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HEALTH AND NUTRITIONAL ASSESSMENT OF FREE-RANGING EASTERN INDIGO SNAKES (*DRYMARCHON COUPERI*) IN GEORGIA, UNITED STATES

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Abstract: Clinical pathology and nutritional parameters are useful in evaluating and monitoring threatened and endangered wildlife populations, but reference ranges for most snake species are lacking. From 2001 to 2005, health assessments were performed on 58 eastern indigo snakes (EIS) (*Drymarchon couperi*) captured in the wild in southeastern Georgia, United States. Health and nutritional assessments performed included hematology, serum biochemistry, fat-soluble vitamins, heavy metals, pesticide contaminants, parasitology, and surveys of other pathogens. Significant differences in total solids, packed cell volume, glucose, blood urea nitrogen, albumin : -globulin ratio, amylase, triglycerides, and bile acids between males and females were observed. Additionally, there was a significant difference between liver and kidney concentrations for vitamins A and E. As previously noted in captive EIS, total Ca was elevated in comparison to concentrations reported in other snake species. Parasitism was a common finding in sampled EIS, but the overall health status of this free-ranging population appeared good. A winter-time dermatitis was found in most snakes, which resolved in the summer months. This study represents the first health and nutritional assessment of free-ranging EIS, and provides needed data to guide monitoring and conservation efforts.

Key words: Clinical pathology, *Drymarchon couperi*, eastern indigo snake, Georgia, health assessment, wildlife health.

INTRODUCTION

The eastern indigo snake (*Drymarchon couperi*), one of the largest North American snake species, reaches 2.6 m in total length and exhibits male-biased sexual size dimorphism.[7,20] Eastern indigo snakes (EIS) were listed as Threatened in 1978 under the Endangered Species Act due to population declines attributed to habitat loss, dwindling gopher tortoise (*Gopherus polyphemus)* numbers, rattlesnake roundups, road mortalities, and over-collection for the pet trade.[26,50,76,77] Review by the U.S. Fish and Wildlife Service determined that continued listing as Threatened was warranted.[77]

The EIS prefers sandhill habitats and gopher tortoise burrows.[8,26,38] In southern Georgia, EIS require these burrows for cool-season shelters and protection from fire.[39,44,45,50,70] Eastern indigo snakes forage and nest in gopher tortoise burrows,

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and commonly use these burrows for extended periods prior to ecdysis.[38,39,53,62,64,65]

There are few health evaluations of free-ranging snakes in general, and limited information on health of EIS, despite its status as a federally listed species.[3,4,14,34,48,54,72,76,78,80] Most available information was obtained from captive specimens. Eastern indigo snakes are predators that feed on a variety of vertebrates (other snakes, anurans, rodents, and juvenile gopher tortoises).[69] Whole prey composition of anurans, rodents, and selected prey items of free-ranging EIS contain higher overall macromineral (calcium [Ca], phosphorus [P], and sodium [Na]) concentrations compared with whole prey species (laboratory-reared rodents) typically fed to captive snakes.[28,59,74] Differences in nutritional profiles between native and commercially raised prey may underlie the clinical data previously reported for captive EIS.[30,31]

In addition to habitat loss, environmental contamination may play a role in decline of EIS.[9,10,38] Exposure to contaminants may be significant in reptiles due to slower metabolic rate and slower clearance of toxins compared with mammals. Health effects of pesticides (polychlorinated biphenyls [PCBs], organochlorines) include endocrine disruption, reproductive impairment, thin-shelled eggs, birth defects, sex reversal, and other effects.[6,8,24,56,57] Reports of endocrine-disrupting chemicals in aquatic reptiles suggest PCB exposure can have an effect on fitness.[8] Given the consequences of these pollutants on wildlife and human health, it was hypothesized that free-ranging EIS would show evidence of exposure to these chemicals.

During a field study of free-ranging EIS from southeastern Georgia (2001–2005), 58 specimens were evaluated for baseline health, nutrition, toxicology, parasites, and disease. Data are provided from physical examinations, morphometric measurements, hematology, biochemistry, toxicology, parasite, pathogen, and nutritional parameters on plasma and selected tissues from EIS.

MATERIALS AND METHODS

Sample collection

From 2001 to 2005, health assessments were performed on free-ranging, captured EIS at the Fort Stewart Military Base and private lands in southeastern Georgia.[41,42,70] Fifty-eight live EIS (35 adult males, 23 adult females) were captured and examined. Some snakes were evaluated on more than one occasion for a combined total of 84 exams (Tables 1, 2). Exams were opportunistic,

Table 1. Male snake capture events.

Snake	Date(s) capture events recorded[a]	Number of capture events
1	29 Jan 2001	2
	17 Dec 2001	
2	17 Dec 2001	1
3	29 Jan 2001	4
	17 Dec 2001	
	25 Jan 2002	
	3 Feb 2003	
4	20 Dec 2001	2
	Date not recorded	
5	25 Jan 2002	2
	10 Feb 2003	
6	24 Sep 2002	2
	31 Jul 2003	
7	24 Sep 2002	1
8	31 Jul 2003	2
	Date not recorded	
9	31 Jul 2003	1
10	31 Jul 2003	1
11	31 Jul 2003	2
	Date not recorded	
12	10 Dec 2003	3
	Date not recorded	
	Date not recorded	
13	22 Dec 2003	1
14	Date not recorded	1
15	7 Feb 2003	1
16	10 Feb 2003	1
17	10 Feb 2003	1
18	31 Jul 2003	1
19	31 Jul 2003	1
20	31 Jul 2003	2
	Date not recorded	
21	8 Sep 2003	1
22	12 Oct 2003	1
23	12 Oct 2003	1
24	18 Dec 2003	2
	29 Jan 2004	
25	24 Nov 2003	4
	29 Jan 2004	
	3 Mar 2004	
	Date not recorded	
26	29 Jan 2004	1
27	2 Dec 2003	1
28	2 Dec 2003	2
	Date not recorded	
29	2 Dec 2003	1
30	2 Dec 2003	2
	29 Jan 2004	
31	29 Jan 2004	1
32	29 Jan 2004	2
	19 Jan 2005	
33	19 Jan 2005	1
34	19 Jan 2005	1
35	19 Jan 2005	1

[a] Not all capture event dates were logged, but data from capture events indicated snake was handled on more than one occasion.

Table 2. Female snake capture events.

