut of the tick, sexual reproduction occurs as *H. americanum* gamonts form paired gametes that result in a zygote following fertilization (Mathew *et al.*, 1999; Baneth *et al.*, 2003). The oocysts that develop during sporogony, an asexual process, contain the sporozoites that infect a canid

when the tick is ingested (Ewing *et al.*, 2000). Once ingested, sporozoites disseminate to various tissues, predominantly skeletal and cardiac muscle (Vincent-Johnson *et al.*, 1997; Panciera *et al.*, 1998), within the canine host for the process of merogony (Vincent-Johnson *et al.*, 1997; Panciera *et al.*, 1998; Baneth *et al.*, 2003). Merogony occurs within a host cell as the parasite asexually multiplies. Two forms of lesions associated with merogony are an 'onion-skin' cyst and an inflammatory granuloma, the latter results from the liberation of merozoites from the previously encysted host cell (Panciera *et al*, 1999; Baneth *et al.*, 2003). Merozoites either form secondary tissue meronts or complete gamontogony and circulate in the vascular periphery of the canid where they may be acquired by a blood-feeding tick (Panciera *et al.*, 1998; Baneth *et al.*, 2003).

Clinical Signs and Symptoms

American canine hepatozoonosis is a debilitating and often fatal disease of domestic dogs with a poor prognosis (Macintire *et al.*, 1997 and 2001). Severe bone and muscle pain is apparent in affected dogs. Muscle pain results from the release of merozoites and the formation of pyogranulomatous lesions and bone pain results from proliferation of periosteal bone (Craig, 1990; Panciera *et al.*, 1997; Baneth *et al.*, 2003). New bone growth is most pronounced along the diaphysis of long bones, although irregular and flat bones may be involved to a lesser extent (Panciera *et al.*, 2000). Periosteal exostosis of bone is grossly visible as elevated plaques or thickened regions on affected bones which are actually expanded with the addition of new bone. Experimentally infected dogs and coyote pups exposed to a dose of 100 *H. americanum* oocysts each developed bone lesions similar to those seen in naturally infected domestic dogs (Kocan *et al.*, 2000; Panciera *et al.*, 2000) whereas bone lesions in naturally infected adult coyotes have not been observed (Kocan *et al.*, 1999, 2000).

Justification

The opportunity to further explore the biology of *H. americanum* in coyotes would strengthen understanding of the role these wild canids may play in the maintenance and transmission of this organism and help determine their role as a wild reservoir for ACH.

Objectives

Objective 1: To identify and characterize the clinical disease produced in adult coyotes and juvenile coyotes each exposed to 50 *Hepatozoon americanum* oocysts isolated from GCT acquisition-fed on either naturally infected wild coyotes or domestic dogs.

Objective 2: To quantitate the muscle and bone lesions in adult and juvenile coyotes each exposed to 50 *Hepatozoon americanum* oocysts isolated from GCT acquisition-fed on either naturally infected domestic dogs or wild coyotes.

II. MATERIALS AND METHODS

Experimental Animals

Eleven coyotes were obtained as pups from the Predator Damage Management Unit, United States Department of Agriculture, Animal Damage Control. Six adult coyotes (Nos. 188, 189, 190, 191, 192, and 193) were 12-18 months of age and five juvenile coyotes (Nos. 26, 27, 28, 29, and 30) were six months of age at the time of the present study. Coyotes were housed outdoors at the Wild Animal Research Facility of Oklahoma State University (OSU) in shaded 24.38m x 41.76m concrete-floored chain-link pens and cared for in accord with standards of the OSU Institutional Animal Care and Use Committee. Animals were observed daily and maintained on commercially dry dog food; water was available *ad libitum*. Tick control was established through monthly application of fipronil (Frontline®, Merial) and efficacy was evaluated weekly. Muscle biopsy of experimental animals obtained prior to exposure was negative for the presence of *H. americanum*.

Isolates of *Hepatozoon americanum*

Sources of *H. americanum* isolates were four naturally infected animals that included 2 adult wild coyotes captured by the Predator Damage Management Unit (USDA) and 2 naturally infected domestic dogs. Of the two wild coyotes, one was live-captured in Osage County, Oklahoma and the other one was shot and killed in northern Payne County, Oklahoma. One of the two domestic dog sources of *H. americanum* (Dog No. 3048) is maintained by Laboratory Animal Resources (LAR) of OSU and cared for in accord with conventional laboratory animal practices. The other domestic dog source of *H. americanum* is privately owned in Stillwater, Oklahoma. Confirmation of infection in domestic dogs and wild coyotes was by microscopic detection of *H. americanum* in hematoxylin and eosin-stained sections of skeletal muscle tissue obtained through surgical biopsy of the biceps femoris.

Ticks

Laboratory-reared *Ambylomma maculatum* ticks, obtained as nymphs from the tick-rearing facility of the Oklahoma Agricultural Experiment Station (Stillwater, Oklahoma) were allowed to feed to repletion on two *H. americanum* naturally infected domestic dogs and one *H. americanum* naturally infected wild coyote. Replete or partially engorged nymphal *A. maculatum* ticks were removed from the carcass of an adult coyote killed in Payne County. Replete nymphs were maintained in an appropriate chamber with light:dark cycles of 14:10h and relative humidity at 90%, in accordance with procedures described by Mathew *et al.* (1998) and allowed to molt to the adult stage.

Oocyst Collection

Newly molted adult *A. maculatum*, previously fed as nymphs on naturally infected dogs or wild coyotes, were placed in a glass petri dish that contained 0.85% buffered saline and submerged with a metal probe placed against the basis capituli. The ventral surface of the tick was pierced with a scalpel-tip and gentle pressure was applied at mid-dorsum with another probe. The pressure applied mid-dorsum allowed some gut, hemolymph, and oocysts to be expressed through the puncture site and out into the surrounding saline. When the contents of the tick ceased to flow from the puncture site, the ventral portion of the tick was removed and the internal remains gently teased with forceps to force any remaining oocysts into the saline for collection.

Animal Exposure and Experimental Design

Due to limitations in the availability of experimental animals and *H. americanum* oocysts, the present study was conducted in three separate trials. In Trial 1 (Figure 1), one adult coyote (No.189) was exposed to the domestic dog isolate (referred to in Figure 1 as "Domestic Dog Isolate No.1") obtained from LAR Dog No. 3048, one adult coyote

(No.191) was exposed to the *H. americanum* domestic dog isolate ("Domestic Dog Isolate No. 2") obtained from a privately owned domestic dog, and one adult coyote (No. 190) was exposed to the *H. americanum* isolate ("Wild Coyote Isolate No. 1") obtained from a live-caught coyote. Also in Trial 1, one adult coyote (No. 192) served as an unexposed control. Coyotes in Trial 1 were exposed to *H. americanum* through hand-feeding each coyote a mixture of canned dog food and 50 *H. americanum* oocysts. In Trial two (Figure 2), two juvenile coyotes (Nos. 26 and 27) and one adult coyote (No. 193) were exposed to the domestic dog isolate (referred to in Figure 2 as "Domestic Dog Isolate No. 1") obtained from LAR Dog No. 3048. The coyotes in Trial 2 were exposed while sedated and 50 *H. americanum* oocysts placed into the stomach of each coyote through oral intubation. In Trial 3 (Figure 3), two juvenile coyotes (Nos. 28 and 29) and one adult coyote (No. 188) were exposed to *H. americanum* oocysts (referred to in Figure 3 as "Wild Coyote Isolate No. 2") obtained from molted adult ticks (collected as nymphs) from the naturally infected coyote carcass. Exposure to *H. americanum* in Trial 3 was accomplished by pouring the oocysts down into the throat of each sedated coyote. One juvenile coyote (No. 30) served as an unexposed control in Trial 3. The duration of each Trial was approximately 100 days. Analyses of clinical disease and quantification of muscle and bone lesions were compared between the age of the coyotes at the time of exposure (juvenile vs. adult) and between coyotes exposed to different *H. americanum* isolates (domestic dog isolate vs. wild coyote isolate).

