FEEDING ELECTROGRAMS AND SALIVARY FLUID SECRETION OF HARD TICKS (ACARI: IXODIDAE)

By

ROBIN DIANE HICKEY

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FEEDING ELECTROGRAMS AND SALIVARY FLUID SECRETION OF HARD TICKS (ACARI: IXODIDAE)

Thesis Approved:

J. Alexander Hair
Thesis Advisor

John R. Sauer

Mark E Payton

D G Wagner

Thomas C. Collins
Dean of Graduate College

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CHAPTER I

THE USE OF ELECTRONIC MONITORING SYSTEMS TO COMPARE FEEDING RHYTHMS OF FEMALE IXODID TICKS

ABSTRACT The fluid exchange of three genera of ixodid ticks, Amblyomma americanum (L.), Dermacentor variabilis (Say), and Rhipicephalus sanguineus (Latreille), feeding on an ovine host, was observed using an electronic monitoring system (EMS). From these recordings, four basic feeding patterns were determined: 1. resting, 2. sucking, 3. salivation, and 4. expulsion of saliva with pool formation. It was determined that A. americanum and D. variabilis spend approximately the same amount of time exhibiting sucking patterns, and A. americanum and R. sanguineus displayed expulsion of saliva with pool formation to the same degree. However, the resting phases and periods of salivation expressed by D. variabilis and R. sanguineus were almost identical.

TICKS ARE ECONOMICALLY IMPORTANT PARASITES due to their tenacious feeding behavior, their ability to harbor and transmit disease-causing organisms, induce toxicosis, and induce tick paralysis (Sutton & Arthur 1962, Binnington & Kemp 1980). Ticks rank second to mosquitoes as vectors of human diseases and surpass all other arthropods in the number and variety of diseases in which they can transmit to domestic animals (Obenchain & Galun 1982).

The transmission of disease pathogens and the induction of tick paralysis and tick toxicosis are related in that they result from the injection of saliva into the host by the

feeding tick (Bertram et al. 1962, Gregson 1967, Tatchell & Moorhouse 1968, Balashov 1972, Burgdorfer 1975, Sauer 1977, Brown & Askenase 1983). Because these economic factors are directly related to the feeding processes, and the nature of the feeding patterns of ixodid ticks are poorly understood, "normal" feeding patterns need to be established. An assessment of each pattern is required in order to determine its relative importance in the feeding cycle. This will facilitate in the initiation of biochemical studies to measure potential antagonism by these chemicals against normal feeding.

Electronic monitoring systems (EMS) have been developed and used to observe and document the normal feeding patterns of some ticks (Sweatman & Gregson 1970, Tatchell et al. 1972, Sweatman et al. 1976, Waladde et al. 1979), as well as other arthropods (Kashin 1966, Friend & Smith 1971, Smith & Friend 1971, Brown & Holbrook 1976, Kimsey & McLean 1987, Wayadande & Backus 1989, Blust & Hopkins 1990). In these studies, many tick feeding processes have been superficially described, and a correlation and comparison of one species to that of another has not been made.

Because the relative importance of each of these feeding processes change during the tick feeding cycle (Gregson 1967), typical feeding patterns need to be established throughout the ixodid family and correlations among these patterns should be determined. It is desirable to ascertain

the relative significance of each phase of the feeding process in order to quantify the effect that any alteration of these processes may have on the completion of the tick feeding cycle. The alteration of these feeding processes might be achieved through the use of various pharmacological agents, giving insight into the development of a new means of tick control.

This study was designed to document and compare similarities of the feeding processes of selected genera of ixodid ticks, attached and feeding on a suitable ovine host. This author presumes that Amblyomma americanum (L.), Dermacentor variabilis (Say) and Rhipicephalus sanguineus (Latreille) are analogous in their feeding behavior due to homologies of the general feeding components, but differ in the amount of time spent performing each process. These genera were chosen for this study due differences observed in the appearance of the waste excreted by these ticks while feeding on the host. Differences were also seen in the total attachment time (from first attachment through repletion) (Hickey, unpublished data). Documentation of the feeding cycle of mated female ticks was accomplished through the use of an electronic monitoring system in conjunction with a strip chart recorder.

Materials and Methods

Host Animals. Two female ovine, believed not to have been

previously exposed to tick infestation, were used as host animals through the duration of the trial. Each ewe was of the Suffolk breed and weighed approximately 175 lbs. The sheep were brought indoors, stanchioned, washed and sheared. The facility was maintained at an average temperature of 22°C and was cleaned and disinfected twice daily (approximately 0800 and 1500 hr) in an effort to provide aesthetic, sanitary and humane conditions for the test animals. The sheep were provided with fresh water and feed *ad libitum*. Nutritional requirements were met with a mixture of SH-019 (Oklahoma State University Feedmill, Stillwater, Oklahoma). The feed consisted of 55% rolled corn, 3.5% black strap mollasses, 15% soybean meal, 25% dehydrated alfalfa pellets, .5% trace-mineral salt, .02% Chlorotetracycline-50, and 1% ground limestone. Host animal health was monitored daily and a log book was kept to record any illnesses. (No illnesses were observed and no medication was administered.)

The dorso-lateral portions on each animal were closely shaven and eight cells made of 6 inch orthopedic cotton stockinette (Melton Company, Oklahoma City, Oklahoma) - four cells per side - were affixed with 3-M #4799 Industrial Adhesive (Adhesive, Coatings and Sealer Division/3M, St. Paul, Minnesota).

Monitoring System. The electronic monitoring devices used in this study were modifications of the Electronic

Monitoring Systems (EMS) of Brown & Holbrook (1976) with line amplifiers (Kendow Technologies, Perry, Okla.), (Fig. 1). These systems consisted of a series electrical circuit involving a 20 Hz oscillator, the tick and ovine host, and the input of the tuned 20 Hz amplifier (Fig. 2). Each monitor was connected to two cable leads; an input lead and and output lead. The input cable was secured to a modified polyvinyl chloride (PVC) ring by means of a banana jack (male connection). The PVC ring was 6 inches in diameter and fit inside the stokinette cell. Each PVC ring had a 0.25 mm silver wire extending into the interior of the ring, also secured by a banana jack female connection. The silver wire was inserted into the dorso-posterior portion of the abdomen of the attached, feeding female tick (Fig. 3). The output cable extended from the ovine host to the monitor by means of a hook-shaped vacutainer needle that was embedded subcutaneously into the host, adjacent to the PVC ring. This lead was secured to the needle by an alligator clamp and provided completion of the circuit. Strip Chart Recorders, series D-5000 (Houston Instrument Inc.) were used in conjunction with the modified EMS to document the normal tick feeding cycle.

The EMS was supplied power by two 9 volt batteries. The specific output gain level was set at 0.050 v and was measured by a Micronta LCD Digital Multimeter (Radio Shack, Stillwater, Oklahoma) with digital settings on AC 3. The

Strip Chart Recorder was set at a chart speed of 12.5 cm per min and the baseline was set at 1.

Ticks. A. americanum, D. variabilis and R. sanguineus used for this study were acquired from the tick colony maintained at the Oklahoma State University Tick Rearing Facility. Immature ticks were reared to adults as described by Patrick & Hair (1975). Adults used were approximately 90 d post-nymphal molt. Through observations made during a preliminary mating study (Hickey, unpublished data), it was determined that A. americanum generally reached their sexual maturity 9 d after infestation; D. variabilis in 5 d, and R. sanguineus in 6 d. (The preliminary study was conducted in the late fall of the year. Because of this, days to sexual maturity are more numerous than those typically seen in the spring.) Sexual maturity was determined by calculating the percent of successful copulations when a sexually mature male was introduced to an attached and feeding virgin female. A copulation was considered successful if the male probed the dorsum of the female, maneuvered to the mating position (sternum to sternum) and remained there for a period of at least 2 h.

In order to reduce feeding variations due to differences in physiology, phase of feeding, and overall time to repletion, this study was set up so that all ticks reached sexual maturity at Day 0. The host sheep was infested with 20 male and 20 female A. americanum on Day -10, 20 pair of

R. sanguineus on Day -7, and 20 pair of D. variabilis on Day -6. Tick infestation was completed in this manner to eliminate the variation in days needed to reach sexual maturity, allowing all ticks to reach sexual maturity by Day 0. Only the feeding processes performed by mated females of each species were monitored.

After all females attached, their positions within the cell were recorded on a flow chart by a corresponding number (1-20). (Females remaining unattached after a 24 h period were removed from the cell and destroyed.) After all ticks reached sexual maturity on Day 0, each female was exposed to a mature male. If successful copulation was not observed, both the female and the male were removed and destroyed.

Two mated female ticks from each species were randomly selected and monitored with the EMS each day. After two 15 min monitoring periods per tick were completed, each monitored female was removed and destroyed. The EMS was used to monitor the feeding patterns of six different mated females (two per species) each day from 24 h post-mating through repletion.

All data recorded were properly identified on the individuals' recorded waveforms. Data were recorded as to day, species, tick ID number, cell number, date of recording, and time of day.

After all data had been taken, each chart was individually analyzed to determine the various feeding rhythms present.

The patterns were identified according to relative changes in conductivity of the fluid and variations in frequency and amplitude of individual peaks. (When speaking in terms of conductivity, only the ability of the fluid to carry an electrical potential is being addressed.)

All recordings were quantified over time, by species. Percentages of each feeding pattern were calculated by determining the total length of the recordings for each pattern type expressed, dividing by the total time of the chart recording for that species on that day, then multiplying by 100. A Chi-square test was performed on all data quantitated. Each species was analyzed independently of the other and a comparison of the three was made only after final conclusions were drawn.

Results

Four basic feeding types were observed, with each type demonstrating minor variations within and between species. These variations are depicted as subtypes a, b, or c for each of the basic patterns discribed (Figs. 4-9). After all patterns were categorized, the similarities within pattern types between species were determined (Figs. 10-12). Each pattern type depicted in the appendix represents a one-minute feeding period (chart speed = 12.5 cm/min). The Y-axis of the figures represents the change in conductivity of the feeding response.

General Pattern Types Expressed in Feeding Female Ticks. Each pattern type represents a specific feeding activity: Type I - Resting; Type II - Sucking (or ingestion); Type III - Salivation; Type IV - Expulsion of Saliva with Pool of Saliva Formation (Fig. 5). The criteria used in determining specific feeding patterns, indicated by EMS, were based upon visual comparisons between previously published charts that demonstrated various feeding activities in hard ticks (Gregson 1967, 1969; Sweatman & Gregson 1970, Tatchell et al. 1972, Sweatman et al. 1976, Waladde 1982, Stone et al. 1983).

The level of fluid conductivity was the major factor in determining the pattern type, however, overall pattern length and appearance were also taken into consideration. As conductivity increases, the position of the pattern in relation to the baseline increases, whereas a decrease in conductivity results in a decreased distance from the baseline.

Those patterns depicted as resting were characterized by a flat or straight line with occasional fluctuations in that line (Fig. 4). Pattern Type I typically demonstrated little or no activity along the baseline. Two subtypes were defined in order to demonstrate the level of variation that occurs within pattern Type I; Subtype 1A, and 1B (Fig. 5). The variations depicted by these subtypes were due to increased number of wave peaks (subtype 1A) and increased

magnitude of peaks (subtype 1B).

Sucking activities were characterized by regular wave movements that showed a decreased fluid conductivity with variations in frequency and amplitude of peaks (Fig. 4). A significant increase in activity occurred at the onset of each imbibement period in comparison to the resting phase. Pattern Type II varied in the intensity of the feeding rhythm, the frequency of wave peaks,and the level of fluid conductivity. Each difference was catagorized under subtype 2A, 2B, and 2C respectively. Pattern subtype 2C demonstrates a combination of subtype 2A and 2B, with an additional increase in fluid conductivity.

Salivation was observed to be an intense feeding pattern with random peaks and extremely large increases in fluid conductivity (Fig. 4). Two variations of this activity are demonstrated (Fig. 7) to illustrate the differences commonly seen in the frequency (subtype 3A) and the amplitude of individual wave peaks (subtype 3B).

The expulsion of saliva with pool of saliva formation was depicted by a sudden upward spike, indicating an abrupt increase in the conductivity of the fluid. At this time, the electrical potential of the fluid present in the hypostome remained the same for a short period of time, causing a plateau in the feeding pattern, then the pattern gradually decreased back to the baseline as feeding resumed (Fig. 4). There were several common variations of this

pattern typically seen (Figs. 8 & 9). Each subtype represents a change in the total time of pool formation, the level of the conductivity of the fluid, and in the frequency of wave peaks within the area designated as pool formation.

Percent Pattern Occurrence. Percentages were calculated for each pattern type within each species on each day to indicate the overall level of pattern occurrence throughout the feeding cycle. The feeding cycle monitored for each species only consists of those days from 24 h post-mated to repletion. The Chi-square test statistics calculated a DF of 6 and X^2 value equal to 3196.38. Results demonstrate that the differences in frequency of occurrence for each pattern type, on a day to day basis, within a species, were significant at *P* less than 0.001.

A. americanum spent 8.00% of its total attachment time resting, 33.00% sucking or imbibing a meal, 39.80% salivating, and 19.20% expelling saliva with pool of saliva formation (Table 1). The longest periods of resting and salivation were exhibited on day +2 post-mating with a total time of 40.00% and 58.00% respectively (Fig. 13). The highest frequency of sucking (62.00%) occurred on day +4 post-mating, and the greatest period of expulsion of saliva with pool formation was 21.00% on day +1 post-mating (Fig. 13). On the average, sucking activities decreased and expulsion of saliva activities increased from day +5 through day +7, possibly indicating lubrication of the hypostome to

facilitate detachment.

D. variabilis spent 38.00% of its total attachment time resting, 33.17% imbibing a blood meal, 14.67% salivating, and 14.17% expelling saliva with pool of saliva formation (Table 1). The longest period of resting was 100.00%, occurring on day +1 post-mating, and the highest frequency of sucking reached 75.00% and was displayed on day +2 (Fig. 14). The greatest period of salivation was demonstraed at 30.00% on day +4, and the highest occurrence of short bursts of saliva with pool formation was 27.00%, occurring on day +3 (Fig. 14). On day +5, a slight increase in sucking activity occurred. This may represent a portion of the rapid engorgement phase of the feeding cycle.

