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Immunological Responses in the Prairie Rattlesnake CROTALUS VIRIDIS VIRIDIS Rafinesque 1818, to Laboratory Infection with Tetrathyridia of MESOCESTOIDES CORTI Hoeppli 1925 (Eucestoda : Mesocestoididea)

Gray Bruce Hanson

Abstract

IMMUNOLOGICAL RESPONSES IN THE PRAIRIE RATTLESNAKE Crotalus viridis viridis RAFINESQUE 1818, TO LABORATORY INFECTION WITH TETRATHYRIDIA OF Mesocestoides corti HOEPPLI 1925 (EUCESTODA : MESOCESTOIDIDEA)

by Gary Bruce Hanson

Prairie rattlesnakes, of the species Crotalus viridis viridis, were orally infected with tetrathyridia of Mesocestoides corti to determine the host response to the parasite. Snakes were maintained at constant temperatures of 25, 30, and 35 C for 4 - 24 weeks. Blood chemistry evaluations of the host were made by standard hematological methods. Eosinophil counts, total proteins, and serum gamma globulins increased while packed-cell volumes and hemoglobins decreased. Autopsy studies revealed the first documented asexual multiplication of Mesocestoides corti in a laboratory infected ectothermic host, with the small intestine being the preferred organ. Pseudocyst formation was first observed to occur at 4 weeks and reached maturity at 8 weeks. Immature pseudocysts consisted of one layer of fibroblasts, but mature ones had an outer layer of fibroblasts, an interface layer, and an inner layer of mononuclear cells. Temperature was an important variable in this research, with the most pronounced changes in blood chemistry, rate of proliferation, and immune response seen at 30 C.

LOMA LINDA UNIVERSITY

Graduate School

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Gary Bruce Hanson

A Dissertation in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Field of Biology

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Each person whose signature appears below certifies that he has read this dissertation and that in his opinion it is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES - CONTINUED

INTRODUCTION

Representatives of the Crotalidae have been known for centuries to be receptive hosts for parasites. Tyson (1683) found nematodes in the stomach of a rattlesnake. Beyer (1898) observed a flatworm in the pericardium of a rattlesnake. Widmer (1970) found that Crotalus viridis viridis is a paratenic or ecological host for the third larval stage of the nematode, Physaloptera, and Voge (1953) lists Crotalus viridis oreganus as one of the intermediate hosts of the larval cestode, Mesocestoides variablis. The prairie rattlesnake, C. v. viridis described by Rafinesque (1818), and the Southern Pacific rattlesnake, Crotalus viridis helleri described by Meek (1905) were used as the host animals in this research.

Mesocestoides corti was described taxonomically by Hoeppli (1925). The discovery of asexual multiplication in M. corti was first noted by Specht and Voge (1965). The adults of the cestode M. corti are parasites of humans (Chandler, 1942), birds, and carnivorous mammals (Webster, 1949), while Mesocestoides tetrathyridia larvae are found in snakes, lizards, frogs, toads, and mice (Gleason et al., 1973).

Studies on the immunity of reptiles are few and fragmentary. Timourian et al. (1961) demonstrated that the carpet snake, Morelia spilotes variegatus, can elicit precipitating antibodies against certain parasitic nematodes. Hildemann (1962), in an immunogenetic study of amphibians and reptiles, observed the

capacity of these animals to produce humoral antibodies against a variety of antigens. Lewis (1969) demonstrated that the chuckwalla, a lizard, employed a humoral and cellular immunologic system against a bacterial infection. Kanakambika and Muthukkaruppah (1972) showed that the immune response in a lizard could be altered by splenectomy.

The general purpose of this research was to study the host response of C. v. viridis to laboratory induced infections with Mesocestoides tetrathyridia. The experimental research design included the following: (1) effects of tetrathyridia on the rattlesnake's eosinophil levels, packed-cell volume, hemoglobin, and gamma globulins; (2) anatomical preferences of the tetrathyridia; (3) rate of proliferation of the tetrathyridia, and (4) histopathologic analysis of affected organs. In addition, it was proposed to assess the significance of temperature variation on the above stated immune responses of tetrathyridial biological features.

MATERIALS AND METHODS

Preliminary Procedures

Prairie rattlesnakes, Crotalus viridis viridis, collected in the autumn of 1974 from Weld County, Colorado, and Pennington County, South Dakota, were segregated in individual cages. Body length and weight were recorded. Crotalus viridis helleri, collected in the spring of 1975 from San Bernardino County, California, was used for the penetration studies. The use of this subspecies was necessitated by the unavailability of C. v. viridis.

The initial strain of Mesocestoides corti used in this research was obtained from infected Swiss Albino mice procured from Dr. Marietta Voge at the University of California, Los Angeles. Tetrathyridia from a source mouse were used to establish laboratory infection in albino Swiss Webster male mice, weighing 20 - 25 g. The initial and subsequent maintenance in laboratory mice was by oral transfer of 25 larvae per mouse (Specht and Voge, 1965).

A second mouse from Dr. Voge's laboratory was sacrificed, the larvae removed and placed in physiological saline. Two hundred fifty larvae were counted and placed in individual 5-cm stentor dishes containing 2 ml of saline solution. These were prepared in advance and set aside at room temperature pending preparation of the snakes.

Infection of Host

Tygon tubing, 5 mm O D, was cut to correspond to one-third of

the total body length of the snake. This tubing was then coated with glycerol. The rattlesnakes were immobilized and the tubing inserted into the esophagus. The host first was given a saline oral gavage, accomplished by the use of a 5-ml disposable syringe filled with physiological saline and attached to the tubing.

Two hundred fifty tetrathyridia, previously counted and placed in saline, were drawn into the empty syringe which had been detached from the tubing. The syringe was reattached to the tubing and the larvae introduced into the snake's stomach. The syringe was again detached, refilled with 5 ml of saline, and the flushing process repeated. The tubing was withdrawn and examined to insure that all larvae had been introduced. Control snakes were gavaged with saline only.

Laboratory Maintenance of Host

Three chambers with individual temperatures set respectively at 25, 30, and 35 C ±1 were used for this experiment. Eighteen experimental snakes and four control snakes were maintained in individual cages in each of the environmental chambers.

The snakes were fed preweighed laboratory mice biweekly, commencing 1 week after initial infection. Mice not eaten within 24 hours were removed. The snakes which did not eat were then force fed Alpo (Beef) dog food. This was effected by oral insertion of a sterile rubber catheter (Davol, 24 Fr.) and the attachment of a metal caulking gun (Kenmark Mfg. Co.) used in delivery of the

homogenate dog food. For each force-fed snake, the amount of dog food delivered corresponded to the weight of the rejected food. Body weights of the snakes were determined monthly to observe weight changes during the period of infection.

The cloacal temperature of the snakes was taken monthly by means of a cloacal probe thermometer (Yellow Springs Instrument Co.) to determine variance of the internal body temperature from the temperature of the chamber.

Blood Withdrawal

Biweekly, from the date of infection, blood was drawn from all rattlesnakes by using cardiac puncture techniques (Sooter, 1955). The snakes were held securely in a snake loop and palpated on the upper one-third segment of the abdomen until the heart was located. This area was cleansed with 70% alcohol. Using a sterile 3-cc syringe and a 20-gauge needle, 2 cm of blood were collected directly from the heart. Blood film slides were made and stained with Wright's-Giemsa stain according to standard procedures (MacInnis and Voge, 1970). The blood was put into heparinized test tubes and refrigerated for later use.

Differential white blood cell counts were made from the stained slides. Nineteen individual differential white blood cell counts for the experimental snakes were made for each of the three different temperatures (time intervals are as specified in Table III). Seven counts were made for the control snakes for each of the

different temperatures using identical time intervals.

Autopsy Procedures

C. v. helleri used to determine the penetration time of the tetrathyridia were sacrificed and examined at 3, 4, 5, and 6 days following the techniques described by Widmer (1970). A total of 12 snakes was used, with three snakes killed at daily intervals. These snakes were kept at 30 C. Additional snakes maintained in the three environmental control chambers were removed at varying periods of 4 - 24 weeks post initial infection, sacrificed, and examined following similar techniques.

Data on the total number of parasites found and their relationship to the internal anatomy of the host were recorded. Infected organs were examined and sample sections were preserved in Bouin's fixative.

Whole Blood Analyses

Eosinophil counts of the refrigerated blood were made using a standard hemocytometer and Pilot's staining solution as outlined by MacInnis and Voge (1970). The degree of anemia was measured using the microhematocrit method. Hemoglobin, LDH and phosphatase levels were determined using the Spectronic-70 Spectrophotometer and the procedures outlined in Bausch and Lomb's Clinical Methods Manual (1965).

Serum Electrophoresis

Serum electrophoresis was done to determine the various serum components. A Gelman electrophoretic chamber was used according to procedures outlined by Gelman Technical Bulletin 20 (1975). The cellulose acetate strips were analyzed with a densitometer available at the Loma Linda University Medical Center.

RESULTS

General Information

The snakes kept at the 25 C temperature were observed for a period extending up to 24 weeks. Data on snakes maintained at 30 C extended only up to 20 weeks because of a malfunction of the chamber. Snakes which were in the 35 C chamber did not survive longer than 16 weeks.

Penetration

Autopsies were done to determine parasite attachment time and location. These tests indicated that after 3 days of infection the majority of larvae were found in the small intestine with smaller numbers located in the large intestine and stomach (Table I). No larvae were found in the coelomic cavity, systemic musculature, lungs, or liver. Gross anatomical examination of the intestinal tract after 3 days post-infection revealed that the larvae had not yet penetrated the intestinal folds but were found between them (Fig. 1). A few tetrathyridia penetrated into the mucosal folds at 4 days (Fig. 2) and moved deeper into the intestinal tissue after 5 and 6 days post-infection. At the end of 4 weeks, the larvae were in the serosa. The movement of the larvae from day 6 until the end of the 4 weeks was not recorded.

Site Selection

The majority of the tetrathyridia was found in the small

TABLE I.

Distribution of *Mesocestoides corti* tetrathyridia in 12 Southern Pacific rattlesnakes, *Crotalus viridis helleri*, 3 - 6 days after oral infection. Each snake was given an exposure dose of 250 larvae.

