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HEMATOLOGY AND SERUM CHEMISTRY OF OZARK AND EASTERN HELLBENDERS (*CRYPTOBRANCHUS ALLEGANIENSIS*)

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ABSTRACT: Hellbenders are large aquatic salamanders. The Ozark subspecies is in decline through its range. This is the first comprehensive report on hematologic and serum chemistry for both Ozark and eastern hellbenders. Blood samples were analyzed for 25 parameters in 33 Ozark hellbenders from the North Fork of White River and the Eleven Point River in Missouri and 45 eastern hellbenders from the Davidson River-Looking Glass Creek in North Carolina and the Cooper Creek in Georgia. Each river was considered a population. In general, the majority of the blood parameters analyzed were similar between populations and subspecies for same-sex individuals, although a few significant differences were identified. The baseline data we acquired are important for future monitoring of hellbender populations, particularly as Ozark hellbender populations continue to age.

Key words: Amphibians; *Cryptobranchus*; Hellbender; Hematology; Serum chemistry

HELLBENDERS (*Cryptobranchus alleganiensis*) are large aquatic salamanders that inhabit cold streams and rivers of parts of the eastern United States. Two subspecies occur: eastern hellbenders (*C. alleganiensis alleganiensis*) and Ozark hellbenders (*C. alleganiensis bishopi*). The subspecies currently are distinguished by geographic region, with Ozark hellbenders only occurring in the White, Spring, Current, and Eleven Point Rivers and tributaries of southeastern Missouri and northeastern Arkansas (Nickerson and Mays, 1973; Rogers, 2001). Ozark hellbenders have been declining with rare recruitment of young in the populations (Wheeler et al., 2003). A comparison of historical data from the late 1970s against more recent data from 1998 found a shift in size classes and a decline in population density in both rivers (Peterson, 1985; Wheeler et al., 2003). Though the causes of the decline remain to be elucidated, changes in abiotic and biotic factors have been observed in the hellbender habitat in some populations, including habitat degradation, erosion, chemical runoff, increased gigging activities (fishing with a pronged spear), heavy canoe traffic, and predation (Dundee, 1971; Humphries, 1999; Minton, 1972; Nickerson and Mays, 1973; Smith and Minton, 1957; Trauth et al., 1992; Williams et al., 1981). Although populations of the eastern subspecies are known also to be in decline in Missouri (Wheeler et al., 2003), the status of eastern hellbenders in the southeastern United States is unclear.

Numerous studies have been conducted on various aspects of amphibian hematology and serum chemistry (Altman and Dittmer, 1974; Carmena-Suero et al., 1980; Cathers et al., 1997; Harris, 1972; Jerrett, 1971; Jerrett and Mays, 1973; Pfeiffer et al., 1990; Roofe, 1961; Rouf, 1969; Scarborough, 1931; Schermer, 1967; Singh, 1978; Stone, 1971; Taketa and Nickerson, 1973*a*,*b*; Vernberg, 1955; Wintrobe, 1933; Wright and Whitaker, 2001). Our purpose was to establish baseline information on hematology and serum chemistry, and to compare data for Ozark hellbenders and for southeastern populations of eastern hellbenders. The protocols, hematological results, and serum chemistry established in this study could be incorporated into future monitoring activities and programs for population restoration.

METHODS

Research Sites

Blood samples for Ozark hellbenders were collected from individuals from two populations—the North Fork of the White River and the Eleven Point River—between June and

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November 2003, and between May and September 2004. The eastern hellbender blood samples were collected during July 2003 and June–July 2004 from hellbender population at the Davidson River-Looking Glass Creek, North Carolina and Cooper Creek, Georgia.

