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HEMATOLOGIC, PROTEIN ELECTROPHORESIS, BIOCHEMISTRY, AND CHOLINESTERASE VALUES OF FREE-LIVING BLACK STORK NESTLINGS (*CICONIA NIGRA*)

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ABSTRACT: Hematologic, protein electrophoresis, serum biochemistry, and cholinesterase values were determined in 36 free-living black stork nestlings (*Ciconia nigra*) between 25 and 53 days of age in order to establish normal reference values for this population. The following values were evaluated: white blood cell counts, red blood cell counts, packed cell volume, hemoglobin, heterophils, lymphocytes, monocytes, eosinophils, prealbumin, albumin, α-globulin, β-globulin, γ-globulin, total protein, aspartate aminotransferase, alkaline phosphatase, lactate dehydrogenase, creatine kinase, calcium, phosphorus, iron, cholesterol, glucose, triglycerides, uric acid, urea, creatinine, total solids, bile acids, and butyrylcholinesterase. Sex-dependent differences were observed in hemoglobin, prealbumin, albumin, γ-globulin, total protein, alkaline phosphatase, and triglycerides. Packed cell volume, butyrylcholinesterase, aspartate aminotransferase, creatine kinase, and creatinine increased with age, whereas albumin, mean cell volume, calcium, phosphorus, cholesterol, and total solids decreased with age. These hematologic and serum biochemistry values can be used as reference ranges in free-living black stork nestlings.

Key words: Biochemistry, black stork, butyrylcholinesterase, *Ciconia nigra,* electrophoresis, hematology, nestlings, reference values.

INTRODUCTION

Hematologic and blood biochemistry analyses are valuable tools for evaluating health of wildlife, both in diagnosing disease and clinical monitoring of the patient (Karesh et al., 1997; Toro et al., 1997; Villouta et al., 1997; Wyk et al., 1998; Lanzarot et al., 2001). However, proper interpretation of these parameters requires appropriate reference values for each species to minimize the effect of species differences. Additional factors such as sex or age may also affect the blood profile of avian species (D'Aloia et al., 1996; McInnes et al., 1996; Villouta et al., 1997; Lanzarot et al., 2001; Howlett et al., 2002).

Black stork chicks could be used as an indicator species of exposure to environmental contaminants, such as cholinesterase inhibitors, since they do not move from the nest and are fed a diet based on aquatic prey, mainly fish and amphibians, exclusively from the nesting territory of the adults (Elliott, 1992). Plasma butyrylcholinesterase (BChE) activity has been used to monitor exposure of wildlife to cholinesterase inhibitors (Mayack and Martin, 2003); however, the proper interpretation of this parameter requires appropriate reference values.

A previous study reported hematologic and some biochemistry parameters for a small number of 59–68-day-old black storks (Puerta et al., 1989). The objective of this investigation was to report hematologic, serum biochemistry, protein electrophoresis, and plasma BChE reference values from a large population of free-living black stork nestlings.

MATERIALS AND METHODS

Evaluations of individual black storks and sample collections for health analysis were conducted between May and July, the hatching-fledging period for this species in this area, in three consecutive years (2000, 2001, and 2002). The study included 36 free-living black stork nestlings from Comunidad Autónoma de Madrid (Central Spain, 39°52′–41°8′N, 3°6′–4°31′W).

All birds were examined and sampled in their nest between 10:00 AM and 1:00 PM by

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the same veterinarian (M.P.L.). Each bird was handled for approximately 10 min for physical examination, as described by Samour (2000), and a blood sample was obtained near the beginning of the handling period. The nestlings were between 25 and 53 days (±3 days) old based on the approximate hatching date, which was determined by weekly observation beginning with the date eggs were laid.

Blood samples (3 ml) were collected from the ulnar vein using a 21-gauge butterfly catheter. All birds were sampled by the same veterinarian (M.P.L.), for quality assurance control (Bowerman et al., 2000), Immediately after blood sampling, two blood smears from each bird were made and air dried. After collection, blood was mixed immediately in tubes with the anticoagulant dipotassium ethylenediaminetetraacetic acid (1.5 mg/dl). Whole blood was used for the complete blood count. Plasma was harvested by centrifugation (10,000 × G for 5 min) for BChE analysis. Whole blood also was mixed with alkaline phosphatase streptavidin (APS)-buffer for sex determination (Arctander, 1988). A portion of whole blood was transferred to tubes without anticoagulant and serum collected by centrifugation (10,000 × G for 5 min) for biochemistry and protein electrophoresis analyses. The samples were maintained at 4–6 C and in the dark. Blood samples were processed in the laboratory within 12 hr, and hematologic analysis was performed on the day of collection by the same veterinarian (M.P.L.).