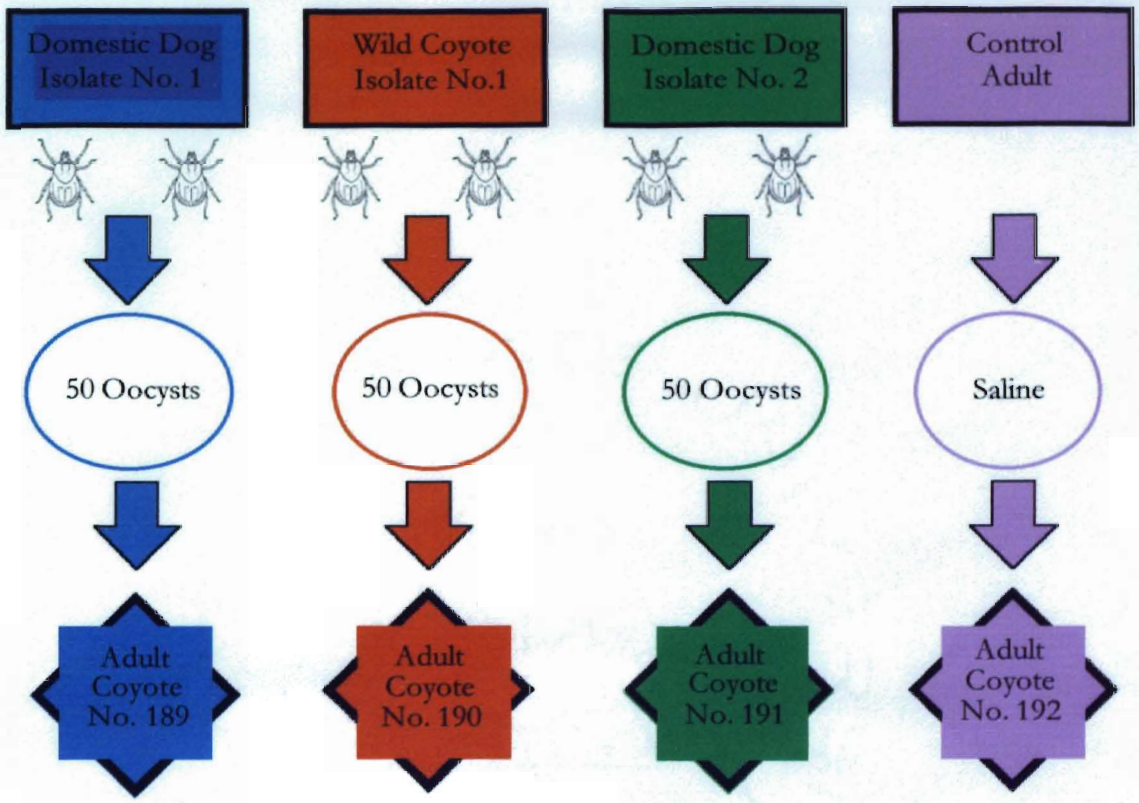

Figure 1. Experimental design for adult coyotes in Trial 1 each exposed to 50 *Hepatozoon americanum* oocysts obtained from adult *Amblyomma maculatum* ticks acquisition-fed as nymphs on naturally infected domestic dogs or a naturally infected wild coyote.

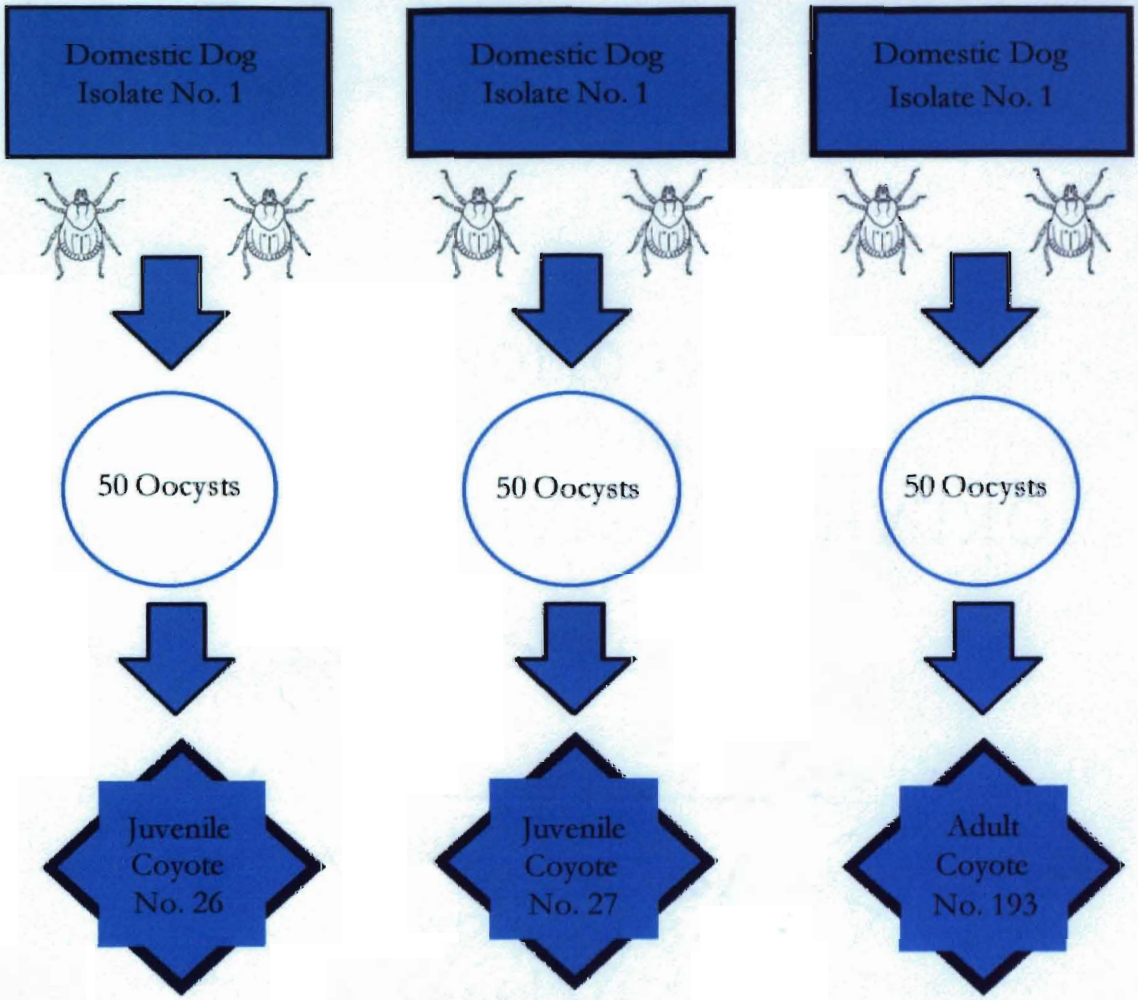

Figure 2. Experimental design for one adult and two juvenile coyotes in Trial 2 each exposed to 50 *Hepatozoon americanum* oocysts obtained from adult *Amblyomma maculatum* ticks acquisition-fed as nymphs on a naturally infected domestic dog.