Of the total time spent on the host, R. sanguineus spent 45.33% resting, 19.67% sucking, 14.33% salivating, and 20.67% expelling saliva with a pool of saliva formation (Table 1). The longest observed period of resting was 80.00% and it occurred on day +2 post-mating (Fig. 15). The highest frequency of blood imbibement (51.00%) occurred on day +6 (Fig. 15). The greatest period of salivation occurred on day +1 with a total time of 28.00%, and the highest frequency of expulsion of saliva with pool of saliva formation (52.00%) occurred on day +3 (Fig. 15). On day +6, a slight increase in sucking activity was observed. This may also demonstrate the rapid engorgement phase of the feeding cycle.

Discussion

Changes in the fluid conductivity in a tick depend upon the opening and closing of the feeding and salivary channels, as well as the ionic composition of the fluid within those channels (Gregson 1969, Sweatman & Gregson 1970). The tick feeding rhythms published in this study are similar to those found in other feeding studies using electronic monitoring (Gregson 1967, Sweatman & Gregson 1970, Tatchell et al. 1972, and Waladde et al. 1979). However, many more variations were commonly observed and categorized as subtypes of those general patterns discribed. These subtypes represent variations in the level of fluid conductivity, frequency of individual wave peaks, amplitude of individual wave peaks, and overall intensity of the feeding pattern.

The resting pattern (Pattern Type I) was established as a period of low activity (Fig. 4), however, the minor wave fluctuations seen in the patterns of subtypes 1A and 1B (Fig. 5) are thought to be attributed to constrictions of the salivarium or pharyngeal pumping (Tatchell et al. 1970, Sweatman & Gregson 1970).

The sucking pattern (Pattern Type II) or the feeding pattern shows a greater fluctuation in the level of fluid conductivity (Figs. 4 & 6) and is thought to result from the opening of the pharyngeal valve with the involvement of the pharynx (Tatchell et al. 1972). Pattern subtypes 2A, B, and

C demonstrate various levels of feeding intensity that may occur during the feeding phase.

The random fluctuations of increased fluid conductivity depicted in Pattern Type III (Salivation), suggests the release of cement, saliva, and/or the ejection of other fluids (Figs. 4 & 7). Gregson (1967) and Stone et al. (1983) visually observed the process of salivation to coincide with electronic monitoring recordings that confirm this.

Pattern type IV demonstrates the expulsion of saliva with the formation of a pool of saliva around the mouthparts (Figs. 4, 8 & 9). This pattern was noted by a sharp increase in the conductivity of fluid within the hypostome. This increase demonstrates a fluid conductivity that is approximately three times higher than that of the sucking activity. The sharp increase in conductivity results in a peak that typically levels off before suddenly dropping to the baseline, as feeding processes were resumed. It is believed that this action clears the area in front of the mouthparts temporarily (Sweatman & Gregson 1970), in which the salivarium remains open without a lot of salivary secretion (Tatchell et al. 1972). It is also thought that because of the presence of saliva in the food channel, the electrical potential is maintained (Tatchell et al. 1972), resulting in the characteristic plateau of the pattern demonstrated in Fig. 4.

In the day to day comparisons of pattern type occurrence for each species, as illustrated in Figs. 13-15, it was interesting to see that A. americanum were readily feeding 24 h post-mating on Day +1. D. variabilis and R. sanguineus, on the other hand, were not. It is thought that these feeding differences could be attributed to the feeding aggressiveness of A. americanum above that of the other two species.

Other differences could be attributed to specificity of host choice. R. sanguineus could be exhibiting reduced feeding activity and increased resting activities because of exposure to a foreign host. Host specificity can effect the rate of survival, length of life cycle, and fecundity due to the nature of the hosts blood (Galun et al. 1978). Since the study was conducted in early March, D. variabilis and R. sanguineus could also be affected by external factors that may prolong the initiation of feeding.

Because of the large amount of heme present in the fecal excrement of D. variabilis and R. sanguineus, it was believed that their feeding cycles would be most similar. However, surprisingly, D. variabilis and A. americanum were almost identical in the ratio of time spent in the sucking patterns and R. sanguineus and A. americanum were almost identical in the ratio of time spent in expulsion of saliva with pool of saliva formation (Table 1).

It was hypothesized that A. americanum would spend a greater amount of time demonstrating salivation due to the

lack of heme present in the fecal excretions. It was also thought that the lack of heme could be due to an increased activity of concentrating the bloodmeal. If this is occurring, it would seem that A. americanum would spend more time secreting unwanted fluids back into the host tissue. Data supports this theory.

From the EMS charts, a generalized feeding cycle could be formulated for each species. Although these different species had similarities among the appearance in the pattern types, the magnitude and frequency of occurrence for each individual pattern varied within species, as well as between species. Based upon the pooled pattern type demonstrations, R. sanguineus had the highest percent of Pattern Type I (resting) occurring of the three species, and also was leading in the time spent in expulsion of saliva and pool formation (Type IV), but only by a small percentage. D. variabilis was responsible for the highest occurrence of Pattern Type II (Sucking) in comparison to R. sanguineus and A. americanum. A. americanum had the highest occurrence of Pattern Type III (Salivation).

Through the establishment of normal feeding patterns of these genera, it is now possible to detect variations in these patterns following administration of various pharmacological agents into the hemocoel of the feeding female tick. The use of electronic monitoring systems will allow us to determine exactly which portion of the feeding cycle is affected and to what extent.

CHAPTER II

AN INCREASE OF CYCLIC AMP LEVELS IN SALIVARY GLANDS OF *DERMACENTOR VARIABILIS* (SAY) AND *RHIPICEPHALUS SANGUINEUS* (LATREILLE) THROUGH DOPAMINE STIMULATION

ABSTRACT The stimulation of D_1 receptors on the salivary gland plasma membranes of *Dermacentor variabilis* (Say) and *Rhipicephalus sanguineus* (Latreille) was facilitated by dopamine to increase the levels of cAMP through the activation of adenylate cyclase. The Competitive Protein Binding Assay was used to quantify the increasing levels of cyclic AMP, which is critical in the control of salivary gland function. The levels of cAMP were significantly increased after glands were stimulated, *in vitro*, by dopamine. The level of cAMP varied between the two species and among the varied weights of the individual ticks. An increase in fluid secretion was also seen after the injection of dopamine into the hemolymph of partially fed female *D. variabilis* and *R. sanguineus*. These increases were measured through the use of an electronic monitoring system and via the collection of salivary fluid secreted from each injected female. From these studies, it was determined that D_1 receptors are present in the salivary glands of *R. sanguineus* and *D. variabilis* ticks.

THE FEEDING BEHAVIOR of a tick plays a vital role in its life cycle and through the feeding processes, can cause damage to their host (Binnington & Kemp 1980, Waladde & Rice 1982). The success and longevity of a tick depends on its ability to find a suitable host and feed to completion. The amount of time required for tick engorgement is related to

the adeptness of the tick in the secretion of saliva and imbibement of blood and tissue fluids (Sweatman & Gregson 1970). The formation of saliva in the tick salivary glands is necessary to complete the feeding processes (Sauer 1977). Because of the role the salivary gland plays in successful feeding behavior, it is important to fully understand its complete function.

The tick salivary gland is a remarkably versatile organ. It is responsible for secreting cement that secures the mouthparts during feeding (Moorhouse & Tatchell 1966, Moorhouse 1969, 1973; Whitwell 1978), anticoagulants that prevent the host blood from clotting (Balashov 1972, Kemp et al. 1982) and prostaglandins that are believed to increase the blood flow to the mouthparts (Tatchell & Binnington 1973, Dickinson et al. 1976, Higgs et al. 1976) and also suppress the host immune response (Ribeiro et al. 1992, Ramachandra & Wikel 1992). The salivary glands also concentrate the imbibed blood meal by secreting excess water and ions from the hemolymph (Tatchell 1967, 1969; Kaufman & Phillips 1973, Sauer & Hair 1972, Hsu & Sauer 1975, Kaufman & Sauer 1982), as well as take in water from saturated air during periods of diapause or molting (Knulle & Rudolph 1974, McMullen et al. 1976, Giath 1979). The salivation process of ticks is necessary in order for the tick to successfully take a blood meal and molt to the next feeding stage.

Fluid secretion is regulated by dopaminergic nerves at the neuroeffector junction (Megaw & Robertson 1974, Megaw 1977). Dopamine has been shown to be a potent agonist of these nerve receptors (Sauer et al. 1974, Kaufman 1976, 1977; Needham & Sauer 1975, 1979; Schmidt et al. 1982, Hume et al. 1984). Convincing evidence has established that dopamine activates a D_1 receptor on the plasma membrane of the cell (Schmidt et al. 1981). The receptor facilitates the activation of adenylate cyclase and cyclic AMP (cAMP) dependant protein kinase which increases the level of cAMP in the tick salivary gland (McSwain et al. 1985). Cyclic AMP is critical in the control of salivary gland function and therefore participates indirectly in the ability of ticks to feed successfully (Sauer & Essenberg 1984).

The impact of dopamine on the dopamine receptor site and its effect on the feeding processes of Dermacentor variabilis (Say) and Rhipicephalus sanguineus (Laetrille) was assessed through in vitro studies by using the Competitive Protein Binding Assay. To further support the evidence of dopaminergic control of fluid secretion in tick salivary glands, in vivo studies were also initiatied. One phase involved the observation of changes occurring in the electrical potential of the fluid being exchanged between feeding female ticks and their host after the injection of dopamine into the hemocoel of each tick. These measurements were obtained through the use of EMS described in Chapter I

(pg. 4). The impact of dopamine was also ascertained through the collection of fluid secreted after salivary gland stimulation via the injection of dopamine into the hemolymph of partially fed females removed from their host.

The Competitive Protein Binding Assay was selected because of its ability to quantitate the increase of cAMP levels stimulated by the presence of dopamine. With this assay, the ability of dopamine to increase the cAMP levels in the salivary glands of D. variabilis and R. sanguineus was determined and further supported by evidence obtained from the feeding electrograms and the collection of salivary fluid after in vivo stimulation by dopamine.

Materials and Methods

Salivary Gland Tissue Collection. D. variabilis and R. sanguineus were reared according to procedures described by Patrick and Hair (1975). Adult D. variabilis and R. sanguineus ticks were allowed to feed and sexually mature on an ovine host. Females were allowed to mate and partially engorge before being used in the study. Females used for the protein binding assay and for collection of fluid secretions were removed by traction and individual weights were recorded immediately before use. All females that were monitored were allowed to remain on the host to continue feeding during the monitoring process.

Cyclic AMP assay. The standard procedure of this assay is

based upon the competition between unlabelled cAMP and a known amount of tritum labelled cAMP ([^{3}H]cAMP) for a receptor subunit of cAMP dependent protein kinase. The amount of labelled cAMP protein complex formed is inversely related to the amount of unlabelled cAMP present in the assay sample. The concentration of labelled cAMP was determined by a liquid scintillation counter. From this, a standard curve was projected.

The salivary glands were dissected from mated, partially engorged D. variabilis and R. sanguineus females by removing the dorsum and flooding the ticks hemocoel with 20 mM morpholino-propane-sulphonic acid (MOPS)-buffered saline (TS/MOPS) (pH 7.0), as described by Needham and Sauer (1975). The left glands served as the control while the right glands were exposed to dopamine.

After the salivary glands were extracted, the control glands were left in the TS/MOPS solution while the experimental glands were introduced into a buffer solution containing 1% dopamine, 1% DMSO, and 2% theophylline for 5 minutes. Theophylline potentiates the affects of low levels of dopamine through the inhibition of cAMP-dependent phosphodiesterase, the enzyme that inactivates cAMP (Sauer et al. 1979).

After the allotted time, all glands were removed from the TS/MOPS solutions and then put into a final stop solution containing 50 μM Tris buffer solution and 4 μM EDTA (to

prevent enzymatic degradation of cAMP by phosphodiesterase). Both the control and experimental glands were homogenized by sonication to rupture the individual cell membranes, followed by heating for three minutes in a boiling water bath to stimulate protein coagulation.

After protein coagulation, the glands were then removed and centrifuged at 15,000 g for 15 min, after which the cAMP in the supernatant was collected and assayed.

A cAMP assay kit (Code TRK.432) was obtained from the Amersham Corporation and the procedures followed were those described by McSwain et al. (1992). Briefly, 50 μl cyclic [^{3}H]AMP, 100 μl cAMP-binding protein, and 50 μl supernatant were used in the reaction volume. The tubes were incubated at 4°C for 2 hr. After the allotted time, 100 μl of charcoal suspension was added to separate the protein bound cAMP from the unbound nucleotides (Brown et al. 1971). Tubes were then centrifuged for 3 min at 15,000 g. The supernatant (200 μl) was removed and placed in Biocount liquid scintillation cocktail. The concentration of cAMP in the unknown was determined by comparison with the linear standard curve that was generated with each assay.

Collection of Salivary Secretions. Partially fed female D. variabilis and R. sanguineus were removed by traction from ovine hosts and saliva was collected by methods described by Tatchell (1967) and McSwain et al. (1992). Each female was weighed and taped (dorsum down) to a small

petri dish. Saliva was collected into 25 µl micropipettes that had been fitted over the mouthparts of each female. Each micropipette was calibrated at 5 µl increments to aid in the measurement of the fluid secreted by the tick, collected in the micropipette. Dopamine was diluted to a 1 mM concentration by dissolving in 3% DMSO, mixed with 20 mM TS/MOPS (described by Needham & Sauer 1975) Ten µl of this solution were injected into the hemocoel of each female with a 10-µl Hamilton syringe. Injections were repeated every 15 minutes for a total time interval of 1 h and saliva secretion was measured and recorded at each 15 min interval.