Snake	Date(s) capture events recorded[a]	Number of capture events
1	17 Dec 2001	1
2	31 Jul 2003	1
3	31 Jul 2003	2
	Date not recorded	
4	31 Jul 2003	1
5	31 Jul 2003	2
	Date not recorded	
6	13 Mar 2003	1
	15 Aug 2003	
7	31 Jul 2003	1
8	31 Jul 2003	1
9	16 Apr 2003	2
	15 Aug 2003	
10	15 Aug 2003	1
11	15 Aug 2003	1
12	15 Aug 2003	2
	Date not recorded	
13	3 Feb 2004	1
14	Date not recorded	1
15	3 Mar 2004	1
16	16 Apr 2003	4
	15 Aug 2003	
	3 Feb 2004	
	1 Mar 2004	
17	3 Mar 2004	1
18	1 Mar 2004	1
19	1 Mar 2004	1
20	1 Mar 2004	1
21	3 Mar 2004	1
22	3 Feb 2004	1
23	1 Mar 2004	1

[a] Not all capture event dates were logged, but data from capture events indicated snake was handled on more than one occasion.

and time between exams was highly variable. Snakes were captured by hand near tortoise burrows between November and March annually. Specimens were also found alive on roads, in box-funnel traps at drift fence arrays, and opportunistically throughout the year.[39]

Thirty-two of the 58 EIS were tagged with radio transmitters from 2002 to 2004.[41,42,71] Marked EIS were not counted as additional captures while tracked with radio transmitters. Live EIS were evaluated in the field or transported to the St. Catherines Island clinic in Action-Packer® (Rubbermaid, Inc., Fairlawn, Ohio 44333, USA) containers. Captured EIS received an identification marker by implanting a passive integrated transponder (PIT) tag. Gender was determined using a lubricated stainless steel probe with standardized methodology.[68] Snout-to-vent length (SVL) and tail length were measured to the centimeter and were combined for total length (TL). Eastern indigo snakes were weighed using spring scales (Pesola AG, Baar, 6340 Switzerland). Eastern indigo snakes with SVL $\geq$115 cm were considered to be adults, SVL 90–114.9 cm were subadults, and SVL <90 cm were juveniles.[46,47,63–66,71] A physical examination was performed on each EIS, and body condition score was assigned by palpation of the muscle surrounding the vertebrae (1 = emaciated, 2 = thin, 3 = normal, 4 = overweight, and 5 = obese).

After physical examination, a 5-ml blood sample was obtained from the heart using a sodium heparin coated syringe after disinfecting the site with isopropyl alcohol. No more than 5% of the blood volume was taken from a particular individual (<0.5 ml per 100 g). In 35 instances, anesthesia facilitated examination and sampling. Anesthesia was induced by isoflurane in oxygen while the EIS was restrained in a clear plastic tube. Once relaxed, snakes were intubated and maintained on isoflurane in oxygen. An ultrasonic doppler was used to monitor the heart rate. If the EIS was manually restrained, at least three handlers were used. Following examination and sample collection, EIS were released at their capture sites within 48 hr of capture, or 2 wk later in EIS that were surgically implanted with radiotransmitters.[40]

Sample analysis

Blood smears were made within 30 min of sample collection and were fixed in methanol and stained with Wright Giemsa (Sigma-Aldrich, St. Louis, Missouri 63103, USA). Whole blood was kept on ice and a portion was centrifuged for plasma collection within 60 min of sample collection. Hematology samples were analyzed manually.[15] White blood cell counts were performed using Eosinophil Unopette (Becton-Dickson, Rutherford, New Jersey 07070, USA) hemocytometer technique by a veterinarian (TMN).[19] White blood cell differentials and hemoparasite examination were performed by a veterinary technician (MO).

Plasma and whole blood were placed in liquid nitrogen within 90 min and transferred to a –70°C ultralow freezer. Samples were shipped overnight on dry ice within 3 mo. Whole blood and plasma were submitted for physiologic assessment including hematology (n = 64), biochemistry (n = 57), ionized Ca (n = 23), bile acid (n = 57), and plasma protein electrophoresis (n = 57). Biochemistry profiles were performed on plasma using standard dry slide determinations on an Ortho

Vitros 250 analyzer (Ortho Diagnostics, Rochester, New York 14626, USA) by the Department of Pathology, University of Miami (Miami, Florida 33124, USA). Protein fractions were evaluated by protein electrophoresis.[21,81,85] Plasma ionized Ca was measured using an ion selective electrode method at the Michigan State University College of Veterinary Medicine (Lansing, Michigan 48824, USA).

Plasma vitamin D (25-hydroxycholecalciferol; $n = 39$) was measured by radioimmunoassay at Boston University (Boston, Massachusetts 02215, USA) using described methods.[19] Plasma vitamin A (retinol; $n = 5$) and vitamin E (α-tocopherol; $n = 5$) concentrations were determined by high performance liquid chromatography (HPLC) at the Wildlife Nutrition Laboratory, Wildlife Conservation Society (Bronx, New York 10460, USA).[18] The smaller sample size for vitamin A and E concentrations was due to limited blood sample volume.

Mercury, lead, arsenic, and cholinesterase (biomarker for organophosphates) levels ($n = 22$) were measured on whole blood, and selected contaminants (organochlorine insecticides and polychlorinated biphenyls) ($n = 7$), copper, zinc, iron, magnesium, cobalt, and selenium ($n = 22$) were measured on plasma. Toxicology assays were performed by a toxicologist (RP; 2001–2005 College of Veterinary Medicine Toxicology Laboratory, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA). The detection limit was 0.05 μg/ml for all metals except mercury, which was 0.01 μg/ml. Total lead was measured by atomic absorption spectrophotometry (AAS). Mercury and arsenic were measured by hydride-generation inductively coupled plasma–optical-emission spectroscopy (ICP-OES). Copper and zinc were measured by ICP-OES or inductively coupled argon plasma–mass spectrometry (ICP-MS). Cholinesterase levels were measured in whole blood with enzyme kinetic spectrophotometry.[75] Organochlorine insecticides (OCIs) and polychlorinated biphenyls (PCBs) were analyzed in plasma by gas chromatography with electron capture detection (GC-ECD). Presence of OCIs or PCBs identified by GC-ECD was confirmed by gas chromatography–mass spectrometry (GC-MS).

Paramyxovirus ($n = 17$), West Nile virus (WNV; $n = 11$), and *Cryptosporidium serpentis* antibody titers ($n = 5$) were measured on plasma by plaque reduction neutralization test (PRNT) and ELISA.[5,35,43,60] For WNV, titers were expressed as the reciprocal of serum dilutions reducing plaques 90% (PRNT90). Samples with PRNT90 titers to WNV of 10 were considered seropositive for WNV.[5] Gastric washes were performed ($n = 9$) by flushing 20 ml of 0.9% saline into the stomach via red rubber catheter followed by aspiration and endoparasite evaluation. An immunofluorescent monoclonal antibody (IFA) test (Merifluor, Meridian Bioscience, Inc., Cincinnati, Ohio 45244, USA) was used to screen gastric and fecal samples ($n = 5$) for *Cryptosporidium* and *Giardia*.[35,36,43] Fecal flotation, direct wet mount, and enteric bacterial cultures were performed (Para-Pak Stool System, Meridian Bioscience, Inc., Cincinnati, Ohio 45244, USA). Ectoparasites were stored in 70% ethanol, mounted following standard techniques and identified.[11,81]

Necropsies were performed on road-killed EIS ($n = 16$) and tissues collected for diagnostics, including nutritional analysis. Liver ($n = 5$) and kidney ($n = 5$) were collected for vitamin A and E analyses.[30,73] Samples were processed and shipped to Arizona State University (Tempe, Arizona 85287, USA) where they were analyzed using HPLC for vitamin E (as α-tocopherol).[30,49,73] Vitamin A (as all-*trans* retinol) was measured with absorbance spectrophotometry at λ max = 277 nm. Vitamin E activity was calculated as 1 mg α-tocopherol = 1.49 IU vitamin E, 1 mg γ-tocopherol = 0.1 IU vitamin E; Vitamin A activity was calculated as 0.3 μg all-trans retinol = 1 IU vitamin A.