Location of larvae*	Mean number of larvae recovered+			
	Days post-infection			
	3	4	5	6
Stomach	12(10-15)	7(6-8)	9(7-11)	10(10)
Small intestine	143(135-149)	164(160-168)	157(150-164)	171(165-181)
Large intestine	35(32-37)	27(21-31)	23(22-26)	13(10-16)
Fecal material	17(16-18)	9(6-12)	12(11-13)	11(7-15)
Flushed in saline purge	13(12-14)	16(13-18)	9(9)	9(6-12)
Total recovered/Percent recovered	666/89	669/89	633/84	642/86

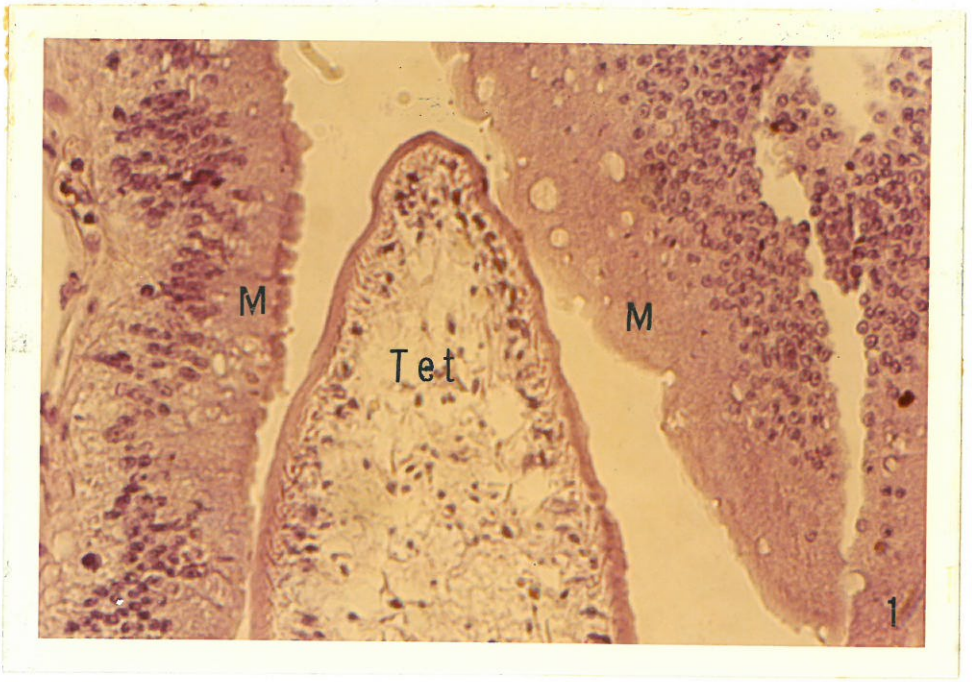
* No larvae were found in the coelomic cavities, systemic musculatures, lungs, or livers.

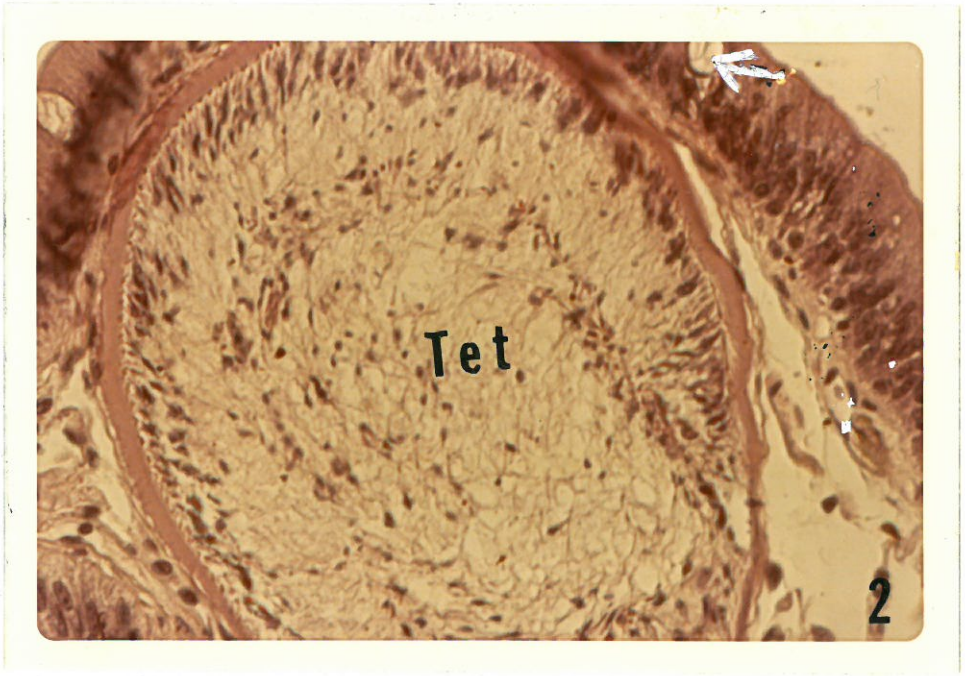
\+ Mean numbers, (range) and percent recovery for each post-infection day represent three snakes.

Figure 1. Tissue section of mucosa (M) of small intestine demonstrating tetrathyridium (Tet) at 3 days post-infection (400X). *

Figure 2. Tissue section of tunica propria (T) of small intestine demonstrating tetrathyridium (Tet) at 4 days post-infection (400X). Note goblet cells (Arrow).

* Note: All tissue sections are from 30 C snakes and except where indicated are stained with hematoxylin and eosin.


M
Tet
M
1


Tet
2

intestine (Table II). The remainder of the larvae, with decreasing mean numbers, were found in the large intestine, stomach, liver, and kidney. No larvae were found in the coelomic fluid, lungs, or systemic musculatures.

The majority of the parasites was located in the serosa of the gastro-intestinal tract of the host. The larvae were most often located in close proximity to the blood vessels (Fig. 3). In the most advanced stages of infection, larvae were not found in the lumen of the intestine. Liver infections were minimal, with mean numbers lower than the other organs, as the infection progressed (Table II). Larvae were found in either the capsule or parenchyma of the liver (Fig. 4).

One snake maintained at 30 C had a tetrathyridial kidney infection. This snake was autopsied 16 weeks post-infection (Fig. 5). This was the only kidney infection observed during the research period. Of the temperatures tested, none showed an apparent effect on site preference. At no time were any tetrathyridia found in any of the control snakes.

Asexual Multiplication

As the infection progressed, the number of Mesocestoides corti larvae increased. There were consistently more larvae at 30 C than at 25 or 35 C (Fig. 7).

TABLE II.

Specific organ preference of tetrathyridia as a function of temperature and post-infection time. A total of 18 snakes was autopsied at each temperature.

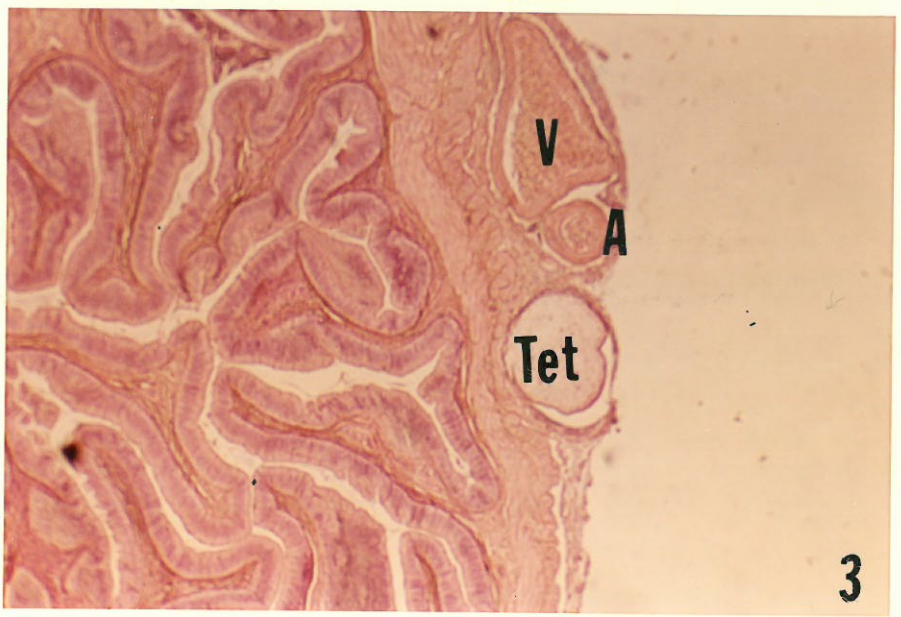
Location of larvae***		Mean number of larvae recovered+					
		Weeks post-infection					
		4	8	12	16	20	24
25 C	Liver	0(0)	9(0-14)	30(19-24)	12(4-21)	39(11-66)	43(18-67)
	Stomach	28(23-34)	38(31-56)	96(53-148)	63(27-114)	62(20-87)	202(182-227)
	Small intestine	127(117-142)	211(189-249)	219(99-289)	378(361-396)	398(388-421)	495(481-507)
	Large intestine	46(27-56)	153(112-181)	93(81-101)	155(98-178)	294(239-342)	478(427-481)
	Total / Percent recovered	653/87	833/111	1338/178	1826/243	1886/251	3656/481
30 C	Liver	38(22-53)	44(14-92)	60(42-132)	33(20-87)	115(105-125)	----------
	Stomach	59(42-73)	36(12-63)	93(40-134)	43(23-63)	123(119-129)	----------
	Small intestine	157(87-187)	194(131-281)	193(113-279)	335(238-429)	427(410-442)	----------
	Large intestine	81(77-84)	158(124-194)	169(143-229)	198(112-290)	263(260-265)	----------
	Total / Percent recovered	1007/134	1781/178*	2062/206*	2430/243*	2380/317	----------
35 C	Liver	0(0)	3(1-7)	28(20-38)	16(14-18)	----------	----------
	Stomach	3(0-10)	35(33-82)	78(29-142)	98(91-103)	----------	----------
	Small intestine	96(61-159)	145(41-211)	342(287-387)	227(220-235)	----------	----------
	Large intestine	50(10-62)	93(20-187)	88(70-171)	220(205-235)	----------	----------
	Total / Percent recovered	490/65	1280/102**	2230/178**	2805/224**	----------	----------

* Four snakes were autopsied at 30 C at this time interval. ** Five snakes were autopsied at 35 C at this time interval. Three snakes were autopsied at all other temperatures and time intervals.

*** A single kidney infection was observed. + Numbers represent the mean and the (range).

Figure 3. Tissue section of serosa of small intestine at 8 weeks post-infection. Note tetrathyridium (Tet) near artery (A) and vein (V) (400X).

Figure 4. Tissue section of liver 20 weeks post-infection. Note tetrathyridium (Tet) (400X).


