BIOSYSTEMATICS OF *NEOTOMA FLORIDANA* AND *NEOTOMA MICROPUS* IN OKLAHOMA

By

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BIOSYSTEMATICS OF NEOTOMA FLORIDANA
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CHAPTER I

INTRODUCTION

The two common species of woodrats found in the state of Oklahoma, the Eastern woodrat, Neotoma floridana (Ord), and the Southern Plains woodrat, Neotoma micropus Baird, have an interesting generally allopatric distribution pattern within the state (Fig. 1). Spencer (1968) found that the two species were sympatric in at least two areas of their ranges in West-Central Oklahoma, and that there was an indication of natural hybridization occurring. He also speculated that this area of sympatry is possibly an area of secondary intergradation of two previously isolated populations that have not yet reached the species level. However, he considered the morphological characteristics of the two forms distinct enough to warrant the retention of the two forms as species, even though they seemed to behave reproductively as subspecies. His comparisons of the two species and their hybrids were made using several cranial characters, bacular shape, and general morphological characteristics plus an evaluation of habitat and nest construction.

Because the question of the status of the two species remained unanswered it was apparent that a more thorough analysis of characteristics would be in order using other taxonomic techniques. The employment of these techniques in an attempt to determine the relationship of the two species is the basis for this investigation.

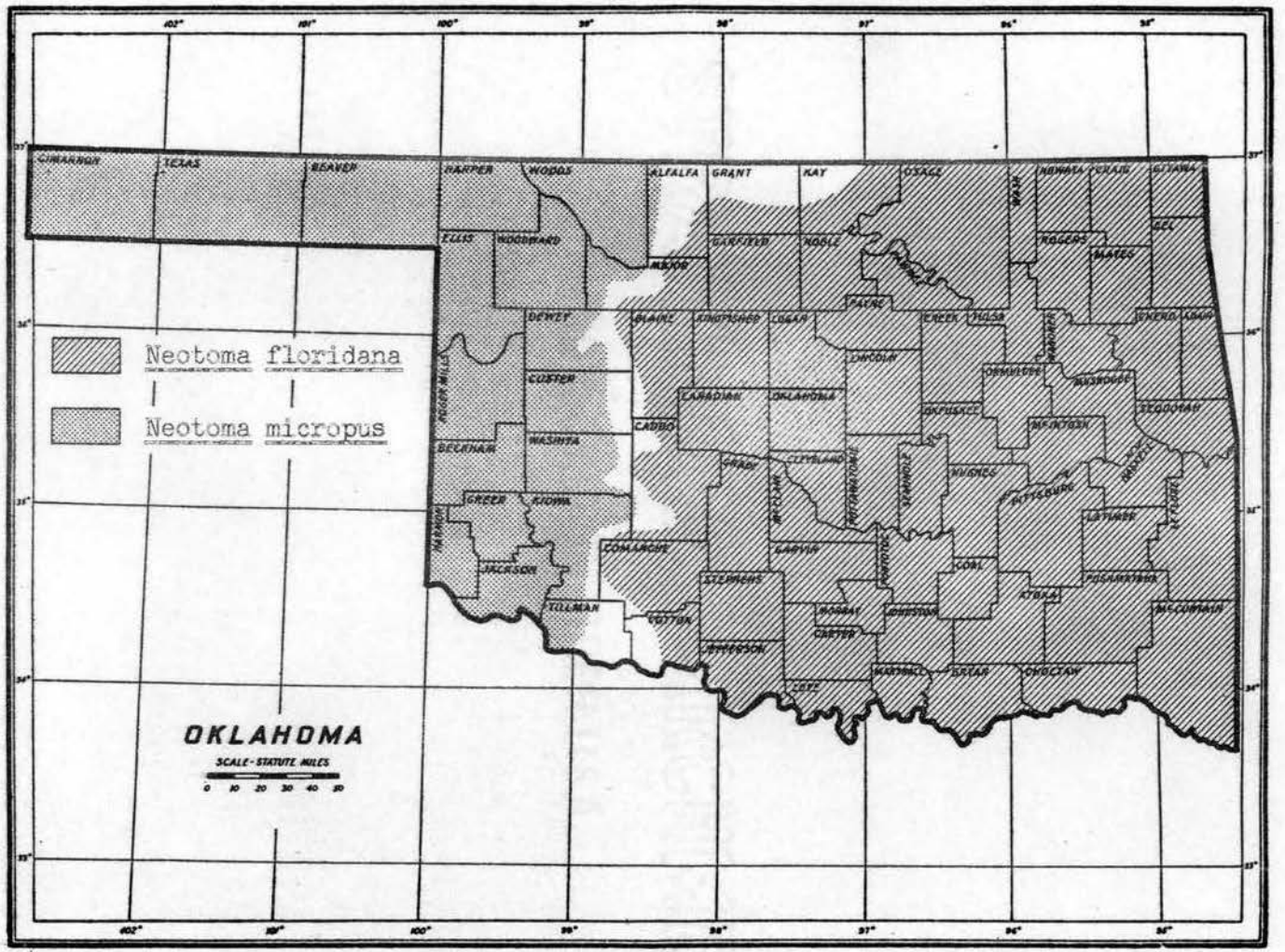


Figure 1. Distribution of the Eastern Woodrat, Neotoma floridana, and the Southern Plains Woodrat, Neotoma micropus, in Oklahoma (after Spencer, 1968).

The primary problem of species validity was broken down into two separate studies each of which employed different procedures currently being used in taxonomy. The first study was the use of statistical analysis to differentiate between the two populations; the second study was a comparison of the blood plasma proteins and hemoglobins by electrophoresis.

Preliminary Identification of the Species

Neotoma floridana is reported to be the larger of the two species (Hall 1955, Finley 1958, Hall and Kelson 1959) but, because there is such an overlap of external measurements and because most of the specimens were collected by students who, for the most part, were inexperienced in the preparation of study skins, only qualitative characteristics were used to identify the two species.

The following are the characteristics of the two species that were used to identify material for this study:

Neotoma floridana. Upper parts buffy gray to brownish, tail bicolored white below, brownish above. Skull with a forked anterior palatal spine (Figs. 2 and 3), narrow sphenopalatine vacuities, and a median posterior palatal notch (Figs. 2 and 4).

Neotoma micropus. Upper parts steely to slaty gray, tail bicolored gray below, blackish above. Skull with wide sphenopalatine vacuities (Fig. 3), single-tipped anterior palatal spine (Figs. 2 and 3), and a median posterior palatine projection (Figs. 2 and 4).

These characteristics are essentially those given by Hall (1955), Finley (1958), and Hall and Kelson (1959).

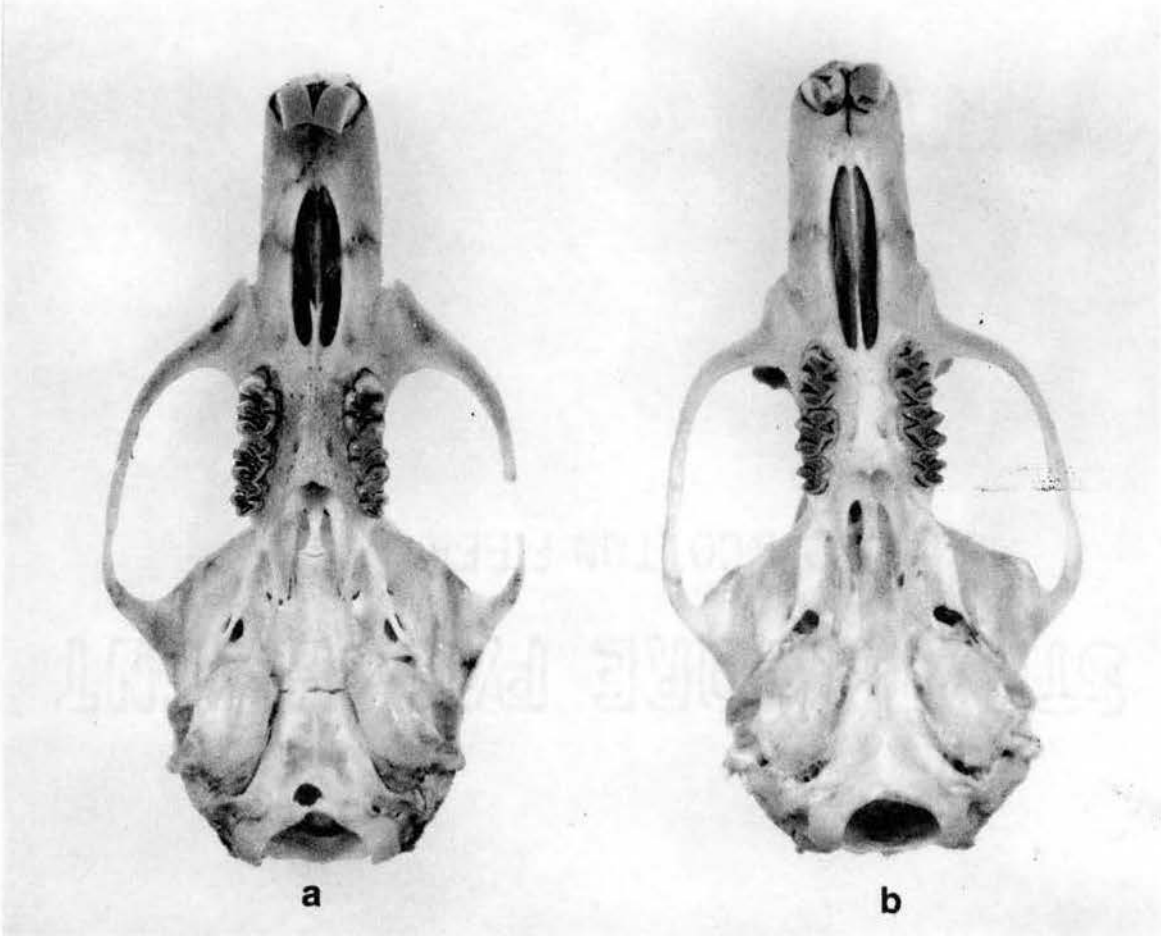


Figure 2. Ventral View of Skulls of (a) Neotoma floridana and (b) Neotoma micropus.