Statistical analysis

As a cross-sectional study, one sample from each snake was analyzed. The first sample collected for each outcome (biochemistry, vitamin, etc.) was selected. A $P \leq 0.05$ determined statistical significance. SPSS 19.0 (SPSS Inc., Chicago, Illinois 60606, USA) was used to analyze data.[23,79] The distribution of continuous data was evaluated using Shapiro-Wilk test, skewness, kurtosis, and q-q plots.[23] Normally distributed data are reported by mean, standard deviation (SD), and minimum–maximum (min–max), while nonnormally distributed data are reported by median, 10–90%, and min–max. The 95% binomial confidence intervals were calculated for all proportions. When prevalence was 0%, methods described by van Belle and Millard were used to estimate 95% CI.[23,79] Levene's test was used to determine if the assumption for homogeneity of variances was met. The Mann-Whitney test was used to determine if sex had affected different dependent variables that were nonnormally distributed, while an independent sample *t*-test was

Table 3. Physical examination findings in free-ranging eastern indigo snakes (*Drymarchon couperi*) in southeastern Georgia, United States (2001–2005). Data are from 84 exams; 51 male exams and 33 female exams (some individuals were examined more than once).

Morphometric parameter	Median	Mean	10–90%	SD	Min–max	*P* value
Body condition score	2.5/5		2.0–3.0		1.25–3.0	
Weight[A]	1.81kg		0.82–3.61		0.31–4.41	
Male		2.3kg		1.0	0.72–4.42	0.006
Female		1.5kg		0.52	0.31–2.28	
Snout to vent length[A]		152.3cm		22.48	92.1–193.0	
Male		160.0cm		21.5	114.0–193.0	0.0001
Female		137.4cm		17.0	92.1–155.5	
Total length[A]		180.6cm		26.9	111.1–226.1	
Male		190.8cm		23.7	137.2–226.1	0.0001
Female		160.5cm		21.8	111.1–186.0	

[A] Denotes significant difference between sexes.

used to determine if sex affected normally distributed dependent variables. Pearson's test was used to perform correlation analysis.

RESULTS

Physical exam

Mean values for body weight, morphometric measurements, and body condition score for the 58 EIS sampled are listed in Table 3. Males were significantly heavier and longer than females ($P = 0.006$, and 0.001, respectively). Most common abnormalities detected were crusting, pustular to ulcerative skin lesions ($n = 58$), and red mite (Trombiculid) infestation ($n = 9$). Skin lesions were only found in cooler months (November through April), while Trombiculid red mites (chiggers) (identified by LD) were only found in warmer months (May through October). Oral trematodes and vertebral osteomyelitis (confirmed with radiographs) were diagnosed in one case. Additionally, two EIS died with severe cutaneous masses, which were confirmed histologically to be fungal granulomas. Necropsy findings will be reported elsewhere (Stedman, unpubl. data).

Table 4. Hematology values for free-ranging eastern indigo snakes (*Drymarchon couperi*) in southeastern Georgia, United States (2001–2005). Data from 64 samples.

Hematology parameters	Median	Min–Max
PCV (%)	32	23–45
Total solids (g/dl)	7.8	6–11.6
WBC ($\times 10^3$/ul)	16.3	4–46
Heterophils ($\times 10^3$/ul)	3.09	0.32–7.82
Heterophils (%)	19.5	2–48
Lymphocytes ($\times 10^3$/ul)	5.29	0.65–13.5
Lymphocytes (%)	32.5	4–83
Azurophils ($\times 10^3$/ul)	6.92	1.79–12.06
Azurophils (%)	42.5	11–74
Basophils ($\times 10^3$/ul)	0.32	0.16–2.12
Basophils (%)	2.0	1–13
Eosinophils ($\times 10^3$/ul)	0.0	0.0
Eosinophils (%)	0.0	0.0

Clinical pathology

Males exhibited significantly lower TS concentration ($P = 0.001$) and higher PCV ($P = 0.0001$) than females. The predominant WBC of indigo snakes was the azurophil, followed by the lymphocyte, heterophil, and basophil. Heterophil counts of males were significantly higher than that of females ($P = 0.0008$). Hematology values are reported in Table 4.

There were significant differences in glucose (Glu) ($P = 0.01$), potassium (K) ($P = 0.04$), total protein (TP) ($P = 0.04$), albumin : globulin ratio (AG ratio) ($P = 0.003$), triglycerides (TG) ($P = 0.0001$), amylase (Amy) ($P = 0.005$), bile acids (BA) ($P = 0.018$), and blood urea nitrogen (BUN) ($P = 0.01$) between sexes (Table 5). Remaining biochemistry parameters did not significantly differ between male and females. Biochemistry values are reported in Table 5.

Fat-soluble vitamins

Plasma vitamin E concentrations ($n = 5$), measured as α- and γ- tocopherols, were typical of obligate carnivores (Table 6). Retinol concentrations (a measure of vitamin A activity) averaged 0.009 ± 0.005 μg/ml (mean ± SD) and 25-OH vitamin D had a median of 150 ng/ml ($n = 39$) with no sex difference detected ($P = 0.166$).

Table 5. Plasma biochemistry concentrations for free-ranging eastern indigo snakes (*Drymarchon couperi*) in southeastern Georgia, United States (2001–2005). Data from 57 samples.