Electronic Monitoring of Feeding Female Ticks. The electronic monitoring devices used in the course of this research phase were the same as those previously described in Chapter I (pg. 4). The procedures used in the maintenance and care of the sheep were also the same as those described in Chapter I (pg 3).

Two orthopedic cotton stockinette cells were affixed to an ovine host using industrial adhesive and cells were numbered for identification purposes.

The sheep host was infested with ten pair of adult D. variabilis in cell #1 and ten pair of R. sanguineus in cell #2. Females were allowed to mate and partially engorge. Each female was then connected to the EMS using procedures described in Chapter I, and were monitored for 10 min before being injected with a 1 mM dopamine solution (described in

the salivary fluid collection procedure above). Ten μl of the dopamine solution was injected into the hemocoel of the feeding female at four 15 min intervals, during which time, electric recordings of fluid exchange were recorded. The injection time was noted by an asterisk on each of the electrograms to aid in identification of any change in conductivity resulting from the injection.

Results

The Effect of cAMP on Fluid Secretion. To determine the effect of dopamine on the D_1 receptor linked to adenylate cyclase, isolated salivary glands of female D. variabilis and R. sanguineus ticks were bathed in a buffer solution containing dopamine. Through the use of the Competitive Protein Binding Assay, it was shown that dopamine (1%) stimulated a significant increase in cyclic AMP in D. variabilis ($P = 0.0405$) and R. sanguineus ($P = 0.0029$), according to a paired-t test (Table 2).

In D. variabilis, the average difference seen between the dopamine stimulated gland and the control gland was 0.653 ± 0.296 pmole cAMP per gland. The overall increase in cAMP for D. variabilis was 31.40%. R. sanguineus demonstrated an average difference of 0.887 ± 0.266 pmole cAMP per gland between the dompamine stimulated and control glands. The overall increase in cAMP for R. sanguineus was 45.02%. The standard deviation is large, but this is most likely due to

the fact that not all of the glands exposed to dopamine had an increase in salivation. This caused the range of the data to be large as well. A significant difference in the overall increase of cAMP in response to dopamine between D. variabilis and R. sanguineus was not found (Table 1).

Salivary Fluid Collection. Partially fed female ticks were stimulated to secrete salivary fluid after being injected with 10 μl of a 1% dopamine solution. The weight of each female was determined before glandular stimulation and a linear regression was performed to compare weight to the total volume of fluid secreted (Tables 3 & 4). A significant correlation of weight versus total volume of fluid secretion was not found in D. variabilis ($P = 0.2798$), but was found in R. sanguineus ($P = 0.0001$). A regression was also performed using a dummy variable to determine whether or not the slopes of the two lines were equal. It was found that the slope of D. variabilis did not equal that of R. sanguineus ($P = 0.0204$).

The average volume of saliva collected from each D. variabilis female was 12.25 μl and the average volume of salivary fluid collected from R. sanguineus females was 3.60 μl.

The Effect of Dopamine on Feeding Electrograms. Through the use of electronic monitoring systems, the conductivity of fluid exchange between the feeding female ticks and their host were recorded. Normal feeding patterns established

before the injection of dopamine were most typically altered immediately after injection. After the injection, however, a increase in fluid conductivity was typically seen and the patterns demonstrated were those of salivation, similar to those described in Chapter I (Figs. 18 & 19). Some ticks, however, did not demonstrate a drastic response to dopamine. These ticks were monitored continuously as dopamine was injected at 15 min intervals up to 45 min after the first injection.

Discussion

The occurrence of cyclic AMP in salivary glands and its role in the function of fluid secretion in ixodid ticks is well documented. There is a D_1 receptor located on the salivary gland plasma membrane (Schmidt et al. 1981). When dopamine binds to this receptor, the adenylate cyclase system (ACS) is stimulated (Schmidt et al. 1981, 1982). The activation of this system causes ATP to be converted to cyclic AMP. The increase in cAMP levels activates the cAMP-dependent protein kinase that begins the phosphorylation of proteins (Needham & Sauer 1975). The build up of phosphorylated proteins aids in the facilitation of fluid transport. The overall result is fluid secretion (McSwain et al. 1985).

The results from this study indicate the presence of cAMP in the salivary glands of D. variabilis and R. sanguineus

through the use of the Cyclic AMP Competitive Protein Binding Assay. This leads to the conclusion that dopamine receptors, most likely of the D_1 subtype, are present and involved in the control of salivary fluid secretion.

Further evidence of dopaminergic control of fluid secretion was also demonstrated through *in vivo* studies by the simple collection of salivary fluid and through the alterations of feeding electrograms after dopamine stimulation via the injection of dopamine into the hemocoel of partially engorged or feeding females.

A significant correlation was observed between the weight of *R. sanguineus* females and the level of fluid secretion after dopamine stimulation. Sauer et al. (1979) found that the weight of the tick and salivary gland fluid stimulation in *A. americanum* were highly correlated. This correlation was not, however, seen in *D. variabilis* females. Several factors could be responsible for these results. One factor could be the initiation of salivary gland degeneration within those females greater than 200 mg that were most likely close to repletion. Further investigation in the stimulation of fluid secretion of *D. variabilis*, however, is needed to determine why a correlation was not found.

It was observed throughout the study that the level of cAMP (pmole/gland) in the control glands were higher than those levels seen in the experimental glands after exposure to dopamine. It was also demonstrated in the *in vivo*

studies that the injection of dopamine did not always result in fluid secretion (Table 3 & 4). These findings may be explained by the presence of another dopamine receptor on the salivary gland membrane.

It has been thought that there is a more complex system involved in dopaminergic control of fluid secretion than the simple explanation of a single dopamine receptor. In 1981. Wong & Kaufman potentiated the effects of dopamine on the fluid secretion of isolated salivary glands through the use of spiperone. Lindsay and Kaufman (1986) proposed two models in the explanation of salivary fluid secretion. The first model suggested that the dopamine receptor is allosterically stimulated by spiperone to increase the rate of fluid secretion. The second model involves the inhibition of dopamine by spiperone, a dopamine D_2 receptor antagonist, through a putative inhibitory pathway. Results show that the activation of a dopamine D_2 receptor, in the absence of a D_2 antagonist, can inhibit adenylate cyclase and decrease cAMP levels (Andersen et al. 1990). This would decrease fluid secretion.

The presence of another receptor on the salivary gland membrane would explain why dopamine did not demonstrate a stimulatory effect on fluid secretion in some of the glands. Another possibility could be due to the fact that the receptors on the salivary gland membrane had already been stimulated by indogenous dopamine and were in a "refractory"

state, therefore, being unable to respond. Evidence of additional dopamine receptors, however, needs to be further investigated. The evidence for a dopamine D_1 receptor presented in this report will help to provide the foundation for further studies involving the regulation of salivary control in D. variabilis and R. sanguineus ticks.

CHAPTER III

SUMMARY

Normal feeding patterns of Amblyomma americanum (L.), Dermacentor variabilis (Say), and Rhipicephalus sanguineus (Latreille) were established through the use of electronic monitoring systems. Each female was monitored for a total time of 30 minutes while attached and feeding on an ovine host.

All electrograms were quantified over time for each species. Four basic feeding types were expressed within each species, and deviations (subtypes) of each feeding pattern were determined. After all patterns were categorized, a comparison of pattern types was made between species.

Resting patterns, designated pattern Type I, were characterized by a flat or straight line with minor wave fluctuations. Two subtypes were defined in order to demonstrate the degree of variation that occurs within Pattern Type I between the species.

Sucking patterns (Pattern Type II) were characterized by an increase in conductivity with peaks varying in degrees of frequency and amplitude. Three subtypes were defined

according to the level of variation in frequency and amplitude of individual peaks.

Salivation patterns were designated as Pattern Type III. This pattern was observed to have major fluctuations in fluid conductivity with variations in frequency and amplitude of peaks. Two subtypes were assigned, depending upon level of conductivity and frequency, as well as amplitude of peaks.

Pattern Type IV, expulsion of saliva with pool of saliva formation, was depicted by a sudden increase in conductivity, a plateau demonstrating the continuing electrical potential of fluid within the hypostome, and a gradual decrease in conductivity back to the baseline. Four subtypes of this pattern were determined by the initial increase in conductivity and the length of the plateau within the pattern.

After all recordings were quantitified, comparisons were made on the day to day activities expressed by each species. It was found that A. americanum spent a significantly more amount of time salivating in comparison with the other two species. This supports the hypothesis that A. americanum spends more time secreting unwanted fluids back into its host, possibly concentrating the imbibed blood meal to a greater degree. Another possibility for the increased salivation activity, however, could be attributed to an increased number of receptors on the salivary gland

membrane. D. variabilis spent a significantly more amount of time feeding and R. sanguineus spent a significant amount of time resting. Further investigation, however needs to be initiated to determine possible physiological and/or environmental factors as to why these results were obtained.

After the establishment of the normal feeding patterns, through the use of EMS, it was demonstrated that these patterns can be altered by injecting dopamine into the hemocoel of the female as she feeds. Patterns observed before the injection demonstrated a combination of all four pattern types. After the injection, an increase in conductivity was observed with random variations in frequency and amplitude of peaks. This pattern resembled those depicted as salivation - Pattern Type III. These results provide evidence that helps to solidify the accuracy of Pattern Type III, as well as providing further evidence that dopamine stimulates fluid secretion in the salivary glands of ixodid ticks.

To further demonstrate the affects of dopamine on salivary fluid secretion, partially fed D. variabilis and R. sanguineus females were injected with dopamine and the volume of fluid secreted was measured. Results were somewhat surprising in the fact that D. variabilis secreted a significantly greater amount of saliva then did R. sanguineus. This was not indicated in the comparison of feeding rhythms. In fact, there was only a minor difference

in the amount of time these two species spent demonstrating Pattern Type III, and these differences were not significant. It was also determined that a linear relationship occurrs between female weight and total volume of fluid secreted in R. sanguineus. A correlation was not found in D. variabilis. This needs to be further investigated.

Another means of determining the effect of dopamine on salivary fluid secretion was carried out through the use of the Competitive Protein Binding Assay by measuring levels of cAMP. Through the use of this assay, it was demonstrated that dopamine stimulated fluid secretion in the isolated salivary glands of D. variabilis and R. sanguineus. The degree of stimulation was measured by the amount of tritiated cAMP present in the final aliquot after glandular exposure to dopamine. An increase of cAMP stimulates the adenylate cyclase pathway which leads to salivary fluid secretion. Therefore, the increased levels of cAMP in glands stimulated by dopamine demonstrate initiation of fluid secretion through a dopamine receptor on the salivary gland membrane.

From these results, several conclusions can be made: 1) distinct feeding patterns are expressed throughout the ixodid family, but several variations of these patterns exist, 2) the relative importance of each of these patterns to the completion of the feeding cycle differs from species

to species, 3) dopamine stimulates salivary fluid secretion *in vivo* and *in vitro* in the ixodid family, 4) a correlation between tick weight and volume of fluid secreted via dopamine stimulation exists, and 5) evidence of D_1 receptors on the salivary gland plasma membrane of *D. variabilis* and *R. sanguineus* has been established.

Results from this study need to be expanded upon. One such study would be to determine the effect of host specificity on the feeding patterns expressed between different species. Another area of investigation would be to determine the effect males have on the total feeding patterns exhibited. Other studies need to look at the effect of various pharmacological agents on the established feeding rhythms of several different species. Another area of study would be to determine whether or not it is an increased number of D_1 receptors on the salivary gland membrane of *A. americanum* that causes it to secrete larger volumes of fluid in comparison to the other species. Work also needs to be done to determine whether or not a D_2 receptor is present in the salivary glands of ixodid ticks. If D_2 receptors are found, then the control of the salivary processes of ticks will be better understood. If studies such as these can be initiated, a deeper insight into the mechanisms controlling feeding behavior will be gained and new methods of tick control can be established.

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APPENDIX: Tables and Figures

Table 1. Percent Occurrence for Each Pattern Type Within the Feeding Cycle of Adult Female *Amblyomma americanum* (L.), *Dermacentor variabilis* (Say), and *Rhipicephalus sanguineus* (Latreille).

Species	Pattern I	Pattern II	Pattern III	Pattern IV
A. amer.	$8.00_{a,A}$	$33.00_{a,B}$	$39.80_{a,B}$	$19.20_{a,C}$
D. var.	$38.00_{b,A}$	$33.17_{a,A}$	$14.67_{b,B}$	$14.17_{a,B}$
R. san.	$45.33_{b,A}$	$19.67_{b,B}$	$14.33_{b,B}$	$20.67_{a,B}$

A comparison was made to determine differences in the percent occurrence between species. These differences are represented vertically by the lower case letters. A comparison was also made to determine differences in the percent occurrence withing species. These differences are represented horizontally by the upper case letters.

Table 2. Levels of Cyclic AMP Observed in Isolated Salivary Glands of *Dermacentor variabilis* (Say) and *Rhipicephalus sanguineus* (Latreille) in Response to Dopamine.

Species	Average Diff. (DA - CTRL) pmole/gland	Observed Significance (*P* value)	Average Increase in cAMP (pmole)
D. var.	0.653 ± 0.296	0.0405	31.40%
R. san.	0.887 ± 0.266	0.0029	45.02%

Table 3. Volume of Fluid Secreted After Salivary Gland Stimulation by Dopamine via Injection into the Hemocoel of Partially Engorged *Dermacentor variabilis* (Say).

Weight (mg) of individual tick	Volume of Fluid Collected (μl)
33	9.5
35	7.0
52	13.0
59	5.5
66	15.0
76	19.5
80	15.0
97	8.5
101	16.5
133	21.5
147	23.5
331	0.0
514	14.0
529	3.0

A linear regression was run to determine whether or not a correlation existed between weight of the female and volume of fluid secrected. No correlation was found ($P = 0.2798$).

Table 4. Volume of Fluid Secreted After Salivary Gland Stimulation by Dopamine via Injection into the Hemocoel of Partially Engorged Rhipicephalus sanguineus (Latreille).