V
A
Tet
3

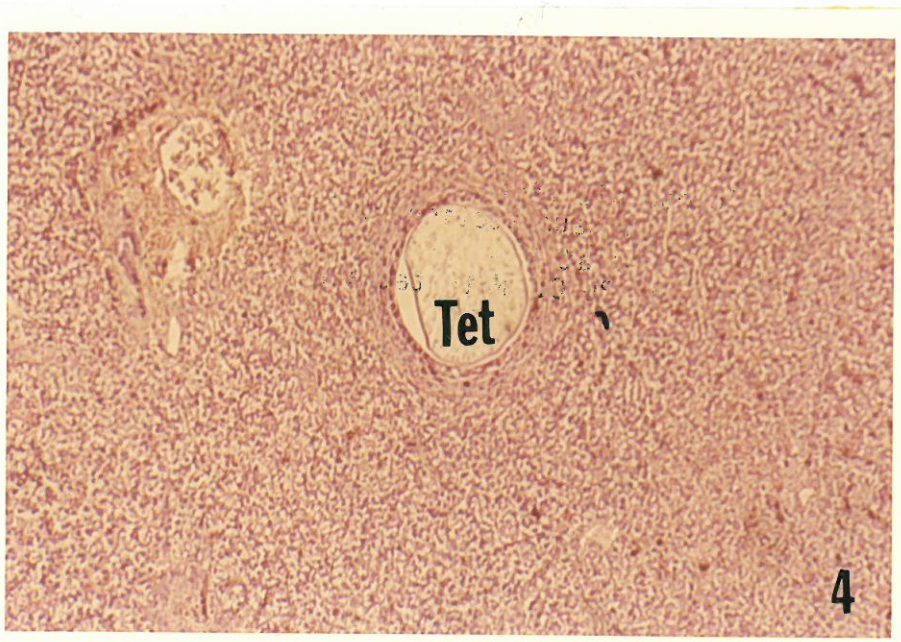
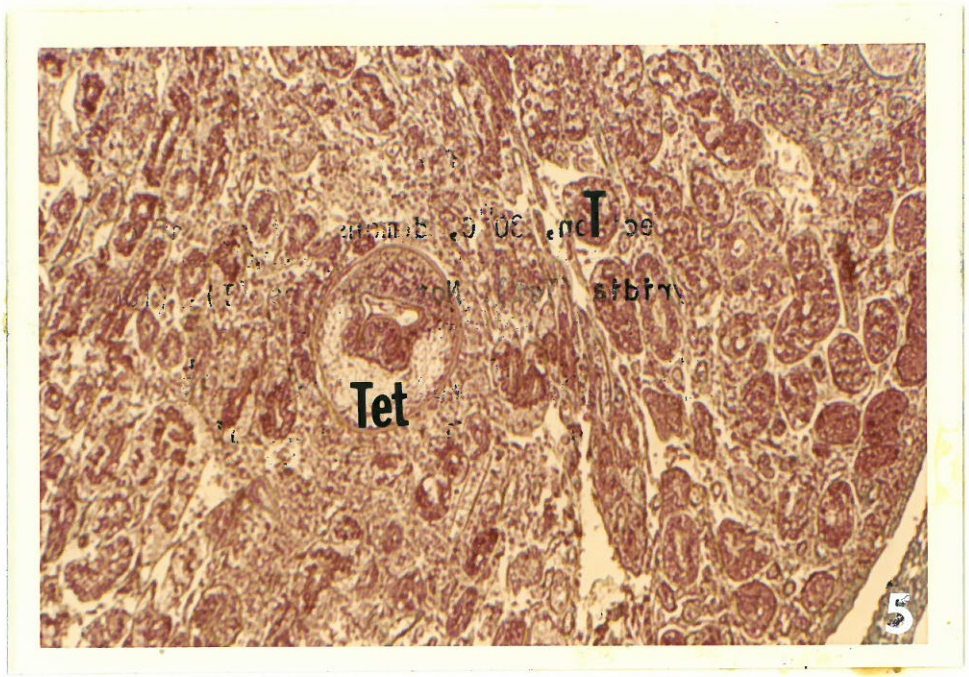

Tet
4

Figure 5. Tissue section of kidney at 16 weeks post-infection demonstrating infection with tetrathyridium (Tet). Note tubules (T) (100X). Masson's Stain.

Figure 6. Tissue section of small intestine at 4 weeks post-infection. Note location of tetrathyridium (Tet) in serosa (Ser) (100X).


Tet
5

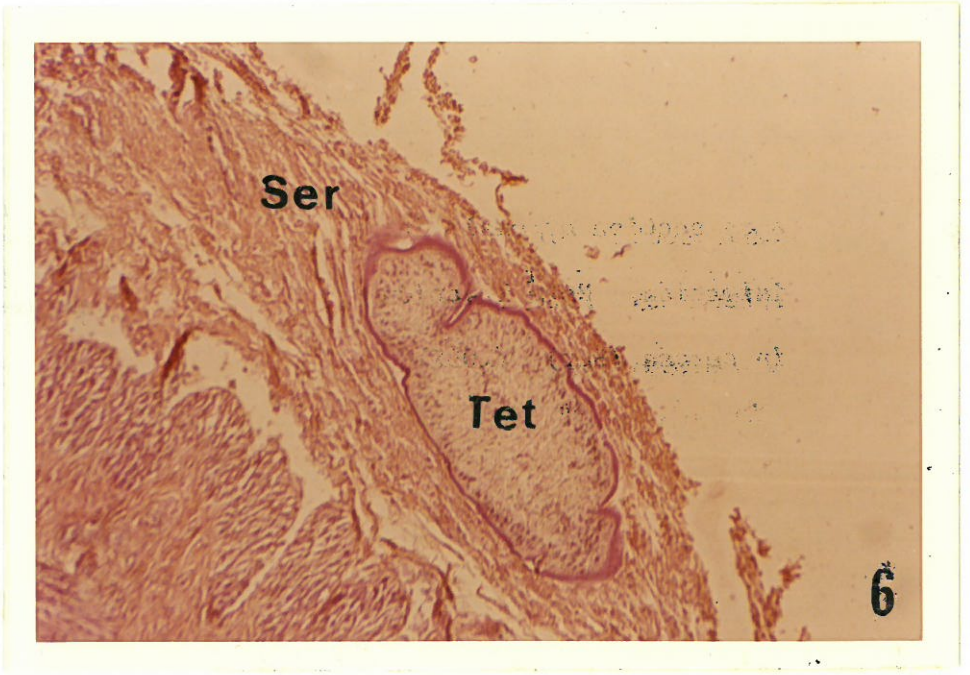
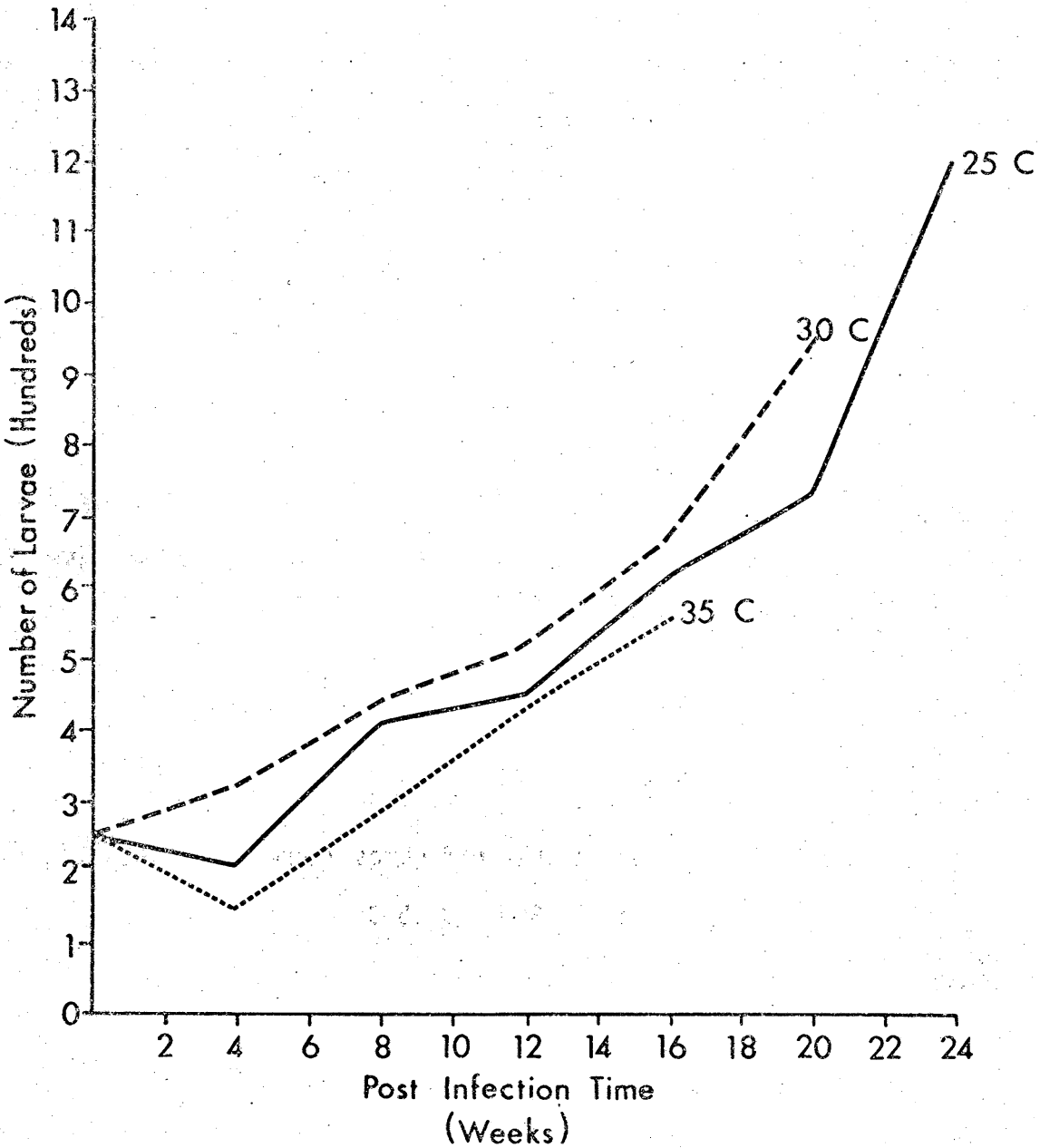

Ser
Tet
6

Figure 7. Asexual reproduction of Mesocestoides corti tetrathyridia as a function of temperature and post-infection time. *

*Note: Temperature in figures 7 and 12 through 16 were 25, 30, and 35 C.


Number of Larvae (Hundreds)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
2
4
6
8
10
12
14
16
18
20
22
24
Post Infection Time
(Weeks)
25 C
30 C
35 C

Histopathology

Differential blood counts, done from blood smears collected during the early period of infection, indicated a minimal demonstrable variation from the controls (Table III). Minimal changes in neutrophils were seen for days 3 - 6 compared to the controls. Tissue sections of these snakes revealed no characteristic blood cellular changes due to the presence of the tetrathyridia.

Tissue sections of the serosa revealed fibroblasts, lymphocytes, and macrophages in the immediate vicinity of the parasite (Figs. 6 and 8).

Pseudocyst formation began as early as 1 month at 30 C, with no discernible reaction noted at that same post-infection time for the other temperatures. The recognition of a pseudocyst was based on an encirclement of the tetrathyridia by the discernible fibrous connective tissue layer. A pseudocyst is a cyst with material for the cyst derived from the host. Initial pseudocyst formation involved a layer of fibroblasts (Fig. 9).