Collecting Blood Samples

A total of 84 animals were collected by hand after removing them from underneath rocks of the river bottom. Scuba gear was used to sample underwater in deeper areas. Seventy-eight animals were subject to blood collection whereas blood samples were not drawn in six juveniles due to their small sizes. Upon capture, the animals were anaesthetized in a weak solution of MS-222 (3-aminobenzoic acid ethyl ester) using a concentration of 2.5 g/8 L water (pH 6.8). Once the animals were rendered nonmobile, a radio-wave scan was performed to determine the identity of the animal. Animals were tagged with an all-weather, extended-range, multi-tag reader (AVID Power Tracker IV) implanted in the upper part of the tail if they were not tagged previously. Body mass (BM), total length (TL), snout–vent length (SVL), location of capture, sexual state, morphological abnormalities and sex were recorded. Sex was identified by external examination or by the records of historically marked hellbenders. Blood was drawn from the caudal vein approximately 2.5 cm posterior to the cloaca. One milliliter of blood was obtained for every 100 mg of body weight, as recommended by Dr. Randy Junge of the St. Louis Zoo (personal communication) and by Wright and Whitaker (2001). After the blood was drawn, it was placed in heparinized vacutainers and centrifuged at 3000 g for 10 min. The plasma was then aliquoted into cryoviales and stored in liquid nitrogen until bioassays were performed. After the blood was drawn, animals were placed in a shallow tub with fresh water to recover from anesthesia. Hellbenders were then released at the site of capture. No animals were permanently removed.

Analysis of Blood Samples

An aliquot of plasma was sent to Antech Diagnostics Laboratory (Memphis, Tennessee) to run the Comprehensive Chemistries and the Complete Blood Counts (CBC). A HITACHI 747-200 or a HITACHI 717 Chemistry Analyzer was utilized to perform the photometric assays. The 13 parameters were glucose, urea nitrogen, total protein, albumin, aspartate aminotransferase (AST), calcium, phosphorus, globulin, creatine phosphokinase (CPK), uric acid, sodium, potassium, and chloride. For the CBC's, a spun packed cell volume (Hct) was performed and then the blood smear was reviewed under a 40× lens to estimate the number of white blood cells (WBC), the 100 cell differential, the presence of parasites, and the red blood cell (RBC) morphology. Thrombocytes were present in all Ozark and eastern hellbenders, mostly as clusters, with a few present individually. Because of the clustering effect, no count was performed. Erythrocytes were not counted.

The parameters analyzed included immunological, excretory, regulatory, and homeostatic functions (Table 1). The description of the physiological functions of these parameters corresponds to the general knowledge for a variety of amphibians and reptiles (Boutilier et al., 1992; Constanzo et al., 1993; Divers, 2000; Elkan, 1976; Mitruka and Rawnsley, 1981; Turner, 1988; Wright and Whitaker, 2001). Some functions of a parameter may be universal across species, whereas, other functions may be more species-dependent.

Statistical Analysis

A single variable Analysis of Variance (ANOVA) was performed comparing the four populations using Systat 9.0. The alpha level was set at $P \leq 0.05$. Statistically significant differences were further analyzed using least squared differences. Data were analyzed separately for male and female animals.

Results

General Appearance and Parasites

Seventy-two percent of the Ozark hellbenders (10 males and 7 females from the North Fork of White River and 5 males and 2 females from the Eleven Point River) and 55% of the eastern hellbenders (12 males and 2 females from the Davidson River and 3

TABLE 1.—Description of the physiological parameters analyzed in a variety of amphibian-reptilian species.