Plasma Biochemistry Parameter	N	Mean ± SD	Min–Max
Glucose (mg/dl)[A]	56	102.7 ± 42.5	42–247
Male	36	106.44 ± 45.53	47–247
Female	20	83.55 ± 31.79	42–148
Sodium (mM/L)	51	166.8 ± 5.7	158–180
Male	32	166.90 ± 5.57	158–180
Female	19	166.37 ± 6.12	158–178
Potassium (mM/L)[A]	51	4.4 ± 0.77	3.2–6.1
Male	32	4.18 ± 0.73	3.2–5.8
Female	19	4.67 ± 0.75	3.2–6.1
Phosphorus (mg/dl)	49	9.4 ± 2.6	5.8–16.6
Male	30	9.4 ± 2.25	6.2–14.5
Female	19	10.07 ± 3.4	5.8–16.6
Uric acid (mg/dl)	56	6.7 ± 5.6	1.5–18.3
Male	38	8.12 ± 6.32	3-3–18.3
Female	18	4.91 ±2.27	1.5–8.5
Calcium (mg/dl)	56	50.0 ± 26.4	13.6–115.5
Male	33	53.25 ± 24.59	17.9–95
Female	23	45.03 ± 28.3	13.6–115.5
Ionized calcium (mM/L)	23	1.89 ± 0.21	1.39–2.39
Male	16	1.9 ± 0.11	1.72–2.07
Female	7	1.52 ± 0.8	1.39–2.39
Total protein (g/dl)[A]	56	7.64 ± 1.4	5.2–12.1
Male	36	7.48 ± 1.36	5.2–10.3
Female	20	8.53 ± 1.58	6–12
Albumin (g/dl)	56	2.3 ± 0.43	1.40–3.27
Male	36	2.3 ± 0.41	1.40–2.96
Female	20	2.41 ± 0.5	1.48–3.27
Globulins			
Alpha 1 (g/dl)	56	0.8 ± 0.51	0.37-1.5
Male	36	0.73 ± 0.26	0.37-1.38
Female	20	0.85 ± 0.75	0.43-1.5
Alpha 2 (g/dl)	56	1.32 ± 0.4	0.4-2.16
Male	36	1.29 ± 0.37	0.4-2.06
Female	20	1.41 ± 0.38	0.8-2.16
Beta (g/dl)	56	1.89 ± 0.48	1.1-3.7
Male	36	1.79 ± 0.42	1.1-3.42
Female	20	2.22 ± 0.67	1.29-3.7
Gamma (g/dl)	56	1.09 ± 0.53	0.23-3.73
Male	36	1.09 ± 0.55	0.23-2.99
Female	20	1.38 ± 0.84	0.45-3.73
A : G ratio[A]	56	0.49 ± 0.11	0.24-0.74
Male	38	0.5 ± 0.11	0.26-0.7
Female	18	0.44 ±0.13	0.24-0.77
Aspartate aminotransferase (U/l)	55	47 ± 41.5	3–215
Male	37	53.95 ± 51.6	3–203
Female	18	45.9 ± 46.76	3–215
Alanine aminotransferase (U/l)	46	30.4 ± 37.5	10–259
Male	28	24.00 ± 13.25	10–73
Female	18	39.22 ± 57.23	12–259
Lactate dehydrogenase (U/l)	52	1005.3 ± 628	100–2809
Male	35	1047.45 ± 680.19	100–2809
Female	17	904.21 ± 556.91	165–1986
Gamma glutamyl transferase (U/l)	54	8.7 ± 1.5	6–12
Male	36	9.0 ± 1.23	6–10
Female	18	8.11 ± 1.94	6–12
Creatine phosphokinase (U/l)	55	378.4 ± 343.4	27–1607
Male	37	393.94 ± 332.65	71–1310
Female	18	391.9 ± 391.8	27–1607
Cholesterol (mg/dl)	54	254.5 ± 55.3	138–423
Male	36	265.23 ± 60.73	138–423
Female	18	243.58 ± 51.04	171–336
Triglycerides (mg/dl)[A]	53	154.5 ± 173.3	12–552
Male	35	55.71 ±83.73	12–349
Female	18	319.25 ± 172.66	21–552
Amlylase (U/l)[A]	54	1106.9 ± 819.9	532–3887
Male	36	877.63 ± 560.86	532–3837
Female	18	1450.95 ± 1023.78	728–3887
Lipase (U/l)	54	460.74 ± 116.7	55–704
Male	36	465.74 ± 110.93	55–665
Female	18	427.89 ± 129.35	292–704
Bile acid (μM/L)[A]	54	11.4 ± 9.9	0–39
Male	36	11.69 ± 9.32	0–38.8
Female	18	6.85 ± 9.67	1.1–39
Blood urea nitrogen (mg/dl)[A]	24	2.3 ± 0.92	1–5
Male	16	1.77 ± 0.44	1–3
Female	8	3.25 ± 1.75	3–5
Creatinine (mg/dl)	56	0.49 ± 0.42	0.1–2.8
Male	36	0.54 ± 0.48	0.1–2.8
Female	20	0.40 ± 0.22	0.1–1
Blood urea nitrogen : creatinine ratio	24	6.4 ± 5.13	2–25
Male	15	4.84 ± 2.79	2–10
Female	9	6.43 ± 2.21	3–25
Total carbon dioxide (mM/L)	49	16.1 ± 4.4	8–25
Male	30	15.83 ± 4.9	8–25
Female	19	16.53 ± 3.59	13–24

[A] Denotes significant difference between sexes.

Liver and kidney concentrations of vitamins E and A, obtained from necropsy specimens ($n = 5$), ranged widely. There was a significant difference ($P = 0.05$) between liver and kidney tissue concentrations of both vitamins (Table 6).

Table 6. Plasma, liver, and kidney concentrations of vitamins A (measured as all-trans retinol), E (measured as α- and γ-tocopherol), and D (25-OH metabolite) sampled in free-ranging eastern indigo snakes (*Drymarchon couperi*) in southeastern Georgia, United States (2001–2005).

Tissue-vitamin	N	Median	Mean	10–90%	SD	Min–max	*P* value
Vitamin E							
Plasma γ-tocopherol (μg/ml)	5		0.69		0.33	0.47–1.25	
Plasma α-tocopherol (μg/ml)	5		25.1		7.2	17.2–36.5	
Liver α-tocopherol (μg/g)	5		34.1		15.4	18.6–58.5	0.05
Kidney α-tocopherol (μg/g)	5		17.6		6.3	9.7–27.2	
Vitamin D							
Plasma 25-hydroxy vitamin D (ng/ml)	39	150		108–190		46–210	
Vitamin A							
Plasma retinol (μg/ml)	5		0.009		0.005	0.004–0.016	
Liver retinol (μg/g)	5		601.2		330.1	91.4–1016.0	0.05
Kidney retinol (μg/g)	5		8.1		9.0	0.33–23.7	

Metals

Blood metal concentrations revealed that magnesium and copper were the dominant metals present. Complete results are shown in Table 7.

Contaminants

Concentrations of organochlorine contaminants including alpha chlordane, aldrin, dieldrin, endrin, gamma chlordane, heptachlor, heptachlor epoxide, lindane, and methoxychlor, p,p'DDD, chlordane, p,p'DDE and p,p'DDT, toxaphene, and PCBs as arochlor #1260 were below detection limits in all plasma samples tested. Mean (±SD) whole blood cholinesterase activity was 2.5 ± 1.0 μM/ml/min.

Pathogens

Paramyxovirus ($n = 17$) and West Nile virus ($n = 11$) antibody titers were negative in EIS sampled using described plaque reduction neutralization tests (PRNT).[5,35,36,43] A single (1/5) snake showed a positive *Cryptosporidium* titer. Fecal examinations identified Pentastome, strongylate nematode, *Physaloptera* sp. and *Rhabdias* sp. ova in four of five (80%) snakes. *Salmonella* sp. was cultured from one out of five (20%) fecal samples and gram-negative bacteria (considered normal fecal flora) were cultured from all fecal specimens. Intraerythrocytic *Hepatozoon* sp. was identified on blood smears of 38 of 61 individuals (62.3%). Two species of chiggers (Acari: Trombiculidae) were identified: *Eutrombicula cinnabaris* (Ewing) and *Parasecia gurneyi* (Ewing). Six snakes were infested with chiggers (mean 19.5; range 4–46 mites per infested snake), which are shown in Table 8. Trombiculid mites were found on all EIS evaluated during the summer months.

DISCUSSION

Health assessments performed in this study established baseline information including physical examination and morphometric data, clinical pathology, nutrition, toxicology, parasitology, and infectious diseases. Without understanding the EIS's baseline health parameters, it is difficult to identify causes of population decline. This information can also aid in captive breeding and

Table 7. Blood concentrations of heavy metal sampled in free-ranging eastern indigo snakes (*Drymarchon couperi*) in southeastern Georgia, United States (2001–2005).

