ESTIMATING AGE IN UNFED ADULT LONE STAR TICKS, *AMBLYOMMA AMERICANUM* (L.)

By

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ESTIMATING AGE IN UNFED ADULT LONE STAR TICKS, AMBLYOMMA AMERICANUM (L.)

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CHAPTER I

INTRODUCTION

Ixodid ticks have been observed to live many years without taking a bloodmeal in the adult stage under field conditions. One species of Argasid tick (a soft tick), Ornithordoros papilles, has been reported by Russian investigators to live over twenty years without feeding when the host population is low (Balashov, 1972). Hard ticks (ixodid ticks) have not been observed to live over twenty years without a bloodmeal, but the lone star tick, Amblyomma americanum (L.) may live up to three years as an unfed adult. Understanding more about physiological age of Amblyomma americanum unfed adults is an important key in developing more effective tick control programs. These ticks survive from one season to the next and there has been no reliable means for determining whether ticks observed in the field are the survivors (old ticks) or newly molted adults. The information gathered in this research project should be helpful in evaluating current control programs and in generating new and more effective control strategies. Recent research has indicated that the unfed adult lone star tick is more susceptible to acaricide treatment as the unfed tick's age increases (Mount, 1980). The primary objective of the present research project has been to determine whether reliable age indicators exist for laboratory-reared Amblyomma americanum, unfed adult lone star ticks.

The present study was a preliminary study of unfed A. americanum

adults that were reared and maintained in the laboratory after a nymphal bloodmeal. Lone star adults have been estimated to live without a bloodmeal for over a year if optimum conditions of temperature, humidity and photoperiod are present. Several parameters were chosen in which age indicators were thought to exist. These parameters were: the depletion of nutrients (hemoglobin and lipid content were tested); the water content of the tick, and related to that, the critical equilibrium humidity (CEH) of the aging tick; the morphological and histological structure of the tick midgut tissue; and the surface morphology of the tick cuticle. Mortality and survivorship were also monitored throughout the research project. All the ticks assayed (about 10,500 ticks were used for this study) were of known chronological age.

Aging is a phenomenon common to all animals and is not only of concern to the gerontologist, but is also important to the entomologist. Rockstein (1973) defines aging as "the sum total of all those changes in structure and function which by their deleterious and degradative nature result in the failure of an individual to survive". Arthropods exhibit aging processes that are common to vertebrate animals (Rockstein, 1973). Honeybees experience a 33 percent decrease in nerve cells from emergence to old age (Rockstein, 1973). Gardner (1940) found that there is a decrease in the number of neurons in the human cerebral cortex with increased age. Similarities like these between arthropods and man, make the arthropod a prime target of many studies on basic aging theories. Arthropods reproduce quickly and in great numbers. Their life spans are generally very short in comparison to many other animals. The aging process may involve a combination of genetic and environmental factors acting in an individual. The aging factors hypothesized for the unfed tick indicate that the physiological age of the tick is linked to the

tick's environment and particularly the metabolism of its last bloodmeal.

Cook (1973) found that Ornithodoros concanesis, a soft tick, showed a gradual decrease in hemoglobin content over time. The hemoglobin is in the form of oxyhemoglobin when it is first ingested by the tick. Within the midgut cells, the oxyhemoglobin is transformed to methemoglobin and finally to hematin crystals (Smit et al., 1977). Balashov (1972) observed that older unfed ticks were without hemoglobin inclusions and that the hematin crystals formed aggregates (Balashov intreprets this as the concentration of the bloodmeal). Smit et al. (1977) studied the hematin crystals in the tick, O. moubata porcinus (this tick feeds on guinea pigs) and concluded that the crystals have an important role in the preservation of the nutrient reserves. After prolonged starvation, the hematin crystals disappeared but the mechanism of how the midgut cells utilize the nutrients in the hematin was not known. The crystallization was suggested as a possible mechanism for further concentrating the bloodmeal.

The key to finding a reliable method to determine the physiological age in the unfed lone star tick may be consideration of the histological changes that occur in the midgut (and other organs) of the tick over time. Balashov (1972) proposed three criteria for determining physiological age from tick midgut tissue. The criteria he used were that of gut ceacal distention, the hemoglobin and hematin content of the cells, and the malpighian tubule contents. He suggested that midgut cell glycogen and fat content might also provide possible clues in determining tick age. Balashov classified the unfed H. asiaticum and I. ricinus into four categories: 1) recently molted ticks in postmolting development, 2) ticks that have completed postmolting development, 3) ticks that have been active for a "long time", and 4) ticks that have starved

for a long period. Balashov described the changes that occurred in the midgut cells during each of the stages that he proposed. The ceacae are described as stretched in category one, in contrast to slender ceacae with many constrictions in the fourth category. The lumen was filled with the distal ends of hypertrophied digestive cells in the recently molted ticks. The active tick had smaller gut cells and a large lumen with constrictions. In emaciated ticks, the lumen disappeared as a result of severe constriction in the midgut walls. Balashov also described the changes that occurred in the malphighian tubules over the four degrees of unfed ticks. Guanine spherules in the lumen of the tubules became progressively larger in each stage.

In the context of the categories used by Balashov, Razumova (1977) devised a method for estimating the physiological age of the unfed tick without using histological techniques. Razumova estimated the size of the midgut diverticula, the color changes in the diverticula and the changes in the malpighian tubules based on the dissection and gross observation of these structures in unfed Dermacentor pictus. Razumova also described a "rapid method" for estimating the age of the unfed tick based on the characteristics of the cuticle. He assessed the changes in general body fatness and flatness, and the softness and darkness of the cuticle. With the stadia of the unfed adult tick, subtle cuticle changes are ocurring with increasing age. Some degree of irreversible external changes proceed in the aging unfed tick much like many of the more widely documented age changes seen in other arthropods. For example, winged insects have been observed to have frayed wings in the older animals (Service, 1977).

While most of the factors involved in the tick aging mechanism have not been fully or even partially researched, some suggestions have

been made on some factors that cause the unfed tick to age. The critical equilibrium humidity or CEH is the relative humidity at which a tick can absorb water from unsaturated air (Sauer and Hair, 1971; Lees, 1946, 1964). Ticks and a few insects are the only animals known to possess this unusual capability. The characteristic is thought to be a key factor enabling the tick to survive long periods without feeding. Sauer and Hair estimated the critical equilibrium humidity of A. americanum adults (adults from the field) to be about 85 percent relative humidity. Lees (1946) found that the critical equilibrium humidity of Ixodes ricinus increased as food reserves were depleted. Based on his results, Lees suggested that the CEH increases as the unfed tick becomes older, increasing to a point where older ticks can only absorb water from saturated air.

Water content and the lipid content have also been speculated to change in some manner in the older tick. Cook (1973) found no pattern of decrease or increase in the lipid content of O. concanesis over a three-month period of starvation. In other arthropods, the lipid content has been shown to decrease in starving arthropods. Aged and starved cave roaches (Pychoscelus striatus, Dictyoptera: Blattidae) showed a decrease in total lipid content while water content rose over time (Tan, 1973). Aging Drosophila melanogaster also show a decrease in fat content and an increase in water content (Fairbanks and Burch, 1970).

Glycogen content in the tick midgut might be a factor that changes in older ticks (Balashov, 1972). Processes involved in the utilization of glycogen in the unfed tick may have some similarity to the biochemical processes involved in the utilization of glycogen in aging flight muscles. Rockstein (1972) deals in detail with the time-related changes that occur with the enzymes and in the trehalose content in aging muscle

tissue. Simon et al. (1969) also described a gradual decrease in the amount of glycogen in the muscle tissue to a complete disappearance in the older houseflies.

Age changes in the muscles of arthropods have been widely documented, especially the age changes found in the Dipteran flight muscles. Age-related muscle degeneration has importance even to the wingless arthropod. Fairbanks and Burch (1970) suggested that the muscles which control the opening and closing of the spiracles degenerate in older arthropods. The flight muscles of D. melanogaster have been photographed with the electron microscope at different ages in the adult stage (Takashahi et al., 1970c). The mitochondria showed progressive degeneration of the I bands, Z bands and sarcotubular elements. The myofibrils showed a progression toward disorganization and in some cases, the myofibrils completely lacked orientation. In some instances, sarcotubular banding patterns were absent. Similar patterns of flight muscle degeneration have been shown in the African mosquito, Eretmapodites chrysogaster (Hylton, 1966) and in the housefly, Musca domestica (Simon et al., 1969).

