Comparative Hematology of the Hellbender, *Cryptobranchus alleganiensis* in Missouri

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This study compares the hematology of two cryptobranchid salmander populations in Missouri; *Cryptobranchus alleganiensis alleganiensis* (Daudin) from the Niangua River in Dallas County, and *Cryptobranchus alleganiensis bishopi* Schmidt from the North Fork of the White River in Ozark County. Determinations included blood cell size, blood cell counts, leucocyte differentiations, pH, hematocrit values, hemoglobin, erythrocyte fragility and coagulation time. Values for leucocyte size, thrombocyte size and number, pH, coagulation time and erythrocyte fragility as well as blood cell photomicrographs are presented for *Cryptobranchus* for the first time. Mean erythrocyte total length was 49.8 μ for the Niangua population and 43.5 μ for the North Fork population. Erythrocyte counts varied from 27,000 per mm^3 to 87,850 per mm^3 ($\bar{x}$ 72,544) for the Niangua population and from 48,750 per mm^3 to 145,000 mm^3 ($\bar{x}$ 92,713) for the North Fork population. Significant differences between the two populations were also noted for erythrocyte total width, erythrocyte nuclear length, monocyte total length, hemoglobin and pH values. Environmental, seasonal and sexual variation appeared to be insignificant. No basophils were observed for either population.

THERE are two extant forms of North American cryptobranchid salamanders (Brame, 1967). They are the hellbender, *Cryptobranchus alleganiensis alleganiensis* (Daudin), which ranges from Kansas to New York, and the Ozark hellbender, *Cryptobranchus alleganiensis bishopi* Schmidt, whose populations appear restricted to heavily spring-fed portions of the Black and White River drainages in southern Missouri and northern Arkansas (Dundee and Dundee, 1965; Nickerson and Mays, 1972).

Except for the studies of Wintrobe (1933) and Vernberg (1955), there are relatively few hematological studies of salamanders, and only the former discusses *Cryptobranchus*. This lack of information prompted our investigation.

Determinations for leucocyte size, thrombocyte size and number, pH, coagulation time and erythrocyte fragility, as well as blood cell photomicrographs are presented for the first time for *Cryptobranchus*.

Materials and Methods

Specimens were obtained from two drainage systems in Missouri; the Niangua River, near the entrance of Bennett Springs, Dallas County, and the North Fork of the White River, within 2.4 km of its merger with Spring Creek, Ozark County. These sites were chosen because of their similarity, i.e., both are heavily spring-fed (Beckman and Hinchey, 1944) and capable of maintaining trout. In addition, both forms of *Cryptobranchus* could be studied.

All specimens were kept in glass holding tanks with a water temperature of 14 C prior to use. Blood samples were obtained from anesthetized animals by making an incision on the ventrum ca. 2.5 cm in length opposite the heart. A solution of 1 part saturated chloretone (Chlorobutanol, U.S.P., Hydrous; Park, Davis & Company) in 2.5 parts distilled water was used for anesthesia. Cardiac puncture was used to obtain 1 cc of blood from the ventricle. The first two drops were discarded to insure that foreign particles were removed.

Hayem's solution and standardized Neubauer hemocytometers were used to determine erythrocyte counts. Erythrocyte size was measured with a Bausch and Lomb ocular micrometer. *In vivo* cells were measured in the Neubauer counting chamber, while *in vitro* cells were measured from Giemsa's differential stained blood smears (Galigher and Kozloff, 1964).