Heavy metal	N	Median	Mean	10–90%	SD	Min–max
Mercury	22	0.54 ppm				
Iron	22		3.20 ppm		1.31	1.82–5.15
Cobalt	22		0.067 ppm		0.06	0.024–0.172
Lead	22		0.091 ppm		0.039	0.037–0.179
Arsenic	22	0.06 ppm		0.031–0.1		0.03–0.1
Magnesium	22		60.60 ppm		6.28	54.2–69.6
Zinc	22		33.50 ppm		10.36	14.0–42.4
Copper	22		1.10 ppm		0.43	0.52–1.91
Selenium	22		0.62 ppm		0.25	0.27–0.94

Table 8. Chigger species and number of larvae detected on six snakes.

Chigger species:	*E. cinnabaris*	*P. gurneyi*
Snake 1	2 larvae	2 larvae
Snake 2	5 larvae	2 larvae
Snake 3	5 larvae	3 larvae
Snake 4	—	16 larvae
Snake 5	36 larvae	—
Snake 6	45 larvae	—

reintroduction programs, which are currently being conducted with this species. In comparison to other reptile species, EIS have a diverse diet with a preference for snakes and amphibians and have unusually high plasma Ca, P, and vitamin D levels. Elevated total protein and globulins, and a predominance of azurophils may be evidence of chronic inflammation, possibly related to seasonal dermatitis and/or parasitism. As ectotherms, snakes exhibit dramatic variation in hematologic, nutritional, contaminant, and biochemical parameters depending on hydration status, season, metabolic rate, reproductive activity, and nutritional status.[15,16] The potential effect of anesthesia and handling on the results is unknown and was not investigated here.

Clinical pathology

The azurophil is the second most common leukocyte in most snake species after heterophils. In this study, azurophils were the most common leukocyte in EIS, as has been reported in other snake species.[4,67] The azurophils of snakes are cytochemically similar to neutrophils, whereas those of lizards are more similar to monocytes.[67] Monocytes generally occur in low numbers in normal reptiles (0–10%), whereas azurophils can account for up to 35% leukocytes in some snakes.[67] The total and percentage of azurophils was much higher in EIS evaluated in this study (mean 42.5%) when compared with free-ranging garter snakes where hematological data is available (mean 15%).[81] An elevation of azurophils may be suggestive of inflammatory diseases, especially acute or granulomatous inflammation.[47] In this study, it is suspected that the elevation in azurophils may be due to a combination of the dermatitis and parasitic migration which were found in most EIS. Interspecies variation is known to occur in number and percentage of leukocytes in snakes, and it's possible that the high circulating azurophil count is normal for EIS.[67]

Total protein (TP), total solids (TS), and globulins were higher than in other snake species (TP of most healthy reptiles 3–7g/dl) but similar to captive EIS (mean 8.9g/dl).[15,31] The elevated globulin portion of total protein is likely the result of inflammation, or contributed in part by folliculogenesis in female EIS.[15] Significant differences found between males and females regarding glucose, potassium, TS, TP, BUN, albumin to globulin ratio, amylase, triglycerides (TG), and bile acids may be the result of gender-based differences in metabolic rate, nutritional, and/or reproductive status.[15]

Calcium, phosphorus, 25-hydroxy vitamin D

Previous studies revealed unusually high concentrations of plasma Ca and P in captive EIS (mean Ca 158.9mg/dl, mean P 35.3mg/dl) when compared with other snake species (mean Ca 9mg/dl, mean P 3mg/dl).[15,31] Similarly in this study, mean plasma total Ca concentrations were high compared with baseline normal concentrations established for other free-ranging and captive snakes.[14,15,80] The most dramatic differences between male and female reptiles are expected to occur in females during vitellogenesis, where Ca, cholesterol, triglycerides, and P concentrations are higher in females compared with males.[16] Similar to avian species, female reptiles exhibit hypercalcemic states associated with follicular development, estrogen, and reproductive activity. Calcium levels during reproductive states may increase two to four fold.[15] Though no difference in Ca levels between males and females was observed, one individual female had total Ca 115.5mg/dl (free-ranging EIS mean 50 ± 26.4 mg/dl) in April when, on one occasion, snakes were documented by ultrasonography to be developing eggs.[61] A larger sample size including more ovulating females would likely have changed this trend. Plasma Ca and P concentrations were higher in captive EIS than the free-ranging EIS in this study.[31] The normal plasma concentration of Ca for most reptiles (not ovulating or laying eggs) ranges between 8 and 11 mg/dl.[15] Ionized Ca concentration provides a clinical measurement of the physiologically active Ca in the circulation. In this study, mean plasma ionized Ca concentration was 1.89 mM/L, SD 0.21, range 1.39–2.39, which is similar to other reptiles (mean ionized Ca concentration in green iguanas 1.47 ± 0.105 mM/L).[15,25] Elevated levels of Ca and P in whole prey eaten by EIS likely reflects a high skeleton to body mass ratio in the prey-snake species consumed.[28] The Ca : P ratio remains approximately

1.7 : 1 in EIS prey-snakes, yet concentrations of Ca and P (ranging from an average of 4.1– 7.3% DM, and 2.4– 4.0% DM, respectively, in snakes) are considerably higher than Ca (0.9–5.9% DM) or P (1.1–3.4%) reported in other vertebrate prey.[28]

Phosphorus concentrations were lower in free-ranging EIS of this study (mean 9.4 mg/dl) when compared with captive EIS (mean 35.3 mg/dl), however this was still elevated compared with healthy reptiles of other species (1–5 mg/dl).[15,31] While total Ca levels were fivefold higher in these EIS compared with reported ranges for healthy snakes (8–11 mg/dl), ionized Ca levels remained similar to most other reptile species.[16] This implies that despite high levels of Ca and vitamin D compared with other snake species, associated lesions were not observed in this study clinically or on postmortem examination. Radiographic evidence of aberrant calcification in captive EIS has not been reported.[31]

The relationship between Ca, P, and vitamin D can be related to environmental factors, primarily exposure to UVB radiation. Exposure to UVB radiation stimulates formation of vitamin D3, which tightly regulates the levels of Ca and P in the blood. Typically, captive snakes are not provided UVB light, whereas in the wild it is presumed that free-ranging snakes are exposed to UVB radiation to varying degrees, whether a physiologic requirement or not.[1] Snakes, which consume whole prey, appear to maintain normal Ca and P metabolism even without UVB light, in contrast to herbivorous reptiles that typically have a requirement for UVB exposure.[1] Plasma 25-hydroxy vitamin D concentrations in both sexes of free-ranging EIS were high (median 150 ng/ml) when compared with other colubrid snakes (corn snakes) exposed to UVB light (mean 78.4, SD 6.69 ng/ml).[1] Plasma 25-hydroxy vitamin D levels have not been reported in captive EIS, but a previous study in corn snakes demonstrated that exposure to UVB radiation significantly elevated vitamin D levels compared with control snakes not exposed to UVB radiation.[1] This suggests that corn snakes (and perhaps other colubrids) are able to utilize UVB light, and this radiation may play a role in Ca and P metabolism. It is still unclear why EIS have such significant elevations in total plasma Ca and 25-hydroxvitamin D concentrations, but it may be related to a uniquely high Ca diet.[69]

Parathyroid hormone (PTH) analysis was not performed in this study but should be evaluated in future research to achieve a more complete picture of Ca metabolism in this species. Since PTH is intricately involved in Ca metabolism by mobilizing it from bone, increasing renal absorption, and activating vitamin D, further research measuring PTH may contribute to further understanding of Ca metabolism in EIS. High Ca, P, and vitamin D compared with other snake species is not understood, but could be due to diet, UVB exposure, or PTH levels. All these factors should be evaluated in future research to further understand Ca metabolism in this species.