The most universally significant arthropod-aging research is that which shows the accumulation of pigments in the arthropod cell. The same type of pigments are found in the cerebral cortex of higher animals, including man (Brody, 1960). D. melanogaster has many electron dense bodies in the fly's cells that take the form of concentric circles within the muscle cells (these bodies were identified as degenerating mitochondria in the muscle tissue). In the midgut globlet cells, these were identified as small dense granules in association with the mitochondria. These bodies were also found in the oencytes, and in the adipose tissue

(Takashahi et al., 1970a, b, c). Haydak (1957) observed and sampled honeybees of known age and described granular matter in distribution around the nucleus of the oenocytes in older bees (one-day old bees had been transparant oenocytes), increasing to the point where the nucleus was no longer visible. All these results showed a gradual accumulation of dense bodies in aging insect cells. These pigments have not yet been researched in the tick.

An entire gambit of factors have been shown to contribute to the aging processes in many different species of arthropods. Almost every organ in the arthropod has been researched to some degree in relationship to arthropod age. Haydak (1957) made many observations on the aging honeybee. He observed a degeneration in the pharyngeal glands and in the malpighian tubules. The pharyngeal glands changed from white to yellow and the malpighian tubules became a yellowish-green color in the old bees. Arnold (1959) found poor circulation and occluding veins in older Blaberus giganteus cockroaches. Reproductive organs of Drosophila melanogaster suffer a gradual decline with age (Miquel et al., as cited by Rockstein, 1973). Another intriguing arthropod aging phenomenon is the dimming of the firefly's lantern with ensuing age (Press et al., 1966). All these changes are effected by the genetic makeup of the organism and the environment of the organism. Only a small amount is known about the aging factors of the unfed tick. The possibilities for age-related studies on the tick are quite numerous. The aging study on the unfed lone star tick touches only the surface of the research problem. For this reason, this research seeks to elucidate on just a few of many age indicators that may help in understanding more about the general

biology of the unfed lone star tick and thereby contribute to the development of more effective and intelligent tick control strategies.

CHAPTER II

CHANGES IN WATER, LIPID, HEMOGLOBIN AND CRITICAL EQUILIBRIUM HUMIDITY

Experimental Design

Two groups of experimental ticks were established. Group A ticks were all fed on rabbits in relatively homogenous physiological condition (Table I). Group B ticks were fed on a wider variety of rabbits over a longer period of time in various physiological states (Table II). Both experiments were conducted as randomized blocks with two criteria of classification - the age of the ticks and the replicate. Three replicates were completed for each experimental group. The two experimental groups were designed to indicate any differences due to seasonality or due to laboratory techniques.

Materials and Methods

In each of three replicates for Group A, two groups of nymphs previously held at 25 $\pm$ 3°C, 95 $\pm$ 3% relative humidity and 14 hours of light were fed on four rabbits to yield about 1500 replete nymphs (Table I). Rabbits were obtained from local suppliers. The engorged nymphs were collected and placed in a growth chamber maintained at 95 $\pm$ 3% relative humidity, 25 $\pm$ 3°C and 14 hours of light. After four weeks and completion of molting, ticks were counted, sexed and placed in five paper cartons.

TABLE I

EXPERIMENTAL DESIGN FOR GROUP A TICKS REPLICATES 1,2,3;
POPULATIONS OF 1500 IN UNITS OF 300 TICKS
(150 MALES AND 150 FEMALES)

REP 1	REP 2	REP 3
AUGUST	SEPTEMBER	OCTOBER
analyze 300 ticks of 1500	analyze 300 ticks of 1500	analyze 300 ticks of 1500
0 month-old ticks	0 month-old ticks	0 month-old ticks
NOVEMBER	DECEMBER	JANUARY
analyze 300 ticks of 1500	analyze 300 ticks of 1500	analyze 300 ticks of 1500
3 month-old ticks	3 month-old ticks	3 month-old ticks
FEBRUARY	MARCH	APRIL
analyze 300 ticks of 1500	analyze 300 ticks of 1500	analyze 300 ticks of 1500
6 month-old ticks	6 month-old ticks	6 month-old ticks
MAY	JUNE	JULY
analyze 300 ticks of 1500	analyze 300 ticks of 1500	analyze 300 ticks of 1500
12 month-old ticks	12 month-old ticks	12 month-old ticks

TABLE II

EXPERIMENTAL DESIGN FOR GROUP B TICKS. REPLICATES 1,2,3;
UNITS OF 300 TICKS (150 MALES AND 150 FEMALES)
SET ASIDE EACH MONTH

REP 1	REP 2	REP 3
AUGUST	SEPTEMBER	OCTOBER
300 ticks set aside	300 ticks set aside	300 ticks set aside
12 month-old ticks	12 month-old ticks	12 month-old ticks
NOVEMBER	DECEMBER	JANUARY
300 ticks set aside	300 ticks set aside	300 ticks set aside
9 month-old ticks	9 month-old ticks	9 month-old ticks
FEBRUARY	MARCH	APRIL
300 ticks set aside	300 ticks set aside	300 ticks set aside
3 month-old ticks	3 month-old ticks	3 month-old ticks
AUGUST	SEPTEMBER	OCTOBER
analyze all units set aside	analyze all units set aside	analyze all units set aside

The containers were covered with clear plastic film (Saran^{R} Wrap) and secured with a rubber band. One of the five cartons was chosen at random for the first 0-month replicate. The remaining cartons were returned to the environmental chamber to age. Three months later, another unit of ticks was chosen at random for a 3-month replicate. This procedure continued for 12 months until all five populations were assayed for each replicate.

Approximately 800 nymphs were fed on two rabbits for Group B (Table II). One carton (less than 400 ticks) was placed in a control chamber. These ticks were left undisturbed for the next 13 months (one month was allowed for the molt to the adult stage). Three months later, another group of nymphs was fed on two rabbits. This unit of ticks was set aside in the control chamber for the next 10 months. The process continued until five units (cartons) of ticks were set aside for each replicate. Four weeks after the last group of replete nymphs was collected, assays were completed simultaneously (same day) for all five age groups.

Experimental ticks were counted to determine survivorship and then, dessicated (0% relatively humidity, 25^{o}C) for a 48 hour period before assays were done. All weighings were made on a Mettler balance (sensitive to 0.01 mg). The plastic film on each carton was replaced by a piece of vinyl coated screening to contain the ticks and to assure their dessication.

Water Content

Five ticks of each sex were chosen at random, and placed in a 10 ml glass beaker. The beaker was covered with a small piece of the vinyl screening. The ticks were placed in drying oven at 60^{o}C and dried to

constant weight. A constant weight was considered the dry weight of the group and the initial weight was considered the wet weight of the ticks. The dry weight was substracted from the initial weight to determine the water content of the ticks. Percent water content was calculated by dividing the water content of the ticks by the initial weight of the ticks.

Lipid Content

Lipid content was determined by a simple methanol and chloroform extraction adapted from a method described by Keleti and Lederer (1974). The method was modified to accommodate the small size of the tick (the method used by Keleti and Lederer required 10 g of biological material). Ten ticks of each sex were chosen at random. The ticks were homogenized in glass Pyrex homogenizers containing 1 ml chloroform. After the ticks were completely homogenized (the cuticle separates into two parts and all the contents of the tick are in solution with the chloroform), 0.5 ml of methanol and 0.5 ml of deionized water were added and mixed thoroughly. The homogenate was filtered through a Waltman Number 1 microfilter. Vacuum was used to draw the homogenate through the filter quickly. The filtered solution was transferred by Pasteur pipet to a graduated glass centrifuge tube. The phases were allowed to separate and the volume (V_1) of the chloroform-lipid layer (the bottom layer) at the mantissa was noted. The top layer of methanol and water was drawn off with a Pasteur pipet and discarded. The volume (V_2) of the chloroform-lipid layer was noted. This volume was transferred to a pre-weighed 5 ml glass beaker (W_b) and the chloroform was allowed to evaporate in a hood overnight. The lipid residue and beaker were weighed (W_1) and lipid content was

calculated from this formula:

$$\text{Total Lipid Content} = (W_1 - W_b)\ V_1/V_2 \qquad (1)$$

Percent lipid content was determined by dividing the calculated lipid content by the initial weight of the ticks.