Leucocyte counts were determined with

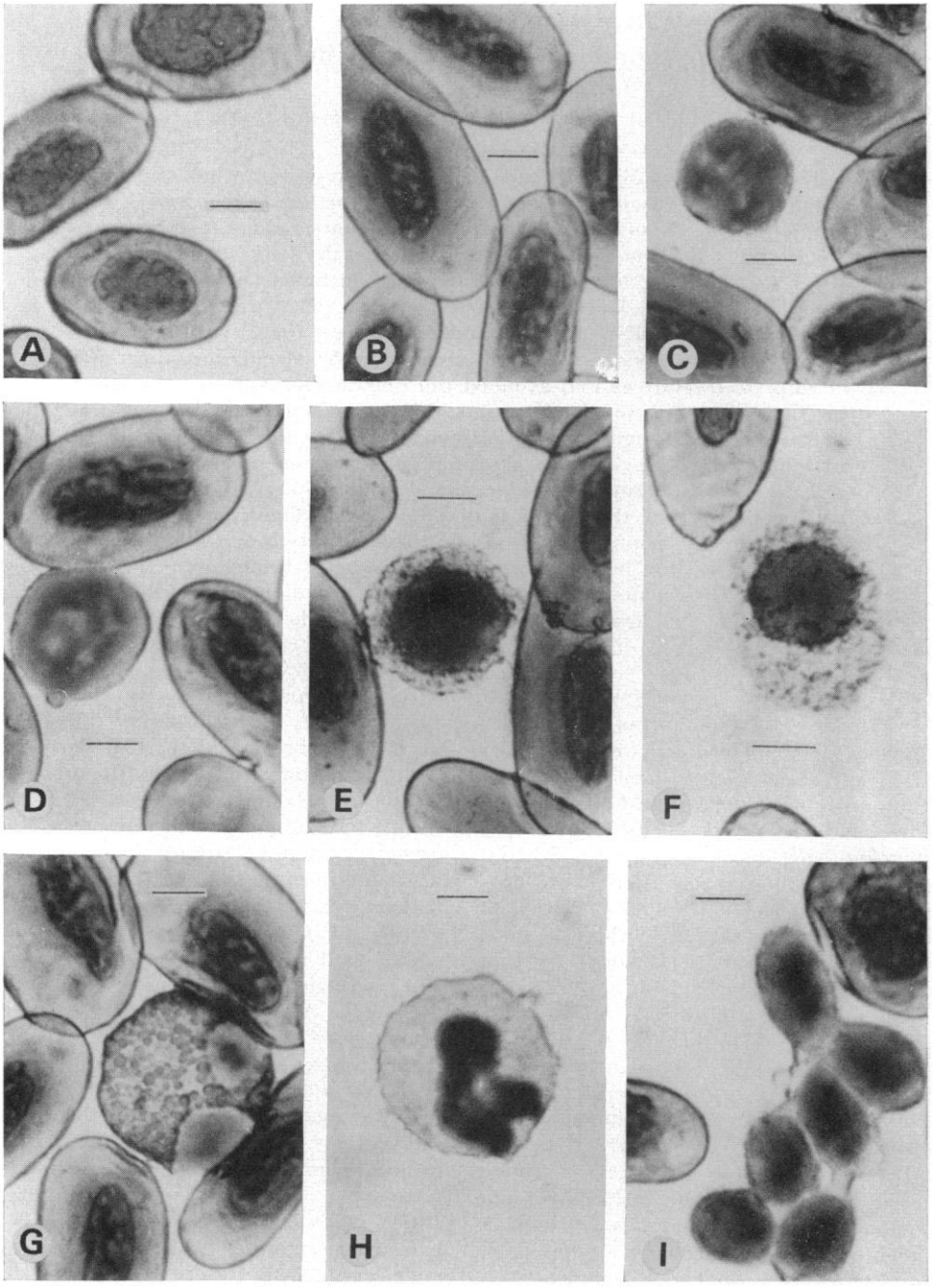

Fig. 1. *Cryptobranchus* blood cells. Each line represents 10 μ. A. North Fork erythrocytes; B. Niangua erythrocytes; C. Small Niangua lymphocyte; D. Large Niangua lymphocyte; E. Niangua monocyte; F. North Fork monocyte; G. Niangua eosinophil; H. North Fork neutrophil; I. North Fork thrombocytes.

the aid of a Neubauer hemocytometer and a modification of Blain's method (Sturkie, 1954). A 1:1 mixture of neutral red (diluted 1:5000 in cold-blooded saline solution) and 12% formalin (in cold-blooded saline solution) was used as the leucocyte dilutant. The Giemsa differential staining technique, mentioned previously for erythrocytes, was used to make leucocyte size determinations.

Differential leucocyte counts were made from blood smears stained with Giemsa's. The preparation was scanned from edge to edge and three hundred leucocytes were counted according to Seiverd (1964).

Thrombocyte counts and size were also calculated from a Giemsa stained blood smear. The indirect method of counting as reported by Seiverd (1964) was used.

Hemoglobin percentage was determined with a Sahli-Adams hemometer. With this method, 100% corresponds to 17.0 g hemoglobin per 100 cc whole blood.

Hematocrit values were determined in heparinized hematocrit tubes (Chase Instruments Corp.). Blood samples were centrifuged in an Adams MP Readacrit (CT-3400, Clay-Adams, Inc.) at 23 C for five minutes.

Blood pH was recorded within 0.05 tolerance with a Sargent combination microelectrode and Sargent pH meter (Model PB-S-30007).