As the infection progressed, the number of pseudocysts increased. Tissue sections at 30 C revealed two separate pseudocyst layers as early as 8 weeks post-infection. The inner pseudocyst layer next to but not attached to the parasite consisted of mononuclear cells, and the outer layer, primarily of fibroblasts (Fig. 10). An interface layer between these two layers was occasionally noted in some of the infections of longer duration. This interface

TABLE III.

Differential white blood cell counts from 57 experimental and 21 control snakes.

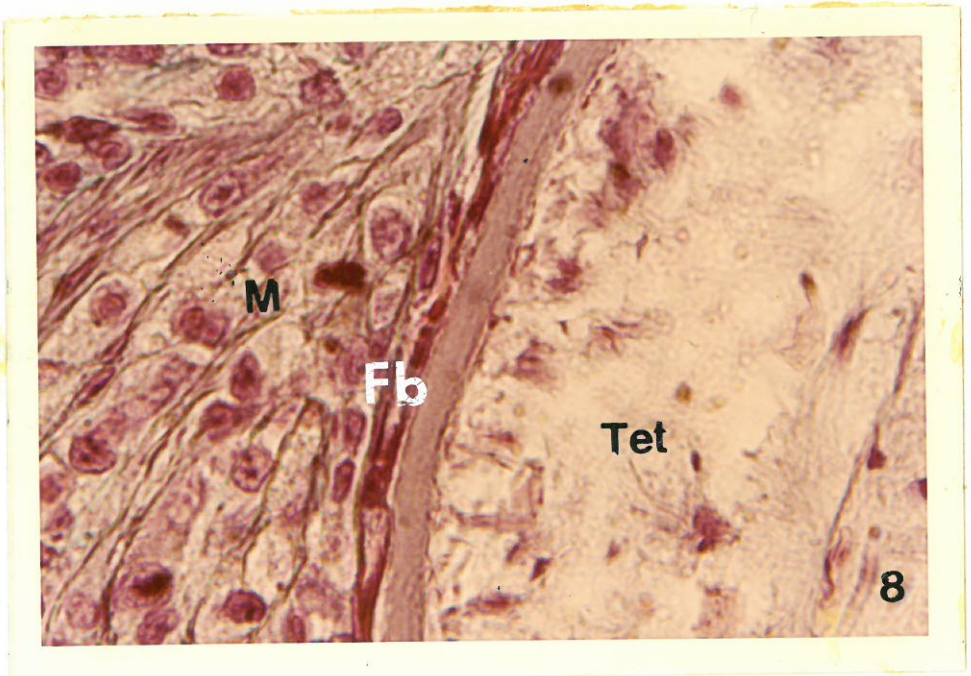
Numbers represent percent.

Temperature	Days post-infection						
	3	4	5	6	7	11	14
25 C							
Number of snakes examined*	3	3	3	3	2	2	3
Lymphocytes	51	51	53	52	51	50	50
Monocytes	1	0	1	2	1	1	0
Eosinophils	13	14	12	11	12	13	13
Basophils	12	12	10	11	11	10	11
Neutrophils	23	23	24	24	25	26	26
30 C							
Number of snakes examined	3	2	3	3	2	3	2
Lymphocytes	51	53	51	50	50	52	52
Monocytes	0	1	1	1	1	1	2
Eosinophils	14	13	12	12	14	13	13
Basophils	15	13	14	11	11	9	8
Neutrophils	20	20	22	26	24	25	25
35 C							
Number of snakes examined	3	3	3	3	3	2	3
Lymphocytes	50	51	51	52	52	53	54
Monocytes	1	0	0	2	2	1	1
Eosinophils	13	11	14	11	13	14	12
Basophils	16	15	15	11	13	10	11
Neutrophils	20	23	20	24	20	22	22
Controls							
Number of snakes examined	3	3	3	3	3	3	3
Lymphocytes	50	50	53	51	52	52	50
Monocytes	1	1	1	1	1	2	1
Eosinophils	14	15	15	13	13	11	13
Basophils	15	14	10	12	9	11	10
Neutrophils	20	20	21	23	25	24	26

*Four separate white cell counts were made per snake at each post-infection day indicated.

Figure 8. Tissue section of small intestine demonstrating fibroblasts (Fb) and macrophages (M) at 4 weeks post-infection. Note tetrathyridium (Tet) (1000X). Masson's Stain.

Figure 9. Tissue section of early pseudocyst formation in small intestine at 4 weeks post-infection. Note fibroblasts (Fb) and tetrathyridium (Tet) (1000X). Mallory's Stain.


M
Fb
Tet
8

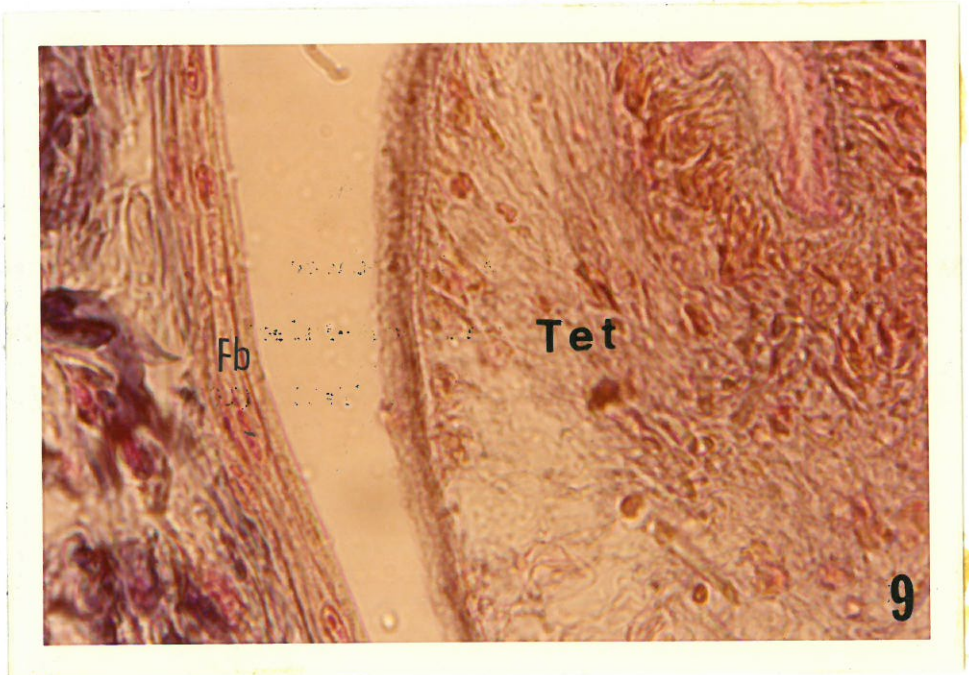
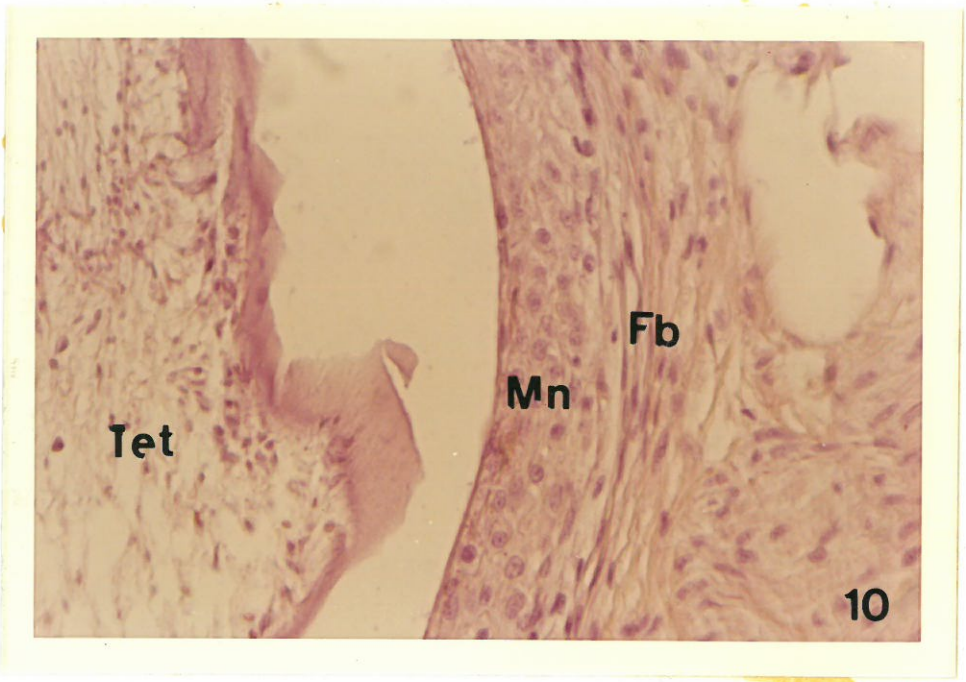
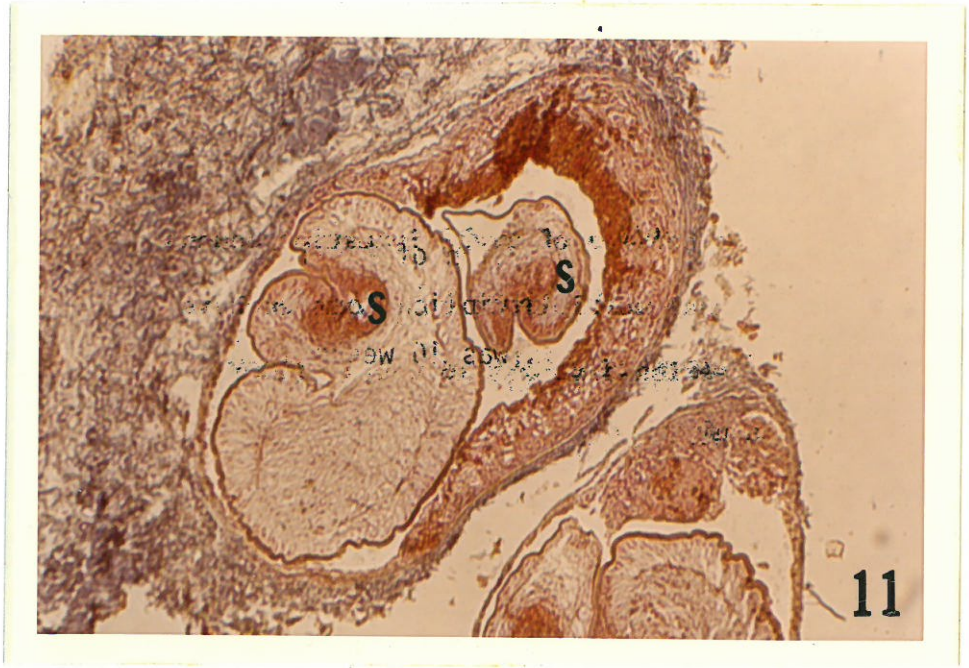

Fb
Tet
9

Figure 10. Tissue section of small intestine demonstrating an outer layer of fibroblasts (Fb) and an inner layer of mononuclear cells (Mn). Note tetrathyridium (Tet). Post-infection time was 16 weeks (400X).