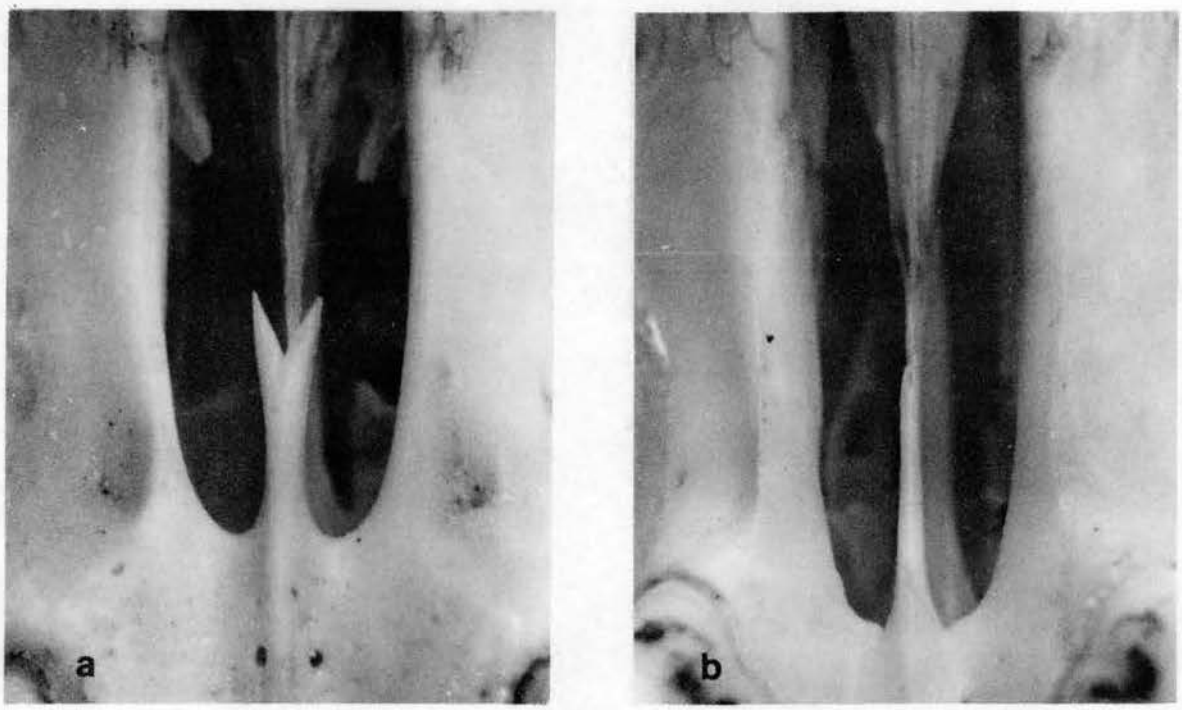


Figure 3. Detail of Anterior Palatal Spine of (a) Neotoma floridana and (b) Neotoma micropus.

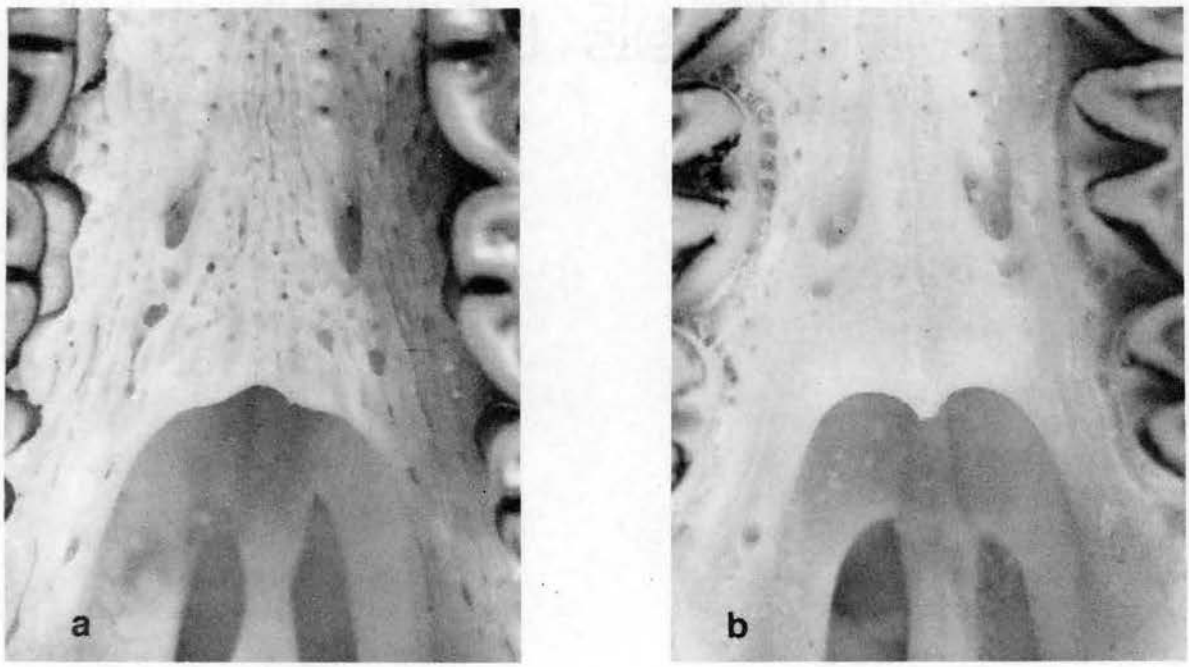


Figure 4. Detail of (a) Posterior Palatal Notch of Neotoma floridana and (b) Posterior Palatal Spine of Neotoma micropus.

The prepared material examined was from the Oklahoma State University Museum collection, and the live material was obtained at Stillwater, Payne County, Oklahoma (Neotoma floridana) and at the Southern Plains Experimental Range near Fort Supply, Harper County, Oklahoma (Neotoma micropus).

CHAPTER II

STATISTICAL ANALYSIS OF CRANIAL CHARACTERS

Neotoma floridana and Neotoma micropus are similar in size, shape, and body proportions. Although Neotoma floridana is reportedly the larger of the two species (Hall 1955, Finley 1958, Hall and Kelson 1959), the characters of the two species overlap to such a degree that the measurement of one character or group of characters can neither define nor separate the two species by simple comparisons.

In order to examine the variation that is present within each species and then to find the differences that exist between the two, a series of statistical analyses were performed on the samples of the two species which were available in the Oklahoma State University Museum collection.

The original design for a portion of these analyses called for each sample to be divided according to sex, and these were to be subdivided into at least three subgroups according to the degree of wear on the cheek teeth. This would have enabled a more realistic comparison of the two species, as young animals of one species would be compared only with young of the other species and not with old animals. Unfortunately the small sample sizes made the groupings by sex the only groups amenable to analysis other than the entire sample.

The computer programs used for this study were Biomedical Data Programs of the Health Sciences Computing Facility, U.C.L.A., which

were in the library of the Oklahoma State University Computer Center. These were: Simple Data Description - BMD01D, Discriminant Analysis for Two Groups - BMD04M, and Analysis of Variance for One-Way Design - BMD01V.

Cranial Characters

Fourteen cranial characters were recorded for each specimen. They were all taken with the same pair of Mitutoyo dial calipers graduated to 0.05 mm. All measurements were recorded to the nearest 0.1 mm. The fourteen cranial characters were:

1. Greatest length of skull - from the anterior tip of the nasals to the posterior projection of the occipitals.
2. Condylo-incisive length - from the anterior projection of the premaxillaries between the incisors to the posterior projection of the occipital condyles.
3. Zygomatic breadth - greatest distance across the zygomatic arch perpendicular to the long axis of the skull.
4. Breadth of brain case - breadth of the cranium measured immediately above the zygomatic processes of the squamosals.
5. Mastoidal breadth - breadth of the cranium between the mastoid processes.
6. Interorbital constriction - least distance across the top of skull between the orbits.
7. Rostral breadth - distance across rostrum at the point of the dorsalmost arch of the incisors.

8. Length of nasals - from the anterior projection of the nasals to the posterior projection of the naso-frontal suture.
9. Length of auditory bulla - from the anterior point lateral to the base of the styliform process to the posterior border of the inflated portion of the auditory bulla.
10. Crown length of maxillary toothrow - from the anterior crown margin of the first molar to the posterior crown margin of the third molar.
11. Breadth of palate - least distance between the closest aveoli of the first molars measuring perpendicular to the long axis of the skull.
12. Breadth of molars - breadth of the first molar measured perpendicular to the long axis of the skull.
13. Length of diastema - from the anterior alveolar border of the first molar to the posterior alveolar border of the incisor.
14. Length of anterior palatine foramina - from anterior inside border to the posterior inside border of the foramina.

Discriminatory Analysis

Using the BMD01D program (Simple Data Description) the mean, standard deviation, standard error of the mean, and range was computed for each character of the two groups and for males and for females of each group. The means and their corresponding standard deviations are shown in Table I. To ascertain if it would be possible to separate the two species using multivariate linear analysis the BMD04M program

(Discriminant Analysis for Two Groups) was used. As there could be no missing data in the analysis, it was necessary to determine the combination of characters that would provide the largest possible number of individuals for the computations. As only 57 per cent of the total number of specimens had all 14 characters measurable (Table II) 8 characters were eventually selected by elimination which then included 72 per cent of the specimens in the original sample (Table III). Two characters, crown length of maxillary toothrow and breadth of molars, were considered to contain a large amount of sampling error that was introduced by the configuration of the toothrow and structure of the skull and, although present in all individuals, were eliminated from the cranial analysis.