Hemoglobin Content

Cook (1973) used a cyanmethemoglobin assay on Ornithodoros concanesis to determine the hemoglobin content of this tick. This method was adapted for use in Amblyomma americanum unfed adults. A standard curve was determined for each month in Group A (a total of 15 standard curves were made) and for each replicate in Group B (three standard curves were made). A certified hemoglobin standard (Sigma, lot No. 109C-6137) was serially diluted with Drabkin's reagent (a cyanmethemoglobin reagent, Sigma, lot No. 99C-6072) to achieve the standard curve. The same batch of hemoglobin standard solution was used for the duration of the research project.

The detergent action of saponin in the Drabkin's reagent lysed the erthrocytes releasing hemoglobin (many of the erthrocytes had already been lysed following ingestion from the sample). The released hemoglobin (Fe II) was oxidized by potassium ferri-cynanide to the ferric state (Fe III) (methemoglobin). Potassium cynanide ions reacted with the methemoglobin to yield cyanmethemoglobin. The cyanmethemoglobin was measured spectrophotometrically on a Spectronic 20 at a wavelength of 540 nm.

Ten ticks of each sex were chosen at random and homogenized in exactly 2 ml of Drabkin's reagent. Supernatant was centrifuged. The

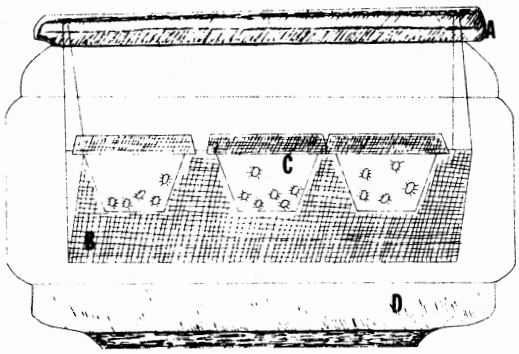
supernatant was centrifuged, a second time and the second supernatant was centrifuged a third time. Each centrifugation was done for 15 minutes with an Eppendorf 5412 centrifuge at 5676 g. The final supernatant was read at 540 nm and recorded. The amount of hemoglobin in a sample was determined by fitting the absorbance value into the equation for the line of the standard curve for hemoglobin. Micrograms of hemoglobin per milligram tick weight were determined by dividing the concentration of hemoglobin in the sample by the initial weight of the sample.

Critical Equilibrium Humidity

Nine humidity chambers were designed from small one gallon clear glass containers (Fig. 1) purchased locally. A different saturated salt solution was placed in the bottom of each bowl. The salt solutions (Winston and Bates, 1960) maintained a range of humidities from 33% to 98% at 25^{o}C. Wire mesh basket was suspended from the rim of the container which was sealed with one or two layers of Parafilm.^{R}

Eighteen plastic containers with screened tops were used to hold the ticks for each replicate and age. Five ticks of each sex were weighed in the plastic containers and placed randomly in one of the nine humidity chambers. All nine humidity chambers were placed in an environmental chamber held at 25^{o}C and 14 hours of light (light was maintained by a 15 watt light bulb). After a period of seven days, the ticks were removed and weighed. The initial weight less the second weight indicated the amount of weight gain or loss for the sample of five ticks. These points were plotted to determine the trends and the critical equilibrium humidity at each age, in each replicate for each sex.

Fig. 1 Humidity Chamber Design. A. Parafilm^{R} layer. B. Wire mesh Basket. C. Tick Containers. D. Standard Salt Solution.


A
C
B
D

Survivorship

Survivorship was determined through the duration of this research project and in each composite group of ticks (one unit per replicate per age). Both the number of dead ticks and the number of live ticks were counted. The percent survivorship was calculated by dividing the number of dead ticks by the total number of ticks in the unit and subtracting this value from 100.

Results

Survivorship

A gradual decline in survival with age was apparent in Group A ticks, but it was at least 90% in all populations (Table III). Survival in Group B was more variable. For example, in the 6-month population survival dropped to less than 85% and the 12-month population survival decline to about 72%. Although the 6- and 12-month populations in Group A and B were significantly different, the 0-, 3-, and 9-month populations were relatively similar and an overall statistical analysis indicated no significant differences between groups.

Water Content

Water content remained constant during the period of starvation in both groups (Table IV). It was slightly more variable in males (55.4 $\pm$ 10.9%) than in females (53.9 $\pm$ 2.5%) in Group A. Similarly, in Group B it was more variable in males (54.4 $\pm$ 4.8%) than in females (50.8 $\pm$ 1.4%). Statistical analysis showed that none of these differences were significant at the 5% level although in 9 of 10 experiments the mean for males

TABLE III

PERCENT SURVIVORSHIP IN *AMBLYOMMA AMERICANUM* UNFED ADULTS

Age in Months	Percent Mortality	
	Group A[1]	Group B[2]
0	100.0*	100.0*
3	96.3	98.7
6	96.1	84.4
9	93.9	93.0
12	91.3	72.1

1, 2 No significant differences between ages at .05 significance level.

* Mean of 3 reps.

TABLE IV

PERCENT WATER CONTENT IN AMBLYOMMA AMERICANUM,
UNFED ADULTS FOR FIVE AGES

Age in Months	Percent Water Content*			
	Group A[1]		Group B[2]	
	Females	Males	Females	Males
0	54.1±2.3	60.1±10.5	52.2±1.4	52.0±2.3
3	56.9±1.7	62.1±2.8	48.7±2.4	51.0±9.3
6	55.0±3.4	58.7±6.4	50.2±3.2	62.8±16.8
9	53.5±2.7	36.0±15.4	51.9±1.1	54.1±4.1
12	50.0±3.1	60.3±10.0	51.0±6.4	52.0±3.8

* Means ± S.D. (3 reps). One rep was 5 ticks.

1,2 No significant difference between ages at .05 significance level.

exceeded that of females (Table IV).

Lipid Content

Lipid content ranged from less than 1% to 55% with much variation between all populations (Table V). Thus statistical analysis did not confirm any real differences. Nevertheless, the lipid content of both males and females seemed to be reduced at 9 and 12 months to about one-half to one-fourth of these at 0 to 6 months. Also, there was less variation between Group A populations than between Group B populations (Table V).

Hemoglobin Content

Hemoglobin content dropped during the first 3 months of starvation to 10-12 ug/mg from an initial content of 16 to 25 ug/mg. Another decrease occurred during the 9 to 12 month period when it reached levels of 6 to 8 ug/mg (Table VI). There was a 30 to 50% decrease during the first 3 months and another reduction of 50% to 69% after 12 months (Fig. 2).

There were differences between Group A and Group B populations (Fig. 3). In Group A initial content was about 16 ug/mg whereas, in Group B it was 25 ug/mg. Also in Group A hemoglobin content remained high through 9 months but in Group B it dropped to the 12-month level after 6 months (Table VI).

Critical Equilibrium Humidity (CEH)

Recently fed ticks did not take up water from unsaturated air. An ability to absorb water from unsaturated air was measurable after 3

TABLE V

PERCENT LIPID CONTENT IN AMBLYOMMA AMERICANUM,
UNFED ADULTS FOR FIVE AGE GROUPS

Age in Months	Percent Lipid Content*			
	Group A[1]		Group B[2]	
	Females	Males	Females	Males
0	6.18* (5.96-6.58)**	8.22* (7.14-9.63)**	20.16* (3.56-57.58)**	20.73* (6.81-48.38)**
3	4.97 (00.10-8.31)	6.29 (00.44-10.73)	4.12 (3.45-4.56)	21.35 (3.45-55.27)
6	6.81 (4.36-11.51)	6.47 (4.48-9.34)	6.12 (4.39-841)	7.40 (4.61-9.64)
9	3.51 (2.59-4.39)	4.31 (1.72-6.84)	1.92 (1.55-2.33)	3.81 (1.95-5.24)
12	2.53 (2.20-278)	1.69 (00.82-2.17)	2.39 (1.74-3.47)	3.17 (0.81-4.83)

* Mean of 3 reps. One rep was 10 ticks.

** Range

1, 2 Significant difference between ages at .10 significance level.

TABLE VI

HEMOGLOBIN CONTENT IN AMBLYOMMA AMERICANUM, UNFED ADULTS FOR FIVE AGE GROUPS

Age in Months	Hemoglobin Content (ug/mg tick wt.)			
	Group A[1]		Group B[2]	
	Females	Males	Females	Males
0	16.02*	15.51*	25.78*	23.95*
3	13.42	9.34	9.57	10.25
6	7.48	13.66	11.55	14.16
9	11.74	11.34	7.84	6.58
12	7.65	6.87	5.56	7.87
Mean Weight of Ticks	35.57 mg		35.45 mg	

1,2 Significant difference between ages at .05 significance level.