Parameter	Physiological function
Hematocrit	Percentage by volume of packed red blood cells.
WBC estimate	Count of leukocytes, erythrocytes and thrombocytes (Wright and Whitaker, 2001). Raised during inflammation-infection; depressed or low during hibernation/posthibernation (Divers, 2000)[1].
Thrombocytes	Blood coagulation and inflammatory reactions (Turner, 1988; Wright, 2001).
Het (Heterophils)/Poly (Polymorphonuclearcytes)	Phagocytize bacteria; increase numbers during bacterial infection (Wright and Whitaker, 2001).
Neutrophils	Protect body from bacterial infections; no production of antibodies; number varies (Mitruka and Rawnsley, 1981).
Lymphocytes	Immunological functions: resistance to infection, antibody production and tissue rejection. (Mitruka and Rawnsley, 1981; Turner, 1988; Wright and Whitaker, 2001).
Monocytes	Phagocytic to protect against infection; process the antigen for lymphocytes. (Mitruka and Rawnsley, 1981; Wright and Whitaker, 2001).
Eosinophils	Not effective for all life stages (Elkan, 1976). Poorly phagocytic (Wright and Whitaker, 2001). Elevated during helminth and protozoal infections (Divers, 2000)[1]. Respond to metazoan parasites (Turner, 1988).
Basophils	Process blood degranulation; surveillance role, recruit eosinophils during helminthes infection (Wright and Whitaker, 2001).
Glucose	Varies according to metabolic state, nutrition, stress (Divers, 2000)[1] raise osmotic pressure of body fluids (Constanzo et al., 1993).
Urea nitrogen	Production and excretion usually low and variable, highly soluble in water (Boutilier et al., 1992; Divers, 2000).
Total protein	Decreased with malnutrition, blood loss, intestinal disease, chronic liver and kidney disease (Divers, 2000)[1].
Albumin	Decrease due to prolonged anorexia, protein-losing enteropathies or nephropathies or chronic hepatic disease; rise due to dehydration (Divers, 2000)[1].
Globulin	Basis for antibodies, glycoproteins (protein-carbohydrate compounds), lipoproteins (proteins involved in fat transport), and clotting factors.
AST (SGOT, aspartate aminotransferase)	Enzyme found in liver, heart and skeletal muscle; non-specific indicator of liver disease (Divers, 2000)[1].
Calcium	Affected by nutrition, vitamin D and albumin level; decreased during chronic renal disease, but elevated levels cause renal disease (Wright and Whitaker, 2001).
Phosphorus	Affected by nutrition, and vitamin D; increased during renal disease (Wright and Whitaker, 2001).
Sodium	Possibly affect acid-base balance, ion exchange, maintenance of concentration and charge differences across cell membranes, determinant of extracellular fluid volume (Boutilier et al., 1992).
Potassium	Ion exchange, determinant of extracellular fluid volume and osmotic balances (Boutilier et al., 1992).
Chloride	Possibly affect acid-base balance, increase or decrease ionic concentrations, and maintenance of concentration and charge differences across cell membranes, determinant of extracellular fluid volume (Boutilier et al., 1992).
CPK (Creatine phosphokinase)	Diagnosis and monitoring of liver, kidney and heart disease. Found in cardiac, smooth and skeletal muscles. Elevated during stress (Divers, 2000)[1].
Uric acid	Occurs in reptiles, birds and insects, raised during dehydration, highly variable, low solubility (Boutilier et al., 1992; Divers, 2000).

[1] Reptile species; all other species are amphibians.

males and 1 female from Cooper Creek) presented some signs of injuries. These injuries included missing limbs, toes, digits, notches in the tail, bone exposure at digits and tail, and missing eyes. Similar injuries were also reported by Nickerson and Mays (1973). Only one female from the North Fork of White River out of the 33 Ozark hellbenders studied, was positive for blood parasites (filarid larva). None of the eastern hellbenders tested positive for blood parasites. Previous studies indicated that parasites in the blood system seemed to be common in salamanders and other amphibians (Anver and Pond, 1984; Marcus, 1981; Nickerson and Mays, 1973; Reichenbach-Klinke and Elkan, 1965; Wright

and Whitaker, 2001). Leeches were found in at least 50% of the Ozark hellbenders in the two streams we studied; however, just one leech was found in an eastern hellbender. Leeches were attached to injury sites, back, abdomen, toes, tail, and cloaca. Two male eastern hellbenders and one female eastern hellbender from the Davidson River had "trails" either on their back or in the abdomen, which might be the result of possible infections by nematodes such as *Dracunculus* worms.

Morphological Measurements

Hellbenders collected during the study ranged from 36.9 to 956.9 g body mass and 190 to 572 mm total length. No significant differences were found between the female hellbender populations in terms of body mass, total length, or snout–vent length (Table 2). Male hellbenders from Cooper Creek were significantly lighter than males from the other populations ($P = 0.009$; Table 2). In terms of total and snout–vent length, males from the North Fork of the White River were significantly larger than males from the Davidson River and Eleven Point River, while males from Cooper Creek were the smallest ($P < 0.01$; Table 2). The two juvenile Ozark hellbenders averaged 109.4 g, with a TL of 259.0 mm. Four juvenile eastern hellbenders averaged 47.2 g, with an average TL of 169.8 mm.