TABLE I

FOURTEEN CRANIAL CHARACTERS OF TWO SPECIES OF NEOTOMA SHOWING MEAN AND ONE STANDARD DEVIATION

Character	Neotoma floridana	Neotoma micropus
Greatest length of skull	48.834 ± 2.402	47.365 ± 2.071
Condylo-incisive length	45.861 ± 2.708	44.522 ± 2.084
Zygomatic breadth	25.742 ± 1.550	25.088 ± 1.440
Breadth of brain case	18.679 ± 0.554	18.297 ± 0.614
Mastoidal breadth	18.662 ± 0.793	18.448 ± 0.691
Interorbital constriction	6.537 ± 0.311	6.146 ± 0.302
Rostral breadth	7.521 ± 0.441	7.500 ± 0.441
Length of nasals	18.490 ± 1.165	18.306 ± 1.180
Length of auditory bulla	6.960 ± 0.233	7.215 ± 0.290
Crown length of maxillary toothrow	8.593 ± 0.304	8.670 ± 0.416
Breadth of palate	3.141 ± 0.374	3.240 ± 0.446
Breadth of molars	2.520 ± 0.098	2.468 ± 0.085
Length of diastema	13.576 ± 1.094	13.186 ± 0.992
Length of anterior palatine foramina	9.654 ± 0.667	10.468 ± 0.774

TABLE II

FREQUENCY OF OCCURRENCE OF 14 CRANIAL CHARACTERS IN A SAMPLE OF 100 NEOTOMA FLORIDANA AND 37 NEOTOMA MICROPUS

Character	Frequency
Greatest length of skull	.72
Condylo-incisive length	.81
Zygomatic breadth	.83
Breadth of brain case	.89
Mastoidal breadth	.78
Interorbital constriction	.98
Rostral breadth	1.00
Length of nasals	.90
Length of auditory bulla	.86
Crown length of maxillary toothrow	1.00
Breadth of palate	.99
Breadth of molars	1.00
Length of diastema	1.00
Length of anterior palatine foramina	1.00

TABLE III

EIGHT CRANIAL CHARACTERS USED IN DISCRIMINANT ANALYSIS OF TWO SPECIES OF NEOTOMA

Character
Condylo-incisive length
Mastoidal breadth
Interorbital constriction
Rostral breadth
Length of auditory bulla
Breadth of palate
Length of diastema
Length of anterior palatine foramina

The number of specimens originally examined were Neotoma floridana, 54 males and 46 females for a total of 100; and N. micropus, 19 males and 18 females for a total of 37. The subsequent elimination of specimens that were broken and, therefore, did not possess all 8 characters brought the totals of individuals for this analysis to N. floridana, 42 males and 32 females for a total of 74; and N. micropus, 17 males and 14 females for a total of 31. An analysis using all 14 characters (the 57 per cent sample) was also performed to ascertain what the addition of more variables would have on the results of the computations.

The principle behind the technique of discriminant analysis is that a linear function of all of the characters measured on each individual of the two groups is calculated. This is a weighted sum of the characters which best separates the groups. The sum of the weighted characters is the discriminant function and the weights determined by the computation are the discriminant function coefficients. The mean discriminant function for each group is obtained by multiplying the mean value of each character of the group by the discriminant function coefficient for the character and then summing these results (Lawrence and Bossert 1967).

The BMD04M program also computes the mean, variance, and standard deviation of the discriminant functions. These are useful for the placing of individuals into their proper groups by first multiplying the characters of the individual by the appropriate discriminant function coefficients, summing the results which will give the individual's discriminant function. This value can then be placed into the group whose mean discriminant function value it most closely approaches.

Mahalanobis' D^2-statistic and its associated F-statistic are also computed by the program. The D^2-statistic is a weighted squared distance in which both the variance of separate characters and the correlations among characters are taken into account (Sokal and Sneath 1963). The calculated F-statistic is used to test the level of significance of the differences between the two groups. These calculated F-statistics were deemed to be significant in this study at the 0.05 confidence level and to be highly significant at the 0.01 confidence level.

The first two uses of discriminant analysis (Analyses M-1 and M-2) were to test the null hypothesis that there was no difference between the males and females in each group. The computations produced algebraically ranked linear discriminant values that were almost completely overlapping (Fig. 5) and calculated F-statistics that were not significant (Table IV). This hypothesis was not rejected, allowing males and females of both groups to be combined in the same sample.

TABLE IV

DISCRIMINANT ANALYSIS OF MALES VERSUS FEMALES IN TWO SPECIES OF NEOTOMA

Analysis	Species	Sample Size ♂♂	♀♀	d.f.	F
M-1	Neotoma floridana	42	32	8, 65	1.792
M-2	Neotoma micropus	17	14	8, 22	1.354

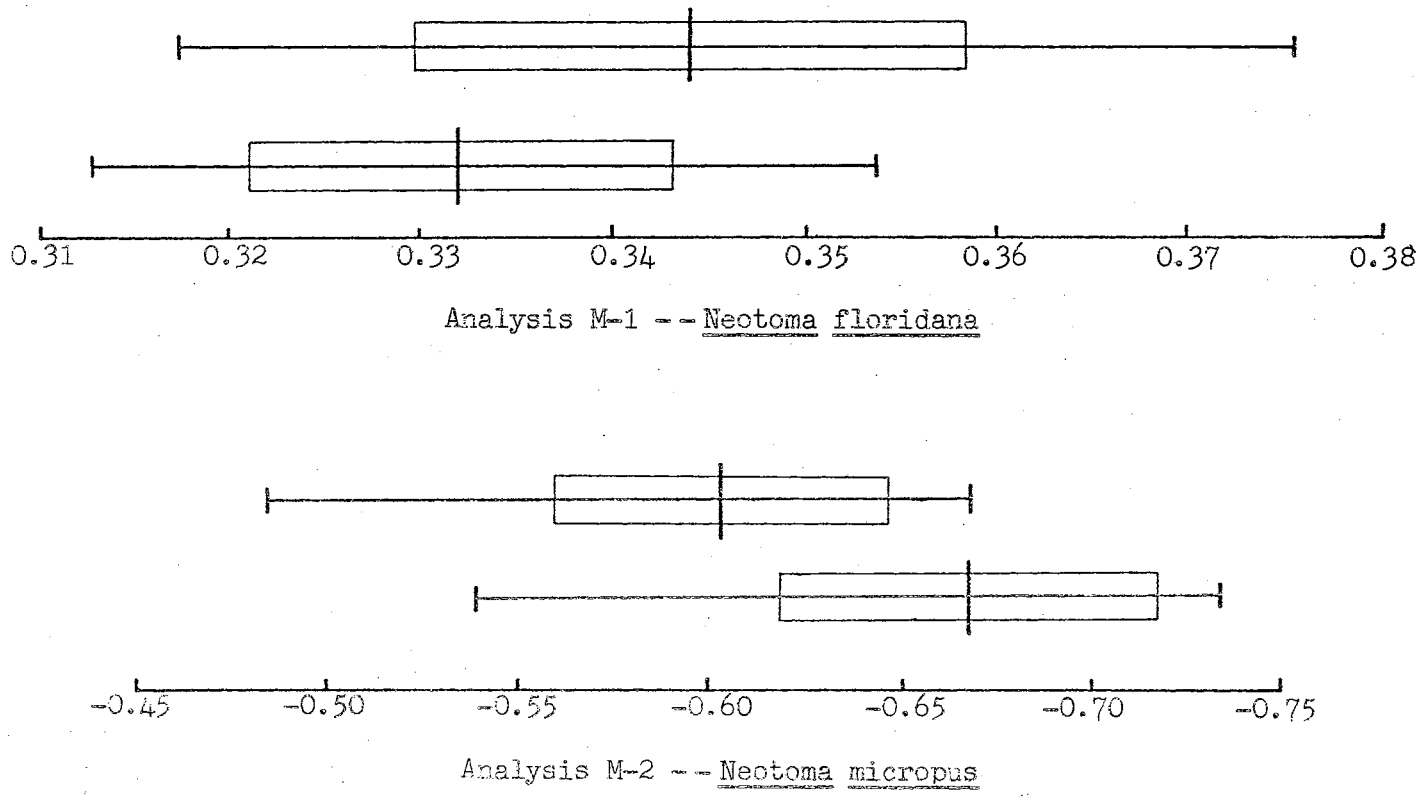


Figure 5. Distribution of Linear Discriminant Values of Males vs. Females of Two Species of Neotoma as Computed in Discriminant Analyses M-1 and M-2. In each graph upper bar represents males, lower bar represents females. Horizontal line indicates range, vertical line the mean, and the closed rectangle one standard deviation either side of the mean.

To ascertain if the two species could be separated by discriminant analysis the third analysis (M-3) was computed using the combined males and females of each group. The null hypothesis that there was no difference between the two groups was tested with the F-statistic that was computed in the program. The computations produced ranked linear discriminant values with only slight overlapping (Fig. 6) and a calculated F-statistic that was highly significant (Table V). Thus, the null hypothesis was rejected and the two groups were considered to be distinct.

TABLE V

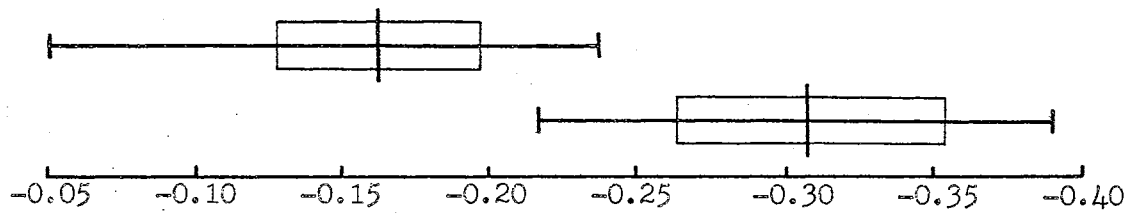
DISCRIMINANT ANALYSIS OF *NEOTOMA FLORIDANA* AND *NEOTOMA MICROPUS* (ANALYSIS M-3)

Species	Sample Size	d.f.	F
Neotoma floridana	74	8, 96	37.864**
Neotoma micropus	31		

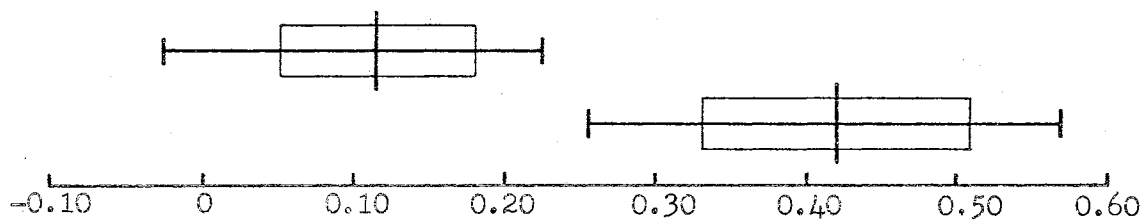
**Probability less than 0.01.