* Mean of 3 reps. One rep was 10 ticks.

Fig. 2 Age Changes in Hemoglobin Mean Values for Two Experimental Populations of Unfed Adult Lone Star Ticks.

% Hemoglobin = amount of hemoglobin per milligram tick weight
Months = the ages of the ticks

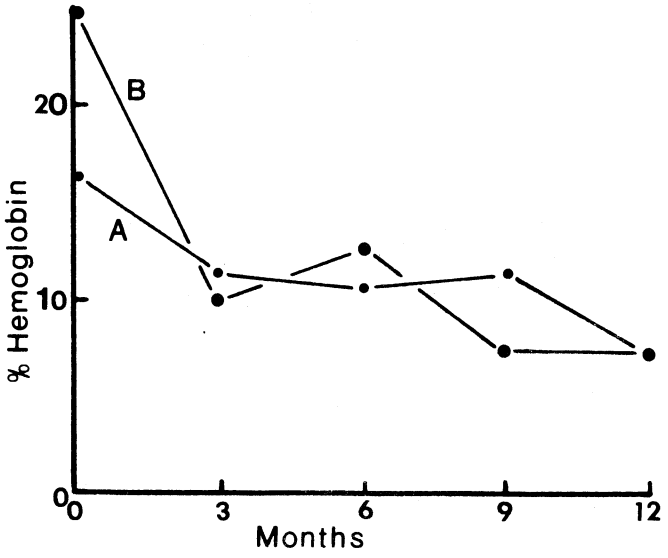
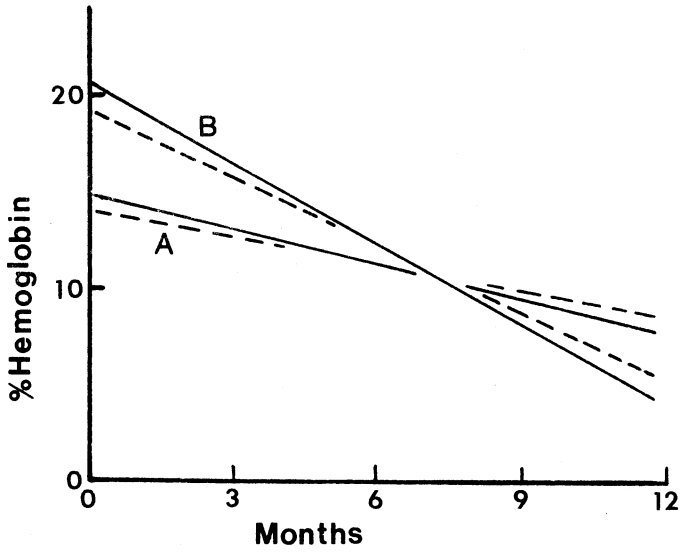

B
A
20
10
0
% Hemoglobin
0
3
6
9
12
Months

Fig. 3 Regression Lines Expressing Differences in Hemoglobin Content Between Two Experimental Populations, (A and B) of Unfed Adult Lone Star Ticks.

Group A Females $Y = -.614 X + 14.946$
Group A Males $Y = -.509 X + 14.400$
Group B Females $Y = -1.339 X + 20.294$
Group B Males $Y = -1.194 X + 19.728$

Solid lines = Female Ticks
Broken lines = Male Ticks
% Hemoglobin = Amount of hemoglobin per milligram tick weight
Months = the ages of the ticks


20
10
0
%Hemoglobin
B
A
0
3
6
9
12
Months

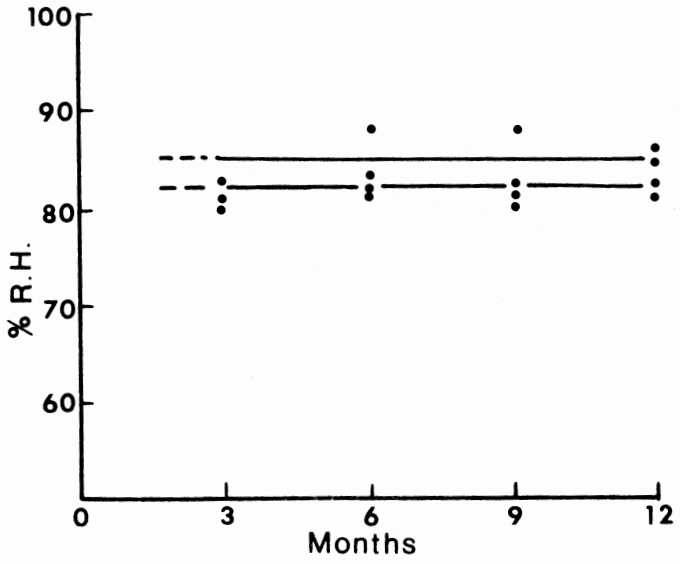
months of starvation and the CEH was 80-85%. It was maintained at this level during the remaining 9 months of the experiments (Fig. 4). The CEH of males appeared to be slightly lower than that of females.

Discussion

Declines in survivorship for the lone star adult were not significant throughout the entire research project (12 months per replicate) indicating that most unfed adult lone star ticks can live longer than one year under optimum conditions of photoperiod, temperature, and relative humidity. Many factors may directly effect the survival of the unfed tick. Our experience suggests that the host animal may play an important role in determining the tick's longevity. The season in which the tick feeds may also affect its survival. Amblyomma americanum nymphs are not generally active in the winter months and do not normally seek a host for a bloodmeal at this time (Semtner et al., 1973). Thus ticks finding a host in the winter months may not live as long as those which find a host during another season because of these seasonal life cycle characteristics of the species.

The water content of the lone star adult may have a significant role in the survival of the tick over long periods of prolonged starvation. Sauer and Hair (1971) found the mean water content of two different groups of Amblyomma americanum adults (taken from the same rearing chamber at the same time on the same day) to be 55.8 $\pm$ 8% and 50.3 $\pm$ 4%, respectively. The results of this project agree closely with these values. Unfed adult Amblyomma americanum maintained at 14 hours of light, 25^{o}C and 95 $\pm$ 3% relative humidity (optimum laboratory conditions) did not exhibit significant changes in the water content with increasing age.

Fig. 4 Critical Equilibrium Humidity (CEH) For Two Experimental Populations of Lone Star Ticks Over Four Ages.


100
90
80
70
60
% R.H.
0
3
6
9
12
Months

Maintaining the water content across the age of the unfed tick may have a role in the continued longevity of a particular tick. Water is essential in carrying out the metabolic processes important to the life of the unfed adult.

The lipid content in the lone star adult showed no definitive pattern of decrease with age. These results are in agreement with the results of Cook (1973). Cook found no pattern of increase or decrease in Ornithodoros cancanesis over time. The results of this project suggest that the lone star tick probably does not utilize lipid as a major energy reserve (or a nutrient) when hemoglobin is still present in sufficent quantities to meet the tick's nutritional needs.

Many endoparasitic organisms do not utilize lipids as an energy source (Read, 1970). In the case of the mosquito, Aedes sollicitans, the amount of hemoglobin in the mosquito can be measured by the synthesis of triglycerides (Von Handel, 1965). The end product of hemoglobin metabolism is often lipid. The unfed adult lone star tick may be storing the lipid (the midgut tissue is the major storage area of the tick--ticks lack significant amounts of adipose tissue) which might be utilized at some point (beyond the first 12 months of starvation) when ingested hemoglobin is no longer available to meet the "starving" tick's nutritional requirements.

Hemoglobin represents the major nutrient reserve in the unfed lone star tick. The results of this project indicate that the hemoglobin content of Amblyomma americanum unfed adults declined with age. Many argasid ticks have been shown to exhibit a gradual decline in the hemoglobin content over time (Cook, 1973; Tatchell, 1963; Grandjean and Aeschlimann, 1973). Argasid ticks are known to live long periods of time possibly

due to an absence of strict synchronization in the digestive process which accounts for the slow digestion of the bloodmeal (Grandjean and Aeschlimann, 1973). They found that the digestive cells in O. Moubata are in different stages of hemoglobin digestion at the same time. Some type of slow utilization of hemoglobin seems to occur in the lone star tick allowing the tick to survive long after a bloodmeal. Lees (1964, 1946) speculated that the critical equilibrium humidity changes as the nutrient reserves decrease and as the ticks age. However, our results indicate that critical equilibrium humidity did not change with age in unfed lone star adults. The maintenance of the CEH in a range of 80-90% relative humidity probably has some significance in the tick's continued survival.