White blood cell counts (WBC) and red blood cell counts (RBC) were determined using an improved Neubauer hemocytometer (Brand, Wertheim, Germany) and Natt and Herrick's solution in blood cell dilution pipettes (Campbell, 1995). Packed cell volume (PCV) was determined by centrifugation at 10,000 × G for 5 min (Howlett et al., 2002). Hemoglobin (Hb) content was determined by cyanide-free hemoglobin determination (Campbell, 1995). Mean cell volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated using the following equations (Campbell, 1995): MCV=(PCV/RBC)·10; MCH=(Hb/RBC)·10; MCHC=(Hb/PVC)·100. Thin blood smears were stained with a Diff-Quick stain (Panreac, Barcelona, Spain), and a 200-cell differential was performed.

Protein electrophoresis was performed using a cellulose acetate methodology (Lumeij, 1987; Cray and Tatum, 1998). Total protein (TP) was determined with the Biuret method (Lumeij and MacLean, 1996). Total solids (TS) were measured using a portable refractometer (C-6 Comecta, Ivimen, Barcelona, Spain) as described by Fudge (2000a).

Serum biochemistry values were measured at 37 C by an automated analyzer (Shimazu CL-7200, Kyoto, Japan) using commercially available test kits (ITC Diagnostics, Izasa S.A., Barcelona, Spain). The chemistry and enzyme panel included iron, aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactic dehydrogenase (LDH), creatine kinase (CK), uric acid, urea, creatinine, total calcium, phosphorus, glucose, cholesterol, and triglycerides. Bile acid concentrations were measured by spectrophotometric procedures using commercially available reagents (Sigma Diagnostics Inc., St. Louis, Missouri, USA).

Plasma butyrylcholinesterase (EC 3.1.1.8) was determined at 37 C in plasma with a test kit whereby butyrylcholine was used as substrate (Test Combination 124125, Boehringer Mannheim, Mannheim, Germany) (Tietz, 1990).

Determination of the sex was performed by Laboratorio de Genética Molecular, Departamento de Producción Animal, Facultad de Veterinaria, Madrid, Spain, using a polymerase chain reaction (PCR)-based single-stranded conformational polymorphism assay (Cortés et al., 1999).

Data are reported as arithmetic mean, standard deviation (SD), median, and inner limits of the percentiles P_5 and P_{95} (for 36 variables). Normality was assessed with the Kolmogorov-Smirnov statistic with Lilliefors significance correction, the skewness statistic, and visual examination of data distribution. Comparisons between sex and age groups were performed using a Mann-Whitney *U*-test (nonparametric analysis) or by one-way analysis of variance (parametric analysis). Differences were considered significant at $P<0.05$ (Bolton, 1997). Statistical calculations were performed using SPSS 10.0 (SPSS Inc., Chicago, Illinois, USA). To determine age-related changes, chicks were divided into two subgroups of ≤40 and ≥41 days old for selected analytes.

RESULTS

Thirty-six free-living nestling black storks (50% males and 50% females) were studied. All birds appeared to be in good condition, and no abnormalities were noted during physical examination or in hematologic analyses throughout the observation period (70 days); all fledged successfully.

Hematologic, blood biochemistry, protein electrophoresis, and BChE values are shown in Tables 1 and 2. Packed cell vol-

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TABLE 1. Hematologic findings in free-living black stork (*Ciconia nigra*) nestlings in Spain.