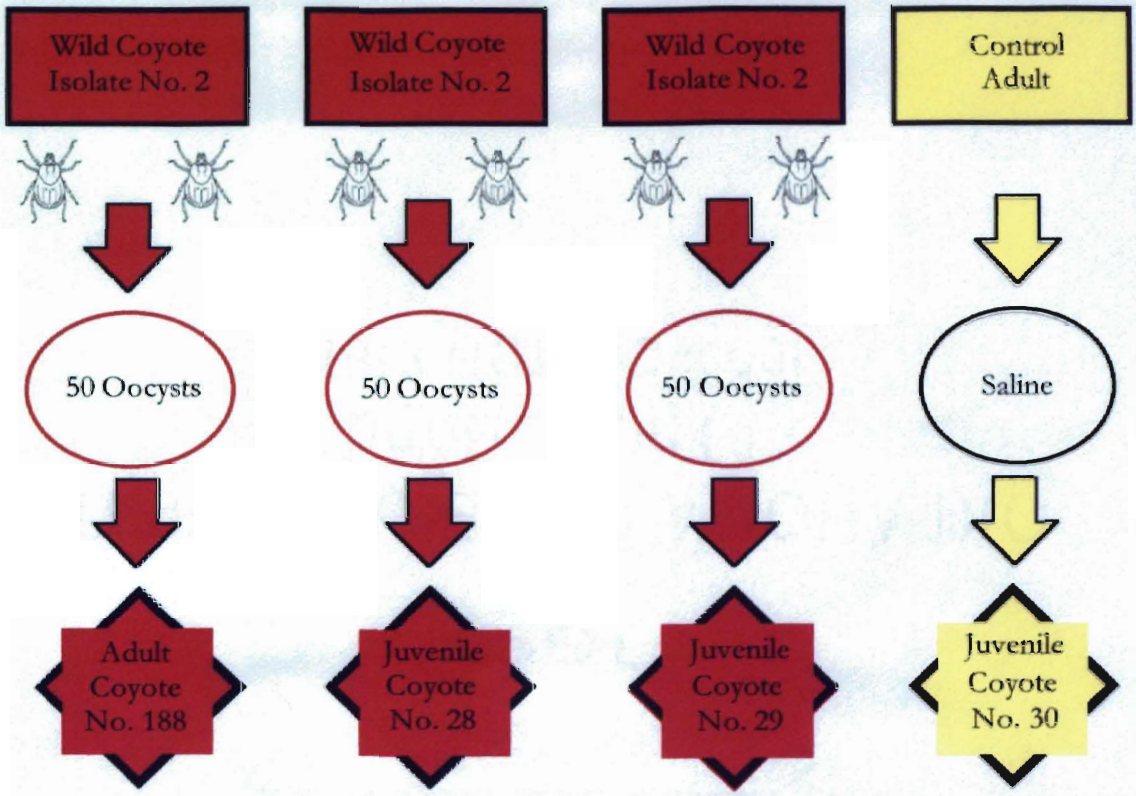

Figure 3. Experimental design for one adult and two juvenile coyotes in Trial 3 each exposed to 50 *Hepatozoon americanum* oocysts obtained from adult *Amblyomma maculatum* ticks collected as replete or partially-replete nymphs from a naturally infected wild coyote carcass.

Characterization of Clinical Disease

Physical Examination

Coyotes were observed daily and gait was graded weekly according to criteria listed in Table 1. Coyotes in Trial 1 were anesthetized for weekly physical examination with a combination of ketamine (4mg/kg; KetaVed™, Vedco) and xylazine (2mg/kg; Tranquived Injection, Vedco) administered intramuscularly (Kreeger *et al.*, 2002). In Trial 2 and Trial 3, coyotes were given a physical examination under anesthesia at the time of exposure and prior to euthanasia at the end of the study.

Physical determinants included general body condition, weight, heart rate, respirations, rectal temperature and capillary refill time. The liver and spleen were palpated for organomegaly and the prescapular, subscapular and popliteal lymph nodes were palpated for enlargement.

Hematology

Complete blood cell counts (CBC) (Cell Dyn, Abbott Diagnostics) and serum biochemical assays (Vitros 950, Ortho-Clinical Diagnostics) were performed weekly only on adult coyotes in Trial 1. Hematologic values of Trial 1 coyotes were compared to baseline CBC and serum chemistry values of pen-raised coyotes that were previously established by Rich and Gates (1979). Differential white blood cell counts and determination of parasitemias were performed manually.

Table 1. Criteria for assessment of gait in adult and juvenile coyotes each exposed to 50 *Hepatozoon americanum* oocysts obtained from naturally infected domestic dog or wild coyote sources.

Observed Characteristics in Evaluation of Gait	Score
Fully functional without impairment	+ + + +
Altered coordination and/or limp	+ + +
Maintains seated position, uses front legs only	+ +
Moves only head or tail	+

Parasitemia was calculated as the number of *H. americanum* gamonts per one thousand leukocytes observed by light microscopy at 1000X in Giemsa-stained blood films. In Trial 1, parasitemia was determined weekly (Table 2) whereas in Trial 2 and Trial 3 the level of parasitemia was only determined on the 100th day post-exposure (Figure 4). Blood was not collected from the adult coyotes in Trial 1 during the 11th week and 13th week post-exposure.

Evaluation of Muscle Lesions

Skeletal muscle samples of biceps femoris, triceps brachii, temporalis, and longissimus dorsi were obtained at necropsy, fixed in neutral buffered formalin and processed for routine histologic examination. Hematoxylin and eosin-stained sections of skeletal muscle (0.5cm x 2cm x 2cm) were examined by light microscopy for the presence of *H. americanum*. Lesions observed within the 2cm^2 area of muscle were enumerated and classified as cysts (Figure 5) or granulomas (Figure 6).

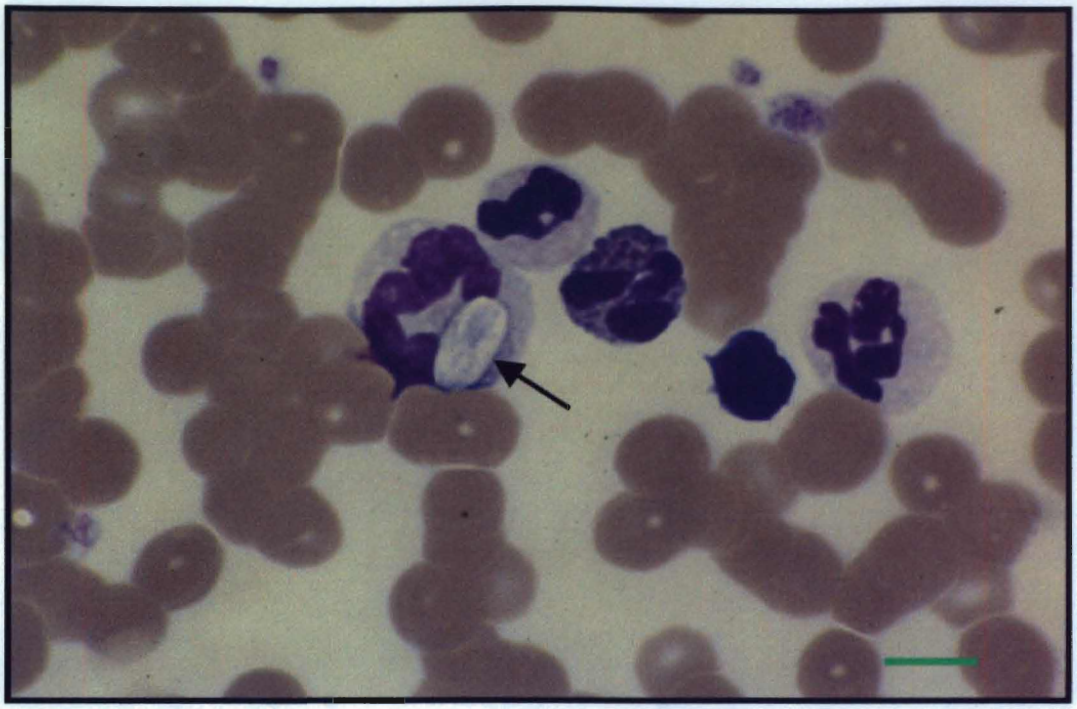

Figure 4. **Stained blood film from an adult coyote experimentally infected with *Hepatozoon americanum*. The arrow points to a cigar-shaped gamont of *Hepatozoon americanum* inside a leukocyte. Giemsa. (Bar = 10μm) Photomicrograph courtesy of Dr. R.J. Panciera.**