Erythrocyte fragility measurements were made by a direct (visual) and indirect (spectrophotometric) method. Two drops of fresh blood were expelled from a 1 cc tuberculin syringe with an 18 gauge needle into 14 test tubes, which contained 5 ml of solution, increasing in 0.02% increments from 0.00% to 0.26% saline (Seiverd, 1964). Each tube was gently agitated to insure an even dispersion of cells. After tubes sat for two hours, a visual determination was made. All tubes were then centrifuged at 500 *g* for 15 min. at 23 C. A spectrophotometric analysis was then made of the supernatant with a Bausch and Lomb Spectronic 20 set at a wave length of 550 nm. Distilled water was used as a control.

After cardiac puncture, the animals were sutured and replaced in their holding tanks where they remained for 10 to 12 days, after which time they were anesthetized and sacrificed by severing the conus arteriosus. Blood samples were again taken and all hematological tests were repeated. In addition, coagulation time was determined using the method of Pace et al. (1947).

Photomicrographs were made at 430 × magnification.

Results

Red blood cells.—Cryptobranchus erythrocytes were usually oval shaped, although this varied from tear drop forms to sphericals. Precursor erythrocytes (polychromatic erythroblasts) were also seen. In the presence of Giemsa solution, erythrocyte membranes and nuclei stained dark blue and the cytoplasm light blue.

Erythrocyte measurements of the Niangua (Fig. 1B) and North Fork (Fig. 1A) populations differed significantly in cell total length, total width and nuclear length (Table 1).

The number of erythrocytes also differed significantly, averaging 72,544 per mm^3 and 92,713 per mm^3 for the Niangua and North Fork populations, respectively (Table 2).

White blood cells.—Direct determinations were made for leucocyte number. The mean count of the Niangua population was 179 per mm^3 and that of the North Fork population was 198 per mm^3 (Table 2).

The results for leucocyte size and leucocyte percentages are given in Tables 1 and 3, respectively.

Lymphocyte nuclei stained purplish-blue and the cytoplasm pale blue with Giemsa's. The nuclei were spherical and centrally located within the cell (Figs. 1C, D). At times, they were indented on one side. The cytoplasm often appeared as a thin ring or halo and was difficult to see. No cytoplasmic granulation was observed. Lymphocytes from the Niangua population had mean dimensions which overlapped those of the North Fork population (Table 1). Of the *Cryptobranchus* leucocytes, lymphocytes were the most abundant, averaging better than 60% for both the Niangua and North Fork populations (Table 3).

Monocyte nuclei comprised one-half to three-fourths of the cell and stained a reddish-lavender, while the cytoplasm, which usually contains dark red granules, stained a faint pink. The nuclei were often round or oval shaped (Figs. 1E, F). Monocytes of the Niangua population had mean dimensions

TABLE 1. BLOOD CELL SIZE (μ) IN *Cryptobranchus* FROM TWO LOCATIONS IN MISSOURI. Included are Measurements (M) for Total Length (TL), Total Width (TW), Nuclear Length (NL), and Nuclear Width (NW); Number of Animals (N); Number of Determinations per Animal (n); Mean ($\bar{x}$); Standard Deviation (s); Standard Error of Mean ($SE_{\bar{x}}$); Confidence Interval (C); and Test of Probability (t).