Parameter[a]	Mean ± SD	Median	P_5[b]	P_{95}[b]
WBC/μl ($\times 10^3$)	14.99±4.14	14.44	8.40	21.64
RBC/μl ($\times 10^6$)	1.61±0.27	1.56	1.15	2.04
PCV (%)	32.25±3.89	33.00	24.6	38.60
≤40[c]	30.88±4.92	31.50	21.00	43.00
≥41[d]	33.35±2.41	33.50	27.20	38.90
Hb (g/dl)	10.54±0.77	10.30	9.52	12.42
Male[e]	10.27±0.69	10.20	9.50	12.00
Female[f]	10.78±0.78	10.70	9.80	12.50
MCV (fl)	205.97±44.44	198.71	147.97	278.61
≤40	189.76±52.59	173.39	135.59	352.45
≥41	218.94±32.51	212.55	174.46	282.99
MCHC (g/dl)	32.12±4.76	31.89	29.24	45.45
MCH (pg)	67.23±11.78	64.10	48.60	90.37
Heterophils (%)	41.75±13.85	42	22.90	72.60
Lymphocytes (%)	50.25±13.14	48	21.50	71.60
Monocytes (%)	1.15±0.95	1	0	3.90
Eosinophils (%)	6.19±3.91	7	1.90	14.00
Basophils (%)	—	—	—	—

[a] WBC = white blood cells; RBC = red blood cells; PCV = packed cell volume; Hb = hemoglobin; MCV = mean cell volume; MCHC = mean corpuscular hemoglobin concentration; MCH = mean corpuscular hemoglobin.
[b] Inner limits of the fifth and 95th percentiles.
[c] $n=16$.
[d] $n=20$.
[e] $n=18$.
[f] $n=18$.

ume, Hb, MCHC, monocytes, ALP, phosphorus, iron, triglycerides, urea, creatinine, and TS did not have a normal distribution. No basophils were identified in blood smears. Sex-dependent differences were observed in hemoglobin, prealbumin, albumin, gamma globulin, TP, ALP, and triglycerides (Tables 1 and 2). Packed cell volume, BChE, AST, CK, and creatinine increased with age, whereas albumin, MCV, calcium, phosphorus, cholesterol, and TS decreased with age.

DISCUSSION

This study determined hematologic values, serum biochemistry, protein electrophoresis, and BChE values from a large population ($n=36$) of free-living nestling black storks providing reference intervals that can be used as baseline information for further studies. It is important to use healthy animals in the establishment of reference values. However, it can be difficult to determine the health of individuals when studying field-caught wild species (Weber et al., 2002). The storks in this study were examined by the same veterinarian and all were clinically normal. Because certain reference value determinations in wild species can be influenced by the effects of stress and handling (Gross and Siegel, 1983; Breazile, 1988; Scope et al., 2002), chicks were managed in their nests to reduce handling time and stress. Although excitability may have influenced our results, the same would likely be true for any sample collection from a wild black stork.

Red blood cell counts, PCV, and Hb values were lower than those described by Puerta et al. (1989) for older black stork chicks (55–68 days old). Age-related increases in RBC, PCV, and Hb concentration have been reported for other avian species (Puerta et al., 1992; Lanzarot et al., 2001; Howlett et al., 2002) and may be

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TABLE 2. Blood chemistry findings in free-living black stork (*Ciconia nigra*) nestlings in Spain.