Hematology and Serum Chemistry

Female eastern Hellbenders from Cooper Creek were significantly different from the other three populations in terms of absolute eosinophils, basophils, and CPK ($P = 0.03$, <0.01, and <0.01, respectively). Female Ozark hellbenders from the North Fork of the White River had significantly lower total protein and uric acid ($P < 0.01$, 0.05, respectively). Females from the two eastern hellbender populations were significantly different from the Ozark hellbender populations in terms of globulin ($P < 0.01$; Table 3).

Male Ozark hellbenders from the North Fork of the White River were significantly different from the other three hellbender populations in terms of lymphocytes, absolute lymphocytes, albumin, and AST ($P < 0.01$, 0.02, 0.05, and 0.05, respectively). Male hellbenders from the Eleven Point River had significantly higher absolute eosinophil levels compared to the other populations ($P = 0.03$). Hematocrit levels were significantly different between Ozark hellbenders and eastern hellbenders ($P < 0.01$; Table 3).

Discussion

Morphological Characteristics

The majority of the hellbenders collected were sexually mature, as defined by Nickerson and Mays (1973) and Peterson et al., (1983) who used both morphology observation and prediction from a length-specific growth rate model. Although historical data have shown that eastern hellbenders were heavier and longer than Ozark hellbenders (Dundee and Dundee, 1965; Fitch, 1947; Grobman, 1943; Jerrett, 1971; Nickerson and Mays, 1973; Topping and Ingersol, 1981), our findings

Table 2.—Morphological characteristics of hellbenders from the North Fork of the White River (NFWR), Eleven Point River (EPR), Davidson River (DR), and Cooper Creek (CC). Values are given as mean ±SD. Different letters within a row indicate significant differences at an alpha of 0.05.

	NFWR	EPR	DR	CC	*P*-value
Males					
n	10	7	21	8	
Body mass (g)	440 ± 90[a]	412 ± 111[a]	372 ± 58[a]	321 ± 101[b]	0.01
Total length (mm)	437 ± 29[a]	379 ± 42[bc]	402 ± 37[b]	379 ± 48[c]	<0.01
Snout–vent length (mm)	290 ± 22[a]	255 ± 28[bc]	260 ± 28[b]	241 ± 30[c]	<0.01
Females					
n	12	4	10	6	
Body mass (g)	605 ± 196	561 ± 120	511 ± 140	435 ± 146	0.22
Total length (mm)	469 ± 64	441 ± 32	453 ± 38	417 ± 44	0.23
Snout–vent length (mm)	321 ± 49	300 ± 19	280 ± 35	287 ± 17	0.1

TABLE 3.—Serum and blood chemistry of hellbenders from NFWR, EPR, DR, and Cooper Creek CC (see Table 2 for abbreviations). Values are given as mean ± SD. Different letters within a row indicate significant differences at an alpha of 0.05.