Population	M	N	n	$\bar{x}$	High	Low	s	$SE_{\bar{x}}$	C	t
					Erythrocytes					
Niangua	TL	11	20	49.78	63.60	43.25	0.753	0.227	0.506	16.89[1]
North Fork		19	20	43.53	54.62	26.29	1.081	0.248	0.521	
Niangua	TW	11	20	26.82	31.38	22.05	1.358	0.410	0.912	4.64[1]
North Fork		19	20	24.61	29.68	16.96	1.206	0.277	0.581	
Niangua	NL	11	20	24.80	28.31	22.41	1.713	0.516	1.151	2.76[2]
North Fork		19	20	23.12	25.98	19.30	1.548	0.355	0.746	
Niangua	NW	11	20	10.83	11.87	9.44	0.717	0.216	0.482	0.22
North Fork		19	20	10.78	11.65	9.95	0.452	0.104	0.218	
					Lymphocytes					
Niangua	TL	10	10	25.08	33.92	17.81	0.885	0.280	0.633	1.32
North Fork		19	10	24.46	31.38	16.96	1.334	0.306	0.643	
Niangua	TW	10	10	23.85	31.38	16.96	0.975	0.308	0.698	0.45
North Fork		19	10	23.63	30.53	16.96	1.360	0.312	0.656	
					Monocytes					
Niangua	TL	10	10	28.11	42.40	21.20	3.188	1.008	2.281	2.60[1]
North Fork		19	10	31.35	42.40	24.59	3.139	0.740	1.561	
Niangua	TW	10	10	26.34	33.92	18.66	1.803	0.570	1.290	0.51
North Fork		19	10	26.78	33.92	18.66	2.555	0.602	1.271	
					Eosinophils					
Niangua	TL	10	10	36.85	50.88	29.68	3.783	1.196	2.706	1.33
North Fork		19	10	35.05	46.64	24.59	3.290	0.755	1.593	
Niangua	TW	10	10	34.01	47.40	26.29	3.228	1.021	2.309	0.16
North Fork		19	10	33.82	44.94	24.59	3.011	0.691	1.458	
					Neutrophils					
Niangua	TL	10	10	36.49	50.03	28.83	5.380	1.701	3.848	0.99
North Fork		19	10	34.96	47.40	29.68	2.943	0.675	1.419	
Niangua	TW	10	10	33.85	41.55	25.44	3.231	1.022	2.311	0.66
North Fork		19	10	33.15	39.01	27.14	2.374	0.545	1.419	
					Thrombocytes					
Niangua	TL	10	7	20.26	26.29	15.26	1.977	0.625	1.414	1.52
North Fork		19	7	21.50	33.92	16.96	2.703	0.620	1.309	
Niangua	TW	10	7	14.31	22.05	11.80	2.650	0.838	1.895	0.138
North Fork		19	7	14.39	18.66	11.87	1.194	0.274	0.578	

[1] Significantly different at 0.01 level.
[2] Significantly different at 0.05 level.

TABLE 2. BLOOD CELL COUNTS (PER MM^3) IN *Cryptobranchus* FROM TWO LOCATIONS IN MISSOURI. Included are Number of Animals (N); Number of Determinations per Animal (n); Mean ($\bar{x}$); Standard Deviation (s); Standard Error of Mean ($SE_{\bar{x}}$); Confidence Interval (C); and Test of Probability (t).

Population	N	n	$\bar{x}$	High	Low	s	$SE_{\bar{x}}$	C	t
				Erythrocytes					
Niangua	9	4	72,544	87,850	27.500	9,722.72	3,240.91	7,473.53	2.43[1]
North Fork	16	4	92,713	145,000	48.750	23,597.31	5,899.33	12,571.47	
				Leucocytes					
Niangua	5	2	179	300	40	99.48	44.49	123.50	0.49
North Fork	8	2	198	300	113	36.90	13.05	30.85	
				Thrombocytes					
Niangua	10	16	5,827	13,682	3,472	3,176.29	1,004.43	2,272.03	0.0004
North Fork	18	16	4,863	9,442	1,436	2,258.49	532.33	1,123.22	

[1] Significantly different at 0.05 level.

significantly smaller than those of the North Fork population (Table 1).

Eosinophils were spherical and had bright red granules in a faint blue cytoplasm. The nuclei stained dark blue and were often bilobed and located at one end of the cell (Fig. 1G). Eosinophil mean dimensions of the two populations did not differ significantly (Table 1).

Neutrophils were spherical and displayed a granular cytoplasm with a lavender to bluish tint. The dark-stained nuclei appeared in various positions and forms, but were usually segmented or lobulated. These lobes, when dispersed, were connected by thin chromatin strands (Fig. 1H). Mean neutrophil dimensions showed considerable overlap between the two populations (Table 1). Neutrophils were second only to lymphocytes in total *Cryptobranchus* leucocyte abundance (Table 3).

No basophils were observed in either population.

Thrombocytes.—These appeared as small spindle-shaped cells, often arranged in clusters (Fig. 1I). The nuclei stained bright blue and the cytoplasm pale blue. There were no significant differences for thrombocyte mean dimensions (Table 1) or counts (Table 2) between the Niangua and North Fork populations.

The results for pH, hematocrit, hemo-

TABLE 3. PERCENTAGES OF LEUCOCYTES IN TWO POPULATIONS OF *Cryptobranchus* FROM MISSOURI. Included are Number of Animals (N); Number of Determinations per Animal (n); Mean ($\bar{x}$); Standard Deviation (s); Standard Error of Mean ($SE_{\bar{x}}$); Confidence Interval (C); and Test of Probability (t).