Weight (mg) of individual tick	Volume of Fluid Collected (μl)
32	3.0
33	0.0
34	0.0
34	1.5
34	2.0
37	0.0
40	2.0
44	3.0
45	4.0
50	6.0
54	2.5
76	7.0
98	10.0
104	10.0

A significant correlation was demonstrated between weight of the female and volume of fluid secreted ($P = 0.0029$).

Figure 1. The Major Electrical Constituents Comprising the Electronic Monitoring System (EMS).

ELECTRONIC MEASUREMENT SYSTEM

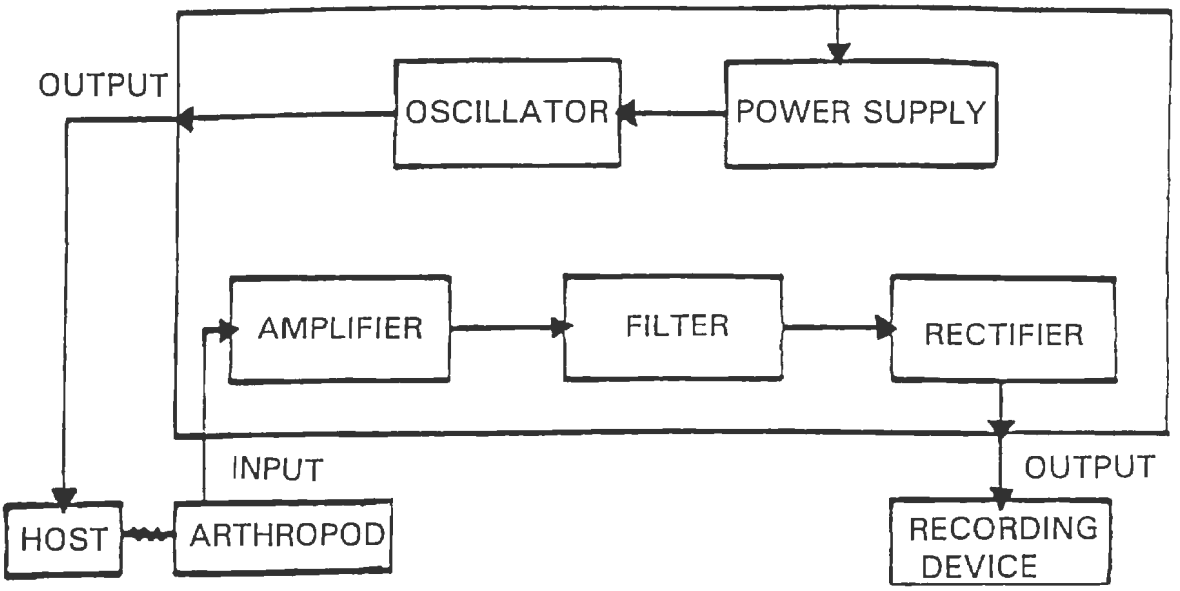


Figure 2. Schematic of the Adapted EMS Technique used for Monitoring Ticks While Feeding on Mammalian Hosts.

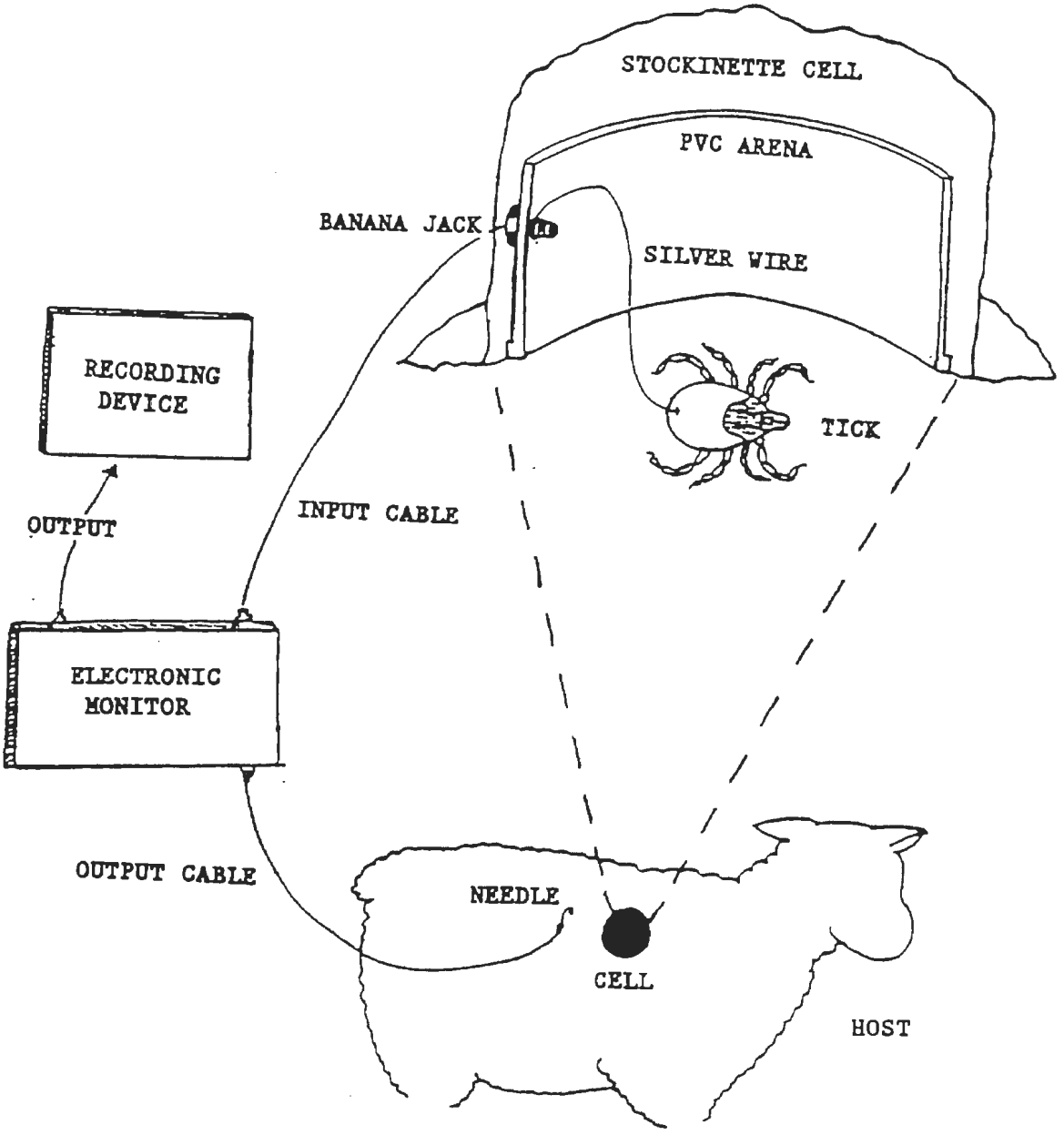
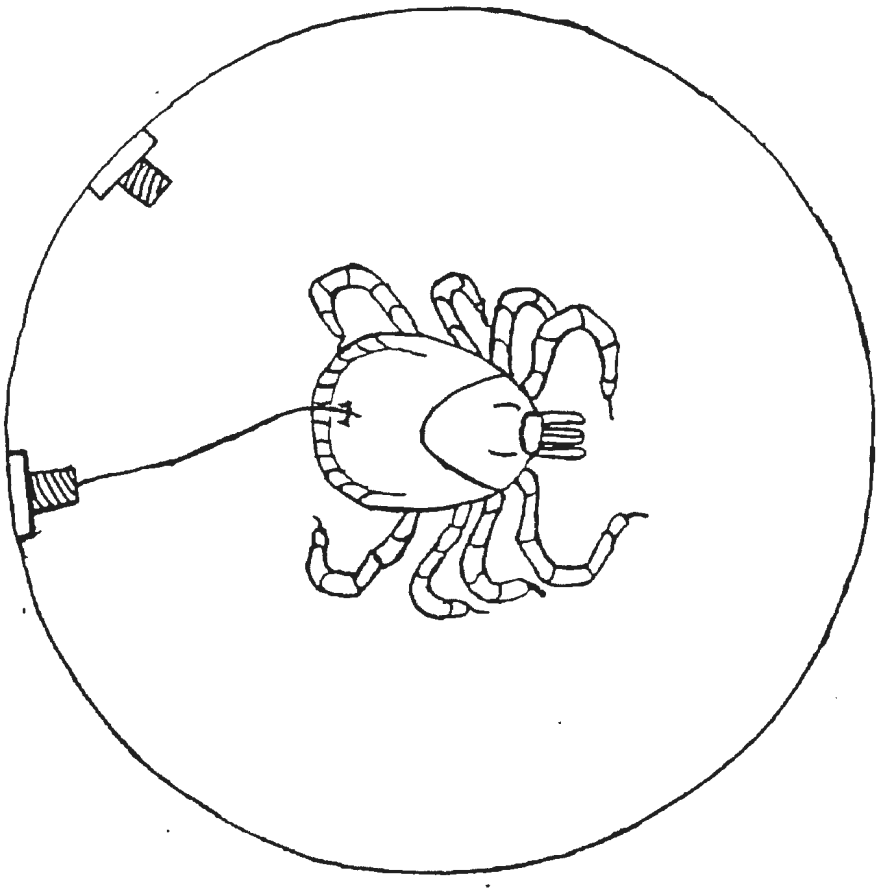

STOCKINETTE CELL
PVC ARENA
BANANA JACK
SILVER WIRE
RECORDING DEVICE
TICK
OUTPUT
INPUT CABLE
ELECTRONIC MONITOR
OUTPUT CABLE
NEEDLE
CELL
HOST

Figure 3. Illustration of Method for Insertion of Silver Wire into Abdomen of Tick.



(Tick shown apporximately 50X actual size)

Insertion of 0.1 mm silver wire
into posterior dorsum of tick.

Figure 4. Four General Pattern Types were Demonstrated by Feeding Female Dermacentor variabilis (Say), Amblyomma americanum (L.), and Rhipicephalus sanguineus (Latreille). Pattern Type I Demonstrates a Recording of Resting Activities; Pattern Type II Illustrates Sucking Activities; Pattern Type III Represents Salivation Phases; and Pattern Type IV Indicates the Expulsion of Saliva with Pool of Saliva Formation.

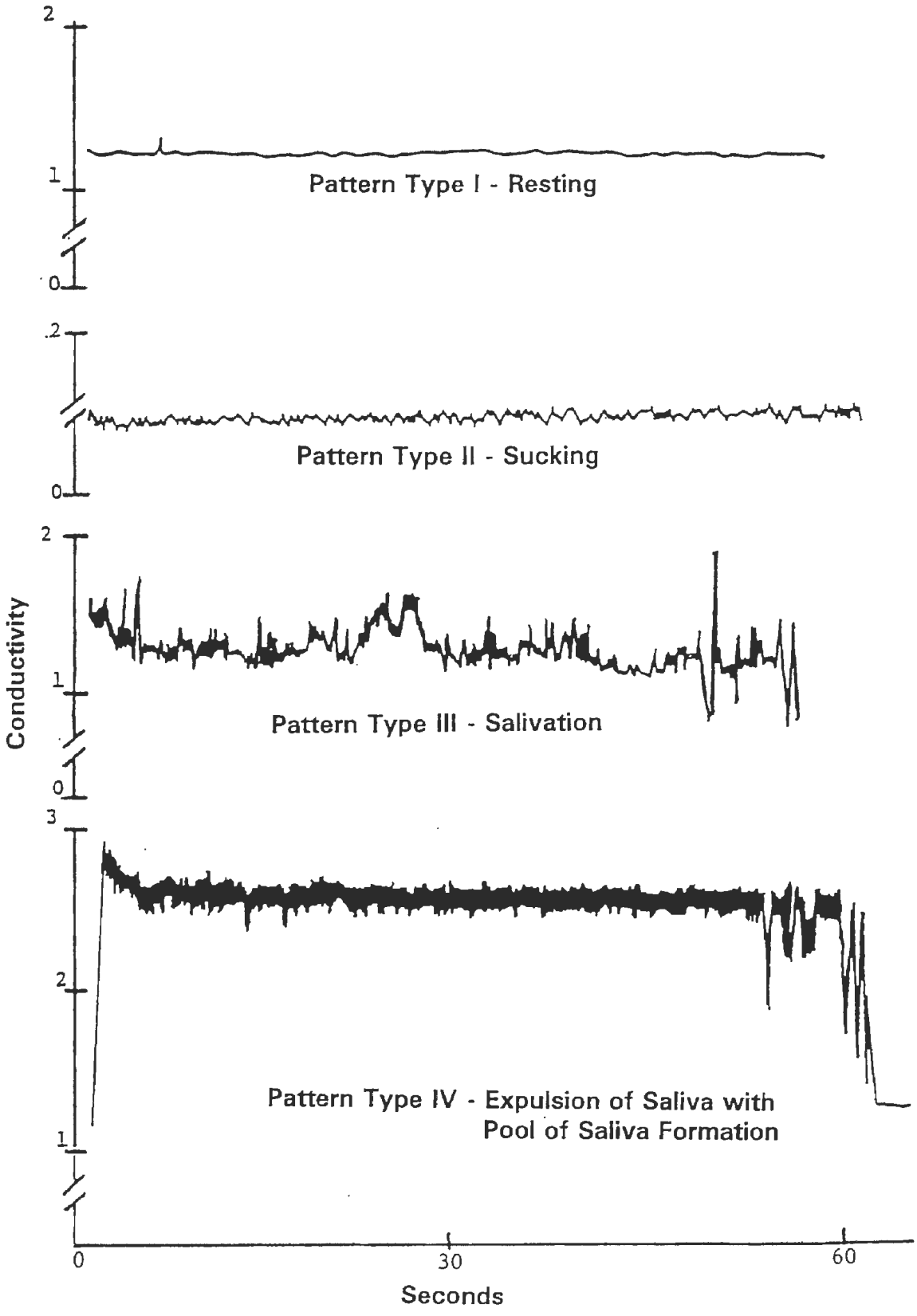

2
1
0
Pattern Type I - Resting
.2
0
Pattern Type II - Sucking
2
1
0
Conductivity
Pattern Type III - Salivation
3
2
1
Pattern Type IV - Expulsion of Saliva with
Pool of Saliva Formation
0
30
60
Seconds

Figure 5. Two Common Variations of Pattern Type I were Seen in Feeding Female Dermacentor variabilis (Say), Amblyomma americanum (L.), and Rhipicephalus sanguineus (Latreille). Pattern Subtype 1A Differs in the Frequency of Wave Peaks. Pattern Subtype 1B Differs in the Amplitude of Wave Peaks.