Although the previous analysis demonstrated the existence of a statistical difference between the two groups, two additional analyses were performed to corroborate the results and to test the null hypothesis of no difference between the group with the sexes separated.

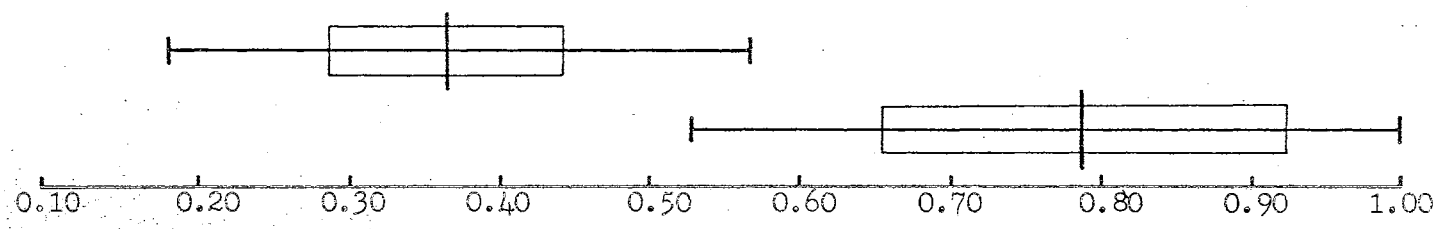
The fourth analysis (M-4) compared males of one group to males of the other; and the fifth analysis (M-5) compared females of one group with females of the other. In both instances the calculated F-statistic was highly significant (Table VI), rejecting the hypothesis of no difference. There was no overlapping of the ranked linear discriminant



Analysis M-3 -- Combined Sexes



Analysis M-4 -- Males vs. Males



Analysis M-5 -- Females vs. Females

Figure 6. Distribution of Linear Discriminant Values of Neotoma floridana and Neotoma micropus in Three Discriminant Analyses, M-3, M-4, and M-5. In each graph upper bar represents Neotoma floridana, lower bar represents Neotoma micropus.

values of the males and only slight overlapping of the linear discriminant values of the females (Fig. 6).

TABLE VI

DISCRIMINANT ANALYSIS OF TWO SPECIES OF NEOTOMA (MALES VS. MALES AND FEMALES VS. FEMALES)

Analysis		Sample Size		Degrees of Freedom	F
		N. floridana	N. micropus		
M-4	♂♂ vs. ♂♂	42	17	8, 50	22.868**
M-5	♀♀ vs. ♀♀	32	14	8, 37	19.001**

**Probability less than 0.01.

Three analyses were performed to determine the results of changing the number of variables and the number of specimens in the two groups. The null hypothesis that there is no difference between the two groups was tested. The first analysis (M-6) used the four significant characters and the second analysis (M-7) used the three of these which had the highest F-statistic values in the analysis of variance (see page 20). This reduction in the numbers of variables employed enabled a larger number of individuals to be included in the calculations. The calculated F-statistics remained highly significant (Table VII). However, there was greater overlapping of the ranked linear discriminant values (Fig. 7). Although extensive overlapping of the two groups of linear discriminant values can be graphically illustrated, the null hypothesis of no significant difference between the two groups was rejected.

TABLE VII

DISCRIMINANT ANALYSIS OF *NEOTOMA FLORIDANA* AND *NEOTOMA MICROPUS* SHOWING EFFECT OF DECREASING AND INCREASING THE NUMBER OF VARIABLES USED IN THE ANALYSIS

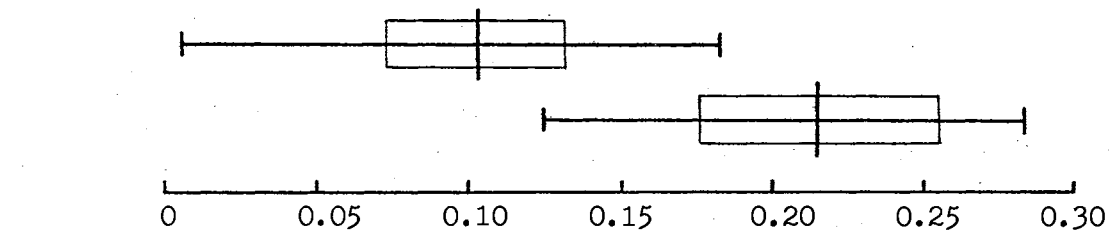
Analysis	Number of Variables	Sample Size		Degrees of Freedom	F
		N. floridana	*N. micropus*		
M-6	4	74	31	4, 100	61.541**
M-7	3	83	34	3, 113	31.716**
M-8	14	57	25	14, 67	20.366**

**Probability less than 0.01.

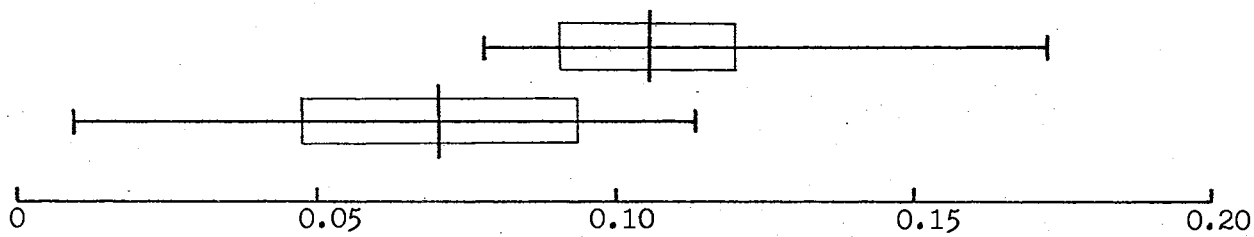
The final analysis (M-8) was to ascertain what would happen if the number of characters was increased. All fourteen characters were used in the computation to test the null hypothesis of no difference between the two groups. The resulting calculated F-statistic remained highly significant (Table VII), and there was complete separation of the ranked linear discriminant value of the two groups (Fig. 7). It was concluded that an increase in the number of variables used in the computation would produce a greater separation of the two populations.

Analysis of Variance

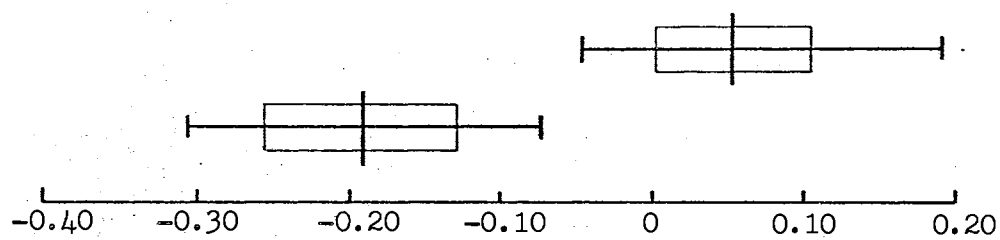
Spencer (1968) found that only one cranial character exhibited a significant difference at the 0.05 confidence level from an analysis of variance; this was interorbital constriction. Because of the clear separation of the two species by discriminant analysis, it seemed logical that some of the characters used in the present study that were not in the study by Spencer might be highly significant. The results of the Simple Data Description (Table I) were again consulted, and



Analysis M-6 -- Four Significant Characters



Analysis M-7 -- Three Highly Significant Characters



Analysis M-8 -- Fourteen Original Characters

Figure 7. Distribution of Linear Discriminant Values of Neotoma floridana and Neotoma micropus Showing the Effect of Decreasing the Number of Variables (M-6 and M-7) and Increasing the Number of Variables (M-8).

nine characters were selected for an analysis of variance which was run on the BMD01V program (Analysis of Variance for One-Way Design). The results of this analysis are illustrated in Table VIII. There were five characters other than the interorbital constriction that were highly significant; they were: greatest length of skull, breadth of brain case, length of auditory bulla, and length of anterior palatine foramina. Two other characters, condylo-incisive length and zygomatic breadth were significant.

From these results the interorbital constriction, length of auditory bulla, length of anterior palatine foramina, and condylo-incisive length were used in the discriminant analysis M-7.

The significant F-statistic values produced by the different analyses of variance demonstrated that there is a real difference between the two groups. This evidence, combined with that produced by the discriminant analyses sufficiently demonstrated that the two groups are biologically distinct and are, in fact, real species.

TABLE VIII

ANALYSIS OF VARIANCE OF NINE CRANIAL CHARACTERS OF *NEOTOMA FLORIDANA* AND *NEOTOMA MICROPUS*

Character	Sample Size		Degrees of Freedom	F
	N. floridana	*N. micropus*		
Greatest length of skull	70	29	1, 97	8.270**
Condylo-incisive length+	79	32	1, 109	6.295*
Zygomatic breadth	80	34	1, 112	4.436*
Breadth of brain case	89	34	1, 121	11.045**
Interorbital constriction+	97	37	1, 132	43.271**
Length of nasals	89	35	1, 122	0.624
Length of auditory bulla+	85	33	1, 117	24.992**
Length of diastema	100	37	1, 135	3.600
Length of anterior palatine foramina+	100	37	1, 135	36.820**

* Probability less than 0.05.
** Probability less than 0.01.
\+ Characters used in discriminant analyses.

CHAPTER III

ELECTROPHORETIC COMPARISON OF BLOOD PROTEINS

The difference between the two species of Neotoma which can be shown in an analysis of cranial characters might also be manifest in the materials which make up the tissues of the organism. The blood contains many proteins which can be readily collected and analyzed to provide genetically controlled characters which, for the most part, are conservative and represent products one or two chemical steps removed from the gene.