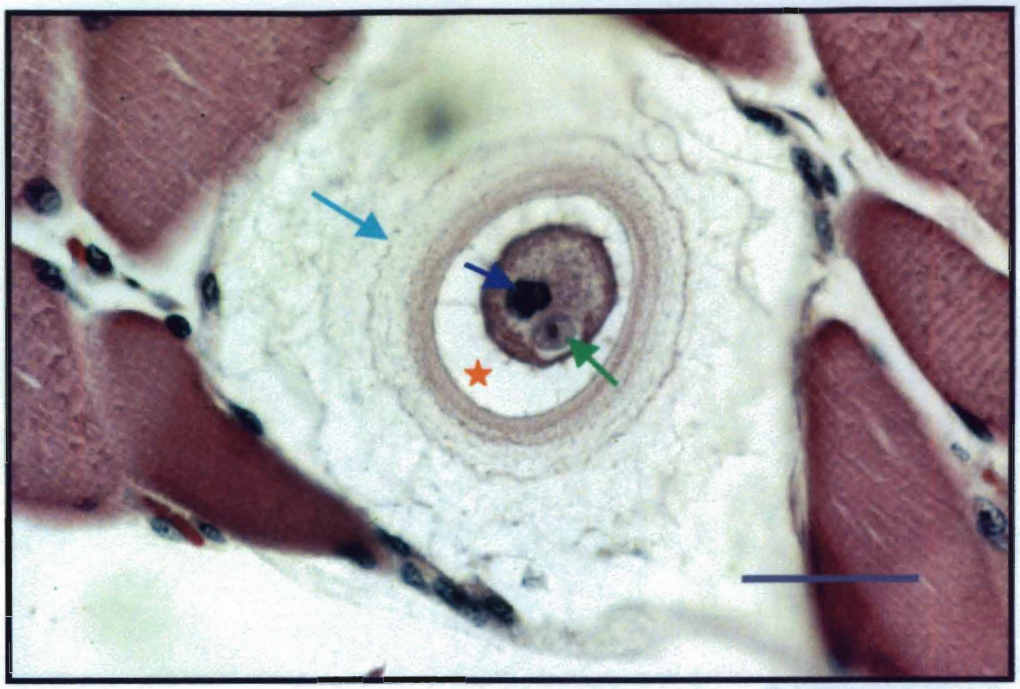

Figure 5. "Onion-skin" cyst of developing *Hepatozoon americanum* in skeletal muscle from an experimentally infected adult coyote obtained at 100 days post-exposure. The oval-shaped parasite is indicated by the bright green arrow and the dark nucleolus of the cell that is host to the parasite is indicated by the bright blue arrow. A light area of vacuolated cytoplasm, denoted by the orange star, surrounds the host cell nucleus. The light blue arrow points to a well-defined mucopolysaccharide layer that surrounds the host cell and gives this lesion an 'onion-skin' appearance. Hematoxylin-Eosin. (Bar = 50μm) Photomicrograph courtesy of Dr. R.J. Panciera.

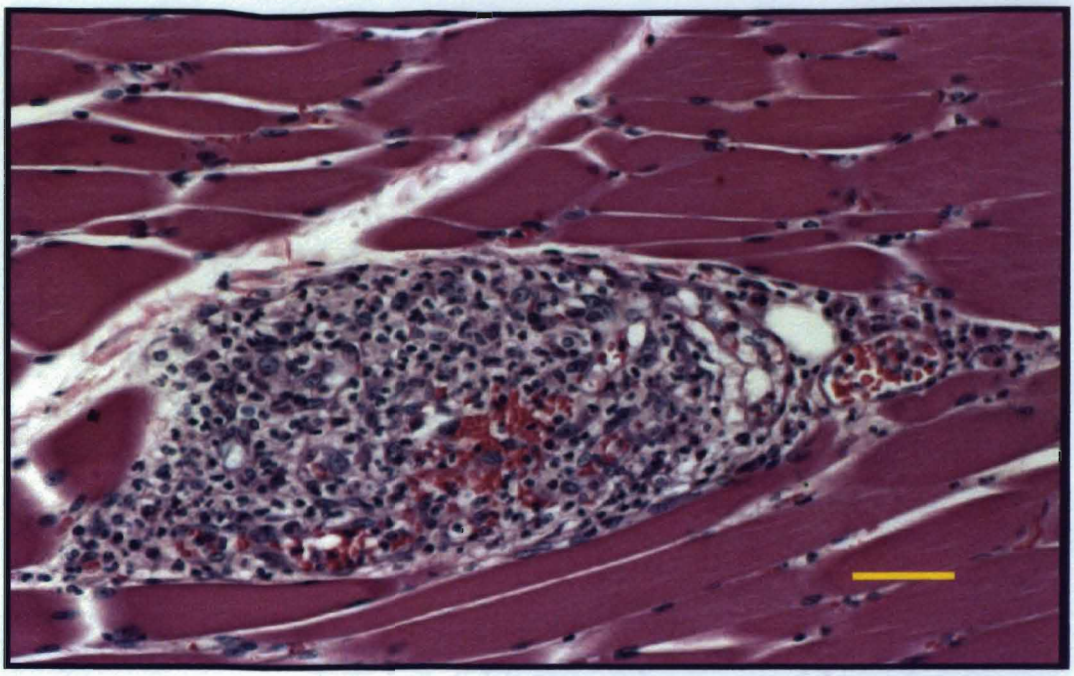

Figure 6. Granulomatous lesion in skeletal muscle from an adult coyote 100 days after exposure to 50 *Hepatozoon americanum* oocysts. This highly vascularized pyogranuloma is caused by the release of *Hepatozoon americanum* merozoites that matured inside an 'onion-skin' cyst. Hematoxylin-Eosin. (Bar = 100μm) Photomicrograph courtesy of Dr. R.J. Panciera.

Evaluation of Bone Lesions

Radiographic Evaluation

In all trials, craniocaudal and mediolateral radiographs of the right hind leg were taken at the time of experimental exposure and 100 days later at the end of the study following euthanasia. Radiographs of the right hind leg, pelvis to tarsus, were reviewed and scored according to proliferation (periosteal, endosteal or both), extent (number of bones affected) and whether opacities were present within the medullary cavity (Figure 7). The scoring system assigned "1" point for each characteristic observed in the radiographs of the right hind leg at 100 days post-exposure (Table 2). The total score for each radiograph was obtained by adding the accumulated points. If all three characteristics, proliferation of the periosteum, endosteum and medullary opacities, were observed in the radiographed bones, a score of '3' was assigned, the maximum score possible. Radiographs in which bone lesions were not observed received a score of '0'. Additionally, radiologic evidence of periosteal proliferation was characterized subjectively with regard to degree (mild, moderate, severe) and was further categorized as active or quiescent.

Gross Evaluation

Femurs from each coyote were collected at necropsy and autoclaved at 15psi for 15 minutes in a solution of water and laundry detergent according to procedures described by Panciera *et al.* (2000). Remaining soft tissue was gently scraped away with a metal probe. Processed bones were air-dried for several days and then measured using Vernier calipers. Measurements of femoral bone diameter were taken at 0.25cm increments along the anterior to posterior diaphysis and then along the medial to lateral diaphysis. Diameter measurements from the two diaphyseal planes (anterior to posterior and medial to lateral) were incorporated into Simpson's Rule for Uneven Area: Area = $1/3h\ [(y_0+y_m) + 4(y_1+y_3+y_5\ldots) +$

$2(y_2+y_4+y_6\ldots)]$ (Oberg *et al.*, 1976). For this formula, the ordinates y_1 and y_2 represent the sequential measurements (y_1=1st measurement; y_2= 2nd measurement, etc…) along the diaphysis of the bone separated by a constant distance (h) of 0.25cm. The two resultant areas (one from the anterior to posterior calculation and the other from the medial to lateral calculation) were added together and divided by two and the quotient converted into volume. Comparison with the control bone volume yielded a difference that represented the amount of new bone growth that occurred on the affected bone and that difference was then expressed as a percent. The area of the femur most affected with proliferation was also noted.