	NFWR	EPR	DR	CC	*P*-value
Males					
Hematocrit (%)	45 ± 6[a]	47 ± 10[a]	38 ± 5[b]	35 ± 8[b]	<0.01
WBC estimate (10^3/uL)	4.4 ± 1.6	6.4 ± 1.4	5.1 ± 1.8	5.4 ± 2.2	0.09
Het/Poly (%)	54 ± 21	36 ± 22	32 ± 17	32 ± 21	0.25
Absolute neutrophils	2366 ± 1379	2238 ± 1298	1640 ± 940	1736 ± 1330	0.64
Lymphocytes (%)	28 ± 21[b]	41 ± 16[ab]	51 ± 18[a]	58 ± 21[a]	0.01
Absolute lymphocytes	1240 ± 903[b]	2732 ± 1600[a]	2607 ± 1129[ab]	3154 ± 2127[a]	0.02
Monocytes (%)	0.4 ± 0.7	0.4 ± 0.5	0.7 ± 1.1	0.5 ± 0.8	0.84
Absolute monocytes	25 ± 46	27 ± 35	38 ± 63	22 ± 36	0.84
Eosinophils (%)	13 ± 10	19 ± 17	11 ± 6	6 ± 6	0.16
Absolute eosinophils	548 ± 446[b]	1161 ± 917[a]	624 ± 447[b]	296 ± 239[b]	0.03
Basophils (%)	4.2 ± 4.2	3.4 ± 2.0	4.4 ± 3.9	3.3 ± 1.4	0.90
Absolute basophils	210 ± 229	213.7 ± 130.8	221 ± 183	175 ± 92.3	0.94
Glucose (mg/dL)	25 ± 14	21 ± 9	22 ± 14	11 ± 10	0.12
Urea nitrogen (mg/dL)	4.1 ± 3.7	1.4 ± 0.5	2.4 ± 0.6	7.6 ± 11.2	0.06
Total protein (g/dL)	4.6 ± 3.0	3.0 ± 0.5	3.4 ± 0.8	3.5 ± 0.8	0.12
Albumin (g/dL)	1.9 ± 1.7[a]	1.1 ± 0.4[b]	1.0 ± 0.4[b]	1.3 ± 0.4[ab]	0.05
Globulin (g/dL)	2.7 ± 1.9	1.9 ± 0.5	2.3 ± 0.6	2.2 ± 0.6	0.47
AST (SGOT) (U/L)	205 ± 152[a]	98 ± 39[b]	136 ± 73[b]	99 ± 40 b	0.05
Calcium (mg/dL)	8.7 ± 2.2	8.0 ± 1.6	8.4 ± 1.1	7.8 ± 1.3	0.61
Phosphorus (mg/dL)	14.5 ± 16.5	7.7 ± 3.3	8.4 ± 2.9	6.2 ± 1.9	0.14
Sodium (mEq/L)	101 ± 28	102 ± 7	113 ± 15	105 ± 7	0.27
Potassium (mEq/L)	11.4 ± 15.7	5.7 ± 3.9	4.0 ± 1.3	6.0 ± 5.5	0.12
Chloride (mEq/L)	77 ± 17	75 ± 3	80 ± 9	78 ± 3	0.71
CPK (U/L)	760 ± 622	1410 ± 2300	6193 ± 8908	2327 ± 2548	0.10
Uric acid (mg/dL)	4.1 ± 7.9	0.6 ± 0.6	0.7 ± 0.3	1.1 ± 1.8	0.11
Females					
Hematocrit (%)	42 ± 15	41 ± 9	33 ± 7	31 ± 5	0.23
WBC estimate (10^3/uL)	4.5 ± 1.4	3.9 ± 1.4	3.7 ± 1.2	2.6 ± 1.8	0.13
Het/Poly (%)	33 ± 14	49 ± 29	34 ± 9	41 ± 18	0.34
Absolute neutrophils	1519 ± 1175	1604 ± 175	1242 ± 434	1173 ± 1319	0.82
Lymphocytes (%)	47 ± 22	30 ± 26	48 ± 13	38 ± 16	0.39
Absolute lymphocytes	2141 ± 1050	1430 ± 1373	1784 ± 854	1021 ± 687	0.17
Monocytes (%)	0.7 ± 0.8	0	0.8 ± 0.8	1.3 ± 1.0	0.11
Absolute monocytes	27 ± 42	0	23 ± 25	27 ± 25	0.51
Eosinophils (%)	15 ± 14	18 ± 18	12 ± 7	0.5 ± 0.8	0.06
Absolute eosinophils	578 ± 500[a]	757 ± 681[a]	446 ± 263[a]	8.0 ± 13.0[b]	0.03
Basophils (%)	4.1 ± 3.2[b]	3.0 ± 3.6[b]	5.8 ± 5.4[b]	19.5 ± 18.6[a]	0.01
Absolute basophils	189 ± 166	84 ± 71	182 ± 144	420 ± 421	0.12
Glucose (mg/dL)	23 ± 12	25 ± 10	18 ± 8	21 ± 13	0.70
Urea nitrogen (mg/dL)	4.1 ± 3.8	1.7 ± 0.5	2.5 ± 0.7	3.8 ± 2.6	0.34
Total protein (g/dL)	2.8 ± 0.6[b]	3.2 ± 0.5[ab]	3.8 ± 0.6[a]	3.4 ± 0.7[a]	0.01
Albumin (g/dL)	1.0 ± 0.5	1.3 ± 0.8	1.4 ± 0.2	1.1 ± 0.5	0.37
Globulin (g/dL)	1.8 ± 0.4[c]	1.9 ± 0.4[b]	2.5 ± 0.4[a]	2.3 ± 0.3[ab]	<0.01
AST (SGOT) (U/L)	95 ± 41	111 ± 23	113 ± 59	135 ± 72	0.49
Calcium (mg/dL)	11 ± 2	12 ± 3	13 ± 2	12 ± 2	0.33
Phosphorus (mg/dL)	11 ± 2	12 ± 3	7 ± 2	9 ± 3	0.09
Sodium (mEq/L)	105 ± 8	105 ± 7	107 ± 4	109 ± 6	0.59
Potassium (mEq/L)	4.0 ± 1.2	4.6 ± 1.3	3.2 ± 1.3	5.2 ± 3.3	0.22
Chloride (mEq/L)	76 ± 3	79 ± 0.5	76 ± 2	76 ± 4	0.44
CPK (U/L)	859 ± 859[b]	3397 ± 6043[b]	1888 ± 1667[b]	8869 ± 8752[a]	0.01
Uric acid (mg/dL)	0.5 ± 0.4[b]	0.5 ± 0.2[ab]	1.0 ± 0.6[a]	0.9 ± 0.3[a]	0.05