A purpose of the project was to establish methods for determining the physiological age of the unfed lone star adult. From the results, it can be concluded that water content, lipid content and the critical equilibrium humidity (although providing some function in the tick's survival) are not good indicators of the physiological age of Amblyomma americanum unfed adults (under present laboratory conditions). Hemoglobin seems to be the major nutrient and energy source for the tick's life during the first 12 months following a bloodmeal. This nutrient is slowly depleted and the drop from 0-month to 12-month ticks suggests that hemoglobin in the tick is a reasonable indicator of physiological age. A precise technique that would enable one to detect subtle differences in hemoglobin content in the midgut of single ticks would provide a feasible method for distinguishing relative ages of ticks from a given population.

CHAPTER III

CHANGE IN SURFACE AND MIDGUT MORPHOLOGY

Experimental Design

The same units of ticks were used for the ultrastructure studies of Amblyomma americanum as those used in the previous assays that were analyzed statistically. The experimental design (Tables I and II) remained the same for the ultrastructure study as it was for the other parts of the research project.

Changes in the Midgut Epithelium

Materials and Methods

Two males and two females were used to obtain the midgut tissue for this procedure. The ticks were dissected in buffered glutaraldehyde (2.5 ml glutaraldehyde, 7.5 ml cacodylate buffer, 2.5 ml distilled water and trace amounts of $CaCl_2$). The dissections involved anchoring the tick's appendages and mouthparts in molten wax and removing the dorsal cuticle with a sharp, curved scapel. All the tissue around the midgut was carefully removed (salivary glands, trachea, etc.). The midgut was teased away from the ventral cuticle, placed in a small labeled vial and taken to the Electron Microscopy Laboratory for further processing.

The tissue was processed (Table VII) and after embedding, blocks of

tissue were sectioned with Sorvall MT-2^{R} ultramicrotome and placed on 200-mesh nickel grids. The grids were stained with uranyl acetate. Tissue was viewed with a Philips 200 transmission electron microscope.

Dow Epoxy Resin (DER^{R}) was the embedding medium of choice after many unsuccessful attempts with Polybed^{R} and Araldite^{R} (Table VIII). Polybed did not completely infiltrate some portions of the tissue causing considerable tearing and distortion of the tissue during tissue sectioning. Initially, the tissue was kept in capped vials of Polybed: propylene oxide (1:1) for 4 hours but later the time was increased to 48 hours with improved infiltration and appearance of the tissue. Araldite also improved the appearance of the tissue but sectioning of tissue was difficult because of block softness. Use of Dow Epoxy Resin yielded a much more acceptable and unaltered appearance to the midgut fine structure except for minor tearing around the hematin inclusions.

Results

Figures 5-7 illustrate changes in cellular inclusions of the midgut tissue in the aging unfed lone star tick. Changes in the appearance of hemoglobin inclusions, hematin crystals, lipid droplets, glycogen groups, and the mitochondria were examined carefully in the tissue. Females and males were similar in appearance at each age group examined.

At 0-month, unfed tick midgut tissue had many hemoglobin inclusions (phagosomes with hemoglobin) (Figs. 5A, 5B). Hematin crystals (very darkly staining spherules) were abundant and located toward the luminal side of the cellular epithelium (Fig. 5). Lipid droplets were present in quantity and were located toward the hemolymph-side of the tissue. Glycogen (Fig. 5C) was visible, but only in small quantities. Mitochondria

TABLE VII

PROCEDURES FOR FIXATION, DEHYDRATION, AND EMBEDDING

1.	Fix tissue immediately in buffered glutaraldehyde. Tissue should be a maximum of 1 mm square and the volume of the fixative should be at least 10 x the volume of the tissue.	2 hours
2.	Wash several times in buffer (2 30-minute washes).	
3.	Post fix tissue in 2% O_sO_4 for 4 hours. Mix 1:1 osmium to cacodylate buffer.	
4.	Buffer wash, overnight	
5.	Dehydrate:	
	a. 50% ETOH	20 minutes
	b. 70% ETOH	20 minutes
	c. 90% ETOH	20 minutes
	d. 95% ETOH	20 minutes
	e. 100% ETOH	20 minutes
	f. 100% ETOH	20 minutes
	g. 100% ETOH (remove plastic liner of vial cap)	20 minutes
	h. Propylene oxide	30 minutes
	i. Propylene oxide	30 minutes
	j. 1:1 propylene oxide and DER	48 hours (capped)
	k. 1:1 propylene oxide and DER	12 hours (uncapped) (in cool vacuum oven)
	l. Embed in 100% DER, place in vacuum oven at 60°C for approximately 48 hours.	
	m. Embed in 100% DER, place in vacuum oven at 100°C for approximately 24 hours.	

TABLE VIII

RECIPES AND RESULTS OF RESINS USED TO EMBED TICK MIDGUT TISSUE

RESIN NAME	RECIPE	RESULT
Poly Bed 812	Resin 9.2 ml *DDSA hardner 7.44 ml **NMA hardner 3.36 ml ***DMP-30 catalyst .40 ml add drop by drop	Poor
Araldite 6005 (poly Sciences)		Poor
DER 736 (Dow Epoxy Resin)	DER 332 10 ml DER 736 3 ml *DDSA hardner 7 ml **DMP-30 catalyst .5 ml add drop by drop	Good

* Dodecenylsuccinic Anhydride
** Nadic Methyl Anhydride
*** Dimethylaminomethylphenol

Fig. 5 Midgut Epithelium of 0-month Lone Star Ticks.

A. Hematin crystals (Hm) and hemoglobin inclusions are located toward the luminal side of the midgut tissue. Lipid droplets (L) are found toward the basement membrane. X31,275.