Johnson and Wicks (1959) used paper strip electrophoresis to analyze the blood serum proteins of 89 forms (species and subspecies) of North American mammals. Johnson (1968) reported on further work that included 409 different forms that were analyzed at the subspecies level. In both cases it was reported that each species of mammal studied tended to have its own protein pattern.

Foreman (1960) suggested that hemoglobins should be used instead of serum proteins because the hemoglobins would not be affected by environmental factors such as age, diet, or health, and, therefore, would be much more stable. He found that closely related species may show similarities in the migration patterns of the hemoglobins, but that each species was distinct, and that these hemoglobin patterns could be used to separate morphologically similar species.

The different hemoglobin molecules, as well as being primary products of genic action have similar biological functions, similar physical and chemical properties, and occur as intra- or interspecies variants that can easily be isolated from other biological contaminants for analysis (Popp 1963).

The principle of electrophoretic separation of proteins is that different protein molecules will have different rates of migration through an electrical field. The distance that these molecules migrate depends on the net charge on the molecule. The use of non-liquid media such as the acrylamide gels cause a buffering or sieving effect that will retard the movement of these molecules, and in this way proteins of equal net charges but of different molecular size or shape will separate into different fractions as they migrate through the gel.

Dessauer and Fox (1962) concluded that acrylamide gel electrophoresis was most useful in studies at the intraspecific and specific taxonomic levels, and the techniques with low resolution properties, such as paper strip electrophoresis, were useful at high taxonomic levels as there is not as complete a separation of some of the protein fractions.

The exceptionally high resolutions of the acrylamide gel electrophoresis results from the fact that both the net charge and the size and configuration of the protein molecule will affect its migration. Proteins found in complex mixtures such as blood serum may separate into many fractions because of these different rates of migration. These high resolution properties of gels enabled the identification of individuals in some population studies (Dessauer and Fox 1962).

The methods employed in this study to analyze both hemoglobins and blood plasma proteins were the Canalco disc-electrophoresis system and the Millipore PhoroSlide electrophoresis system.

Disc electrophoresis was developed independently by Ornstein (1964) and Davis (1964) in 1962. The technique uses polyacrylamide gel columns usually of three sections: sample gel, spacer gel, and separation gel. The protein sample is introduced into the large pore sample gel, concentrated in the large pore spacer gel, and separated into fractions in the small pore separating gel. This enables samples as small as 50 µgm to be completely separated into their many fractions.

The Millipore electrophoresis system used a 1x3 inch PhoroSlide that has a microporous surface in which the electrophoretic migrations take place in the same manner as in cellulose acetate strips. These slides, however, are not at all fragile and can be cleared and stored like microscope slides. Two samples can be analyzed on each strip at the same time, each sample requiring only 0.3 µl of material. The resulting protein bands can be either visually analyzed or can be quantitatively analyzed with the Millipore PhoroScope densitometer.

Preparation of Blood Samples

Six specimens of Neotoma floridana were obtained from Stillwater, Payne County, Oklahoma, and five specimens of Neotoma micropus were obtained from the Southern Plains Experimental Range, near Fort Supply, Harper County, Oklahoma.

One blood sample was collected from each individual. Replications were not attempted as the woodrats were not available for sacrifice, and the risk involved with repeated cardiac punctures was considered

to be prohibitive. The blood sample was obtained by first introducing the woodrat into a funnel-type holding cage as described by Thomas (1945). This was constructed of 1/4 inch hardware cloth, 24 inches long, 3 inches wide at one end and 2 1/4 inches at the other. This small end was closed with a rubber stopper. A sleeve, slightly larger than the large end of the funnel, was constructed of hardware cloth and covered with paper. This was passed through and attached to a sheet of cardboard the size of the front of the woodrat's cage. The open end of the funnel was inserted into the sleeve and this was placed in front of the opened cage. The woodrat was then prodded until it entered the funnel. Spikes, 4.5 inches in length, were then passed through the mesh of the funnel directly behind the woodrat to block the open end. The animal was then injected intraperitoneally with 0.5 ml of Combuthal (Sodium thiopental-Sodium pentobarbital) and immediately allowed back into its cage by placing the small end of the funnel into the sleeve after removing the rubber stopper. After 10 to 15 minutes when the animal was completely unconscious, 2 ml of blood was withdrawn by cardiac puncture. The blood was centrifuged for 15 minutes to separate the plasma, which was then collected. The remaining red blood cells were then washed three times in normal saline solution and suspended in 1 ml of saline. The plasma and suspended red blood cells were placed in 10 ml culture tubes and frozen at -15 C for storage.

Hemoglobin Preparation

To prepare the hemoglobin solution for electrophoresis the frozen samples of suspended red blood cells were rapidly thawed to lyse the cells. Distilled water in a ratio of one part water to one part suspended cellular solution was added to insure complete hemolysis. Toluene was added in a ratio of 0.5 ml toluene for each milliliter of hemoglobin solution, and thoroughly mixed by rapid agitation for one minute. This mixture was then centrifuged at maximum speed for 15 minutes. The resulting centrifuge tube had four layers: toluene on the top, a layer of lipids, the hemoglobin solution, and the compacted stroma at the bottom. The hemoglobin solution was removed by passing a hypodermic needle through the toluene and lipid layers into the hemoglobin solution layer and withdrawing it into a syringe. The hemoglobin was then placed in a 10 ml culture tube and refrigerated at -15 C for storage.

Analysis of Proteins

Hemoglobins and plasma proteins were analyzed by disc electrophoresis using the Buchler Polyanalyst apparatus, a Regulated Power Supply Model 50, manufactured by Lambda Electronics Corp., and Canalco premixed chemicals. The reagents were prepared following the instructions for their mixture in the Canalco Model 6 Operating Instructions. The sequence of gel preparation, however, followed Thompson (1967) which is in the reverse order of that given in the Canalco instructions. The separating gel was prepared first; the spacer gel second; and, as hemoglobin prevents polymerization of the sample gel and for simplicity of technique, no sample gel was prepared.

The bottom of the gel tubes were first closed with rubber caps and the tubes were then placed upright in a Buchler polymerization rack. The separating gel was then added to within 6 mm of the top. This was layered with a small quantity of water to prevent the formation of a meniscus and the gel was allowed to polymerize for 30 minutes. The water layer was then removed using a small piece of absorbent paper tissue to insure complete removal. The tube was rinsed with the unpolymerized spacer gel solution and then approximately 1 cm of the spacer gel solution was added, water layered, and photopolymerized for 30 minutes using six 15-inch long, 15-watt fluorescent lights placed 3 to 5 inches from the tubes. When polymerization was complete, the water layer was removed and 5 µl of the protein sample was added. This was then covered by 0.1 ml of 40 per cent sucrose solution to prevent the protein sample from washing away in the upper buffer. Barbital buffer with a pH of 8.6 was then added to the lower buffer chamber. The tubes were placed into position in the base of the upper buffer chamber, and this placed over the lower buffer chamber, allowing the tubes to dip into the buffer. Bromophenol blue tracking dye was added to the buffer in a ratio of 5 ml/liter and thoroughly mixed. This was then carefully added to the upper buffer chamber, taking care not to wash off the sucrose-protein sample. Current was then applied for a prerun at 50 volts until the tracking dye had passed through the upper gel. The voltage was then increased to 100 volts for the remainder of the run. When the tracking dye was approximately 0.5 cm from the bottom of the gel, electrophoresis was terminated. At this time during analysis of hemoglobins the bands could be seen as translucent "rust" bands in the gel. No protein

bands were observed at this time in tubes containing plasma proteins. The gels were then removed from the gel tubes and placed in 10 ml culture tubes and were fixed and stained for several hours with Amido Schwartz dye in acetic acid. The gels were then placed in destaining tubes and the excess stain was removed by electrophoresis at 150 volts with 7.5 per cent acetic acid replacing the buffers. After destaining, the tubes were stored in 10 ml culture tubes containing 7.5 per cent acetic acid.

The blood proteins were analyzed by Millipore PhoroSlide electrophoresis using the Millipore instructions. Each PhoroSlide was first prebuffered using TEB buffer for hemoglobin or barbital buffer for blood plasma proteins. Excess buffer was removed by careful blotting and the slide placed in the PhoroSlide cell. This cell has two chambers, each holding 5.5 ml of barbital buffer; the PhoroSlide was positioned with one end in each chamber. The cell cover assembly was firmly placed over the PhoroSlide and approximately 0.3 µl of protein sample was applied to the PhoroSlide by each of the two applicators for one minute. The applicators were removed, the cell closed, and 100 volt current applied for 15 minutes. After electrophoresis was completed the PhoroSlide was fixed and stained in Ponceau-S dye for 10 minutes. The excess stain was removed by three one minute rinses in 5 per cent acetic acid. The PhoroSlide was then blotted and either allowed to dry with an opaque white background or it was cleared for analysis by densitometry by immersing in 95 per cent ethanol for one minute and then in clearing solution (30 per cent ethyl acetate in acetic acid) for one minute.

Results of Hemoglobin Electrophoresis

The hemoglobin samples analyzed by disc electrophoresis separated into distinct fractions or bands. Two bands were found for Neotoma floridana and three bands were found for Neotoma micropus (Figs. 8 and 9). There was variation among individuals as to the position of these bands, however, the number of bands remained constant for each species.

The hemoglobin analyzed by Millipore PhoroSlide electrophoresis resulted in one broad band for Neotoma floridana and two bands for Neotoma micropus. The cleared PhoroSlides were analyzed using the Millipore PhoroScope densitometer. The electrophoretogram traced on the oscilloscope produced a single large peak for Neotoma floridana and a large peak with a pronounced accessory peak for Neotoma micropus. A representative electrophoretogram for each species was photographed directly from the oscilloscope, and was then traced and is presented as Figure 10.