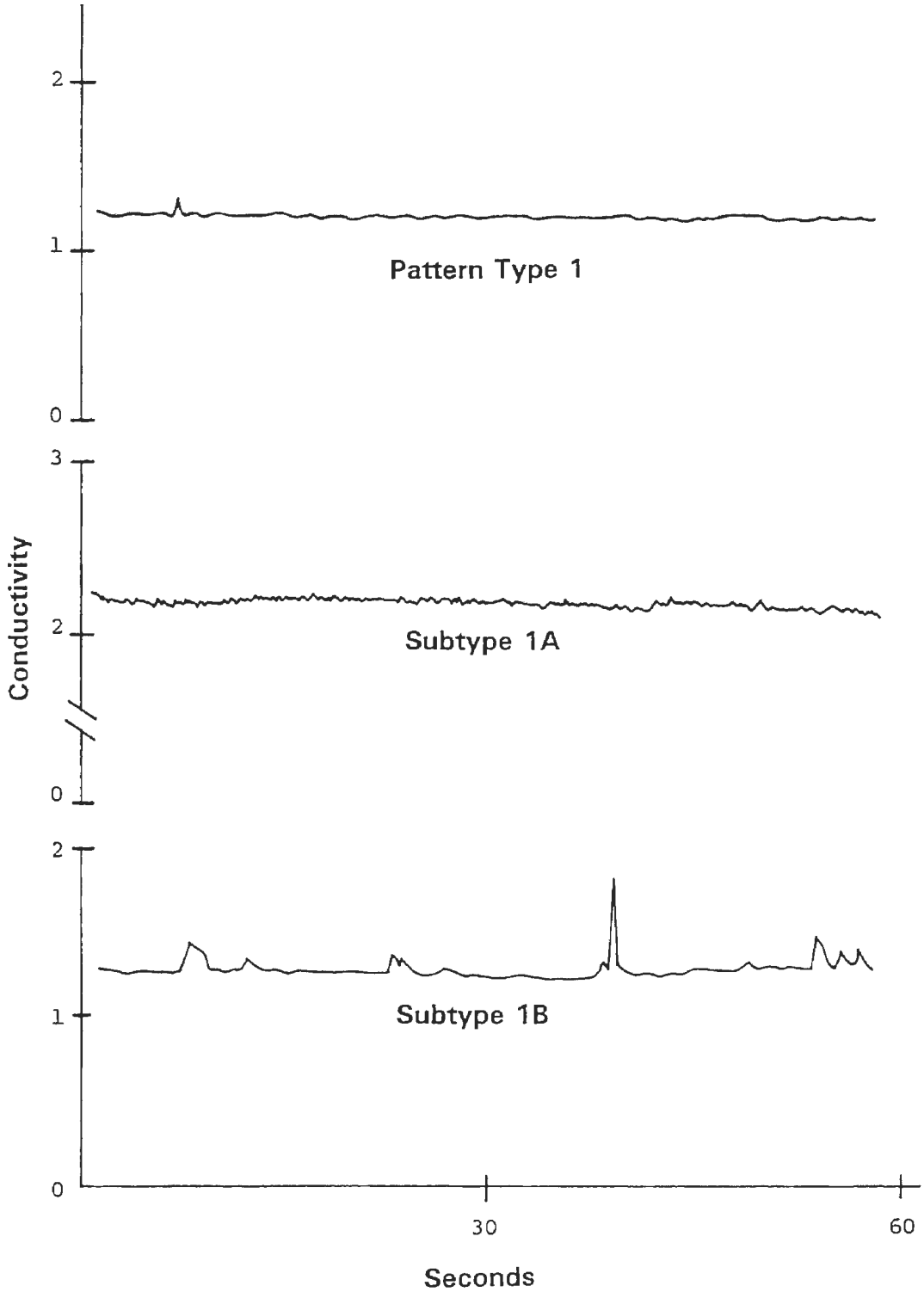

2
1
0
Pattern Type 1
3
2
0
Subtype 1A
2
1
0
Subtype 1B
Conductivity
30
60
Seconds

Figure 6. Three Common Variations of Pattern Type II were Seen in Feeding Female Dermacentor variabilis (Say), Amblyomma americanum (L.), and Rhipicephalus sanguineus (Latreille). Pattern Subtype 2A Differs in the Amplitude of Wave Peaks. Pattern Subtype 2B Differs in the Frequency of Wave Peaks. Pattern Subtype 2C Differs in the Frequency and Amplitude of Wave Peaks.

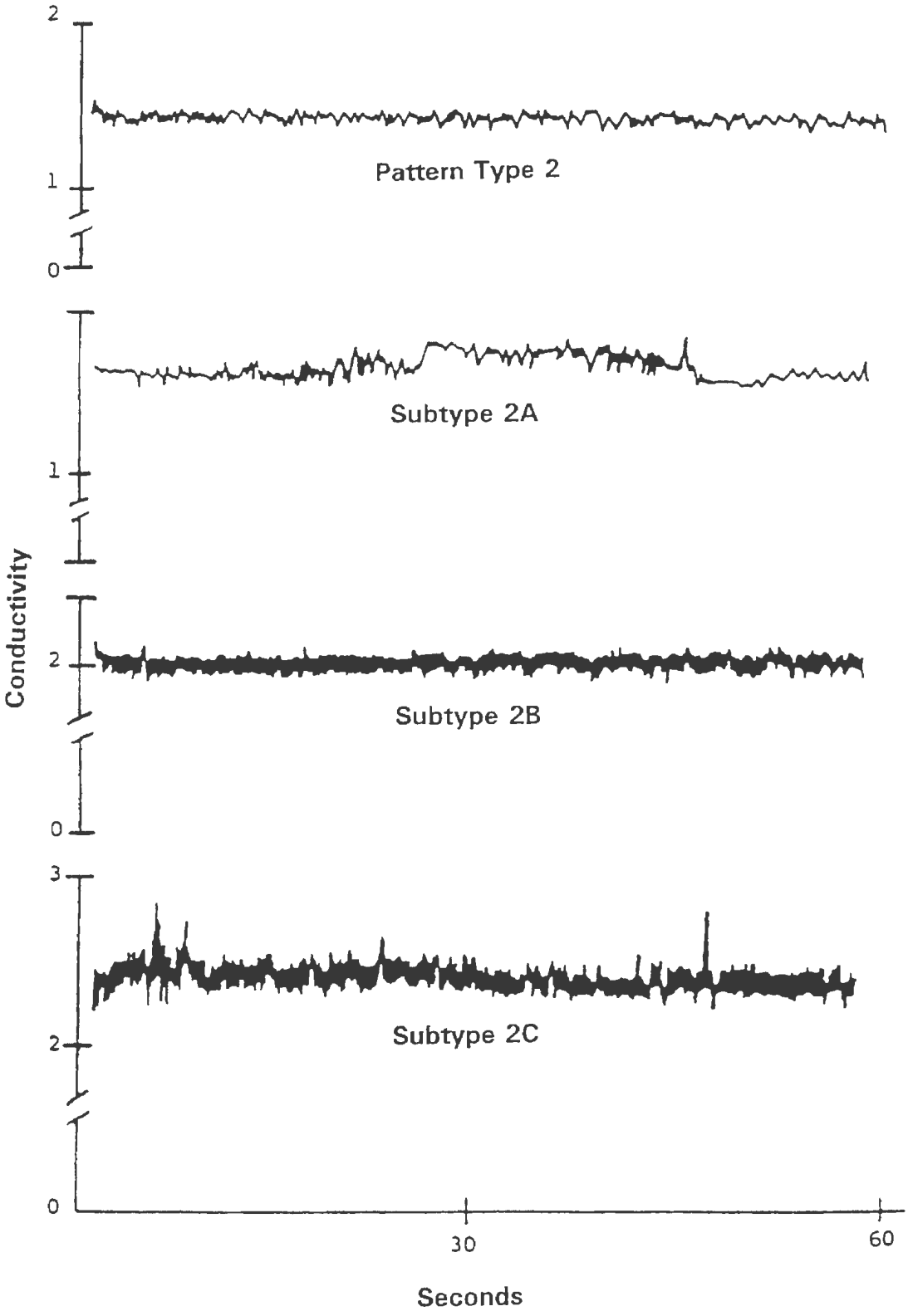

2
1
0
Pattern Type 2
1
Subtype 2A
2
0
Subtype 2B
3
2
0
Subtype 2C
Conductivity
30
60
Seconds

Figure 7. Two Subtypes of Pattern Type III were Identified in Feeding Female Dermacentor variabilis (Say), Amblyomma americanum (L.), and Rhipicephalus sanguineus (Latreille). Pattern Subtype 3A Differs in the Frequency of Wave Peaks. Pattern Subtype 3B Differs in the Amplitude of Wave Peaks.

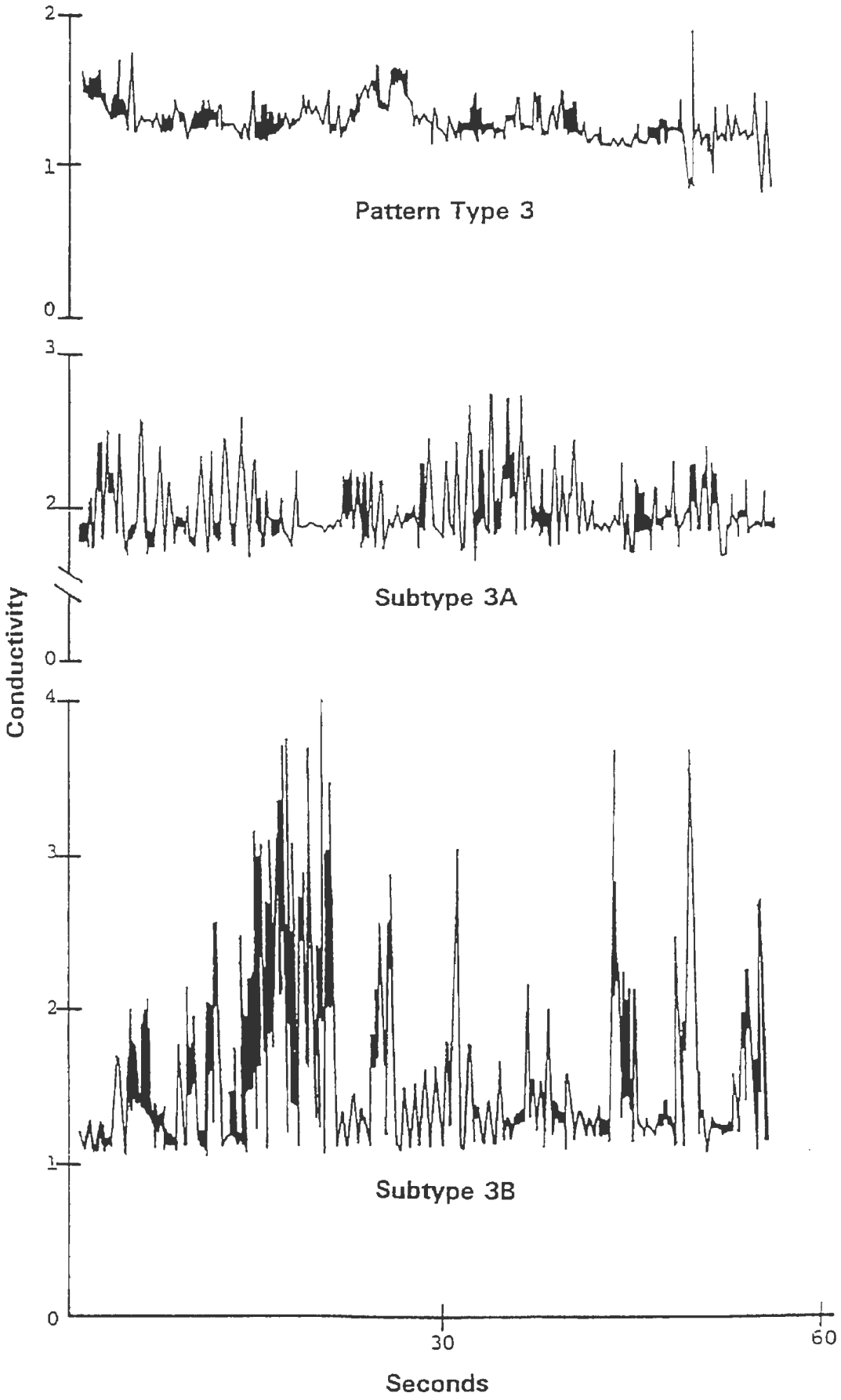

2
1
0
Pattern Type 3
3
2
0
Subtype 3A
Conductivity
4
3
2
1
0
Subtype 3B
30
60
Seconds

Figure 8. Four Subtypes of Pattern Type IV were Demonstrated in Feeding Female Dermacentor variabilis (Say), Amblyomma americanum (L.), and Rhipicephalus sanguineus (Latreille). Pattern Subtype 4A Differs in the Amplitude of Wave Peaks within Pool Formation and Total Length of Pool Formation.