Table 2. Radiographic quantification of bone lesions from adult and juvenile coyotes experimentally infected with 50 *Hepatozoon americanum* oocysts obtained from domestic dog and wild coyote sources.

Observed Characteristics in Radiographic Evaluation of Bone Lesions	Score
Periosteal proliferation present in at least one bone	1 Point
Presence of endosteal proliferation	1 Point
Presence of opacities located within medullary canal of tibia or femur	1 Point

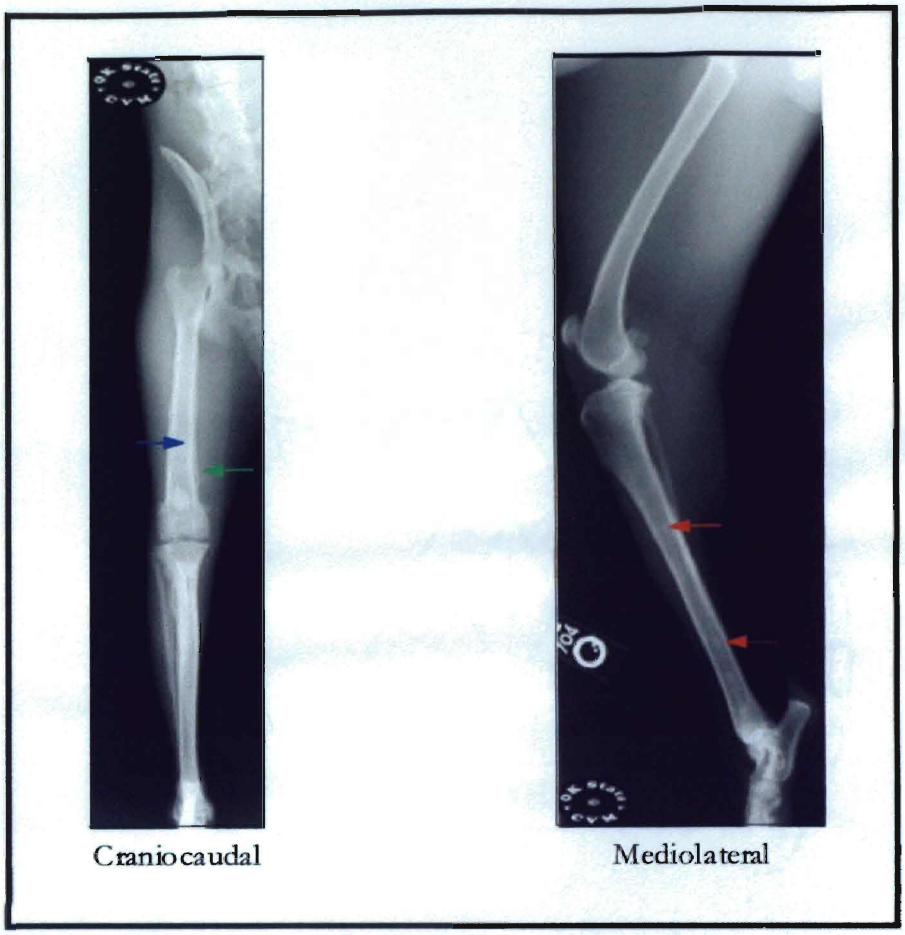

Figure 7. Right-hind leg craniocaudal and mediolateral radiographs of adult coyotes 100 days following exposure to 50 *Hepatozoon americanum* oocysts obtained from wild coyote and domestic dog sources. The blue arrow in the craniocaudal view points to endosteal proliferation while the green arrow indicates periosteal proliferation. The red arrows in the mediolateral view point to mineralizations, which appear as areas of opacity, within the medullary cavity of the tibia. This is the first time endosteal proliferation and medullary opacities have been seen in association with experimental canine heptozoonosis.

Statistical Analyses

The number of cysts and granulomas, percent of new bone growth observed grossly, and radiographic bone lesion scores were compared by t-test evaluation following analysis for normality using the Kolomgorov and Smirnov test (Sokal and Rohlf, 1997). Measurements of muscle and bone lesions were compared between adult and juvenile coyotes and between isolates of *H. americanum*. The Bonferroni adjustment for multiple comparisons was used as a criterion of statistical significance (Sokal and Rohlf, 1997).

III. RESULTS

Clinical Disease

All exposed coyotes became infected and manifested signs of clinical disease consistent with ACH (Vincent-Johnson *et al.*, 1997; Macintire *et al.*, 2001; Panciera *et al.*, 1997). When animals from all trials were combined and compared, the observed disease ranged from mild to moderate in severity regardless of age at the time of exposure or origin of *H. americanum* isolate. Salient features included ocular discharge, fever, wasting, lymphadenopathy, and a reluctance to move. An elevation of body temperature (>39.2C [102.5F]) was detected at 3 weeks post-exposure in all infected coyotes and persisted for approximately 5 weeks. By the tenth week post-exposure, all but one coyote (No. 190) were afebrile. Bilateral mucopurulent eye discharge appeared at 7 weeks post-exposure in juvenile coyotes and at 8 weeks post-exposure in adult coyotes. The average weight of adult coyotes at the time of exposure was 10kg (23.32lbs.) (Range: 8.36 - 12.09kg./18.4 - 26.6lbs.) while juvenile coyotes weighed an average of 4.47kg (9.85lbs.) (Range: 4.46 – 4.90kg/9.2 – 10.8lbs.). Juvenile coyotes gained an average of 1.76kg (3.88lbs.) (Range: 1.09 – 2.18kg/2.4lbs. - 4.8lbs.) during the 100-day study while the control juvenile coyote gained 3.4kg (7.5lbs.) during that same time period. One experimentally infected adult coyote (No. 191) gained 0.09kg (0.02lbs.) over the course of the 100-day study but all other infected adult coyotes lost an average of 1.04kg (2.3lbs.) (Range: 0.59kg – 1.54kg/1.3 – 3.4lbs.). The control adult coyote gained 2.63kg (5.8lbs.). Inappetance was noted on 6 non-consecutive days in two juvenile coyotes (Nos. 27 and 29) 7 and 8 weeks post-exposure. Transient lymphadenopathy (prescapular, subscapular and popliteal) was observed in six of the nine infected coyotes. Hematology data, derived from coyotes in Trial 1, demonstrated an increase in leukocytes in two coyotes (Nos. 190 and 191)(mean WBC count: 50.33cells/μL; reference range: 6,200.0 - 14,100.0cells/μL) seven weeks after experimental exposure. The increase in

white blood cells was composed primarily of mature neutrophils (mean neutrophils: 44.85cells/μL; reference range: 2,728.0 – 12,126.0cells/μL and mean percent neutrophils: 93.5%; reference range: 44 - 86%) (Figure 8). All serum chemistry values from adult coyotes exposed during Trial 1 remained within accepted normal ranges for the duration of the study.

Parasitemia

Gamonts of *H. americanum* were first detected 8 weeks post-exposure in stained blood films from all but one adult coyote (No. 189) in Trial 1. Parasitemias for all animals at 100 days post-exposure, regardless of age or isolate, were <1% (Table 3). Juvenile coyotes infected with *H. americanum* isolated from domestic dogs had a parasitemia of 0.9%; juveniles infected with isolates from wild coyotes had a parasitemia of 0.8% and adult coyotes that received domestic dog isolates had parasitemia levels of 0.2%. Those infected with isolates from wild coyotes had parasitemias of 0.3%.