did not show a clear distinction between eastern and Ozark hellbenders in terms of size, possibly because of the small sample size.

Hematology and Serum Chemistry

Our data indicated that Ozark and eastern hellbender populations have very similar ranges for most of the physiological parameters measured. The few statistical differences observed between Ozark and eastern hellbenders (globulin in females and hematocrit in males) could be the consequence of adaptations to environmental factors such as site-specific seasonal changes, diseases, reproductive stage, temperature, and stress (Harris, 1972; Kollias, 1984; Maniero and Carey, 1997; Robertson, 1978; Roofe, 1961; Wright and Whitaker, 2001). Additional studies are needed to determine if differences in the levels of these chemicals are due to intrinsic differences between the two subspecies and/or environmental factors.

We observed several differences between the four populations that could not be attributed to subspecies, including absolute eosinophils, basophils, CPK, total protein, and uric acid in females and lymphocytes, absolute lymphocytes, absolute eosinophils, albumin, and AST in males. These differences may be due to genetic or environmental factors. For instance, it has been shown that amphibian immune function and physiological condition may be influenced by environmental factors such as temperature (Cooper et al., 1992; Maniero and Carey, 1997; Wright and Cooper, 1981). Due to time and personnel constraints and limited available equipment, Ozark hellbenders were collected on a monthly basis throughout the entire study period while eastern hellbenders were clustered in June–July 2004. Effects on immune parameters due to this sampling discrepancy remain unknown. Levels of leukocytes in the current study are similar to those measured by Jerret and Mays (1973) and Wintrobe (1933). Vertebrates use leukocytes to defend against infections, inflammations and diseases; each leukocyte (basophils, eosinophils, lymphocytes, monocytes, neutrophils) has a specific function (Beutler, 2004; Divers, 2000; Duncan et al., 1994; Mitruka and Rawnsley, 1981; Wright and Whitaker, 2001), and their levels fluctuate significantly in response to the production of others. Lymphocyte levels are usually very constant in healthy animals, although the value may decrease in older individuals (Duncan et al., 1994). Further study is needed to determine if the statistically significant differences that we found among the four populations have biological significance in terms of the function, health, and survival of the populations.

This study establishes baseline information for serum and blood chemistry parameters. Due to small sample sizes, we cannot quantitatively attribute the differences in blood parameters to size, age, or environmental factors, although some blood parameters were different between the two subspecies. As additional data continue to accumulate, factors that contribute to differences in blood parameters among populations can be determined. Ongoing and future monitoring studies should find these data helpful for assessing the physiological and immunological parameters of individual hellbenders and populations. Of particular interest will be to monitor how parameters change over time in Ozark hellbender populations if they continue to decline. In the end, we reiterate the urgency of placing resources and research efforts toward protecting and restoring Ozark hellbenders to sustain their dwindling populations.

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