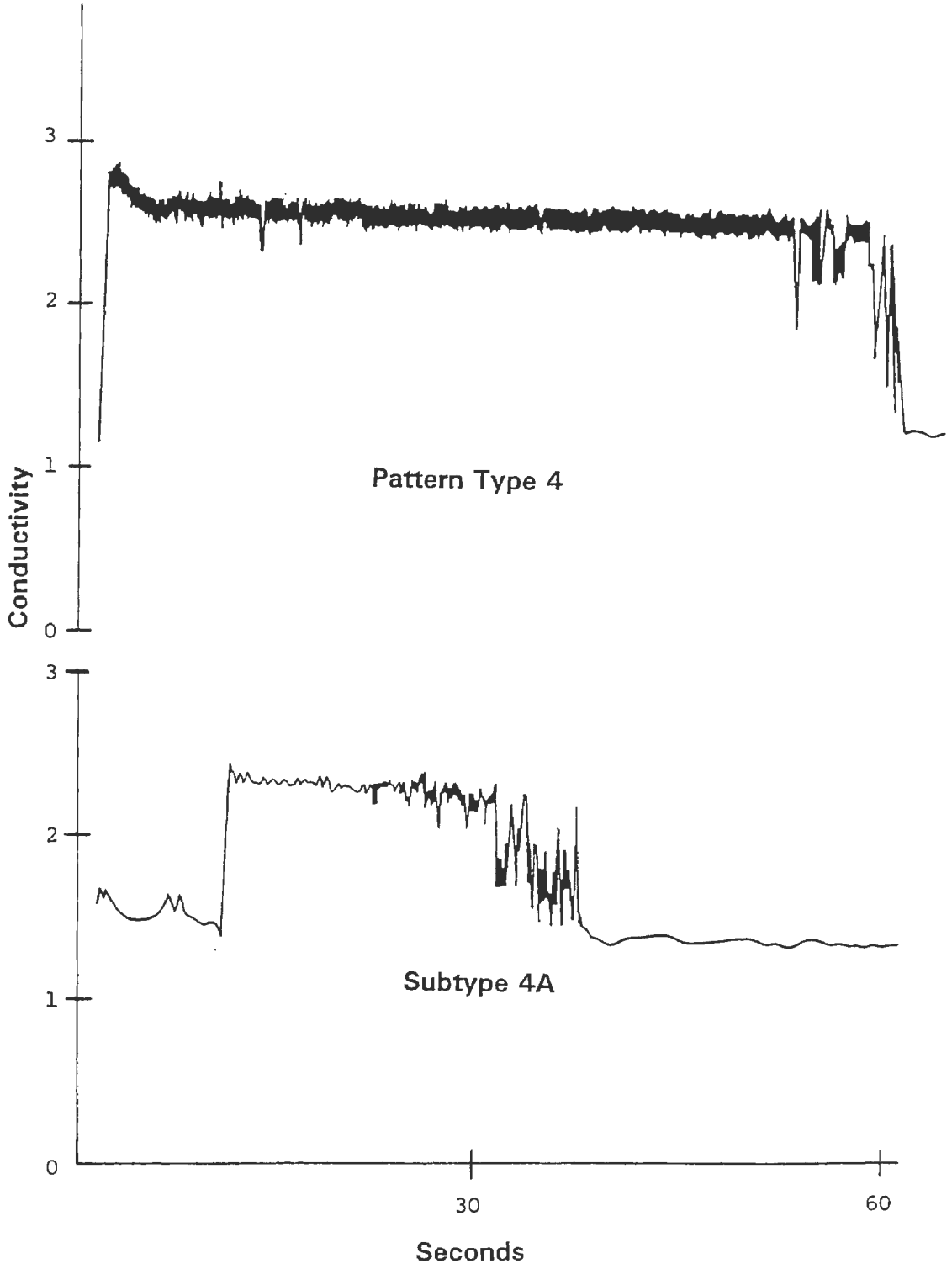

Conductivity
3
2
1
0
Pattern Type 4
3
2
1
0
Subtype 4A
30
60
Seconds

Figure 9. Continuation of Common Variations in Pattern Type IV Seen in Feeding Female Ticks. Pattern Subtype 4B and Pattern Subtype 4C Differ in the Amplitude of Wave Peaks within Pool Formation and Total Length of Pool Formation.

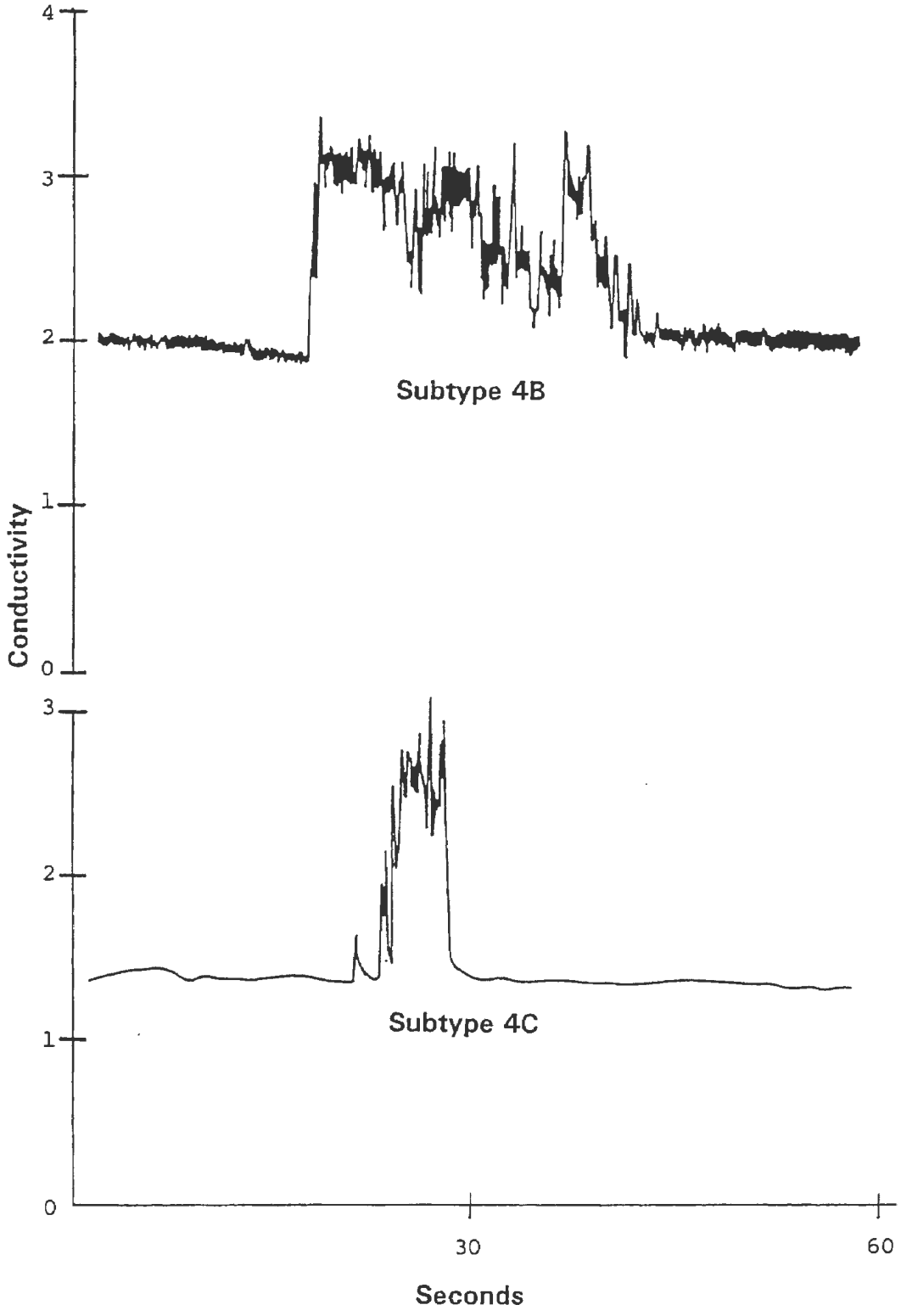

4
3
2
1
0
Conductivity
Subtype 4B
3
2
1
0
Subtype 4C
30
60
Seconds

Figure 10. Similarities in Pattern Types I and II of *Dermacentor variabilis* (Say) and *Rhipicephalus sanguineus* (Latreille) were Compared.

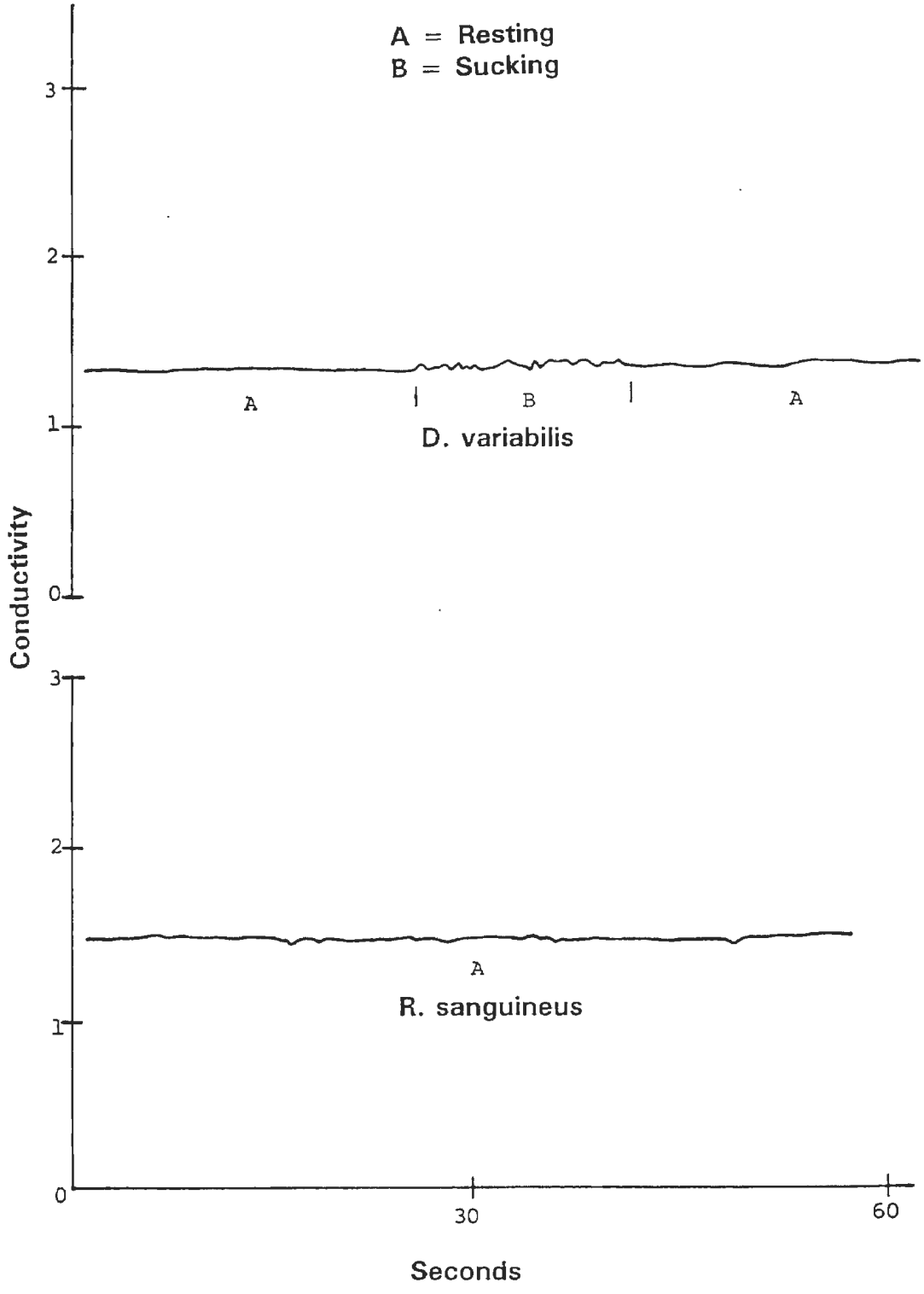

A = Resting
B = Sucking
3
2
1
0
A
B
A
D. variabilis
Conductivity
3
2
1
0
A
R. sanguineus
30
60
Seconds

Figure 11. A Comparison of Pattern Type III in Amblyomma americanum (L.), Dermacentor variabilis (Say), and Rhipicephalus sanguineus (Latreille).