Results of Plasma Protein Electrophoresis

The plasma proteins analyzed by disc electrophoresis produced over 15 fractions which appeared as distinct bands in the gel (Fig. 11). The relative position and magnitude of these bands varied greatly between the two species, and, as no densitometer was available, an attempt to identify the major fractions was made following Clarke's (1964), Davis' (1964), and Ornstein's (1964) methods for the identification of the proteins found in human serum. Figure 12 shows representative gels of Neotoma floridana and Neotoma micropus aligned with Clarke's (1964) suggested identification system for human serum

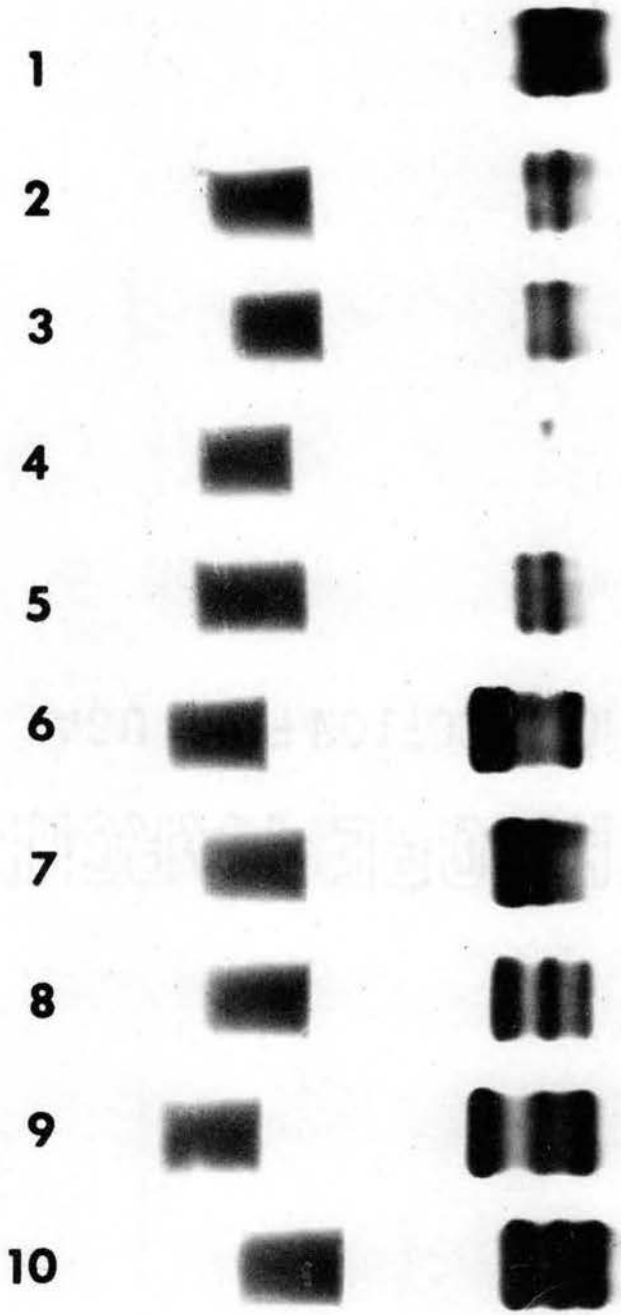


Figure 8. Electrophoretic Patterns of Hemoglobin in Acrylamide Gel. 1-5 Neotoma floridana, 6-10 Neotoma micropus. Origin to the left, anode to the right.

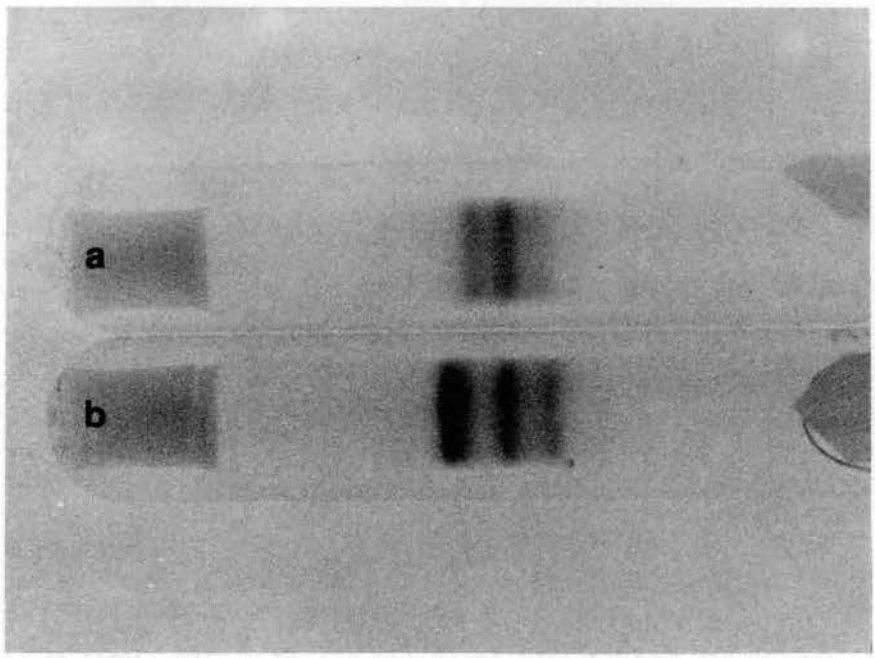


Figure 9. Representative Electrophoretic Patterns of Hemoglobin in Acrylamide Gel. (a) Neotoma floridana (b) Neotoma micropus. Origin to left, anode to the right.

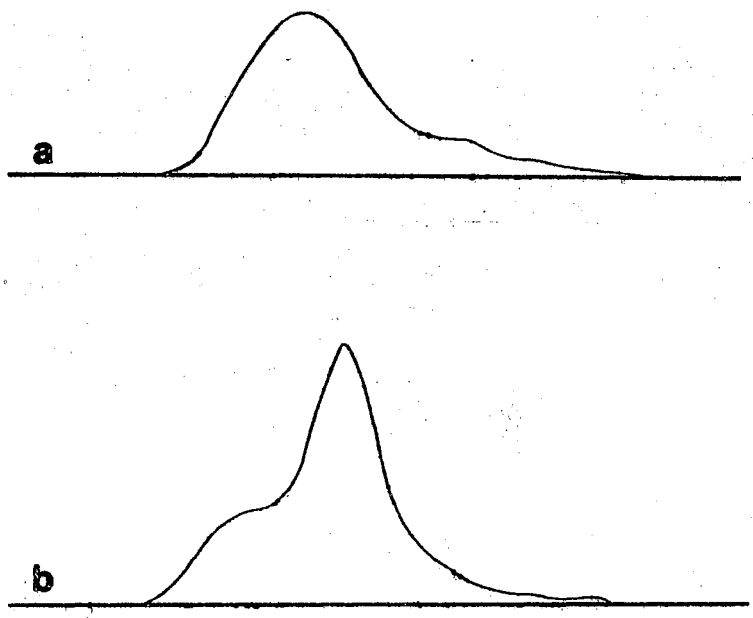


Figure 10. Representative Electrophoretograms of Hemoglobin Using Millipore PhoroSlide System of (a) Neotoma floridana and (b) Neotoma micropus. Origin to the right, anode to the left.

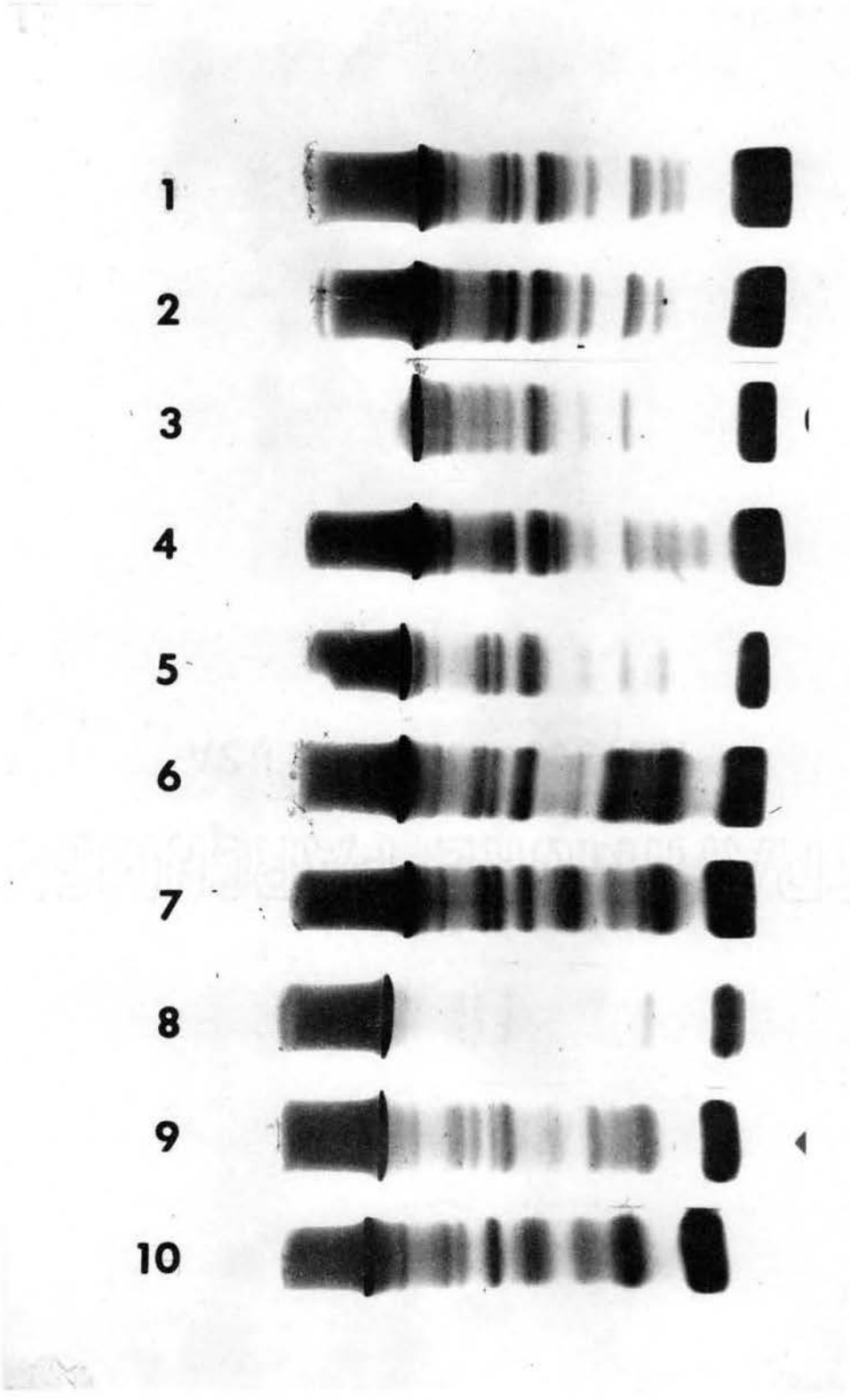


Figure 11. Electrophoretic Patterns of Blood Plasma Protein in Acrylamide Gel. 1-5 Neotoma floridana, 6-10 Neotoma micropus. Origin to the left, anode to the right.