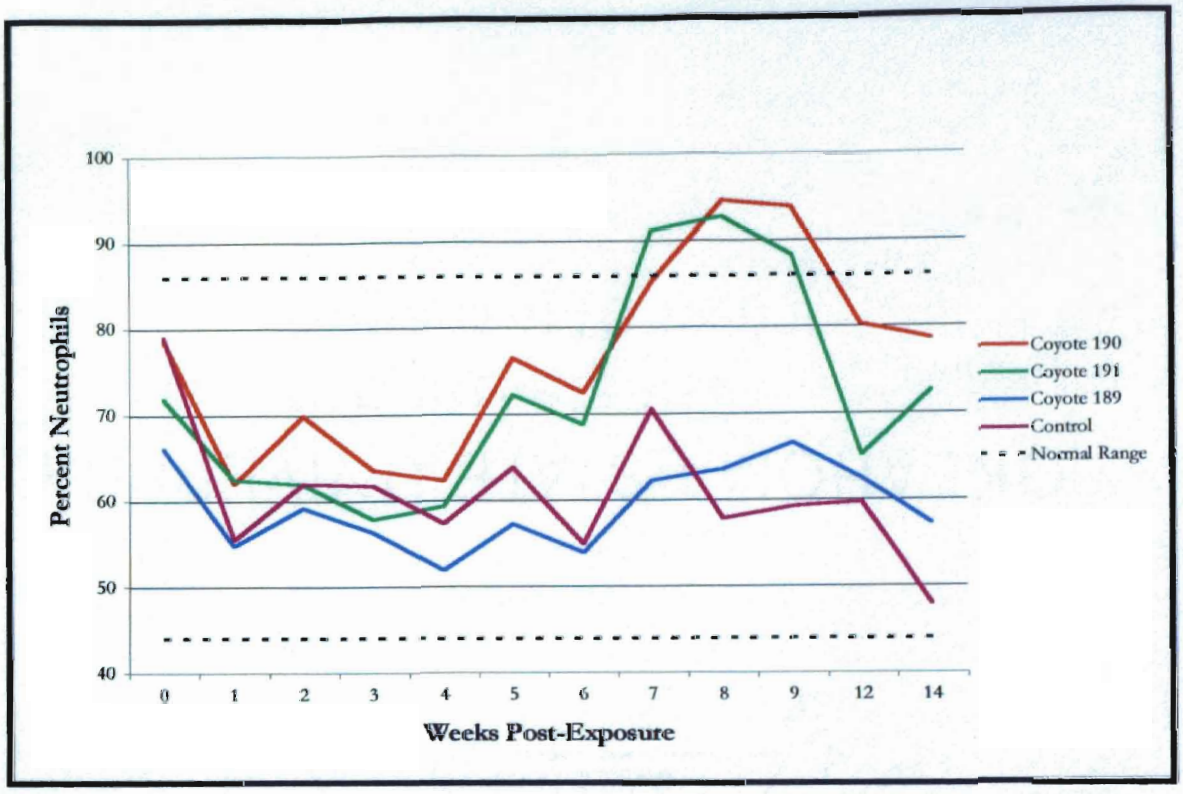

Figure 8. Increase in percentage of neutrophils in white blood cell counts performed weekly from Trial 1 adult coyotes exposed to 50 *Hepatozoon americanum* oocysts obtained from naturally infected domestic dog and wild coyote sources. Adult coyote No. 190 (represented by the red line) received a wild coyote isolate of *H. americanum*, adult coyote No. 191 (green line) received a domestic dog isolate of *H. americanum*, and adult coyote No. 189 (blue line) received a domestic dog isolate of *H. americanum*. Adult coyote No. 192 (pink line) served as an uninfected control.

Table 3. Average parasitemia of adult and juvenile coyotes 100 days following exposure to 50 *Hepatozoon americanum* oocysts obtained from naturally infected wild coyote and domestic dog sources.

Isolate of *Hepatozoon americanum*	Age of Coyote	Average Parasitemia
Domestic Dog	Juvenile	0.9%
Domestic Dog	Adult	0.2%
Wild Coyote	Juvenile	0.8%
Wild Coyote	Adult	0.3%

Table 4. Weekly parasitemia levels from Trial 1 adult coyotes each exposed to 50 *Hepatozoon americanum* oocysts obtained from wild coyote and domestic dog sources.

Weeks Post Exposure	189	190	191	192
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	0	0	0
5	0	0	0	0
6	0	0	0	0
7	0	0	0	0
8	0	0.3	0.2	0
9	0	0.1	0	0
10	0	0.5	0.2	0
11	NA	NA	NA	NA
12	0	0.2	0.3	0
13	NA	NA	NA	NA
14	0	0.5	0.5	0

No. 189 received a domestic dog isolate of *H. americanum*.
No. 190 received a wild coyote isolate of *H. americanum*.
No. 191 received a domestic dog isolate of *H. americanum*.
No. 192 is a control animal.

Quantitative Analysis of Muscle Lesions

Types of Lesions by Frequency

The average number of *H. americanum* 'onion-skin' cysts and granulomas per $2cm^2$ of skeletal muscle from adult and juvenile coyotes is shown in Table 5. In adult and juvenile coyotes that received a domestic dog isolate (n=5), the mean number of total lesions (combined cystic and granulomatous lesions) (± standard error) was 14.3 (± 6.0) lesions/$2cm^2$ of skeletal muscle whereas the mean number of total lesions in both adult and juvenile coyotes that received a wild coyote isolate (n=4) was 19.5 (± 4.5) lesions/$2cm^2$. Coyote pups (n=4), regardless of isolate, were found to have 21.4 (± 5.7) total lesions per $2cm^2$ of skeletal muscle whereas the total number of cystic and granulomatous lesions in adult coyotes (n=5) was 12.8 (± 4.4) per $2cm^2$ of skeletal muscle tissue. No statistical difference was observed in the mean number of total lesions (cysts and granulomas) between juvenile and adult coyotes or between coyotes exposed to different isolates of *H. americanum*.

In all infected coyotes, the average number of "onion-skin" cysts (± standard error) per $2cm^2$ of skeletal muscle (29.3 ± 3.6) was significantly ($t = 6.8$, $df = 16$, $P = <0.001$) greater than the average number of granulomas (3.3 ± 1.1). No statistical difference was observed in the number of 'onion-skin' cysts between adult and juvenile coyotes or between the coyotes exposed to different isolates of *H. americanum*. However, the average number of granulomas was significantly greater ($t = 5.0$, $df = 7$, $P = 0.001$) in juvenile coyotes (7.0 ± 1.1) infected with either isolate compared to those in adults (1.4 ± 0.5) (Table 4). On average (± standard error), 7.0 (± 1.1) granulomas per $2cm^2$ of skeletal muscle were observed in tissues from juvenile coyotes whereas granulomas in adult coyotes averaged 1.4 (± 0.5) per $2cm^2$. No statistical difference was found in the frequency of lesion type between coyotes that received wild coyote isolate and coyotes that received domestic dog isolate.

Location of the Two Types of Lesions

Microscopic observation of stained tissue obtained at necropsy revealed both cystic and granulomatous lesions most frequently present in skeletal and cardiac muscle. All coyotes had cystic lesions in cardiac and skeletal muscle. Encysted parasites were also found in the pancreas of two adult coyotes (Nos. 189 and 191) and in joint and adipose tissue of one coyote (No. 191). One adult coyote (No. 193) had cystic lesions in the muscularis of the small intestine and peri-fat of an adrenal and thyroid. Granulomatous lesions were present in cardiac tissue from three adult coyotes (Nos. 188, 191, and 193) and one juvenile coyote (No. 26) while another juvenile coyote (No. 29) had a granuloma in perisynovial adipose tissue.

Table 5. Average number of cystic and granulomatous lesions per $2cm^2$ of skeletal muscle from adult and juvenile coyotes 100 days post-exposure to 50 *Hepatozoon americanum* oocysts isolated from wild coyote and domestic dog sources.