Parameter[a]	Mean ± SD	Median	P_5[b]	P_{95}[b]
Prealbumin (g/dl)	0.39±0.09	0.41	0.23	0.54
Prealbumin (%)	12.85±2.70	13.60	7.32	17.16
Male[c]	11.35±2.64	11.20	7.10	15.20
Female[d]	14.36±1.80	14.74	10.82	17.30
Albumin (g/dl)	1.27±0.21	1.19	0.98	1.74
≤40[e]	1.37±0.25	1.35	0.98	1.82
≥41[f]	1.19±0.13	1.17	0.98	1.45
Albumin (%)	41.37±5.24	40.49	32.76	51.87
≤40	43.39±5.82	44.95	33.30	53.20
≥41	39.78±4.24	39.40	31.50	50.10
Male	43.72±4.98	43.40	36.10	53.20
Female	39.03±4.49	37.80	31.50	48.81
α-globulin (g/dl)	0.52±0.08	0.52	0.37	0.69
α-globulin (%)	17.27±2.34	17.00	12.91	21.47
β-globulin (g/dl)	0.34±0.09	0.33	0.21	0.57
β-globulin (%)	11.39±2.93	10.60	7.36	18.01
γ-globulin (g/dl)	0.53±0.10	0.51	0.36	0.70
Male	0.47±0.07	0.46	0.31	0.60
Female	0.56±0.10	0.56	0.40	0.76
γ-globulin (%)	17.10±2.89	16.60	12.06	22.46
TP (mg/dl)	3.03±0.30	2.99	2.53	3.38
Male	2.92±0.23	2.90	2.51	3.30
Female	3.14±0.33	3.04	2.63	4.00
A/G ratio	1.15±0.15	1.12	0.83	1.61
AST (IU/l)	174.33±43.76	162	105.8	276
≤40	151.38±27.67	156	102.0	194.0
≥41	192.70±46.10	176	140.05	279.0
ALP (IU/l)	2223.25±660.91	2114	256	3301
Male	1890.12±671.81	2016	240	2760
Female	2521.32±499.56	2712	1556	3399
LDH (IU/l)	1062.94±317.37	944	436	1612
CK (IU/l)	828.72±456.42	986	22	1417
≤40	617.81±537.01	679	22	1457
≥41	997.45±297.74	1041	508	1422
Calcium (mg/dl)	10.52±0.88	10.26	9.27	11.20
≤40	10.99±1.08	10.90	9.20	13.80
≥41	10.14±0.40	10.02	9.57	10.99
Phosphorus (mg/dl)	6.29±0.87	5.97	5.29	8.50
≤40	6.81±0.98	6.65	5.40	8.50
≥41	5.88±0.48	5.75	5.27	7.30
Iron (mg/dl)	193.33±113.64	158	84.2	620
Cholesterol (mg/dl)	197.00±23.00	188	176	225
≤40	208.19±28.09	204	156	266
≥41	188.05±12.76	186	166	215
Glucose (mg/dl)	246.72±17.22	246	219	280
Triglycerides (mg/dl)	113.47±82.43	75	50	407
Male	77.18±35.91	70	42	178
Female	145.95±98.53	110	54	435
Uric acid (mg/dl)	10.97±3.91	9.06	4.88	17.78
Urea (mg/dl)	8.22±4.03	6.07	4.20	24.60

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TABLE 2. Continued.

Parameter[a]	Mean ± SD	Median	P_5[b]	P_{95}[b]
Creatinine (mg/dl)	0.47±0.18	0.45	0.30	1.05
≤40	0.39±0.08	0.39	0.30	0.57
≥41	0.54±0.21	0.48	0.34	1.06
TS (g/dl)	4.17±0.41	4.00	3.12	4.48
≤40	4.33±0.39	4.20	4.00	5.20
≥41	4.04±0.39	4.00	3.03	4.78
Bile acids (μmol/l)	11.94±6.62	10.40	2.72	20.42
BChE (IU/l)	2347.55±570.02	2340	1289	3528
≤40	1808.67±366.39	1860	1289	2280
≥41	2632.85±436.74	2580	2040	3540

[a] TP = total protein; A/G ratio = albumin/globulin ratio; AST = aspartate aminotransferase; ALP = alkaline phosphatase; LDH = lactic dehydrogenase; CK = creatine kinase; TS = total solids; BChE = butyrylcholinesterase.
[b] Inner limits of the fifth and 95th percentiles for *P*.
[c] $n=18$.
[d] $n=18$.
[e] $n=16$.
[f] $n=20$.

due to adaptation to flight, at which time the need for oxygen is greatly increased. Increased hemoglobin content in adults also may be a consequence of decreased blood volume per unit of body weight with age (Palomeque and Planas, 1978; Celdrán et al., 1994).

Total and differential leukocyte counts vary widely in avian species, probably reflecting interspecies variability and different capture, restraint, and blood collection methodologies (Padilla et al., 2003). The mean WBC for these free-living nestling black storks is lower than values reported in slightly older chicks (Puerta et al., 1989), possibly because of different analytic methods employed or age. Lymphocytes were the most numerous leukocytes in the blood of these nestlings, as has been reported in older black stork chicks (Puerta et al., 1989). No basophils were identified in the blood smears, probably because the granules within avian basophils are water soluble and may stain poorly with Diff-Quick-type stains (Phalen et al. 1995).