Vitamins A and E

Vitamin concentrations were evaluated in plasma and tissue because of their critical role in numerous physiologic functions. Efficient storage and utilization of vitamins A and E during cyclical periods of hibernation represents an effective physiological mechanism for temperate snakes that has not previously been examined. Studies in uromastyx lizards and brown bears demonstrated dynamic storage of vitamins, and related antioxidant defense system present during hibernation and activity periods.[2,80] Nutritional analysis of prey items consumed by EIS appear to exceed dietary requirements established for obligate carnivores for these nutrients, thus the possibility exists for hepatic storage as in other species.[27,28,29,51,52] The wide range of retinol concentration in liver tissue (91.4–1016.0 μg/g), and low circulating plasma concentration (0.004-0.016 μg/ml) suggests cycling of retinol in EIS, and warrants further studies to better understand animal vitamin A requirements and metabolism. In contrast, despite being fat-soluble, vitamin E is not particularly stored. If EIS were storing Vitamin E, liver values would be expected to be higher than plasma values, though limited data exist on vitamin storage in snakes. Kidney values were approximately one-half those measured in liver; similar liver and plasma concentrations of vitamin E may indicate that circulating blood levels reflect other body tissue concentrations.

Metals and contaminants

Reference ranges are unknown for metals and contaminants in most snake species. The limited data available in water snakes suggest the results of this study refuted the original hypothesis that the EIS would have high values of heavy metals and other contaminants as mentioned in the introduction. In fact, detected levels of heavy metals were below or similar to values seen in water snakes.[12–14,16,17,37,84] Additionally, organochlo-

rines and PCBs were not detectable, and were not determined to play any significant role in the health status of EIS in this study.[56]

Pathogens

Prevalence of parasites, hemoparasites, and enteric bacteria indicated these were common organisms in the population. Based on necropsies of road-killed specimens found in Florida, EIS are known to harbor at least 18 species of endoparasites; however, the presence of parasites did not compromise health of the EIS.[33] Chiggers (*E. cinnabaris* and/or *P. gurneyi*) were found on six EIS in this study. Other ectoparasites reported to infest EIS (in Florida) are the chigger *Eutrombicula splendens* (Ewing) and the tick *Amblyomma dissimile*.[22,32,33,58,82,83] The level of ectoparasitism recorded for EIS in this study was low, and did not compromise the health of these individuals. Seasonal dermatitis in cooler months suggests environment and seasonality are inciting causes for these lesions. The absence of lesions in spring suggests spontaneous resolution and little impact on overall health of EIS (Stedman, unpubl. data).

CONCLUSIONS

Seasonal dermatitis and secondary inflammation were noted in almost all free-ranging EIS sampled. Elevated Ca and vitamin D concentrations previously reported in captive EIS were demonstrated in free-ranging individuals, which provide insight into normal values for the species. However, greater investigations into Ca regulation, role of parathyroid hormone, and underlying influences of nutrition and natural diet are needed. This health assessment of EIS provides baseline data and reference values needed to more effectively monitor population health and implement conservation efforts.

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LITERATURE CITED

1. Acierno MJ, Mitchell MA, Zachariah TT, Roundtree MK, Kirchgessner MS, Sanchez-Migallon Guzman D. Effects of ultraviolet radiation on plasma 25-hydroxyvitamin D3 concentrations in corn snakes (*Elaphe guttata*). Am J Vet Res. 2008;69(2):294–297.

2. Afifi M, Alkaladi A. Antioxidant system in Uromastyx philbyi during hibernation and activity periods. Cent Euro J Biol. 2012;9:864–868.

3. Allender MC, Dreslik MJ, Wylie DB, Wylie SJ, Scott JW, Phillips CA. Ongoing health assessment and prevalence of Chrysosporium in the eastern massasauga (*Sistrurus catenatus catenatus*). Copeia. 2013;1:97–102.

4. Allender MC, Mitchell M, Phillips CA, Beasley VR. Hematology, plasma biochemistry, and antibodies to select viruses in wild-caught eastern Massasauga rattle snakes (*Sistrurus catenatus catenatus*) from Illinois. J Wildl Dis. 2006;42:107–114.

5. Allison AB, Mead DG, Gibbs SJ, Hoffman DM, Stallknecht DE. West Nile virus viremia in wild rock pigeons. Emerg Infect Dis. 2004;10:2253–2255.

6. Ankley GT, Giesy JP. Endocrine disruptors in wildlife: a weight of evidence perspective. In: Kendall AR, Dickerson R, Suk W, Giesy J (eds.). Principles and processes for assessing endocrine disruption in wildlife. Pensacola (FL): SETAC Press; 1998. p. 349–368.

7. Bauder JM, Macey JN, Wallace MP, Snow F, Safer AB, Stevenson DJ. Natural history note: *Drymarchon couperi* (eastern indigo snake): juvenile observations. In press. Herp Review.

8. Bhandri RK, Deem SL, Holliday DK, Jandegian CM, Kassotis CD, Nagel SC, Tillitt DE, vom Saal FS, Rosenfeld CS. Effects of the environmental estrogenic contaminants bisphenol A and 17α-ethinyl estradiol on sexual development and adult behaviors in aquatic wildlife species. Gen Comp Endocrinol. 2015;214:195–219.

9. Breininger DR, Legare ML, Smith RB. Eastern indigo snakes (*Drymarchon couperi*) in Florida: influence of edge effects on population viability. In: Akcakaya H, Burgman M, Kindvall O, Wood C, Sjögren-Gulve P, Hatfield J, McCarthy M (eds.). Species conservation and management: case studies. New York (NY): Oxford University Press; 2004. p. 299–311.

10. Breininger DR, Mazerolle MJ, Bolt MR, Legare ML, Drese JH, Hines JE. Habitat fragmentation effects on annual survival of the federally protected eastern indigo snake. Anim Conserv. 2012;1–8.

11. Brennan JM, Jones EK. Keys to the chiggers of North America with synonymic notes and descriptions of two new genera (Acarna: Trombiculidae). Ann Entomol Soc Am. 1959;52:7–16.

12. Burger J, Campbell KR, Murray S. Metal levels in blood, muscle and liver of water snakes (*Nerodia* spp.) from New Jersey, Tennessee and South Carolina. Sci Total Environ. 2007;373:556–563.

13. Burger J, Murray S, Gaines KF, Novak JM. Element levels in snakes in South Carolina: differences between a control site and exposed site on the Savannah river site. Environ Monit Assess. 2006;112:35–52.

14. Calle PP, Rivas J, Munoz M, Thorbjamarson J, Dierenfeld ES, Braselton WE, Karesh WB. Health

assessments of free-ranging anacondas (*Eunectes murinus*) in Venezuela. J Zoo Wildl Med. 1994;25:53–62.

15. Campbell T. Clinical pathology. In: Mader DR, Divers SJ (eds.). Current therapy in reptile medicine and surgery. St. Louis (MO): Elsevier; 2014. p.70–93.

16. Campbell KR, Campbell TS. The accumulation and effects of environmental contaminants on snakes: a review. Environ Monit Assess. 2001;70:253–301.