B. A closer view of hemoglobin inclusions and excretory spherules (ES) found in the 0-month tick midgut. X94,000.

C. Glycogen groups (G) are also found at this age. X157,500.

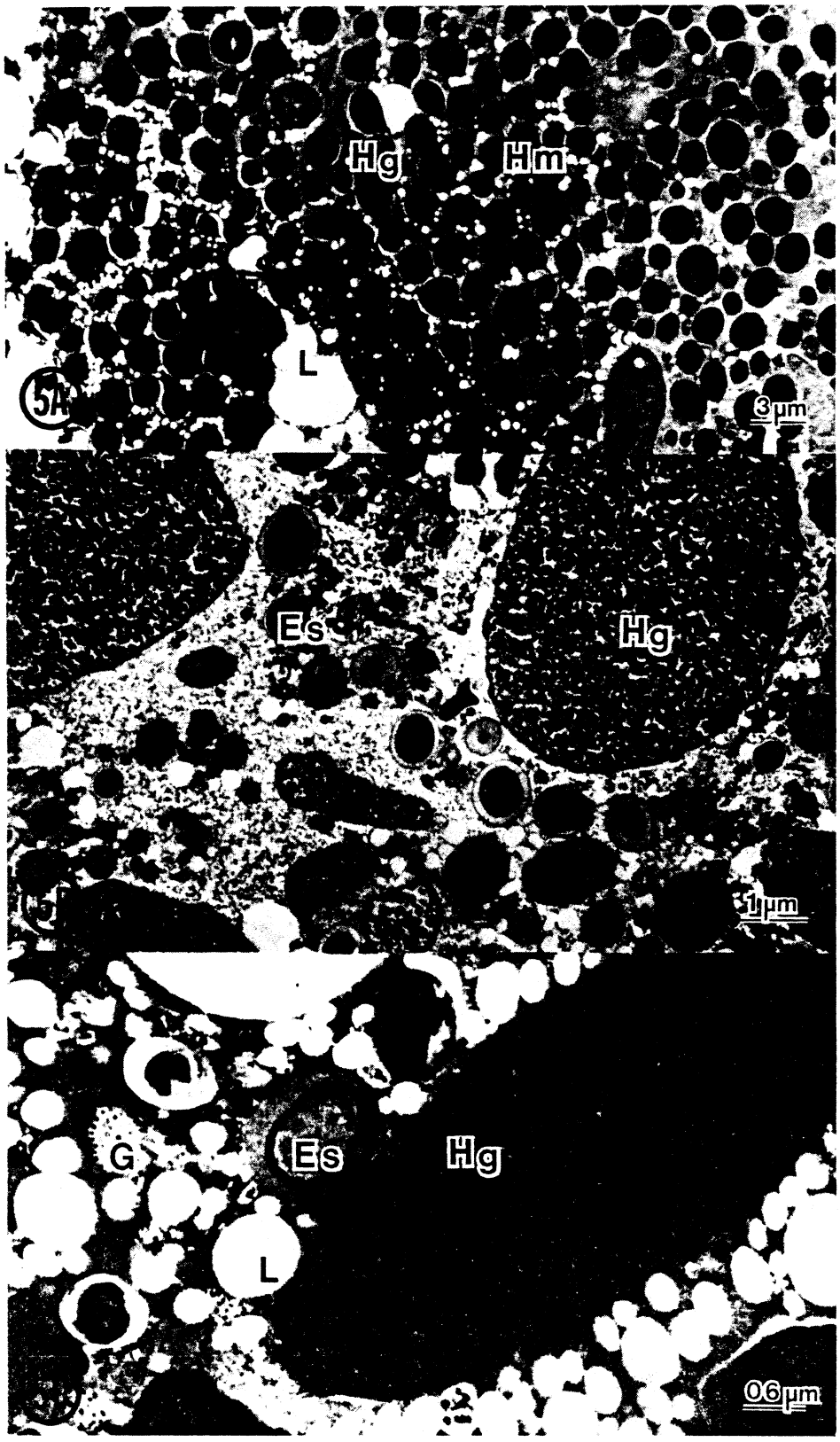

Hg
Hm
L
5A
3μm
Es
Hg
1μm
G
Es
Hg
L
0.6μm

Fig. 6 Midgut Epithelium of 3- and 6-month Lone Star Ticks.

A. Hemoglobin inclusions (Hg) are still found by 3 months. Glycogen group (G) are more visible at this age. Hematin crystals (Hm) and lipid droplets (L) are unchanged. X191,250.

B. A closer view illustrates hemoglobin inclusions (Hg) near a nucleus (N) at 3 months. X354,000.

C. By 6 months, glycogen groups are greatly increased and hemoglobin inclusions, generally, absent. X179,000.

D. Hematin crystals, excretory spherules (Es), and lipid droplets are present in 6-month tick midgut tissue. X123,750.

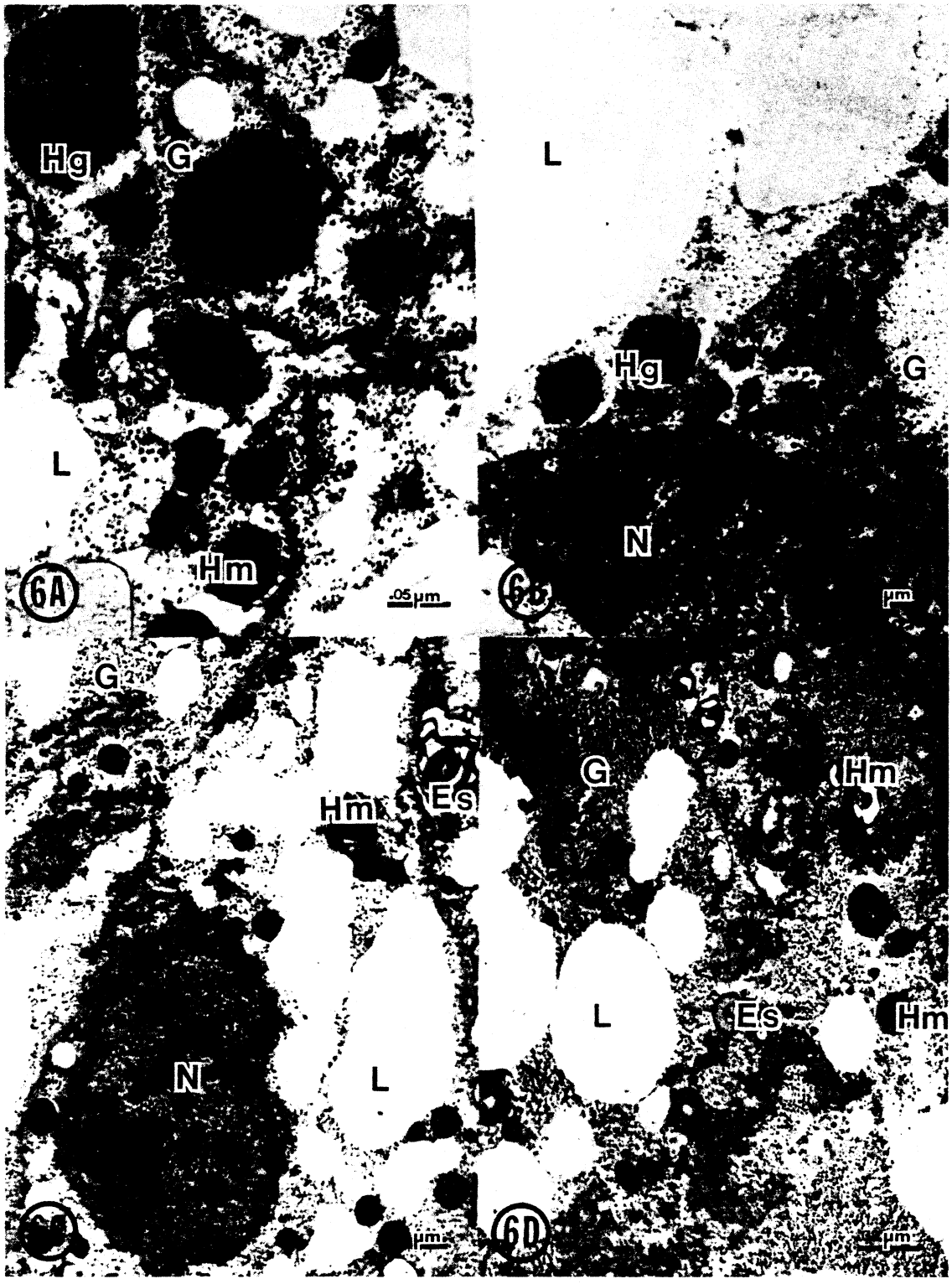

Hg
G
L
Hm
6A
.05 µm
L
Hg
G
N
Hm
G
Hm
Es
N
L
G
Hm
L
Es
Hm
6D
µm

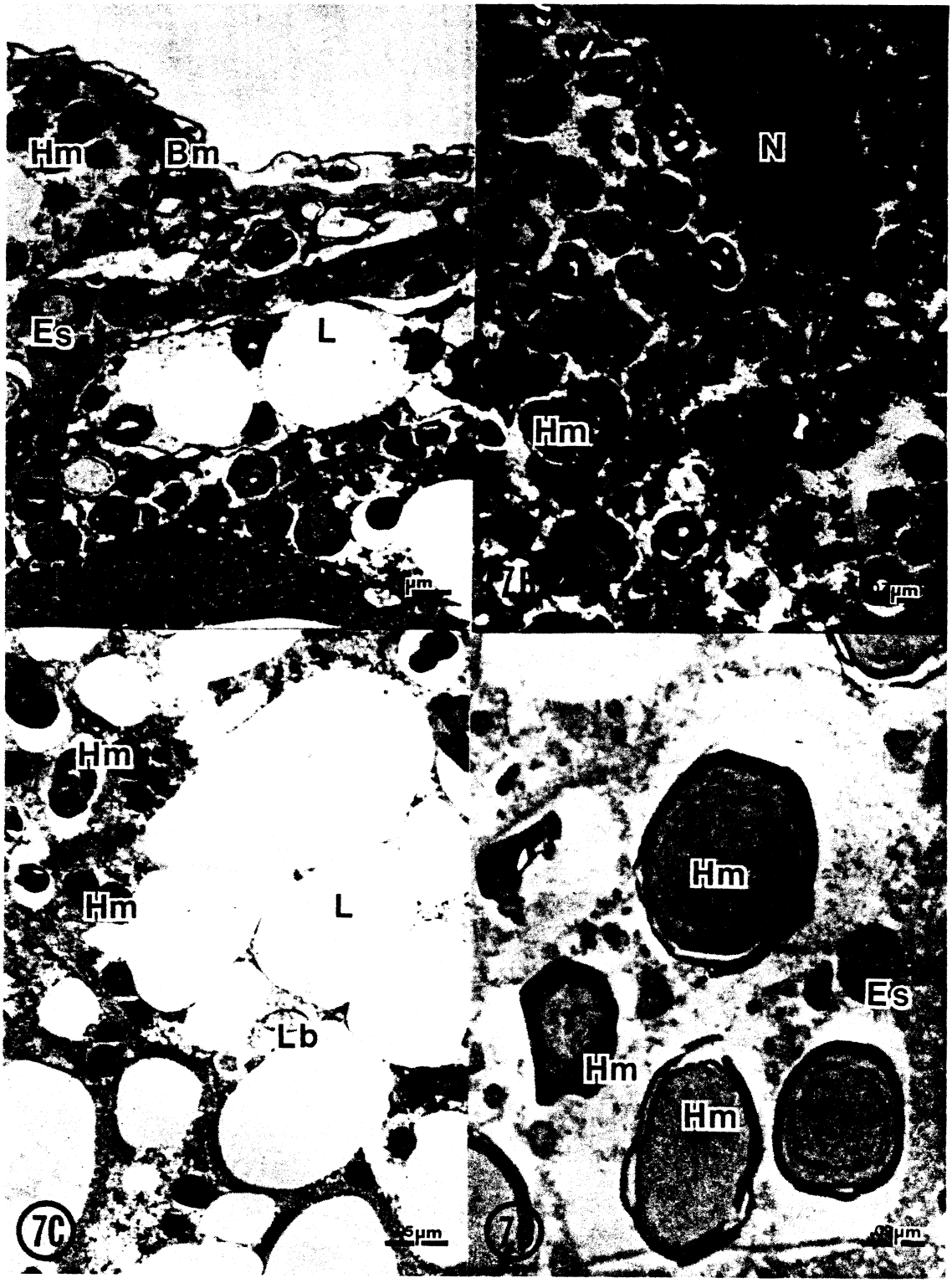
Fig. 7 Midgut Epithelium of 9- and 12-month Lone Star Ticks.