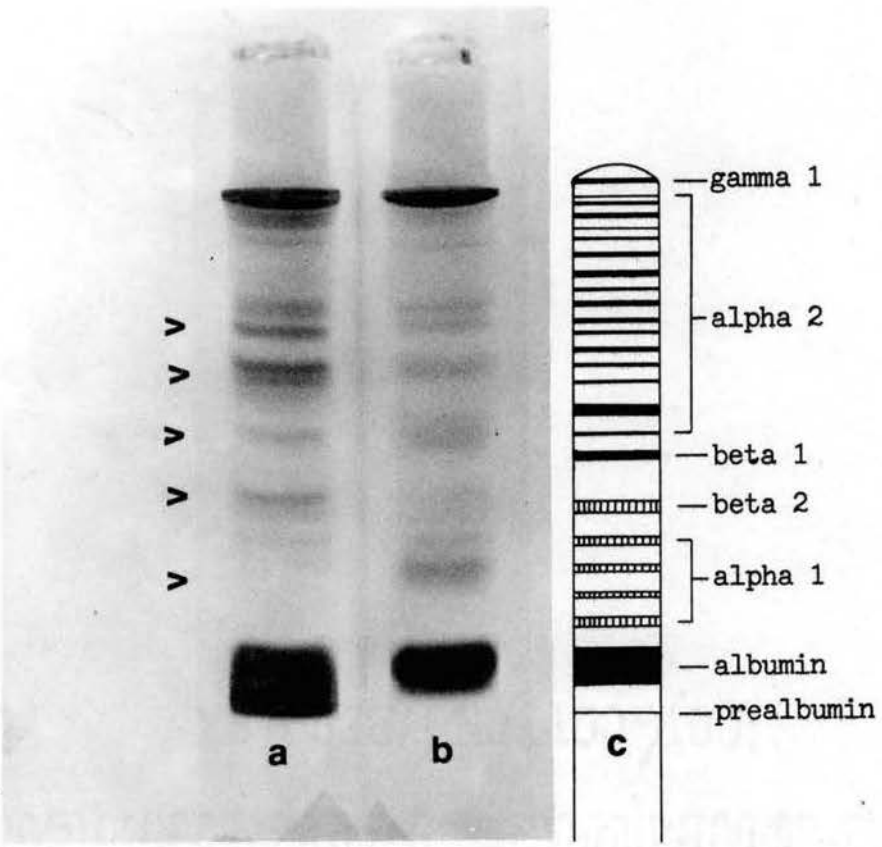


Figure 12. Representative Electrophoretic Patterns of Blood Plasma Protein of (a) Neotoma floridana and (b) Neotoma micropus Showing (c) the Suggested Identification System of Clarke (1964) for Human Serum Protein Bands. Arrows indicate regions of possible difference.

proteins. Both species had a faint band that preceded the albumin band. This was assumed to be a prealbumin fraction. The albumin was present in the same relative position in both species. Several dark bands 2.5 cm from the origin were considered to be the beta-1 or transferrin bands. Using these bands as a base the other fractions were then tentatively identified. There was a wide dark band approximately 7 mm behind the albumin band of Neotoma micropus which was considered to be a postalbumin or an alpha-1 globulin. This band was faintly present but indistinct in Neotoma floridana. Other than this major difference, there was a relative difference in both the number and intensity of bands in the alpha-1, alpha-2, and gamma globulins. Both species had strong alpha-2 bands and a variable number of bands in the gamma region. Figure 12 illustrates these regions of possible differences.

The plasma analyzed using Millipore PhoroSlide electrophoresis produced only 3 or 4 distinct bands on the PhoroSlide. These were analyzed using the Millipore PhoroScope, and the electrophoretogram was photographed directly from the oscilloscope. The negatives were then compared to find if there were any apparent differences. An attempt to identify the different fractions was made using the Phoro-Scope quantitation procedure in the PhoroSlide Principles and Operating Instructions Manual. It should be noted that the identification of the protein fractions using this procedure differs from those of Clarke (1964), Davis (1964), and Ornstein (1964). Figure 13 presents tracings of the electrophoretograms for Neotoma floridana, and Figure 14 presents tracings of the electrophoretograms of Neotoma micropus to show the considerable amount of variation found within

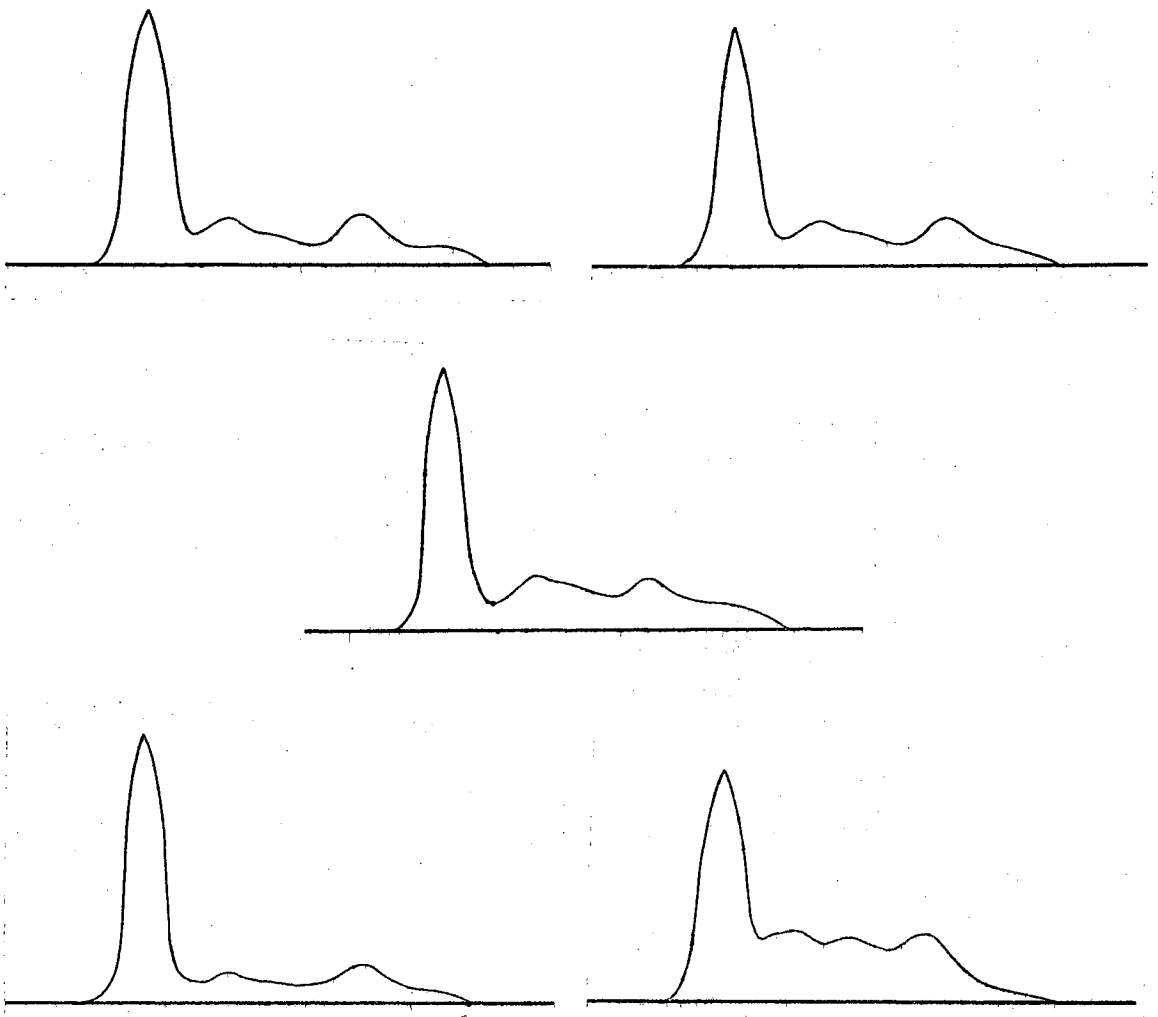


Figure 13. Millipore Electrophoretograms of Blood Plasma Proteins of Neotoma floridana. Origin to the right, anode to the left.