Isolate of *Hepatozoon americanum*	Age of Coyote	Cysts*	Granulomas
Domestic Dog	Juvenile	33.5	8.0†
Domestic Dog	Adult	19.3	0.7
Wild Coyote	Juvenile	38.0	6.0†
Wild Coyote	Adult	31.5	3.0

*Statistically greater number of cysts in all infected coyotes
†Statistically greater number of granulomas in juvenile coyotes

Quantitative Analysis of Bone Lesions

Radiographic Evaluation

Medullary opacities, and periosteal and endosteal new bone growth was visible in radiographs from all but two coyotes (Nos. 27 and 189) exposed to *H. americanum*. One of the two coyotes (No. 189) did not develop any observable proliferation, either endosteal or periosteal, or have any opacities located within the medullary canal and was therefore assigned a score of "0". The other coyote (No. 27) received a score of "2" due to the absence of any opacities. Both of these coyotes (Nos. 27 and 189) received the *H. americanum* isolated from a domestic dog source but no statistical differences in bone lesions based on radiograph scoring was detected between juvenile and adult coyotes or between sources of *H. americanum* isolate (Table 6). Bone lesions in radiographs of adult coyotes were 'active' and 'reactive' whereas lesions in juvenile coyotes were 'inactive' and 'quiescent'. When present, periosteal proliferation in adult coyotes was mild (Nos. 188 and 191) to moderate (No. 190) whereas all juvenile coyotes exhibited moderate proliferation.

Gross Evaluation

No significant difference in the values used to determine gross proliferation was detected between age groups or isolates used to infect coyotes (Table 7). However, one adult coyote (No. 189) did not have any observable new bone proliferation (0%) and another adult coyote (No. 191) exhibited only minimal new bone growth (1.5%). All four of the experimentally infected juvenile coyotes manifested new bone growth that affected nearly half of the femoral bone surface. The greatest amount of proliferation (86.63% and 84.63%) occurred in juvenile coyotes (Nos. 28 and 29, respectively) exposed to wild coyote isolate of *H. americanum*. However, juvenile coyotes (Nos. 26 and 27) that received domestic dog isolate had less new bone growth (48.94% and 59.07%, respectively) than the adult (No. 190) with

the greatest amount of proliferation (67.07%) and exposed to the wild coyote isolate. The other adult coyote that received the wild coyote isolate (No. 188) had less new bone growth (30.94%) than his cohort (No. 190: 67.07%) and less new bone growth than the adult coyote (No. 193) exposed to the domestic dog isolate (42.57%). Proliferative lesions of juvenile coyotes were smooth in texture and uniform in thickness with a gradual transition from unaffected bone to new bone growth whereas the femoral lesions of adult coyotes were extremely rough and "knobby" upon the surface and sharply uneven in thickness, with sudden transitions from unaffected bone to ragged deposits of new bone growth. The distal lateral portion of the femur exhibited the greatest amount of periosteal proliferation.

Table 6. Average bone lesion scores from radiographs of adult and juvenile coyotes 100 days post-exposure to 50 *Hepatozoon americanum* oocysts obtained from wild coyote and domestic dog sources.

Isolate of *Hepatozoon americanum*	Age of Coyote	Average Score
Domestic Dog	Juvenile	2.5
Domestic Dog	Adult	2.0
Wild Coyote	Juvenile	3.0
Wild Coyote	Adult	3.0

Table 7. Average percent of gross femoral periosteal proliferation in adult and juvenile coyotes 100 days post-exposure to 50 *Hepatozoon americanum* oocysts isolated from wild coyote and domestic dog sources.

Isolate of *Hepatozoon americanum*	Age of Coyote	Percent Proliferation
Domestic Dog	Juvenile	54.0%
Domestic Dog	Adult	14.7%
Wild Coyote	Juvenile	85.6%
Wild Coyote	Adult	49.0%

IV. DISCUSSION

All coyotes (n=9) exposed to 50 *H. americanum* oocysts became infected and all infected animals exhibited clinically detectable disease. Morbidity in adult and juvenile coyotes ranged from mild to moderate and clinical signs of disease were consistent with those previously reported for ACH in domestic dogs. Neither adult nor juvenile coyotes died as a result of experimental exposure to 50 *H. americanum* oocysts obtained from either domestic dogs or wild coyotes. All 4 infected juvenile coyotes (100%) developed moderate disease; however, clinical disease varied among adult coyotes as 2 of 5 (40%) infected coyotes exhibited only minimal disease, 2 of 5 (40%) exhibited mild disease and one adult coyote (20%) exhibited moderate clinical disease. At 100 days post-exposure to either domestic dog or wild coyote isolate of *H. americanum*, juvenile coyotes had a higher parasitemia (0.85% vs. 0.25%) than did adult coyotes. Four of five (80%) adult coyotes (80%) were parasitemic whereas all juvenile coyotes (100%) had detectable gamonts. The parasitemia levels observed in adult and juvenile coyotes in the present study are consistent with previous reports of <1% parasitemia in domestic dogs with ACH. Results of the present study demonstrate that both adult and juvenile coyotes are susceptible to experimental infection with *H. americanum* isolates obtained from domestic dogs or wild coyotes and that both adult and juvenile coyotes develop clinical disease.

Microscopic evaluation of striated muscle from infected coyotes revealed the spectrum of previously described lesions associated with ACH: 'onion-skin' cysts and inflammatory granulomas. Cysts were more numerous than granulomas (30.57 vs. 4.42, respectively) in both adult and juvenile coyotes and both types of muscle lesions (cysts and granulomas) were most frequently found in skeletal and cardiac muscle. However, juvenile coyotes had statistically greater numbers of granulomatous lesions than did adult coyotes (7 per $2cm^2$ of skeletal muscle vs. 1.85 per $2cm^2$ of skeletal muscle, respectively).

Bone lesions similar to those associated with ACH in domestic dogs were observed by radiography in 8 of 9 (89%) infected coyotes. Adult coyotes exhibited mild to moderate degrees of periosteal and endosteal proliferation whereas in juvenile coyotes, both types of bone proliferation were moderate in severity. Variation in the intensity of bone lesions was observed between age groups of coyotes as adult coyotes had active lesions while lesions of juveniles were quiescent. Radiographic opacities within the tibial or femoral canals were observed in 7 of 9 (78%) infected coyotes. In the present study, medullary opacities and proliferation of the endosteum are recognized for the first time in association with experimental canine hepatozoonosis, but the significance of these observations remains to be determined.

Gross bone lesions were observed in 8 of 9 (89%) infected coyotes and resembled those previously reported from dogs with ACH (Vincent-Johnson *et al.*, 1997; Panciera *et al.*, 1997; Macintire *et al.*, 2001). Juvenile coyotes exhibited less variation in the amount of gross proliferation (range: 49% - 87%) than did adult coyotes (range: 1% - 67%); however, neither adult nor juvenile gait was significantly affected. Lesions also varied morphologically; juvenile coyotes had smoothly distributed diaphyseal proliferation whereas adult coyotes had rough, circular elevations of new bone growth on the diaphysis.

Experimental exposure to 50 oocysts of *H. americanum* recovered from ticks that fed on wild coyotes or domestic dogs produced clinical disease, parasitemia, merogonic muscle lesions, and bone lesions similar to those observed in natural and experimental canine heptozoonosis. However, the significantly greater number of granulomatous lesions observed in juvenile coyotes in the present study may indicate that the age of the animal at the time of infection influences the resultant disease. Previous studies (Kocan *et al.*, 1999; Panciera *et al.*, 1999; Drost *et al.*, 2003) demonstrated that larger doses of *H. americanum*

oocysts produced severe disease in dogs and coyotes. The 50 oocyst-dose utilized in the present study represents the smallest experimental dose reported to date to produce clinical disease. These findings suggest that the number of *H. americanum* oocysts used to initiate infection may also influence the severity of the disease. Results of the present study also support the hypothesis that coyotes can serve as suitable reservoir hosts for *H. americanum* and that both adult and juvenile coyotes could contribute to the transmission of ACH to domestic dogs.

LITERATURE CITED

Baneth, G., J.S. Mathew, V. Shkap, D.K. Macintire, J.R. Barta and S.A. Ewing (2003) Canine Hepatozoonosis: Two Disease Syndromes caused by separate Hepatozoon spp. Trends in Parasitology. 19(1):27-31.

Baneth, G. (2001) Canine Hepatozoonosis. *In* The Encyclopedia of Arthropod-transmitted Infections of Man and Domestic Animals. M.W. Service (ed.) CABI Publishing, London, England. p215-220.