17. Campbell KR, Campbell TS, Burger J. Heavy metal concentrations in northern water snakes (*Nerodia sipedon*) from East Fork Poplar Creek and the Little River, East Tennessee, USA. Arch Environ Contam Toxicol. 2005;49:239–248.

18. Catignani GL, Bieri JG. Simultaneous determination of retinol and alpha-tocopherol in serum or plasma by liquid chromatography. Clin Chem. 1983;29: 708–712.

19. Chen TC, Turner AK, Holick MP. Methods for the determination of the circulating concentration of 25-hydroxyvitamin D. J Nutr Biochem. 1990;1:315–319.

20. Conant R, Collins JT. A field guide to reptiles and amphibians of eastern and central North America. 3rd ed. Boston (MA): Houghton Mifflin Company; 1998.

21. Cray C, Zaias J. Laboratory procedures. Vet Clin North Am Exot Anim Pract. 2004;7:487–518, viii–ix.

22. Crossley DA, Proctor CW. New records of chigger species (Acarina: Trombiculidae) in Georgia. Ga Entomol Soc J. 1971;6:184–187.

23. Daniel WW. Biostatistics, a foundation for analysis in the health sciences. Balding DJ, et al. (eds.). 8th ed. New York (NY): John Wiley & Sons, Inc; 2005. 782 p.

24. Danstra T. Potential effects of certain persistent organic pollutants and endocrine disrupting chemicals on the health of children. J Clin Toxicol. 2002;40:457–465.

25. Dennis P. Plasma concentrations of ionized calcium in healthy iguanas. J Am Vet Med Assoc. 2001;219:236–328.

26. Diemer JE, Speake JM. The distribution of the eastern indigo snake, *Drymarchon corais couperi*, in Georgia. J Herpetol. 1983;17:256–264.

27. Dierenfeld ES, Alcorn HL, Jacobsen KL. Nutrient composition of whole vertebrate prey (excluding fish) fed in zoos. National Agricultural Library [Internet]. 2002. 20 p. Available from http://www.nal.usda.gov/awic/zoo/WholePreyFinal02May29.pdf

28. Dierenfeld ES, Norton TM, Hyslop NL, and Stevenson DJ. Nutrient composition of prey items consumed by free-ranging eastern indigo snakes (*Drymarchon couperi*). Southeast Nat. 2015;14:551–560.

29. Dierenfeld ES, Traber MG. Vitamin E status of exotic animals compared with livestock and domestics. In: Packer L, Fuchs J (eds.). Vitamin E in health and disease. New York (NY): Marcel Dekker, Inc; 1992. p. 345–360.

30. Douglas TC, Pennino M, Dierenfeld ES. Vitamins E & A, and proximate composition of whole mice and rats used as feed. J Comp Biochem Physiol. 1994; 107A:419–424.

31. Drew M. Hypercalcemia and hyperphosphatemia in indigo snakes (*Drymarchon corais*) and serum biochemical reference values. J Zoo Wildl Med. 1994; 25:48–52.

32. Durden LA, Klompen JH, Keirans JE. Parasitic arthropods of sympatric opossums, cotton rats and cotton mice from Merritt Island, Florida. J Parasitol. 1993;79:283–286.

33. Foster GW, Moler PE, Kinsella JM, Terrell SP, Forrester DJ. Parasites of Eastern Indigo Snakes (*Drymarchon corais couperi*) from Florida, U.S.A. Comp Parasitol. 2000;67:124–128.

34. Gobble H, Dunn D, Wilson S, Thompson T. Coccidioidomycosis in an Eastern indigo snake (*Drymarchon corais couperi*). Vet Pathol. 1999;36(5):516.

35. Graczyk TK, Cranfield MR, Fayer R. A comparative assessment of direct fluorescence antibody, modified acid-fast stain, and sucrose floatation techniques for detection of *Cryptosporidium serpentis* oocytes in snake fecal specimens. J Zoo Wildl Med. 1995; 26:396–402.

36. Graczyk TK, Cranfield MR, Fayer R. Evaluation of commercial enzyme immunoassay (EIA) and immunofluorescent antibody (IFA) test kits for detection of *Cryptosporidium* oocysts of species other than *Cryptosporidium parvum*. Am J Trop Med Hyg. 1996;54:274–279.

37. Hopkins WA, Rowe CL, Congdon JD. Elevated trace element concentrations and standard metabolic rate in banded water snakes (*Nerodia fasciata*) exposed to coal combustion wastes. Environ Toxicol Chem. 1999;18:1258–1263.

38. Hyslop NL. Movements, habitat use, and survival of the threatened eastern indigo snake (*Drymarchon couperi*) in Georgia. PhD Dissertation. 2007. 132 p.

39. Hyslop NL, Cooper RJ, Meyers JM. Seasonal shifts in shelter and microhabitat use of *Drymarchon couperi* (Eastern Indigo Snake) in Georgia. Copeia. 2009;3:458–464.

40. Hyslop NL, Meyers JM, Cooper RJ. Survival of radio-implanted *Drymarchon couperi* (eastern indigo snake) in relation to body size and sex. Herpetologica. 2009;65:199–206.

41. Hyslop NL, Meyers JM, Cooper RJ, Stevenson DJ. Indigo Snake capture methods: effectiveness of two survey techniques for *Drymarchon couperi* in Georgia. Florida Scientist. 2009;72:93–100.

42. Hyslop NL, Meyers JM, Cooper RJ, Stevenson DJ. Effects of body size and sex of *Drymarchon couperi* (eastern indigo snake) on habitat use, movements, and home range size in Georgia. J Zoo Wildl Med. 2014;78: 101–111.

43. Jacobson ER, Origgi F. Use of serology in reptile medicine. Seminars in avian and exotic pet medicine. 2002;11:33–45.

44. Landers JL, Speake DW. Management needs of sandhill reptiles in southern Georgia. In: Proc Ann Conf Southeast Assoc Fish Wild Agen. 1980;34:515–529.

45. Lawler HE. The status of *Drymarchon corais couperi* (Holbrook), the Eastern indigo snake in the southeastern U.S.A. Herpetol Rev. 1977;8:76–79.

46. Layne JN, Steiner TM. Sexual dimorphism in occurrence of keeled scales in the eastern indigo snake (*Drymarchon corais couperi*). Copeia. 1984:776–778.

47. Layne JN, Steiner TM. Eastern indigo snake (*Drymarchon corais couperi*): summary of research conducted on Archbold Biological Station. Report prepared under Order 43910-6-0134 to the U.S. Fish and Wildlife Service. Jackson (MS): U.S. Fish and Wildlife Service; 1996.

48. Martin JC, Schelling SH, Pokras MA. Gastric adenocarcinoma in a Florida indigo snake (*Drymarchon corais couperi*). J Zoo Wildl Med. 1994;25(1):133–137.

49. McGraw KJ, Nolan PM, Crino OL. Carotenoid accumulation strategies for becoming a colorful house finch: analyses of plasma and liver pigments in wild molting birds. Funct Ecol. 2006;20:678–688.

50. Moler PE. Distribution of the eastern indigo snake, *Drymarchon corais couperi*, in Florida. Herpetol Rev. 1985;16(2):37–38.