Figure 11. Tissue section of small intestine demonstrating asexual multiplication. Note suckers (S). Post-infection time was 16 weeks (400X). Mallory's Stain.


Tet
Mn
Fb
10


S
S
11

layer consists of a combination of fibroblasts and mononuclear cells.

Asexual multiplication within the pseudocyst was first noted at 16 weeks (Fig. 11). Two to four larvae were usually found within each pseudocyst. The average diameter of the pseudocyst before multiplication was 400 microns and ranged to 500 microns as the infection progressed. The pseudocyst in snakes kept at 30 C had the greatest increase in diameter (25 - 35 microns) above the initial pseudocyst size.

Few neutrophils, primary indicators of acute inflammatory response, were observed in the microscopic tissue sections obtained from snakes infected 4 or more weeks. Lymphocytes, fibroblasts, and macrophages were characteristically seen after 4 weeks of infection. Reptilian cell morphology, both blood and tissue, was verified by the use of the publications by Gans and Parsons (1970) and Frye (1973).

Blood Analyses

As the post-infection time lengthened, eosinophils, total protein, and serum gamma globulin increased (Figs. 12, 15, and 16), but packed-cell volume and hemoglobin decreased (Figs. 13 and 14).

In each case there was a high ($r \geq .86$) positive or negative correlation respectively with the increasing number of parasites (Table IX, Appendix). This was observed for each temperature considered independently.

Figure 12. Eosinophil counts for the experimental and control snakes 2 - 24 weeks post-infection.

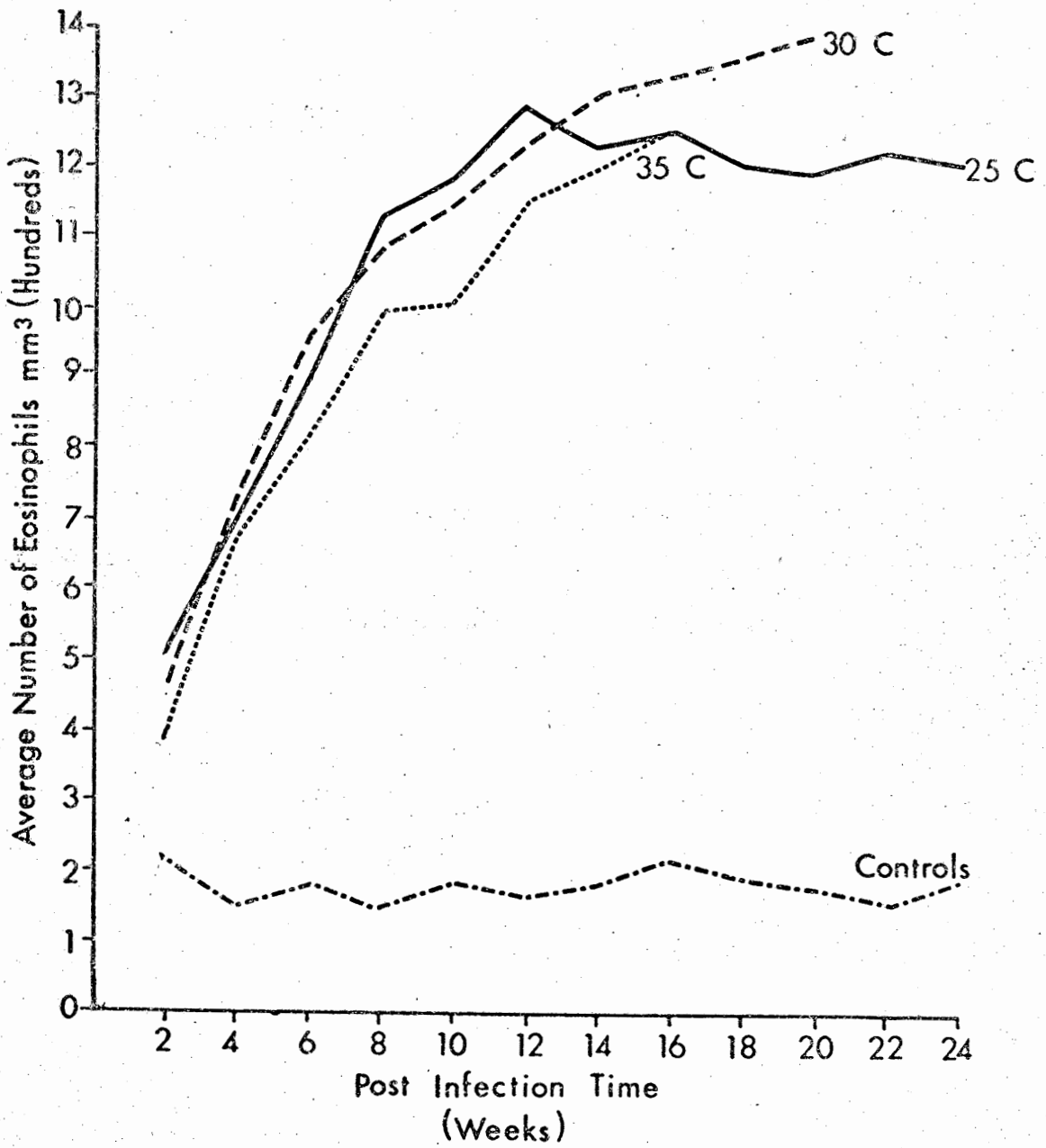

Average Number of Eosinophils mm³ (Hundreds)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
2
4
6
8
10
12
14
16
18
20
22
24
Post Infection Time
(Weeks)
30 C
35 C
25 C
Controls

Figure 13. Packed cell volume for experimental and control snakes 2 - 24 weeks post-infection.

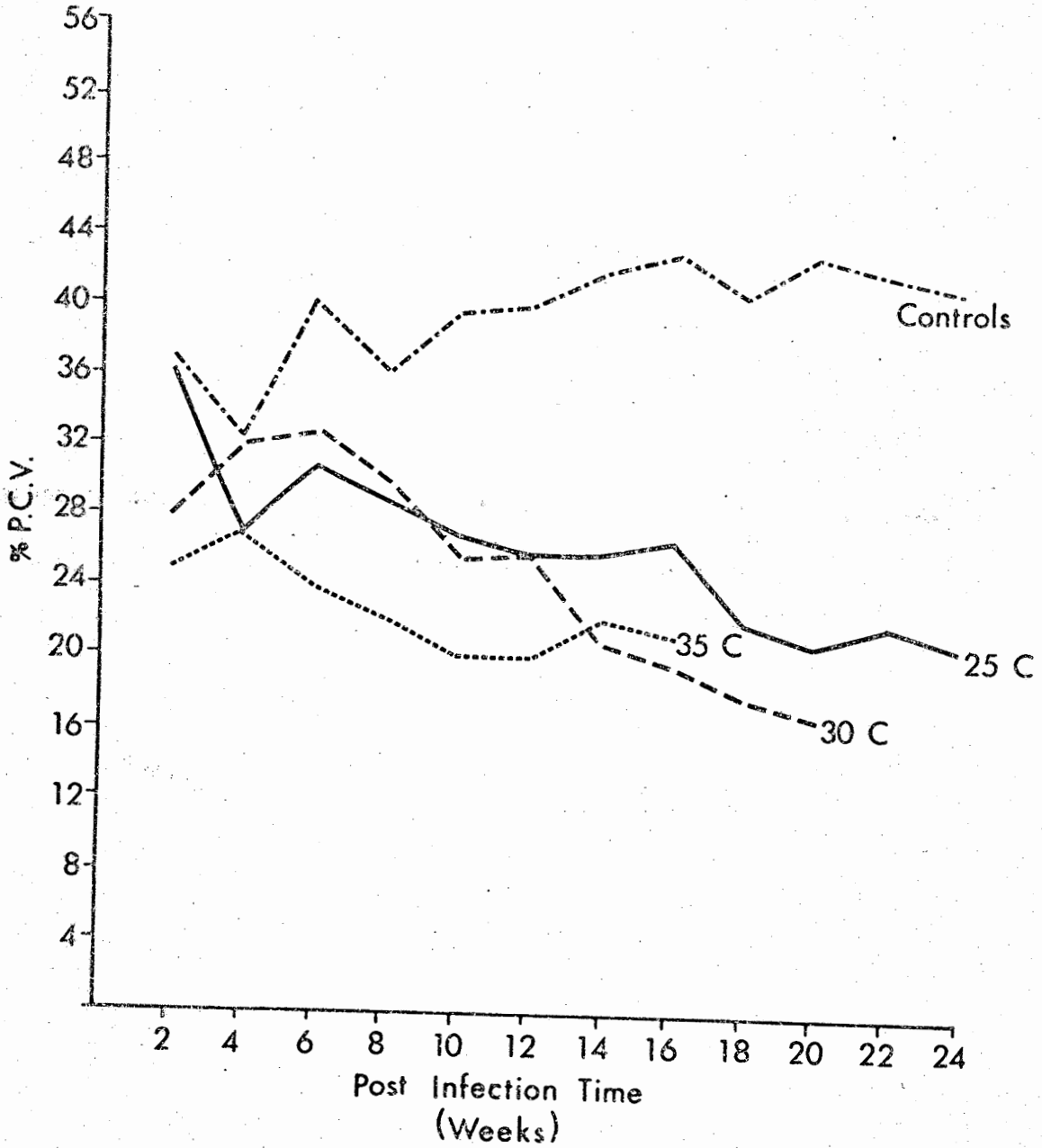

56
52
48
44
40
36
32
28
24
20
16
12
8
4
% P.C.V.
Controls
35 C
25 C
30 C
2
4
6
8
10
12
14
16
18
20
22
24
Post Infection Time
(Weeks)

Figure 14. Hemoglobin readings for experimental and control snakes 2 - 24 weeks post-infection.