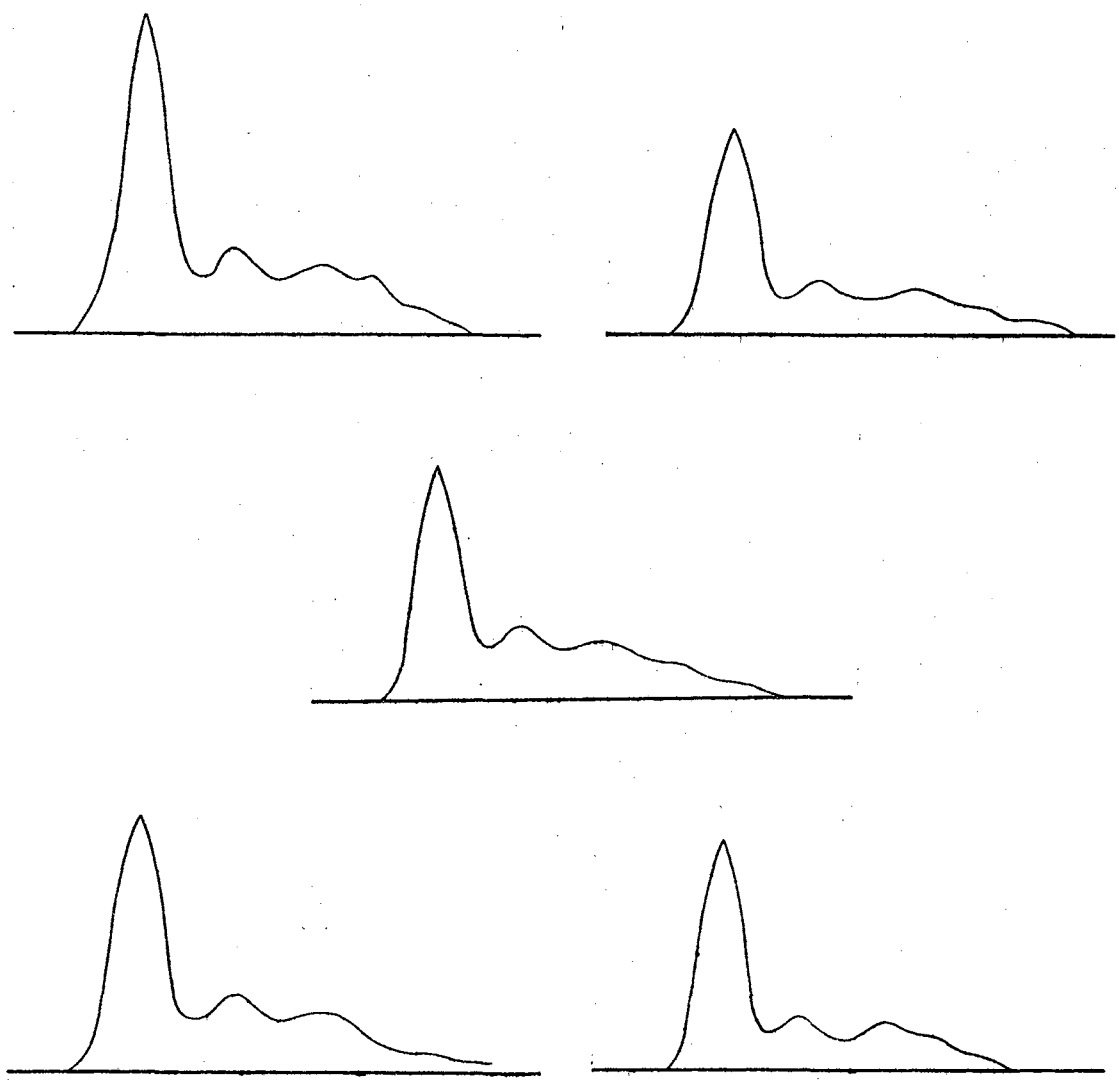


Figure 14. Millipore Electrophoretograms of Blood Plasma Proteins of Neotoma micropus. Origin to the right, anode to the left.

each species. The albumin peak is used as a mark in the analysis so that the electrophoretograms show the different protein fractions in relation to the albumin band, and not in relation to the distance migrated during electrophoresis. Figure 15 presents representative electrophoretograms of Neotoma floridana and Neotoma micropus aligned with the Millipore example of human serum protein to demonstrate the differences observed between the two species in the positions of the alpha and beta globulin peaks.

Conclusions

From the analysis of the hemoglobins by disc electrophoresis it is obvious that there is a distinct difference between the two populations of woodrats. This difference is noticeable but is not as dramatic using Millipore PhoroSlide electrophoresis. The analysis of plasma protein by disc electrophoresis is unfortunately hampered by having no available means to identify the different protein fractions. The differences that were observed could very well be sound taxonomic characters. Millipore PhoroSlide electrophoresis has proven to be of too low resolution to be able to detect specific differences in plasma proteins.

From these results it can be concluded that there is a genetic difference between the woodrat population of Stillwater, Payne County, Oklahoma (Neotoma floridana) and the woodrat population of Fort Supply, Harper County, Oklahoma (Neotoma micropus). However, because of the small sample sizes and because only one locality in the range of each woodrat was sampled it can only be inferred that the differences found are specific. If additional samples collected over the ranges of both

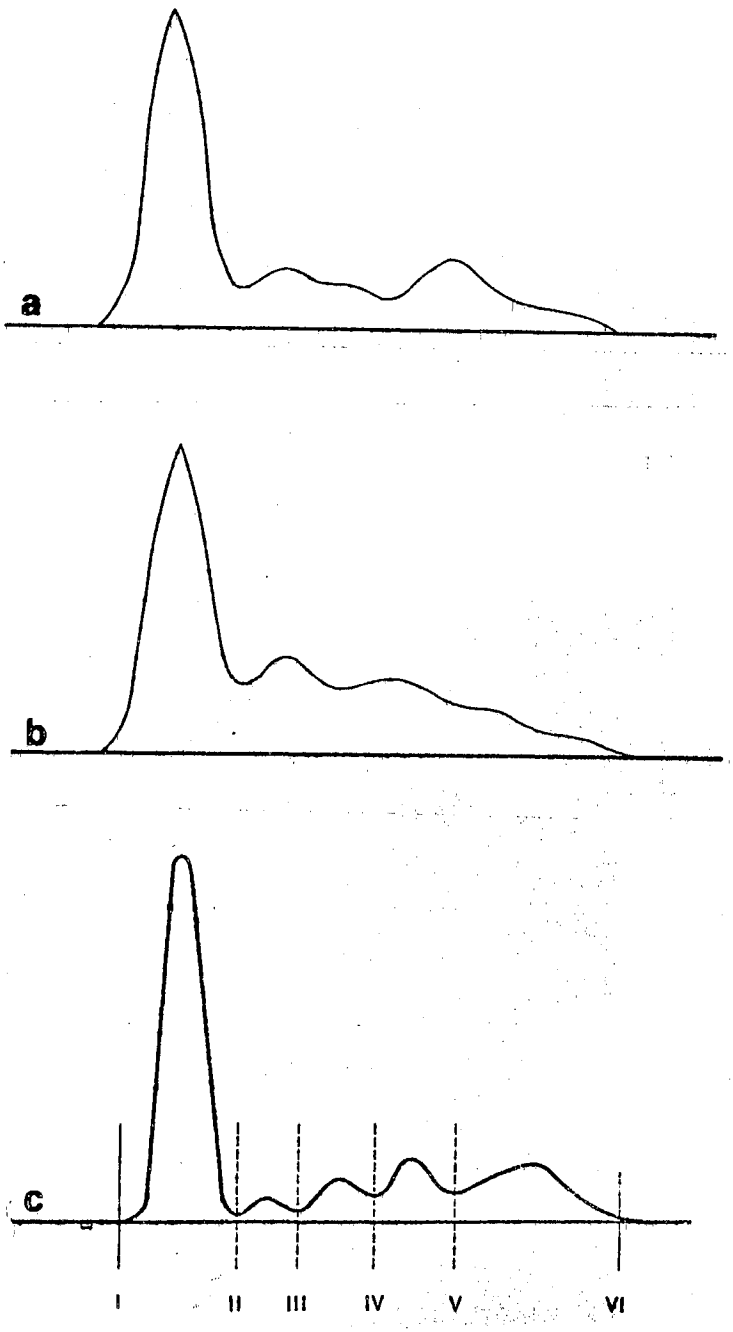


Figure 15. Representative Millipore Electrophoretograms of Blood Plasma Proteins of (a) Neotoma floridana and (b) Neotoma micropus Aligned with (c) the Millipore PhoroScope Example of Human Serum Protein. I-II Albumin, II-III Alpha-1, III-IV Alpha-2, IV-V Beta, and V-VI Gamma Globulins.

species were to be analyzed and if the differences that were demonstrated in this study were found to remain constant within each species, then these differences could be considered as taxonomic characters which would readily separate the two species.

CHAPTER IV

SUMMARY

Spencer (1968) reported the finding of hybrid individuals in an area of contact between the two normally allopatric ranges of Neotoma floridana and Neotoma micropus in Oklahoma. This was supported by breeding experiments where captive animals of the two species produced fertile hybrids.

His analysis of the two species verified the morphological differences that were currently used for the identification of each species. The differences in bacular morphology and pelage coloration also were verified as being distinct for each species.

The analysis of cranial characters that was undertaken by him showed only one character that showed a significant difference using an analysis of variance, that of interorbital constriction.

This present study not only demonstrated that the two species represented two distinct populations using discriminant analysis, but also that there were six cranial characters other than interorbital constriction which showed significant differences using analysis of variance. These were the greatest length of the skull, condylo-incisive length, zygomatic breadth, breadth of brain case, length of auditory bulla, and length of anterior palatine foramina.

The analysis of blood proteins provided additional evidence of the differences between the two species. There was a definite

difference found in the hemoglobins analyzed by electrophoresis and a probability of a difference found in the blood plasma proteins. Unfortunately, the samples were too limited to provide a valid inference as to the actual protein characteristics of each species.

Simpson (1961) in a discussion of the application of the biological species concept states that populations that can produce fertile hybrids are not necessarily conspecific.

> The more stringent criterion [that of complete reproductive isolation] is undesirable, because populations that are in all other respects exactly like unquestionable species do occasionally produce fertile hybrids. It is also impractical, because the possibility of producing fertile hybrids can rarely be judged adequately on available evidence except for species so obviously distinct that the question need not be raised at all.
>
> If there are distinct gaps between ranges of characters, it is sufficiently probable that isolation is at least complete enough to warrant specific separation.

In his discussion of the evolutionary species he further states,

> It is also clear that two species may interbreed to some extent without losing their distinction in evolutionary roles and that this is the really important point for evolutionary taxonomy. The amount of interbreeding allowable by definition is then precisely as much as does not cause their roles to merge. The taxonomic value of the genetic criteria of interbreeding and isolation lies not in those characteristics in themselves but in their evidence as to whether populations are or are not capable of sustaining separate and unitary roles over considerable periods of time.

Mayr (1969) states that "allopatric forms that hybridize only occasionally in the zone of contact are full species." From the evidence furnished by Spencer as to the differences in bacula and coloration and from the new evidence generated in this study, it can probably be stated that the two populations are full species which, by chance, produce occasional hybrids.

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