51. National Research Council. Vitamin tolerance of animals. Washington (DC): National Academies Press; 1987. 96 p.

52. National Research Council. Nutrient requirements of dogs and cats. Washington (DC): National Academies Press; 2006. 424 p.

53. Newberry L, Jensen JB, Stevenson DJ. Natural history notes–nesting habitat and egg depredation: *Drymarchon couperi* (eastern indigo snake). Herpetol Rev. 2009;40:97.

54. Parmer DG. Adenocarcinoma of a female indigo snake. In: Proc Am Assoc Zoo Vet; 1978. p. 238–239.

55. Pearce PA, Peakall DB, Reynolds LM. Shell thinning and residues of organochlorines and mercury in seabird eggs, Eastern Canada 1970–1976. Pesticides Monitor J. 1979;13:61–68.

56. Rainwater TR, Reynolds KD, Canas JE, Cobb JB. Organochlorine pesticides and mercury in cottonmouths (*Agkistodon piscivorus*) from northeastern Texas, USA. Environ Toxicol Chem. 2005;24:665–673.

57. Ratcliffe DA. Decrease in eggshell weight in certain birds of prey. Nature. 1967;215:208–210.

58. Rohani IR, Cromroy HL. Taxonomy and distribution of chiggers (Acarina, Trombiculidae) in north-central Florida. Florida Entomol. 1979;62:363–376.

59. Schairer ML, Dierenfeld ES, Fitzpatrick MP. Nutrient composition of whole green frogs (*Rana clamitans*) and southern toads (*Bufo terrestris*). Bull Assoc of Rept Amphib Vet. 1998;8:17–20.

60. Seva A, Sercundes MK, Martins J, de Souza SO. Occurrence and molecular diagnosis of *Cryptosporidium serpentis* in captive snakes in Sao Paulo, Brazil. J Zoo Wildl Med. 2011;42:326–329.

61. Smith CR, Cartee RE, Hathcock JE, Speake DW. Radiographic and ultrasonographic scanning of gravid eastern indigo snakes. J Herpetol. 1989;23(4): 426–429.

62. Snider AT, Bowler JK. Longevity of reptiles and amphibians in North American collections. Herpetological circular No. 21. Society for the Study of Amphibians and Reptiles; 1992. 40 p.

63. Speake DW. A survey of pesticide residues in selected species of reptiles and amphibians from the southeastern United States. Final Report. U.S.F.W.S. Contract 14-16-0008-1154. 1979. 13 p.

64. Speake DW, McGlincy JA. Response of indigo snakes to gassing of their dens. In: Proc Ann Conf SE Assoc Fish and Wildl Agencies. 1981;35:135–138.

65. Speake DW, McGlincy JA, Colvin TR. Ecology and management of the eastern indigo snake in Georgia: a progress report. In: Odum R, Landers L (eds.). Proc Rare and Endangered Wildlife Symposium. Technical Bulletin WL4. Athens (GA): Georgia Department of Natural Resources, Game and Fish Division; 1978. p. 64–73.

66. Speake DW, Mount RH. Some possible ecological effects of "rattlesnake roundups" in the southeastern Coastal Plain. Proc Ann Conf SE Assoc Game and Fish Comm; 1973. 27:267–277.

67. Stacy NI, Alleman AR, Sayler KA. Diagnostic hematology of reptiles. Clin Lab Med. 2011;31:87–108.

68. Stahl SJ. Techniques for sexing reptiles. In: Proc 13th Ann North Am Vet Conf. 1999;787–788.

69. Stevenson DJ, Bolt MR, Smith DJ, Enge KM, Hyslop NL, Norton TM, Dyer KJ. Prey records for the Eastern Indigo Snake (*Drymarchon couperi*). Southeast Nat. 2010;9:1–18.

70. Stevenson DJ, Dyer KJ, Willis-Stevenson BA. Survey and monitoring of the eastern indigo snake in Georgia. Southeast Nat. 2003;2:393–408.

71. Stevenson DJ, Enge KM, Carlile L, Dyer KJ, Norton TM, Hyslop NL, Kiltie RA. An Eastern Indigo Snake (*Drymarchon couperi*) mark-recapture study in southeastern Georgia. Herpetol Conserv Biol. 2009;4: 30–42.

72. Stiles S, Stiles J, Godwin J, Jenkins C, Rush E, Lock B, Johnson V, Wines M, Guyer C. Repatriation of eastern indigo snakes to conservation lands in South Alabama, USA. The Orianne Society [Internet]. 2013; 37–41. Available from http://www.oriannesociety.org/sites/default/files/Stiles%20et%20al%20%202013%20-%20IUCN%20Global%20Re-introduction%20Perspectives [smallpdf.com]%20(1).pdf

73. Taylor SL, Lamden MP, and Tappel AL. Sensitive fluorometric method for tissue tocopherol analysis. Lipids. 1976;11:530–538.

74. Thomas J, Glatt B, Dierenfeld ES. Proximate, vitamins A & E, and mineral nutrient composition of free-ranging cotton mice (*Peromyscus gossypinus*) from St. Catherines Island, GA. Zoo Biol 2004;23:253–261.

75. Tor ER, Holstege DM, Galey FD. Determination of cholinesterase activity in brain and blood

samples using a plate reader. J AOAC Int. 1994;77: 1308–1313.

76. U.S. Fish and Wildlife Service. Endangered and threatened wildlife and plants. Listing of the Eastern indigo snake as a threatened species. Fed Regist. 1978; 43:4026–4029.

77. U.S. Fish and Wildlife Service. Eastern indigo snake recovery plan. Atlanta (GA): U.S. Fish and Wildlife Service; 1982. 23 p.

78. U.S. Fish and Wildlife Service. Eastern Indigo Snake (*Drymarchon couperi*): 5-Year Review-Summary and Evaluation. Jackson (MS): Mississippi Ecological Services Field Office; 2008. 30 p.

79. Van Belle G, Millard SP. STRUTS: Statistical rules of thumb©. Seattle (WA). 1998. p. 3–14.

80. Vestergaard P, Stoen O, Swenson JE, Mosekilde L, Heickendorff L, Frobert O. PLoS ONE. 2011;6:1–6.

81. Wack RF, Hansen E, Small M, Poppenga, R, Bunn D, Johnson C. Hematology and plasma biochemistry values for the giant garter snake (*Thamnophis gigas*) and valley garter snake (*Thamnophis sirtalis fitchi*) in the central valley of California. J Wildl Dis. 2012; 48(2):307–313.

82. Walter D E, Krantz GW. Collection, rearing and preparing specimens. In: Krantz GW, Walter DE (eds.). A manual of acarology. 3rd ed. Lubbock (TX): Tech University Press; 2009. p. 83–96.

83. Wilson N, Durden, LA. Ectoparasites of terrestrial vertebrates inhabiting the Georgia Barrier Islands, USA: an inventory and preliminary biogeographical analysis. J Biogeogr. 2003;30:1207–1220.

84. Wylie GD, Hothem RL, Bergen DR, Martin LL. Metals and trace elements in Giant garter snakes (*Thamnophis gigas*) from the Sacramento Valley, California, USA. Arch Environ Contam Toxicol. 2009;56: 577–587.

85. Zaias J, Cray C. Protein electrophoresis: a tool for the reptilian and amphibian practitioner. J Herpetol Med Surg. 2002;12: 30–32.

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