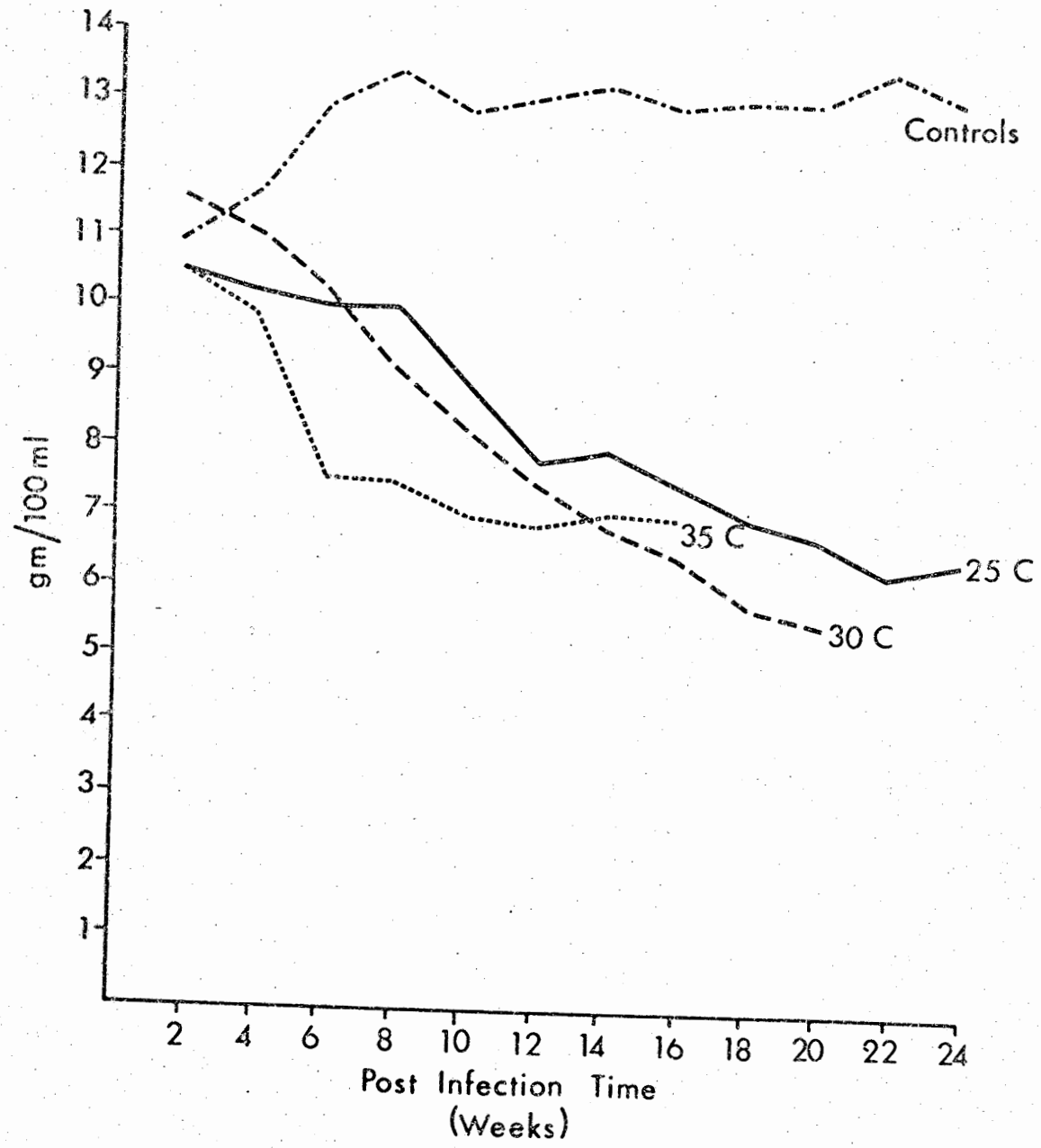

14
13
12
11
10
9
8
7
6
5
4
3
2
1
gm/100 ml
Controls
35 C
25 C
30 C
2
4
6
8
10
12
14
16
18
20
22
24
Post Infection Time
(Weeks)

Figure 15. Total protein values for experimental and control snakes 2 - 24 weeks post-infection.

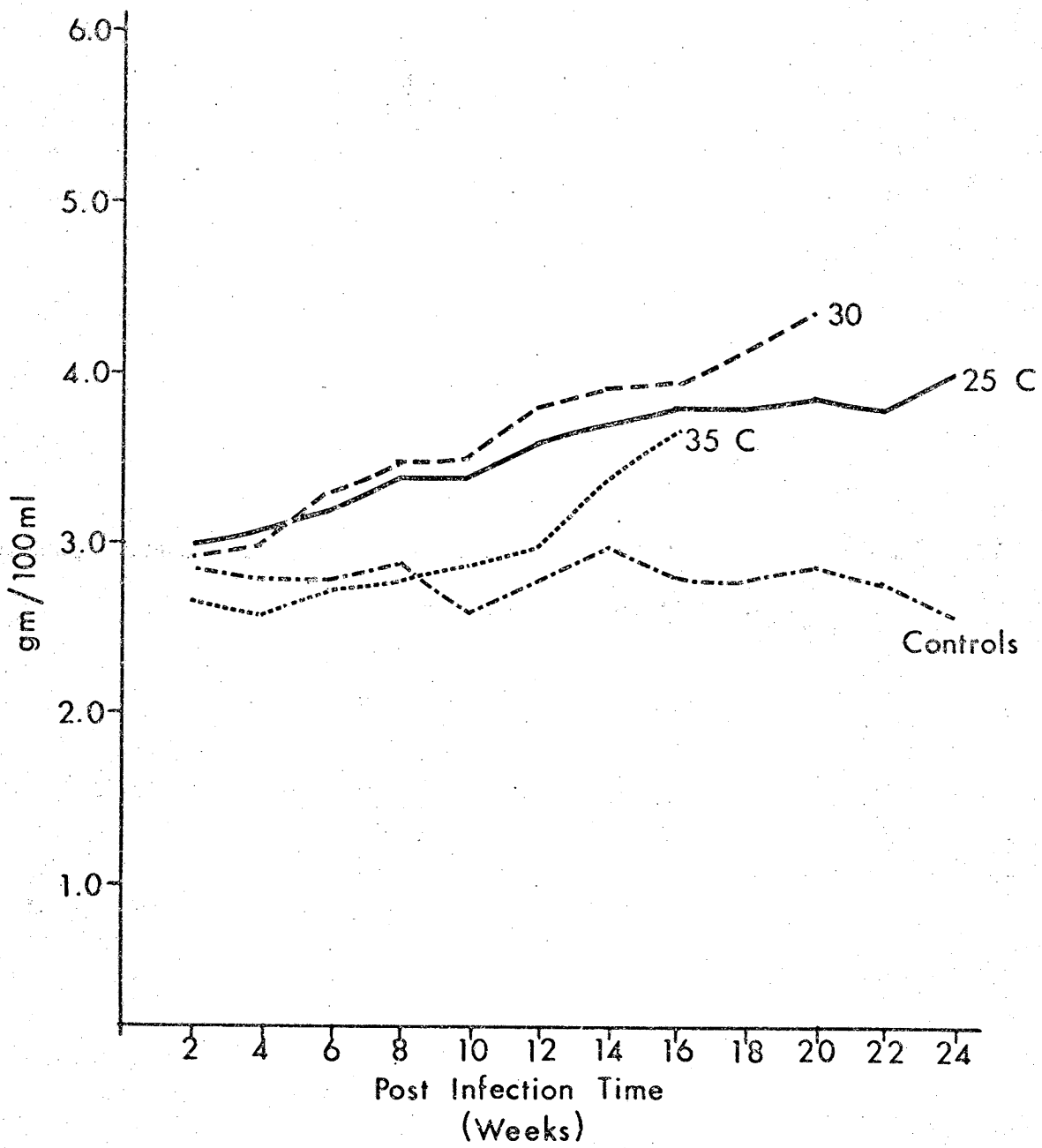
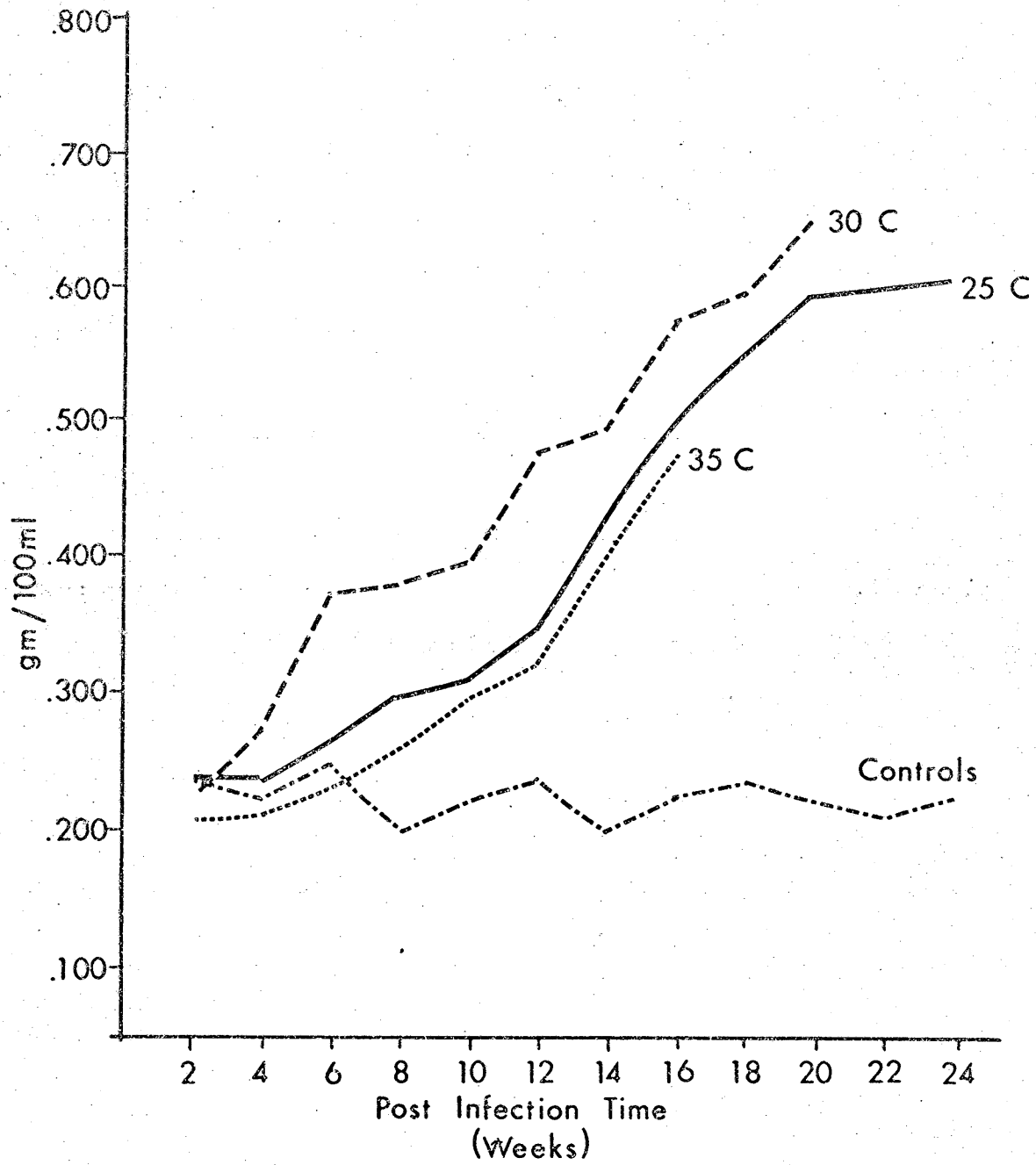

6.0
5.0
4.0
3.0
2.0
1.0
gm/100ml
30
25 C
35 C
Controls
2
4
6
8
10
12
14
16
18
20
22
24
Post Infection Time
(Weeks)

Figure 16. Serum gamma globulin values for experimental and control snakes 2 - 24 weeks post-infection.