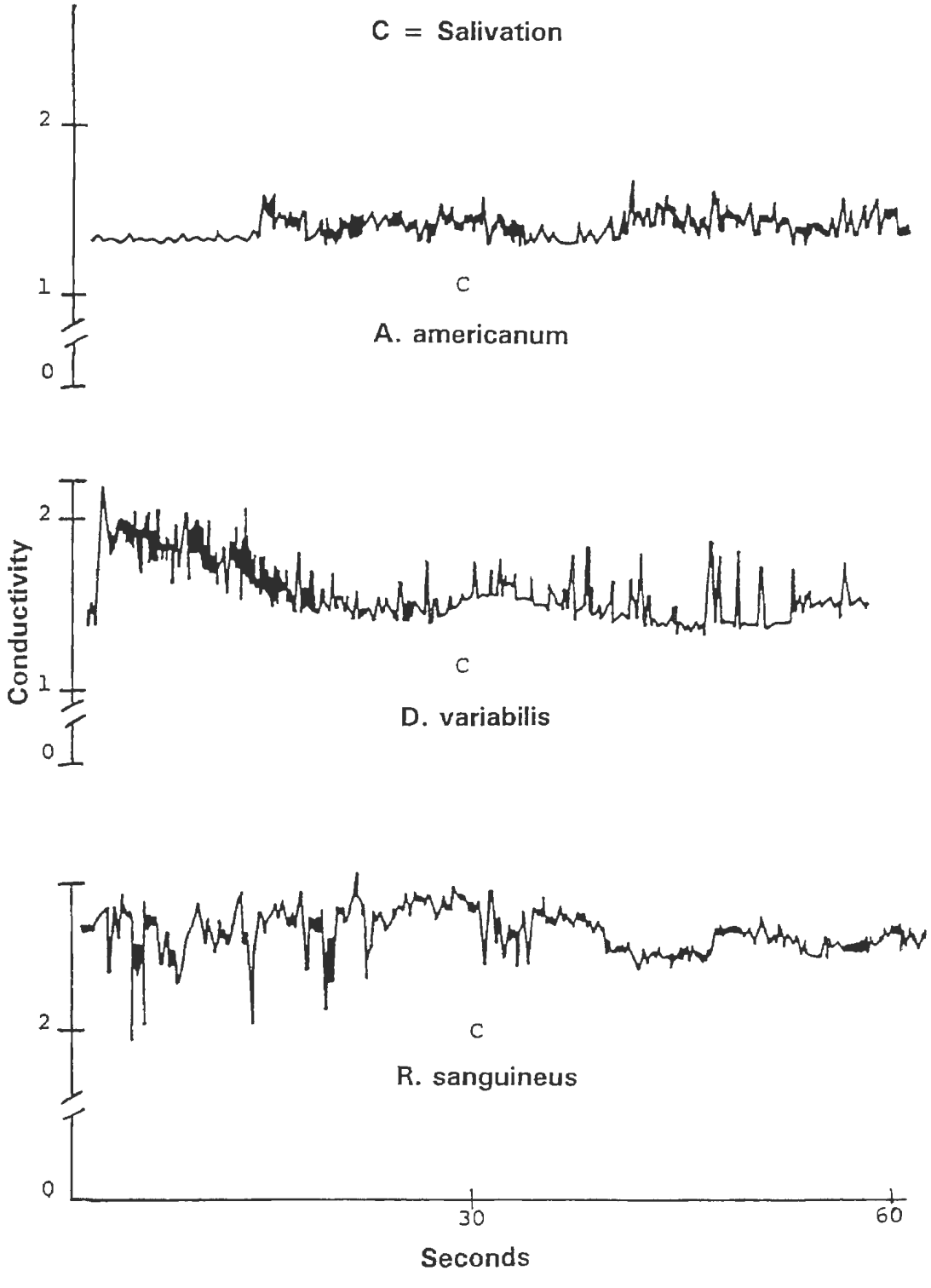

C = Salivation
2
1
0
C
A. americanum
Conductivity
2
1
0
C
D. variabilis
2
0
C
R. sanguineus
30
60
Seconds

Figure 12. A Comparison of Pattern Types I, II, III, and IV in Amblyomma americanum (L.), Dermacentor variabilis (Say), and Rhipicephalus sanguineus (Latreille) and the Relationships to Sequence During Feeding.

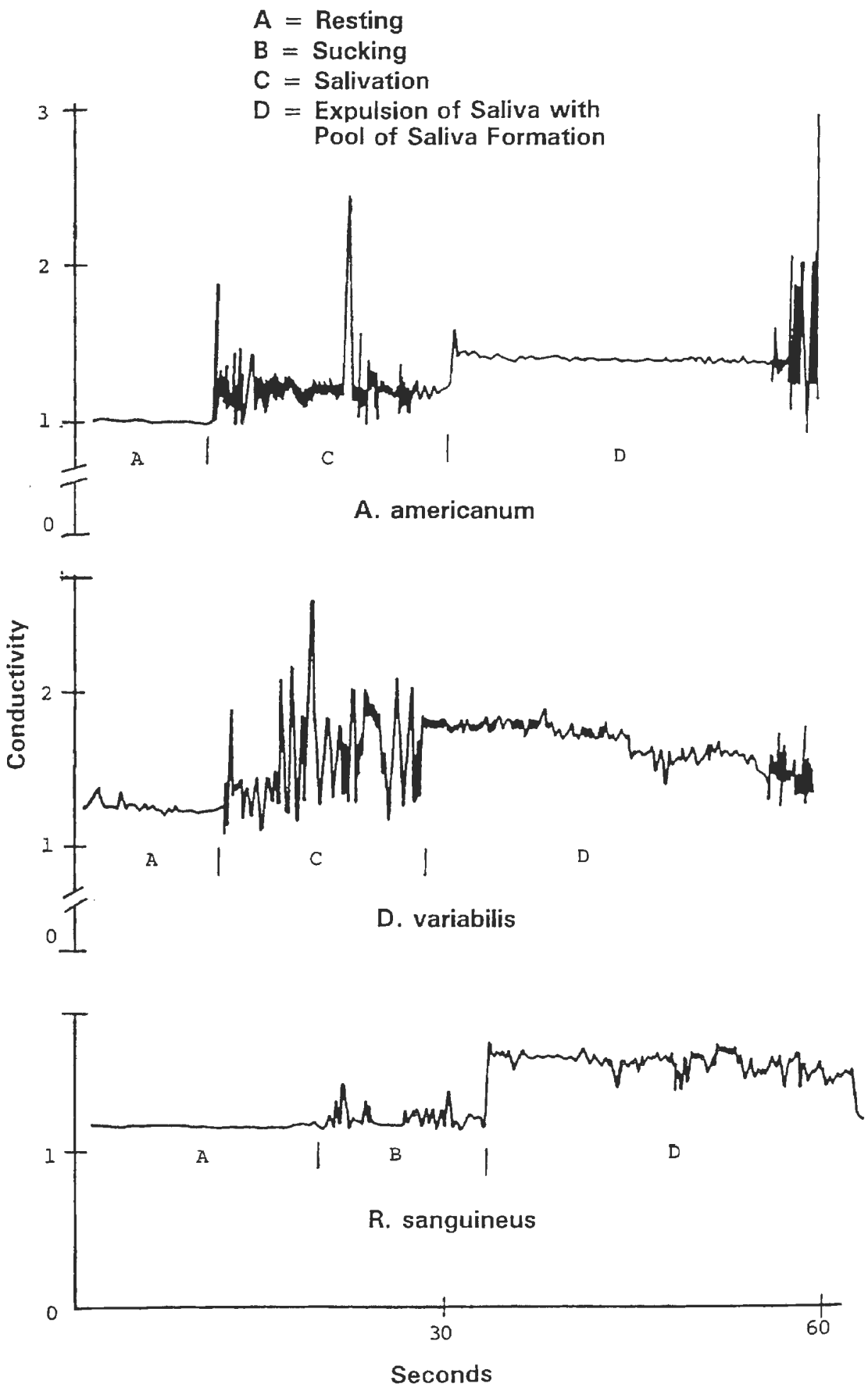

A = Resting
B = Sucking
C = Salivation
D = Expulsion of Saliva with Pool of Saliva Formation
Conductivity
3
2
1
0
A
C
D
A. americanum
2
1
0
A
C
D
D. variabilis
1
0
A
B
D
R. sanguineus
30
60
Seconds

Figure 13. Percent Daily Occurrence for Each Pattern Type Throughout the Feeding Cycle of Adult Female *Amblyomma americanum* (L.).

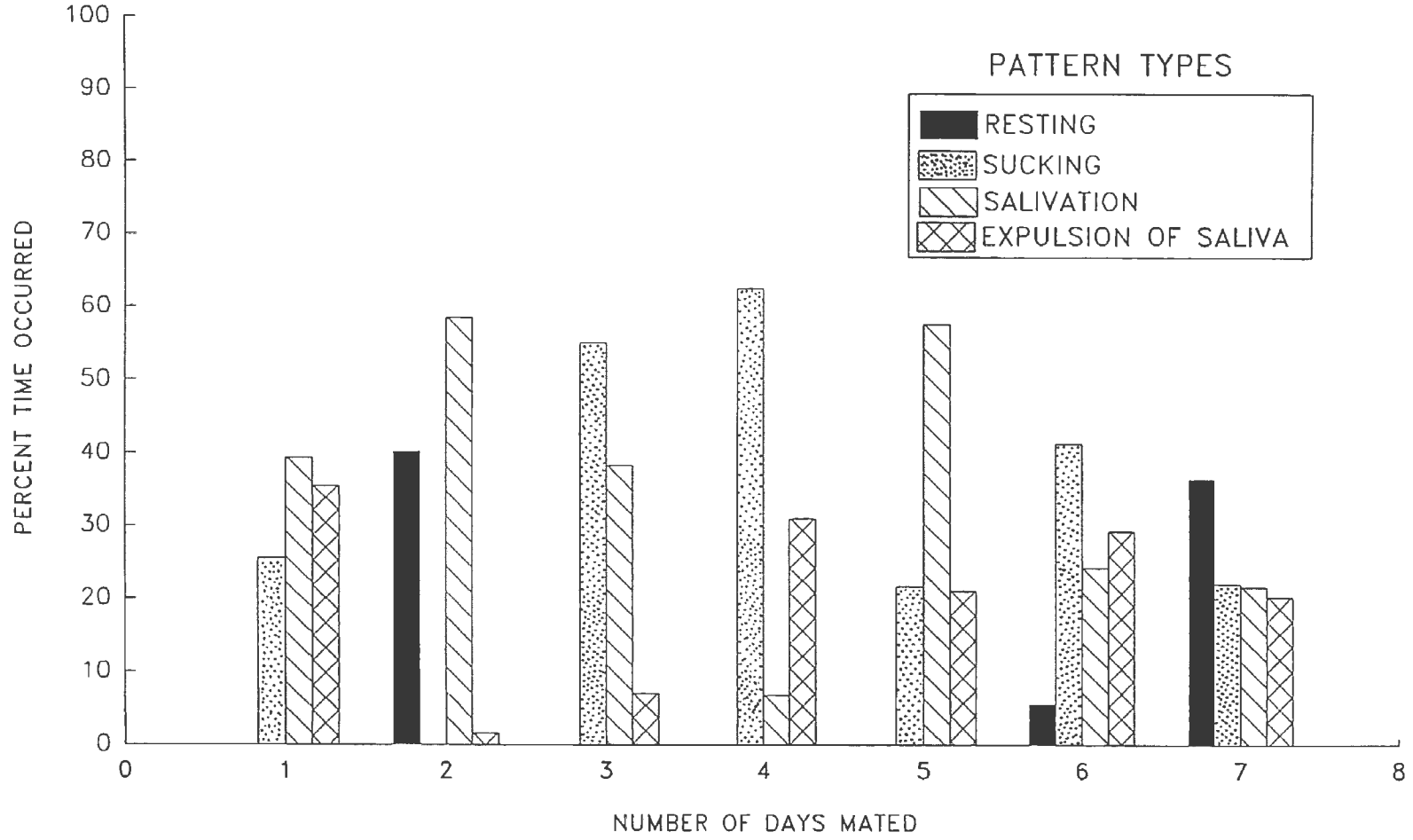

PATTERN TYPES
RESTING
SUCKING
SALIVATION
EXPULSION OF SALIVA
PERCENT TIME OCCURRED
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
NUMBER OF DAYS MATED

Figure 14. Percent Daily Occurrence for Each Pattern Type Throughout the Feeding Cycle of Adult Female *Dermacentor variabilis* (Say).

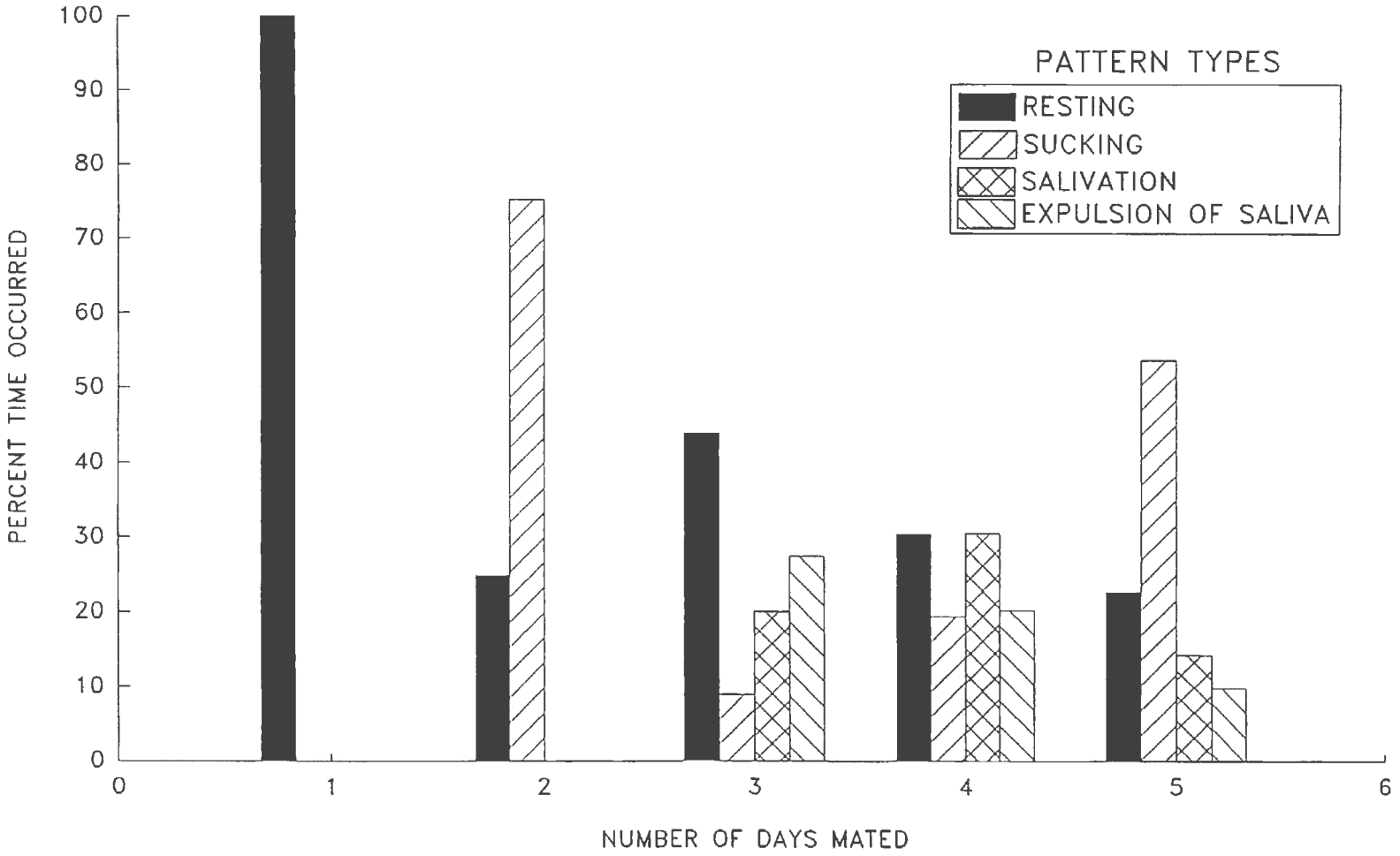

PATTERN TYPES
RESTING
SUCKING
SALIVATION
EXPULSION OF SALIVA
PERCENT TIME OCCURRED
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
NUMBER OF DAYS MATED

Figure 15. Percent Daily Occurrence for Each Pattern Type Throughout the Feeding Cycle of Adult Female *Rhipicephalus sanguineus* (Latreille).