A. This micrograph illustrates degenerating hematin crystals (Hm) found in 9-month midgut tissue. Excretory spherules (Es), lipid droplets (L) are found at this age. Basement membrane (Bm). X90,000.

B. At 12 months, the hematin crystals located near a nucleus (N) continued to degenerate. X146,250.

C. Possible Lipid breakdown products (Lb) near lipid droplets (L) are found at this age. X202,500.

D. These crystalline structures are possibly a form of hematin crystals that have undergone degeneration. X112,500.


Hm
Bm
Es
L
µm
N
Hm
µm
Hm
Hm
L
Lb
7C
µm
Hm
Es
Hm
Hm
7
µm

were difficult to find.

Ticks at 3 months still had some phagosomes containing hemoglobin in the midgut cells (Fig. 6A) with many hematin crystals. The appearance and amount of lipid was not noticeably changed from the 0-month age group (Fig. 6B). Mitochondria were seen more frequently and glycogen groups were more abundant (Fig. 6B). Pinocytotic vesicles were visible in the epithelium of ticks of this age.

By 6 months, hemoglobin inclusions were no longer present in cells of the epithelium (Fig. 6C). Hematin crystals were present, and in some cases, these inclusions appeared to be disintergrating (Fig. 6D). Glycogen groups were abundant and seemed to be the most distinguishing feature at this age (Figs. 6C, 6D). The quantity of lipid seemed to be reduced only slightly as compared to that seen at other ages.

By 9 months, the midgut tissue had many degenerating hematin inclusions (Fig. 7A). The glycogen groups were not as numerous as they were in the 6-month age. Lipid inclusions were present and mitochondria were visible.

The 12-month tick midgut tissue showed continued degeneration of the hematin inclusions (Fig. 7B). These inclusions were often vacant or empty in appearance lacking the darkly staining components seen in earlier ages (Fig. 7D). Lipid inclusions were present and some breakdown of lipid was seen (Fig. 7C). Glycogen groups were present and numerous mitochondria were seen.

Other general observations included hemoglobin inclusions and hematin crystals near the luminal side of the midgut. Lipid droplets were located near the basement membrane. Muscle cells were found around the outside of the basement membrane (Fig. 7A). Tracheoles were also found

outside the basement membrane.

Discussion

Ixodid ticks feed slowly, ingesting blood and other material that is lysed by the salivary gland secretions according to Balashov (1972). Amblyomma americanum nymphs remain attached to the host for 4-5 days (Sauer and Hair, 1971). Ticks differ from other arthropods in that the digestion is mostly intracellular (Balashov, 1972).

Digestion of the blood occurs in phases in both ixodid and argasid ticks. In the argasid tick, the first phase of digestion is associated with rapid agglutination and hemolysis of ingested cells (Balashov, 1972). Assimilation of the major hemoglobin mass into gut cells in the second phase lasts several weeks to several months. The third phase consists of very slow utilization of semi-digested hemoglobin during the free-living stage of the tick. Tatchell (1964) found slightly different results in Argas persicus. He found that no digestion occurs in the first few days (after ingestion) while the new epithelium is developing. The second phase of hemolysis and digestion takes place in the active epithelium (lasting about 20 days). The third stage is typified by slow and constant digestion that continues until another feeding or until the tick dies.

Raikhel (1978) also believes that ixodid digestion has three phases. External digestion occurs as a result of the lytic effect of the salivary gland secretions. Luminal digestion continues the lysis of the blood cells, but it is not known when the process of luminal digestion is complete. Intracellular digestion is the most important and extensive mechanism in the digestive process.

Raikhel (1978) also discussed the ultrastructure of both the unfed nymph and the feeding nymph. His observations did not consider the unfed tick at different ages. Three types of cells form the ixodid tick midgut: undifferentiated, secretory and digestive cells. These cells may differ with the physiological stage of the tick. The midgut epithelium of the unfed nymph consists mostly of digestive cells retained from a previous bloodmeal and in different stages of digestion. Raikhel observed a few undifferentiated reserve cells and some digestive cells undergoing degeneration. Although I did not study the tick gut epithelium in as much detail as Raikhel, my results agree in general with his findings in terms of structures and relationships of organelles within the cell.

In the earliest age group of ticks (0-month ticks), many phagosomes containing hemoglobin were observed. The phagosomes were completely absent by the 6-month age group. The fact that hemoglobin changed and was converted to hematin crystals agrees with that observed by others (Balashov, 1972; Smit et al., 1977). The hematin crystals were also observed to change from darkly staining crystals to lighter crystals by 9 months and by 12 months, breakdown products of the hematin were observed. It appears that hemoglobin may be the major metabolite that fuels the tick's metabolic processes.

Raikhel (1978) observed round, homogeneous, non-membrane bound lipid inclusions. The lipid inclusions seen in the present study are similar. Some lipid breakdown was observed in older ticks (9- and 12-month) but lipid degradation does not seem to be a major source of energy at this point in the life of the unfed tick. Glycogen groups were found in the tick midgut which may serve as a major nutrient reserve for the tick

(Raikhel, 1978). Little glycogen was seen in 0-month ticks. More glycogen groups were observed in the gut cells of 3-month ticks. By 6 months, the glycogen groups were quite abundant with some reduction seen in 9- and 12-month ticks. Glycogen was most visible in 6-month ticks and at a time when the hemoglobin inclusions were no longer conspicuous. Glycogen may be an important storage product of hemoglobin catabolism.

The ultrastructure of the unfed tick midgut appears to be a good indicator of the physiological age of the tick.

Scanning Electron Microscopy of the Tick Cuticle

Materials and Methods

Ticks of various age groups were prepared for scanning electron microscopy (SEM) in the following manner: Two male and two female Amblyomma americanum were frozen, and then sonicated to remove surface contamination. One female was oriented ventral side up and the other dorsally. The same procedures were followed with two male ticks. The ticks were affixed to metal stubs and coated with 100-200 Å gold-palladium (60:40) under vacuum to reduce surface charging (charging is a building-up of electrons on the cuticle) and to prevent possible damage from the SEM electron beam. After coating, specimens were viewed with a Joel JSM-35 scanning electron microscope.

Results

Many different aspects of the tick cuticle were studied without any indication of obvious changes. The festoons, mouthparts, Haller's

organ, anal and genital appertures were observed. The only obvious changes were sizes of two cervical grooves on the dorsal surface of females (Fig. 8). The cervical grooves of the female tick seemed to become longer and possibly, wider and deeper in older ticks. Measurements of the cervical groove and the neck (the anterior margin of the scuttellum) were taken and a ratio was derived which expressed the groove size in relationship to the size of the tick (Table IX). These values indicated an increase in groove size from 0-month to 3-month female ticks and possibly from 6-month to 9- or 12-month ticks. Regression analysis showed the differences over age (Fig. 9).