.800
.700
.600
.500
.400
.300
.200
.100
gm / 100ml
30 C
25 C
35 C
Controls
2
4
6
8
10
12
14
16
18
20
22
24
Post Infection Time
(Weeks)

Autopsies showed minimal liver penetration by tetrathyridia, and insignificant changes in LDH and alkaline phosphatase differential tests substantiated these observations.

Statistical Analysis

One-way analysis of variance indicated significant variation between the temperature groups for eosinophil count, packed-cell volume, hemoglobin, total protein, and serum gamma globulin. This was usually observed by the sixth week of infection (Tables IV - VIII, Appendix). The most pronounced changes were shown by the 30 C temperature groups (Figs. 12 - 16).

All control snakes indicated minimal variations for all tests and can be considered to have remained constant (Figs. 12 - 16). No significant ($P < .05$) differences were observed based on sex, on weight-length ratios, or on feeders versus nonfeeders (one-way Anova).

DISCUSSION

The liver is the primary site for natural infections in lizards (Specht and Voge, 1965) and experimental infection in mice (Specht and Widmer, 1972). Autopsies after 4 weeks post-infection revealed the tetrathyridia preferred the small intestine for colonization, with minimal liver involvement (Table II). The induced infection and controlled environment of this experiment and the metabolic rates of ectothermic hosts may have caused a different site selection.

Evidence for asexual multiplication of larval cestodes has been recorded for Echinococcus granulosus by Heath (1970), Hymenolepis cantaniana by Jones and Alicata (1935), and Multiceps multiceps by Cannon (1942). Asexual multiplication of Mesocestoides tetrathyridia in mice was verified by Specht and Voge (1965). As far as the author is aware, the present research is the first demonstration of asexual multiplication of Mesocestoides tetrathyridia in Crotalus viridis viridis.

The morphological features of pseudocyst formation are comparable to those found by Specht and Widmer (1972). The presence and development of pseudocysts, abscesses, and encapsulation in reptiles have been noted before (Boam et al., 1970; and Donaldson et al., 1975).

This encapsulation and chronic response suggests that the cellular hypersensitivity is the basic defense mechanism against the

tetrathyridia (Larsh, 1967; Larsh et al., 1972). The final cellular immune response is immobilization of the parasite within the pseudocyst (Fig. 11).

Cell-mediated immunity (CMI) to parasitic infections has been documented by Soulsby (1970) when he cites specific examples of CMI in protozoal and helminth infections. Cell-mediated immune reactions in turtles, lizards, and snakes were discussed by Cohen (1971). He found accumulations of lymphocytes and macrophages in skin grafts undergoing rejection in turtles and lizards.

High levels of eosinophilia are symptomatic of selected helminth infections (Ali-Khan et al., 1973; Powell et al., 1966; and Belding, 1965). Platt (1969) indicates that there can be as much as 50% increase in eosinophils in echinococcosis and trichinosis. The pattern of eosinophilia during my experimental period paralleled the level of eosinophilia of other experimenters. Values for control snakes were similar to those reported for Crotalus cerastes (MacMahon and Hamer, 1975a).

Decrease in packed-cell volume can be used to determine the degree of anemia (MacInnis and Voge, 1970). Normal hematocrit readings for C. cerastes was 28 packed-cell volume in healthy snakes (MacMahon and Hamer, 1975b). The control snakes in this study, C. v. viridis, remained well above this figure (32 - 44), but in the host snakes with advancing infections, the readings were 20 - 24. This anemia was apparently a function of the parasite numerical levels. A demand for blood nutrients, as noted by Voge and Coulombe

(1966), which increases as the number of parasites increase, causes a rapid progression of anemia. Although Mesocestoides sp. is not a direct blood parasite, the majority of the larvae were found near blood vessels (Fig. 3), an indication of a possible cause-effect relationship.

Ryerson (1949) indicates the range of normal hemoglobin for reptiles to be from 7.25 and 10.93 grams per 100 ml. MacMahon and Hamer (1975a) demonstrated the hemoglobin values for C. cerastes to be 7.5 grams per 100 ml. The control snakes in this research remained above 10.93, possibly due to uniform controlled temperatures. Experimental snakes had readings from 5.7 to 7.2.

Increased total protein is usually seen in parasitic infections (Sigma Technical Bulletin No. 540, 1974). This was confirmed with the results obtained in this research. Cohen (1954) lists the total protein for C. viridis as 2.74 grams per 100 ml for normal uninfected specimens. Infected animals have higher total protein levels than controls, reflecting an increase in serum gamma globulin production in the host (Wainwright, 1975; Marcial-Rojas, 1971). This increase in serum gamma globulin is an indication of activity of the host's humoral immune system against foreign proteins; i.e., parasite, by making use of its humoral immune system (Eisen, 1974). The data of this study again supported these conclusions.

Reptiles have immunoglobins IgG and IgM, which can be used in the humoral defense mechanism (Cohen, 1971). Timourian et al. (1961) have also shown that the carpet snake Morelia spilotes variegatus

can elicit precipitating antibodies against parasitic nematodes. It is well documented (Cohen, 1971) that reptiles, including snakes, have the capability and potential to use their humoral immune system to ward off invading parasitic infections. Future research hopefully will show if reptiles have the capacity for passive transfer of immunity to Mesocestoides sp. as was demonstrated by Kowalski and Thorson (1972) in endothermic hosts.

Neutrophils, indicators of acute inflammation (Bickley, 1973), were not evident in observable numbers in the blood smears or tissue sections. Ryerson (1949) found an increase in neutrophils in reptiles infected with hemogregarines. Ratcliffe and Geiman (1938) found that the small intestine of reptiles, when undergoing necrosis caused by amebic infections, demonstrated an acute inflammatory response. Boam et al. (1970) indicated that reptiles lack neutrophils similar to those in mammals, and therefore do not form the neutrophil exudate seen in acute inflammatory response. Reptilian neutrophils are not lacking but are somewhat dissimilar from their mammalian counterparts and could easily be mistaken for other white blood cells. Donaldson et al. (1975) also found minimal acute inflammatory response in amebiasis in snakes. It is hypothesized that the snakes in this study had no noticeable acute response because no tissue necrosis occurred in the early infection as noted by Ratcliffe and Geiman (1938). The acute inflammatory response noted in this experiment was of short duration or entirely absent, and the chronic inflammatory response amd encapsulation occurred

as early as 4 weeks post-infection and 30 C.

The most pronounced effects of the infection were observed at 30 C. Klauber (1972) lists the optimum temperature for the Crotalidae to be between 26.5 and 32 C. Snakes at 25 C produced less pronounced changes in blood chemistry because they were outside their optimal temperature range, whereas at 35 C, the animals were close to their lethal temperatures. MacMahon and Hamer (1975a, b) found no significant changes in hemoglobin and hematocrit between rattlesnakes kept at 25 and 30 C. However, snakes were observed for a period of only 2 months and were free of infection. Binyon and Twigg (1965) noted dramatic changes in hematocrit in the grass snake, Natrix natrix, as the seasons changed.

Temperature has a pronounced effect on the formation of antibodies in ectothermic animals such as reptiles (Allen and McDaniel, 1937; Bisset, 1948; and Evans et al., 1965). Cooler temperatures decreased antibody synthesis, while warmer temperatures increased the reaction. Evans and Cowles (1959) demonstrated this with the lizard Dipsosaurus dorsalis. They found that by increasing the temperature from 25 to 35 C, the lizard increased antibody synthesis. Their procedures did not parallel the exact procedures of this study since their temperatures did not remain constant for the host animals. Therefore, the finding that the snakes kept at 30 C (Fig. 16) produced the most marked increase of antibodies may suggest that there is a peaking period within the 25 to 35 C span.

Future investigation hopefully will be in the following areas: effects of *M. corti* on long-term infections of *C. v. viridis*, with specific studies of passive immunity against *M. corti*, and the investigation of specific immunoglobins in serum globulin changes.

SUMMARY

1. A successful infection of the prairie rattlesnake, Crotalus viridis viridis, was achieved using Mesocestoides corti tetrathyridia as the infective agent.
2. The tetrathyridia demonstrated a predilection for the serosa of the small intestine, but were also found in the serosa of the large intestine and stomach, and the liver parenchyma.
3. By the use of autopsy procedures and microscopic tissue examination, asexual multiplication of the larvae was substantiated.
4. Histopathologic sections revealed pseudocyst formation as early as 4 weeks. Immature pseudocysts are composed of a layer of fibroblasts around the tetrathyridia, but mature ones consist of an outer layer of fibroblasts, an interface layer, and an inner layer of mononuclear cells.
5. Increasing numbers of parasites resulted in increases in eosinophils, total protein, and serum gamma globulin, and decreases in packed-cell volume and hemoglobin.
6. Humoral and cellular immune systems were demonstrated in C. v. viridis by experimental infection with tetrathyridia. Humoral response was shown by an increase in serum gamma globulin, and cellular response was substantiated by noting the process of pseudocyst formation.
7. Temperature is an important variable factor in the rate of asexual multiplication of the tetrathyridia and the expression

of the immune response to laboratory induced infections in an ectothermic host. The most pronounced changes were seen at 30 C.

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APPENDIX

TABLE IV.

One-way analysis of variance table for eosinophil counts.

Week	Group*	Sample Size	Group Total	Group Mean	One-Way Analysis of Variance Table				
					Source	Sum Of Squares	D.F.	Mean Squares	F Ratio (P- Value)
10	1	13	15600	1200	Group Mean	148500	2	74250	3.1692
	2	13	14700	1130.7	Within	820000	35	23428.6	5.4E-02
	3	12	12550	1045.8	Total	968500	37		
	Total	38	42850	1127.6					
12	1	13	16550	1273.1	Group Mean	170900	2	85450	5.90357
	2	13	15700	1207.6	Within	506600	35	14474.3	6.17E-03
	3	12	13300	1108.3	Total	677500	37		
	Total	38	45550	1198.6					
14	1	13	15900	1223.1	Group Mean	142700	2	71350	25.456
	2	13	17150	1319.2	Within	98100	35	2802.8	1.49E-07
	3	12	14050	1170.8	Total	240800	37		
	Total	38	47100	1239.5					

(Continued on page 52)

*Note: In Tables IV through IX, group 1 is 25 C, group 2 is 30 C, and group 3 is 35 C.