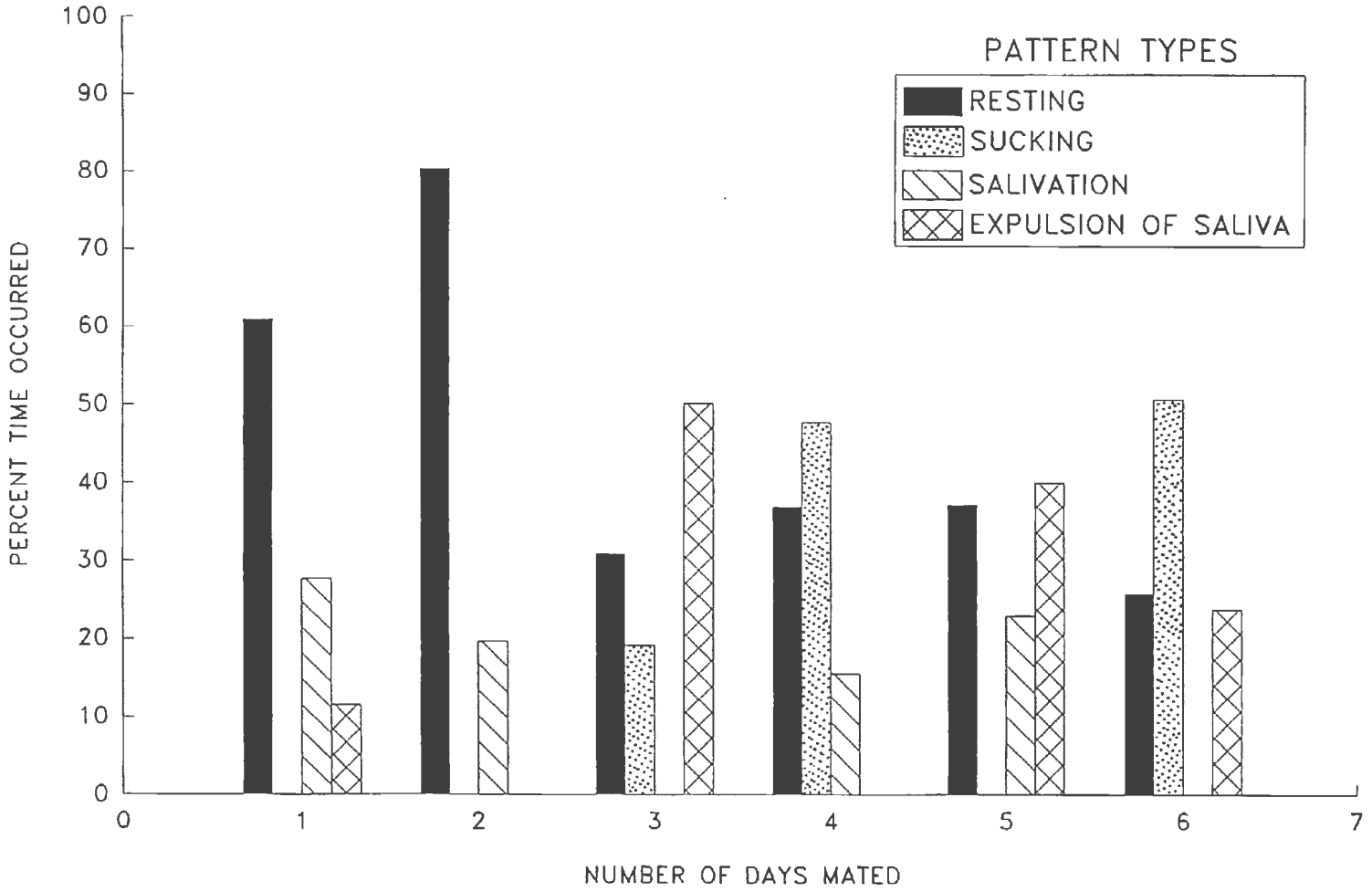

PATTERN TYPES
RESTING
SUCKING
SALIVATION
EXPULSION OF SALIVA
PERCENT TIME OCCURRED
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
NUMBER OF DAYS MATED

Figure 16. The Effects of Dopamine on the Normal Feeding Rhythms of Partially Engorged, Feeding Female *Dermacentor variabilis* (Say).

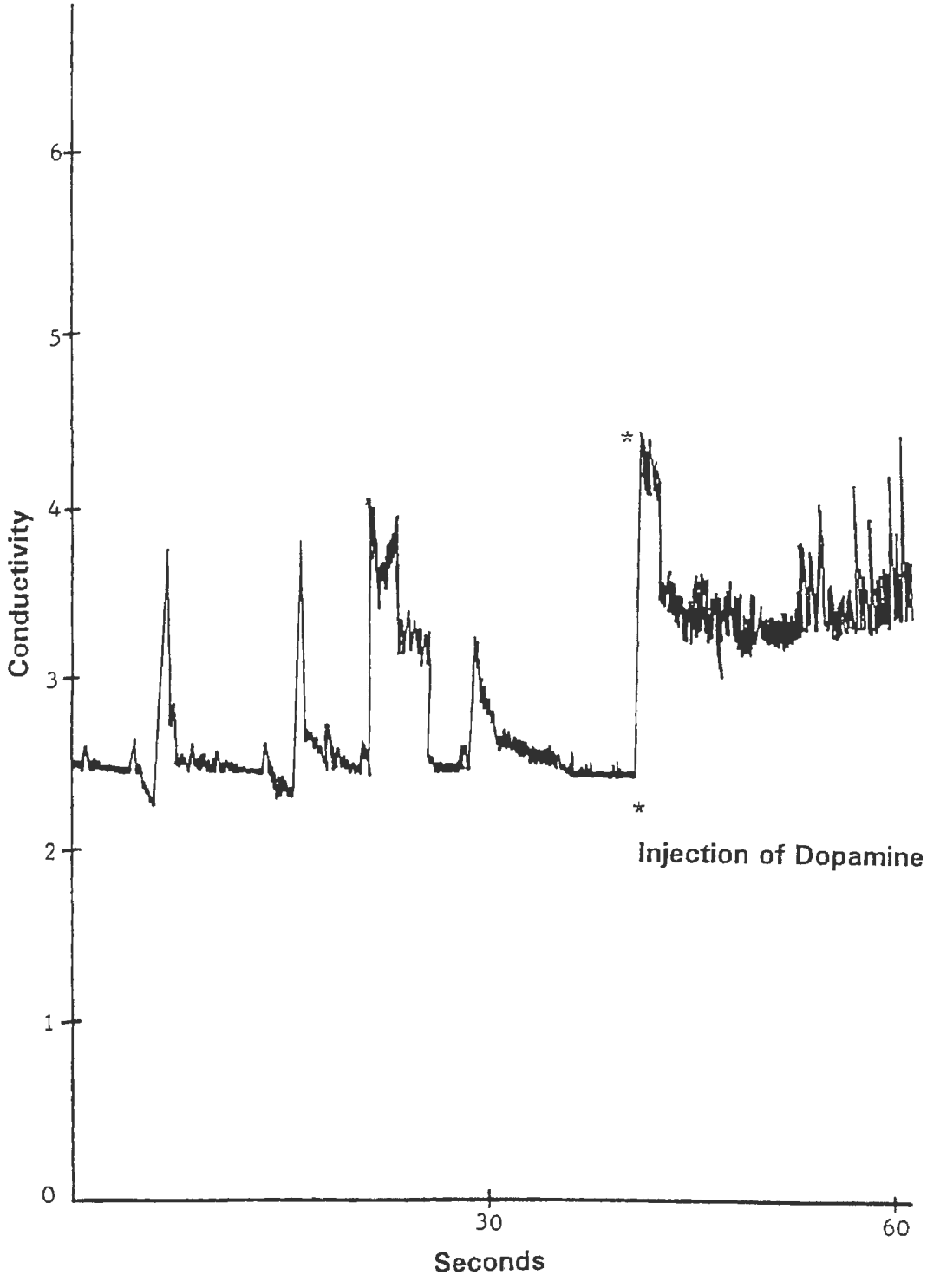
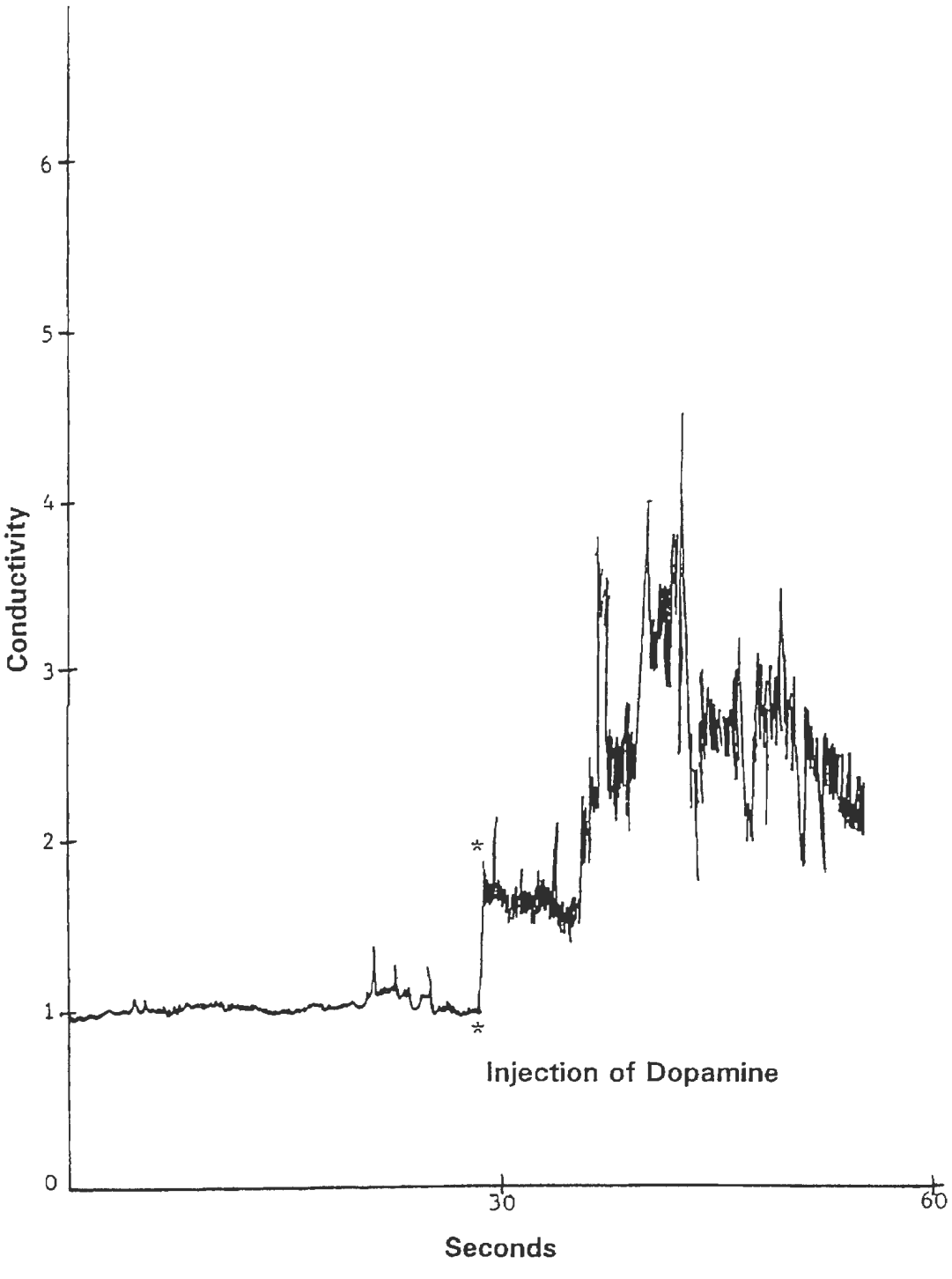

Conductivity
6
5
4
3
2
1
0
30
60
Seconds
*
*
Injection of Dopamine

Figure 17. The Effects of Dopamine on the Normal Feeding Rhythms of Partially Engorged, Feeding Female *Rhipicephalus sanguineus* (Latreille).


Conductivity
6
5
4
3
2
1
0
*
*
Injection of Dopamine
30
60
Seconds

VITA

Robin Diane Hickey

Candidate for the Degree of

Master of Science

Thesis: FEEDING ELECTROGRAMS AND SALIVARY FLUID SECRETION OF HARD TICKS (ACARI: IXODIDAE)

Major Field: Entomology

Biographical:

Personal Data: Born in Oklahoma City, Oklahoma, 16th March, 1968, the daughter of Jerry Lee Hickey and Ginger Hickey.

Education: Graduated from Edmond Memorial High School, Edmond, Oklahoma 1986; Bachelor of Science degree in Agriculture (Animal Science) from Oklahoma State University, May 1991; completed the requirements for the Master of Science degree at Oklahoma State University in May 1993.

Professional Experience: Laboratory assistant, Oklahoma State University Tick Research Laboratory, Department of Entomology, Stillwater, Oklahoma, 1989-1993; Teaching assistant, Oklahoma State University, Department of Entomology, Stillwater, Oklahoma, 1991-1993.

Professional Organization: Entomological Society of America