Barton, C.L., E.A. Russo, T.M. Craig and R.W. Green (1985) Canine Hepatozoonosis: A Retrospective Study of 15 Naturally Occurring Cases. Journal of the American Animal Hospital Association. 21:125-134.

Craig, T.M. (1978) *Hepatozoon canis* Infection in Dogs: Clinical, Radiographic, and Hematologic Findings. Journal of the American Veterinary Medical Association. 173(8):967-972.

Craig, T.M. (2001) *Hepatozoon* spp. and Hepatozoonosis. *In* Parasitic Diseases of Wild Mammals. Samuel, W.M., M.J. Pybus and A.A. Kocan (eds.) Iowa State University Press, Ames, Iowa. p462-468.

Craig, T.M. (1990) Hepatozoonosis. *In* Infectious Diseases of the Dog and Cat. C.E. Green (ed.) W.B. Saunders, Philadelphia. p778-785.

Davis, D.S., R.M. Robinson and T.M. Craig (1978) Naturally Occurring Hepatozoonosis in a Coyote. Journal of Wildlife Diseases. 14:244-246.

Drost, W.T., C.A. Cummings, J.S. Mathew, R.J. Panciera and J.C.H. Ko (2003) Determination of Time of Onset and Location of Early Skeletal Lesions in Young Dogs experimentally infected with *Hepatozoon americanum* using Bone Scintigraphy. Veterinary Radiology and Ultrasound. 44(1):86-91.

Ewing, S.A., R.J. Panciera, J.S. Mathew, C.A. Cummings and A.A. Kocan (2000) American Canine Hepatozoonosis: An Emerging Disease in the New World. Tropical Veterinary Diseases. 916:81-92.

Ewing, S.A., J.G. DuBois, J.S. Mathew and R.J. Panciera (2002a) Larval Gulf Coast Ticks (*Amblyomma maculatum*) (Acari: Ixodidae) as host for *Hepatozoon americanum* [Apicomplexa: Adeleorina]. Veterinary Parasitology. 103:43-51.

Ewing, S.A., J.S. Mathew and R.J. Panciera (2002b) Transmission of *Hepatozoon americanum* (Apicomplexa: Adeleorina) by Ixodids (Acari: Ixodidae). Journal of Medical Entomology. 39(4):631-634.

Kocan, A.A., M. Breshears, C. Cummings, R. Panciera, S.A. Ewing and R.W. Barker (1999) Naturally Occurring Hepatozoonosis in Coyotes from Oklahoma. Journal of Wildlife Diseases. 35(1):86-89.

Kocan, A.A., C.A. Cummings, R.J. Panciera, J.S. Mathew, S.A. Ewing and R.W. Barker (2000) Naturally Occurring and Experimentally Transmitted *Hepatozoon americanum* in Coyotes from Oklahoma. Journal of Wildlife Diseases. 36(1):149-153.

Kreeger, T.J., J.M. Arnemo and J.P. Raath (2002) Handbook of Wildlife Chemical Immobilization. Wildlife Pharmaceuticals, Inc., Fort Collins, Colorado. p168.

Lane, J.R. and A.A. Kocan (1983) *Hepatozoon* sp infection in Bobcats. Journal of the American Veterinary Medical Association. 183(11):1323-1324.

Macintire, D.K., N.A. Vincent-Johnson, A.R. Dillon, B.L. Blagburn, D.S. Lindsay, E.M. Whitley and C. Banfield (1997) Hepatozoonosis in dogs: 22 Cases (1989-1994). Journal of the American Veterinary Medical Association. 210:916-922.

Macintire, D.K., N.A. Vincent-Johnson, C.W. Kane, D.S. Lindsay, B.L. Blagburn and A.R. Dillon (2001) Treatment of Dogs Infected with *Hepatozoon americanum*: 53 Cases (1989-1998) Journal of the American Veterinary Medical Association. 210:916-922.

Mathew, J.S., S.A. Ewing, R.J. Panciera and J.P. Woods (1998) Experimental Transmission of *Hepatozoon americanum* Vincent-Johnson et al., 1997 to dogs by the Gulf Coast tick, *Amblyomma maculatum* Koch. Veterinary Parasitology. 80:1-14.

Mathew, J.S., J.T. Saliki, S.A. Ewing, T.W. Lehenbauer, R.J. Panciera, J.T. Malayer, C.A. Cummings and A.A. Kocan (2001) An Indirect Enzyme-linked Immunosorbent Assay for Diagnosis of American Canine Hepatozoonosis. Journal of Veterinary Diagnostic Investigation. 13:17-21.

Mathew, J.S., S.A. Ewing, R.J. Panciera and K.M. Kocan (1999) Sporogonic Development of *Hepatozoon americanum* (Apicomplexa) in its Definitive Host, *Amblyomma maculatum* (Acarina). Journal of Parasitology. 85:1023-1031.

Mercer, S.H., L.P. Jones, J.H. Rappole, D. Twedt, L.L. Laack and T.M. Craig (1988) Hepatozoonosis in Wild Carnivores in Texas. Journal of Wildlife Diseases. 24:574-576.

Oberg, E., F.D. Jones and H.L. Horton (1976) Machinery's Handbook: A Reference Book for the Mechanical Engineer, Draftsman, Toolmaker and Machinist. 20th edition. Industrial Press Inc., New York, New York. p167-168.

Panciera, R.J., J.S. Mathew, C.A. Cummings, J.C. Duffy, S.A. Ewing and A.A. Kocan (2001) Comparison of Tissue Stages of *Hepatozoon americanum* in the Dog using Immunohistochemical and Routine Histological Methods. Veterinary Pathology. 38:422-426.

Panciera, R.J., S.A. Ewing, J.S. Mathew, T.W. Lehenbauer, C.A. Cummings and J.P. Woods (1999) Canine Hepatozoonosis: Comparison of Lesions and Parasites in Skeletal Muscle of Dogs Experimentally or Naturally Infected with *Hepatozoon americanum*. Veterinary Parasitology. 82:261-272.

Panciera, R.J., S.A. Ewing, J.S. Mathew, C.A. Cummings, A.A. Kocan, M.A. Breshears and J.C. Fox (1998) Observations on Tissue Stages of *Hepatozoon americanum* in 19 naturally infected Dogs. Veterinary Parasitology. 78:265-276.

Panciera, R.J., J.S. Mathew, S.A. Ewing, C.A. Cummings, W.T. Drost and A.A. Kocan (2000) Skeletal Lesions of Canine Hepatozoonosis caused by *Hepatozoon americanum*. Veterinary Pathology. 37:225-230.

Panciera, R.J., N.T. Gatto, M.A. Crystal, R.G. Helman and R.W. Ely (1997) Canine Hepatozoonosis in Oklahoma. Journal of the American Animal Hospital Association. 33:221-225.

Rich, J.E. and N.L. Gates (1979) Hematologic and Serum Chemistry Values of Pen-Raised Coyotes. Journal of Wildlife Diseases. 15:115-119.

Semtner, P.J. and J.A. Hair (1973) Distribution, seasonal abundance and hosts of the Gulf Coast Tick in Oklahoma. Annals of the Entomological Society of America. 66:1264-1268.

Sokal, R. R., and F. J. Rohlf (1997). Biometry 3rd edition. W. H. Freeman, San Francisco, California. p887.

Vincent-Johnson, N.A., D.K. Macintire, D.S. Lindsay, S.D. Lenz, G. Baneth, V. Shkap and B. L. Blagburn (1997) A New *Hepatozoon* species from Dogs: Description of the Causative Agent of Canine Hepatozoonosis in North America. Journal of Parasitology. 83(6):1165-1172.

Williams, H.R., P.D. Teel, O.F. Strey, R.E. Wright and D.E. Mock (2002) Zoogeography of the Gulf Coast Tick, *Amblyomma maculatum* Koch. Texas A&M University Website: http://ticsys.tamu.edu

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