Population	Cell Type	N	n	$\bar{x}$	High	Low	s	$SE_{\bar{x}}$	C	t
Nianuga	Lymphocytes	9	3	66.55	83.66	54.00	10.09	3.36	7.76	1.33
North Fork		18	3	60.75	78.00	46.40	10.68	2.52	5.31	
Niangua	Monocytes	9	3	9.33	14.33	6.00	2.43	0.81	1.86	0.61
North Fork		18	3	8.38	19.67	3.00	4.32	1.02	2.15	
Niangua	Eosinophils	9	3	3.96	9.67	1.00	2.77	0.92	2.13	1.00
North Fork		18	3	4.94	9.67	1.33	2.23	0.53	1.11	
Niangua	Neutrophils	9	3	20.70	32.00	2.66	9.87	3.29	7.59	1.04
North Fork		18	3	25.88	44.67	3.66	13.10	3.09	6.51	

[1] No basophils were observed in either population; N = 9 (Niangua), N = 18 (North Fork).

TABLE 4. HEMATOLOGICAL DETERMINATIONS FOR TWO *Cryptobranchus* POPULATIONS IN MISSOURI. Included are Number of Animals (N); Number of Determinations per Animal (n); Mean ($\bar{x}$); Standard Deviation (s); Standard Error of Mean ($SE_{\bar{x}}$); Confidence Interval (C); and Test of Probability (t).

Population	N	n	$\bar{x}$	High	Low	s	$SE_{\bar{x}}$	C	t
				pH (at 25°C)					
Niangua	9	2	7.43	7.60	7.20	0.133	0.444	0.102	5.35[1]
North Fork	14	2	7.39	7.65	6.90	0.213	0.570	0.123	
				Hematocrit Values (% cells)					
Niangua	8	5	43.33	46.75	38.90	2.685	0.949	2.447	0.174
North Fork	15	5	40.08	55.00	30.00	6.791	1.753	3.761	
				Hemoglobin (g/100 ml)					
Niangua	9	3	10.07	11.45	8.50	0.934	0.311	0.718	2.90[1]
North Fork	16	3	8.32	12.16	6.63	1.667	0.417	0.884	
				Coagulation Time (sec)					
Niangua	9	1	61.94	97.50	45.00	20.378	6.793	15.664	1.82
North Fork	15	1	80.40	120.00	30.00	25.889	6.685	14.338	
				Erythrocyte Fragility (% NaCl for total lysis)					
Niangua	8	2	0.096	0.11	0.08	0.009	0.003	0.008	1.44
North Fork	10	2	0.090	0.11	0.08	0.015	0.005	0.011	

[1] Significantly different at 0.01 level.

globin, coagulation time and erythrocyte fragility are given in Table 4.

DISCUSSION

We noted significant differences between the Niangua (*C. a. alleganiensis*) and North Fork (*C. a. bishopi*) populations in erythrocyte size, erythrocyte number, monocyte size, pH and hemoglobin values.

Our erythrocyte counts for the Niangua population agree closely with those of Wintrobe (1933) for *C. a. alleganiensis*. However, certain differences do exist between Wintrobe's (1933) results and our own. He listed higher leucocyte counts, larger percentages of basophils and neutrophils, higher hemoglobin and hematocrit values, but lower erythrocyte counts and smaller percentages of lymphocytes and monocytes than we found in either of our populations. The great differences in leucocyte counts may have been partly due to methodology. Wintrobe used the blood smear method of determination whereas we used a hemocytometer. Wintrobe could have misidentified monocytes for basophils, which would account for the differences in the two studies.

Various environmental factors are known to affect erythrocyte size (Haden, 1940; Altman and Dittmer, 1961; Harris, 1963). Year round water chemistry data for 1970–71 for the North Fork River and data based on limited sampling from the Niangua River (Nickerson, pers. comm.) indicate similarity of the two rivers. This similarity suggests that the effect of the two rivers is of little significance on the differences of erythrocyte size between the Niangua and North Fork populations.

Hutchinson and Szarski (1965) and Rouf (1969) among others, have noted considerable individual variation in blood cell counts among amphibians. Other authors (Alder and Huber, 1923; Klieneberger, 1927; Schermer, 1967) have reported seasonal variation in certain amphibian cell counts. In this study, neither type of variation appeared significant.

Sexual variation in cell counts, a phenomenon found in certain frogs (Arvy, 1947; Kaplan, 1951), was not observed in this study.

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