TABLE IV. (Continued)

16	1	13	16100	1238.5	Group Mean	167400	2	83700	41.37
	2	13	17700	1361.5	Within	70800	35	2022.8	6.01E-10
	3	12	14500	1208.3	Total	23800	37		
	Total	38	48300	1271.1					
18	1	6	7200	1200	Group Mean	110200	1	110200	91.074
	2	6	8350	1391.6	Within	12100	10	1210	5E-06
	Total	12	15550	1295.8	Total	122300	11		
20	1	6	7150	1191.6	Group Mean	130200	1	130200	183.38
	2	6	8400	1400	Within	7100	10	710	2E-06
	Total	12	15550	1295.8	Total	137300	11		

TABLE V.

One-way analysis of variance table for packed-cell volume.

Week	Group	Sample Size	Group Total	Group Mean	One-Way Analysis of Variance Table				
					Source	Sum of Squares	D.F.	Mean Squares	F Ratio (P- Value)
6	1	14	447	31.92	Group Mean	532.8	2	266.4	5.622
	2	14	466	33.28	Within	1847.7	39	47.37	7.14E-03
	3	14	352	25.14	Total	2380.5	41		
	Total	42	1265	30.11					
8	1	14	412	29.42	Group Mean	487	2	243.5	11.99
	2	14	433	30.92	Within	791.5	39	20.29	8.69E-05
	3	14	323	23.07	Total	1278.5	41		
	Total	42	1168	27.80					
10	1	14	379	27.07	Group Mean	383	2	191.5	8.629
	2	14	386	27.57	Within	865.5	39	22.19	7.89E-04
	3	14	293	20.92	Total	1248.5	41		
	Total	42	1058	25.17					
12	1	14	360	25.71	Group Mean	506.3	2	253.2	12.07
	2	14	373	26.64	Within	817.9	39	20.97	8.30E-05
	3	14	264	18.85	Total	1324.2	41		
	Total	42	997	23.73					

(Continued on page 54)

TABLE V. (Continued)

14	1	14	384	27.42	Group Mean	310.8	2	155.4	23.38
	2	14	291	20.78	Within	259.2	39	6.6	2.11E-07
	3	14	331	23.64	Total	570	41		
	Total	42	1006	22.98					
16	1	14	371	26.5	Group Mean	432.9	2	216.4	28.14
	2	14	262	18.71	Within	299.9	39	7.6	2.7E-08
	3	14	330	23.57	Total	732.8	41		
	Total	42	963	22.92					
18	1	6	139	23.16	Group Mean	126.74	1	126.74	35.03
	2	6	100	16.66	Within	36.18	10	3.61	1.5E-04
	3	-	---	-----	Total	162.92	11		
	Total	12	239	19.91					
20	1	6	137	22.83	Group Mean	176.32	1	176.32	55.65
	2	6	91	15.16	Within	31.68	10	3.16	2.4E-05
	3	-	---	-----	Total	208	11		
	Total	12	228	19					

TABLE VI.

One-way analysis of variance table for hemoglobin data.

Week	Group	Sample Size	Group Total	Group Mean	One-Way Analysis of Variance Table				
					Source	Sum of Squares	D.F.	Mean Squares	F Ratio (P- Value)
6	1	14	141.5	10.1	Group Mean	57.99	2	28.99	7.67
	2	14	143.5	10.2	Within	147.28	39	3.77	1.54E-03
	3	14	107.6	7.6	Total	205.27	41		
	Total	42	392.5	9.3					
8	1	14	143.4	10.2	Group Mean	70.35	2	35.17	5.41
	2	14	130.1	9.2	Within	253.13	39	6.49	8.37E-03
	3	14	100	7.1	Total	323.48	41		
	Total	42	373.4	8.8					
10	1	14	123	8.7	Group Mean	34.54	2	17.27	6.837
	2	14	125.5	8.9	Within	98.5	39	2.52	2.84E-03
	3	14	97.4	6.9	Total	133.04	41		
	Total	42	345.9	8.2					
12	1	14	109.1	7.7	Group Mean	13.79	2	6.89	7.271
	2	14	112.6	8.0	Within	36.98	39	.95	2.07E-03
	3	14	94.1	6.7	Total	50.77	41		
	Total	42	315.8	7.5					

(Continued on page 56)

TABLE VI. (Continued)

14	1	14	110.4	7.88	Group Mean	8.38	2	4.19	7.91
	2	14	95.4	6.81	Within	20.64	39	.529	1.30E-03
	3	14	100.2	7.15	Total	29.02	41		
	Total	42	306	7.28					
16	1	14	108.8	7.77	Group Mean	16.06	2	8.03	14.177
	2	14	87.6	6.25	Within	22.09	39	.599	2.35E-05
	3	14	97.5	6.96	Total	38.15	41		
	Total	42	293.9	6.99					
18	1	6	42	7	Group Mean	7.20	1	7.20	34.73
	2	6	32.7	5.45	Within	2.07	10	.207	1.54E-04
	3	-	----	----	Total	9.28	11		
	Total	12	74.7	6.22					
20	1	6	41	6.83	Group Mean	6.45	1	6.45	37.77
	2	6	32.2	5.36	Within	1.70	10	.1708	1.11E-04
	3	-	----	----	Total	8.16	11		
	Total	12	73.2	6.1					

TABLE VII.

One-way analysis of variance table for total protein.

Week	Group	Sample Size	Group Total	Group Mean	One-Way Analysis of Variance Table				
					Source	Sum of Squares	D.F.	Mean Squares	F Ratio (P- Value)
6	1	9	28.8	3.2	Group Mean	1.228	2	.614	5.380
	2	9	29.3	3.25	Within	2.739	24	.114	1.17E-02
	3	9	25	2.77	Total	3.967	26		
	Total	27	83.1	3.07					
8	1	9	34	3.77	Group Mean	4.409	2	2.20	13.66
	2	9	30.8	3.42	Within	3.87	24	.161	1.09E-04
	3	9	25.2	2.8	Total	8.28	26		
	Total	27	90	3.33					
10	1	9	30.9	3.43	Group Mean	2.081	2	1.04	18.12
	2	9	32.1	3.56	Within	1.378	24	5.74	1.59E-02
	3	9	26.3	2.92	Total	3.45	26		
	Total	27	89.3	3.3					
12	1	9	31.9	3.54	Group Mean	2.79	2	1.39	16.76
	2	9	33.9	3.76	Within	2.004	24	8.5E-02	2.78E-05
	3	9	27	3	Total	4.803	26		
	Total	27	92.8	3.43					

(Continued on page 58)

TABLE VII. (Continued)

14	1	9	31.8	3.53	Group Mean	1.14	2	.570	15.02
	2	9	35.2	3.91	Within	.911	24	3.79E-02	5.86E-03
	3	9	30.9	3.43	Total	2.052	26		
	Total	27	97.9	3.62					
16	1	9	34.5	3.83	Group Mean	1.77	2	.888	1.740
	2	9	36.6	4.06	Within	12.24	24	.510	.196E-02
	3	9	31	3.44	Total	14.02	26		
	Total	27	102.1	3.78					
18	1	6	21.3	3.55	Group Mean	1.41	1	1.41	36.92
	2	6	25	4.16	Within	.309	10	3.09E-02	1.22E-04
	3	-	----	----	Total	1.45	11		
	Total	12	46.3	3.85					
20	1	6	22.6	3.76	Group Mean	1.33	1	1.33	71.28
	2	6	26.6	4.43	Within	.187	10	1.87E-02	1.1E-05
	3	-	----	----	Total	1.52	11		
	Total	12	49.2	4.1					

TABLE VIII.

One-way analysis of variance table for serum gamma globulin.

Week	Group	Sample Size	Group Total	Group Mean	One-Way Analysis of Variance Table: Source	Sum of Squares	D.F.	Mean Squares	F Ratio (P- Value)
6	1	9	2.34	.26	Group Mean	.1211	2	6.05E-02	36.18
	2	9	3.4	.37	Within	4.016E-02	24	1.67E-02	5.69E-03
	3	9	1.98	.22	Total	.1612	26		
	Total	27	7.72	.28					
8	1	9	2.77	.307	Group Mean	7.78E-02	2	3.89E-02	20.05
	2	9	3.51	.39	Within	4.65E-02	24	1.94E-03	7.56E-06
	3	9	2.34	.26	Total	.12439	26		
	Total	27	8.62	.319					
10	1	9	2.79	.31	Group Mean	5.46E-02	2	2.73E-02	18.51
	2	9	3.6	.40	Within	3.54E-02	24	1.47E-03	1.36E-05
	3	9	2.7	.30	Total	9.00E-02	26		
	Total	27	9.09	.336					
12	1	9	3.53	.3992	Group Mean	.1155	2	5.77E-02	10.7
	2	9	4.32	.48	Within	1.29E-02	24	5.4E-02	1.10E-12
	3	9	2.88	.32	Total	.1285	26		
	Total	27	10.73	.397					

(Continued on page 60)

TABLE VIII. (Continued)

14	1	9	3.87	.43	Group Mean	6.43E-02	2	3.21E-02	178.75
	2	9	4.63	.514	Within	4.32E-03	24	1.8E-04	3.84E-15
	3	9	3.59	.398	Total	6.86E-02	26		
	Total	27	12.09	.447					
16	1	9	4.44	.49	Group Mean	7.19E-02	2	3.59E-02	140.75
	2	9	5.29	.58	Within	6.13E-03	24	2.55E-04	5.25E-04
	3	9	4.21	.46	Total	7.80E-02	26		
	Total	27	13.94	.51					
18	1	6	3.27	.545	Group Mean	1.68E-02	1	1.68E-02	61.38
	2	6	3.72	.62	Within	2.75E-03	10	2.75E-04	1.7E-05
	3	-	----	----	Total	1.96E-02	11		
	Total	12	6.99	.582					
20	1	6	3.38	.563	Group Mean	2.70E-02	1	2.70E-02	24.10
	2	6	3.95	.658	Within	1.12E-02	10	1.12E-03	6.17E-04
	3	-	----	----	Total	3.83E-02	11		
	Total	12	7.33	.610					

TABLE IX.

Correlation coefficients between blood analysis and the growth curve of Mesocestoides corti.

Blood Analyses	Group	4 Weeks	8 Weeks	12 Weeks	16 Weeks	20 Weeks	24 Weeks
Eosinophilia	1	.895	.899	.918	.991	.987	.993
	2	.946	.938	.901	.982	.991	----
	3	.957	.977	.912	.993	----	----
Packed-cell volume	1	-.905	-.888	-.898	-.969	-.983	-.990
	2	-.997	-.931	-.911	-.991	-.991	----
	3	-.997	-.879	-.911	-.918	----	----
Hemoglobin	1	-.902	-.901	-.924	-.948	-.983	-.889
	2	-.980	-.930	-.889	-.991	-.991	----
	3	-.954	-.924	-.891	-.924	----	----
Total protein	1	.878	.866	.924	.889	.942	.888
	2	.997	.910	.887	.930	.996	----
	3	.889	.894	.889	.894	----	----
Gamma globulin	1	.878	.866	.924	.977	.942	.888
	2	.994	.990	.887	.899	.994	----
	3	.889	.894	.889	.894	----	----