Discussion

The results suggest that the size of the cervical groove may be a reliable age indicator for distinguishing between newly molted ticks (0-month) and all other age groups. The ratio may be useful in distinguishing between 3- and 6-month ticks and possibly 9-month female ticks and most certainly, 12-month ticks or older.

The cervical grooves are attachment sites for the muscles of the mouthparts (Douglas, 1940). A deepening and lengthening of the cervical grooves (muscle attachment sites) may suggest some deterioration of the muscles attached to the mouthparts.

Fig. 8 Scanning Electron Micrographs of two female Lone Star Ticks Illustrating the cervical grooves located dorsally on the Scutellum.

A. 0-month Tick. Cervical groove (Cg). Anterior margin of the Scutellum (Ams). X120.

B. 12-month Tick. Cervical groove (Cg). Anterior margin of the Scutellum (Ams). X120.

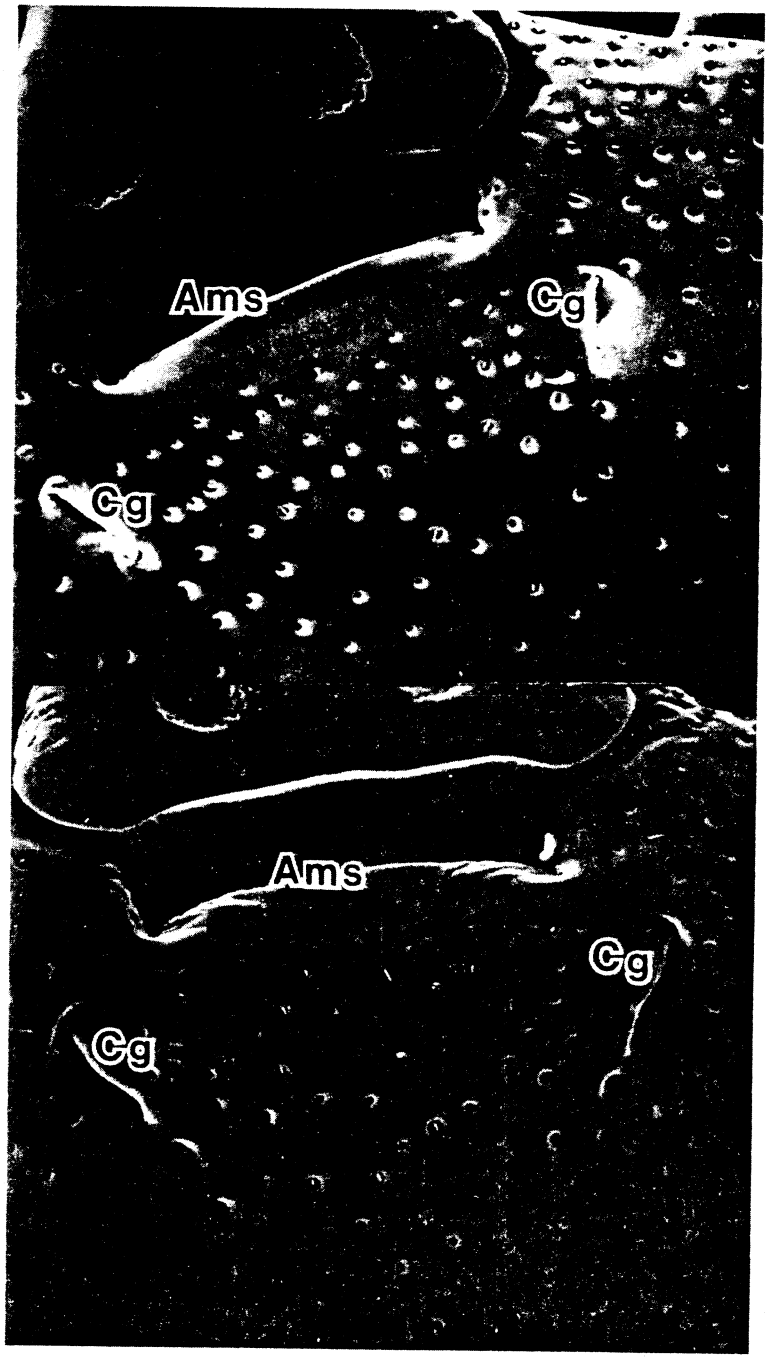

Ams
Cg
Cg
Ams
Cg
Cg

TABLE IX

CHANGE IN THE CERVICAL GROOVE SIZE IN *AMBLYOMMA AMERICANUM*, UNFED ADULT FEMALES FOR FIVE AGE GROUPS

Age in Months	Cervical Groove Ratio*
0	4.057* ± .97
3	2.972 ± .60
6	3.126 ± .80
9	2.688 ± .41
12	2.357 ± 44

* Cervical Groove Ratio = $\frac{\text{Neck Size in Millimeters}}{\text{Groove Size in Millimeters}}$

The higher the ratio the smaller the groove size

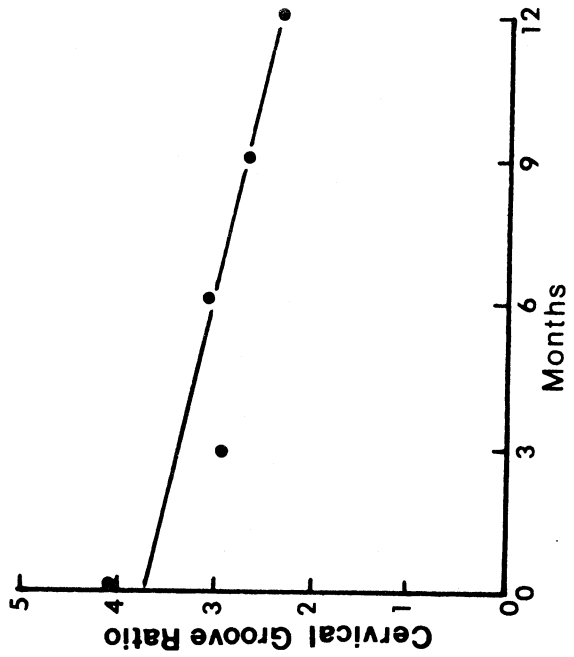
Mean ± S.D. of 3 reps.

Fig. 9 Change in Cervical Groove Size in Amblyomma americanum, Unfed Adult Females for Five Age Groups Expressed by the Line of Regression.

$Y = -0.123\ X + 3.777$

$$\text{Cervical Groove Ratio} = \frac{\text{Neck Size in Millimeters}}{\text{Groove Size in Millimeters}}$$

Months = ages of the ticks


Cervical Groove Ratio
0
1
2
3
4
5
0
3
6
9
12
Months

CHAPTER IV

SUMMARY AND CONCLUSIONS

Survivorship, water, lipid, hemoglobin, critical equilibrium humidity and morphological changes in the midgut epithelium and external surface cuticle were examined for five ages (0,3,6,9,12-month) of reared ticks. Results suggests that a "host" factor may be involved in the continued survival of the tick. Survival may also be effected by the season in which the tick feeds. Ticks feeding to repletion in the winter months exhibited higher mortality than ticks fed at other times.

The water content and the critical equilibrium humidity did not change significantly in the various ages. The critical equilibrium humidities for all ages and sexes were in the range of 80-90%. Although the water content and the critical equilibrium humidity are likely involved in the survival of the tick, neither are good indicators of physiological age.

Tick lipid content decreased only slightly with tick age. Hemoglobin content descreased significantly from about 16 ug/mg to 7 ug/mg in Group A experimental ticks and from about 25 ug/mg to 7 ug/mg in Group B. Hemoglobin content in the tick may be a good indicator of physiological age.

At 0-months, many inclusions containing hemoglobin were present as well as many electron dense hematin crystals in the gut epithelium. After 3 months fewer hemoglobin inclusions were present and by 6 months,

hemoglobin inclusions were absent, glycogen groups abundant. 9- and 12-month ticks had numerous disintegrating hematin crystals in their gut epithelial cells. The ultrastructure of the midgut epithelium is an accurate indicator of physiological age of laboratory reared unfed adults.

Dorsal cervical groove size changes with the age of the unfed female. For example, at 0 months, the cervical groove ratio (anterior margin of the scutellum: cervical groove length) was 4.057 and at 12 months 2.357. Cervical groove size may be a quick but possibly less precise method for estimating tick age.

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VITA

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