Protein electrophoresis is used to determine the exact blood albumin concentration, to evaluate whether a disease process is acute or chronic, and to discern whether a patient has an infection (Rosental, 2000). The electrophoretic parameters of serum versus plasma samples are similar, apart from fibrinogen (Tatum et al., 2000), and can be compared. Thus, the concentration of the five protein fractions resolved by protein electrophoresis (prealbumin, albumin, α-globulins, β-globulins, and γ-globulins) in nestling black stork serum was similar to those described for plasma in white stork (Montesinos et al., 1997). Prealbumin, as per our results, constituted 13.87% of the total albumin in black storks, whereas in some avian species it may represent from 10% to 75% of the total albumin concentration or be entirely absent (Cray and Tatum, 1998).

The range for TP was lower than that of older black stork chicks (Puerta et al., 1989), possibly because of differences in analytic methods employed, sample size, and increasing antibody concentrations with age. Total protein was greater in females than in males, which is in agreement with Hochleithner (1994), who reported sex-related differences in total protein in birds. The TS reference ranges, although providing information that may assist the clinician, should never be used as a sole assessment criterion for disease evaluation (Fudge, 2000a).

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The mean AST activity in this study is similar to that reported for slightly older black stork chicks (Puerta et al., 1989) and for free-living chicks of *Phoenicopterus ruber* (Puerta et al., 1992). Mean CK was significantly higher than the value reported for *Ciconia* (Montesinos et al., 1997). Patient excitement, handling, trauma, and irritating injections can elevate CK (Bollinger et al., 1989; Fudge, 1994). Chicks sampled in this study were managed in their nests by a veterinarian to reduce handling time and trauma, so it is likely that these factors had minimal effect on CK activity. Alkaline phosphatase activity in these nestlings was higher than values described for older chicks (55–68 days old) (Puerta et al., 1989), which were close to their full growth, and may be due to increased osteoblast activity causing elevated ALP in the younger birds (Hochleithner, 1994). Alkaline phosphatase activity was greater in females than in males, which is opposite of reported values in bald ibises (*Geronticus eremita*) (Dutton et al., 2002). The range for LDH was similar to that reported for adult and chick *P. ruber* (Puerta et al., 1992).

In this study, uric acid and urea concentrations were similar to those reported in older black stork chicks (Puerta et al., 1989). Values of creatinine were higher than reported by Hochleithner (1994) for a variety of other avian species investigated.

The serum calcium range was within that described for a wide variety of avian species (Fudge, 2000b). The mean iron concentration was similar to that described for bald eagles (*Haliaeetus leucocephalus*) (Bowerman et al., 2000). As far as we know, values for bile acids are the first reported for Ciconiiformes. The cholesterol concentration was similar to that reported for white stork (*Ciconia ciconia*) (Montesinos et al., 1997) and bald eagles (Bowerman et al., 2000).

Agricultural and silviculture applications of organophosphate and carbamate pesticides with anticholinesterase properties have been responsible for mortality of songbirds, raptors, gulls, and waterfowl (White and Mitchell, 1983; Fairbrother, 1996). Measurement of acetylcholinesterase (AChE) activity in brain tissue or BChE inhibition in brain tissue or blood plasma of birds can be used as an indicator of exposure to ChE-inhibiting insecticides (McInnes et al., 1996; Mayack and Martin, 2003). In birds, the proportion of plasma cholinesterases that is BChE or AChE varies for different species, and different species have different age-dependent patterns of AChE or BChE activity (McInnes et al., 1996; Mayack and Martin, 2003). In eastern bluebirds (*Sialia sialis*), European starlings (*Sturnus vulgaris*), and house wrens (*Troglodytes aedon*) (Gard and Hooper, 1993; Mayack and Martin, 2003), BChE activity in plasma increases steadily with age in nestlings, which is in agreement with our results, where the values obtained also increased over the nestling period and are lower than the values reported by Kiesau and Kummerfeld (1998) from adult white storks.

Normal values for blood constituents vary widely for different species (Wyk et al., 1998). Thus, to assess the physiologic and pathologic condition of wild birds it is of paramount importance to know normal blood values for individual species. The hematologic, blood biochemistry, and BChE values obtained in this study for nestling black storks will likely be useful for the interpretation of laboratory findings in future studies and clinical cases.

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