HEALTH ASSESSMENT OF FREE-LIVING EASTERN BOX TURTLES (*TERRAPENE CAROLINA CAROLINA*) IN AND AROUND THE MARYLAND ZOO IN BALTIMORE 1996–2011

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HEALTH ASSESSMENT OF FREE-LIVING EASTERN BOX TURTLES (*TERRAPENE CAROLINA CAROLINA*) IN AND AROUND THE MARYLAND ZOO IN BALTIMORE 1996–2011

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Abstract: Health data for free-living eastern box turtles (*Terrapene carolina carolina*) at the Maryland Zoo in Baltimore were analyzed. One hundred and eighteen turtles were captured on or near zoo grounds over the course of 15 yr (1996–2011), with recapture of many individuals leading to 208 total evaluations. Of the 118 individuals, 61 were male, 50 were female, and 7 were of undetermined sex. Of the 208 captures, 188 were healthy, and 20 were sick or injured. Complete health evaluations were performed on 30 turtles with physical examination records, complete blood counts (CBCs), and plasma biochemistry profiles. Eight animals were sampled more than once, yielding 40 total samples for complete health evaluations of these 30 individuals. The 40 samples were divided into healthy ($N = 29$) and sick ($N = 11$) groups based on clinical findings on physical examination. Samples from healthy animals were further divided into male ($N = 17$) and female ($N = 12$) groups. CBC and biochemistry profile parameters were compared between sick and healthy groups and between healthy males and females. Sick turtles had lower albumin, globulin, total protein (TP), calcium, phosphorous, sodium, and potassium than healthy animals. Sick turtles also had higher heterophil to lymphocyte ratios. Healthy female turtles had higher leukocyte count, eosinophil count, total solids, TP, globulin, cholesterol, calcium, and phosphorous than healthy males. Banked plasma from all 40 samples was tested for antibodies to *Mycoplasma agassizii* and *Mycoplasma testudineum* via enzyme-linked immunosorbent assay. One sample from a clinically healthy female was antibody positive for *M. agassizii*; none were positive for *M. testudineum*. This study provides descriptive health data for eastern box turtles and CBC and biochemistry profile information for *T. carolina carolina* at and near the Maryland Zoo in Baltimore. It also reports low serologic evidence of exposure to mycoplasmosis.

Key words: Complete blood count, eastern box turtle, health survey, mycoplasmosis, plasma biochemistry, *Terrapene carolina carolina*.

INTRODUCTION

The eastern box turtle (*Terrapene carolina carolina*) is a small chelonian that is native to much of the eastern United States. Its numbers are dwindling due to habitat fragmentation, collection for the pet trade, and road mortality; however, populations persist in urban areas.[5,27] Druid Hill Park in Baltimore, Maryland, USA, is a 745-acre urban park with several fragmented areas providing variations of typical box turtle habitat, including deciduous forests, smaller areas of evergreen and mixed forests, and shrub and scrub lands.[16,29,33] The Maryland Zoo in Baltimore is situated within Druid Hill Park and maintains a population of eastern box turtles in a naturalistic exhibit as part of their Maryland Wilderness section. Additionally, many free-living eastern box turtles live on and around the zoo grounds. To understand potential disease threats to the zoo's box turtle population, the Maryland Zoo staff has collected health data on free-living box turtles found on and near zoo grounds since 1996. These data provide a longitudinal perspective on the health and disease status of a population of eastern box turtles over the course of many years. The objectives of this study were to provide descriptive health data, complete blood counts (CBCs), plasma biochemistry profile concentrations and to determine exposure to *Mycoplasma* spp. in the free-living eastern box turtle population at the Maryland Zoo. Additionally, CBC and plasma biochemistry profiles were compared between healthy and sick turtles and between healthy male and female turtles.

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MATERIALS AND METHODS

Field work was conducted from spring 1996 to winter 2011 as part of an ongoing demographic

study conducted by the Maryland Zoo's reptile curatorial staff. For this study, opportunistic sampling was performed on free-living *T. carolina carolina* found on or near zoo grounds by zoo staff or visitors. All turtle sightings were reported to the reptile curatorial staff, who traveled to the turtle's location and captured it for evaluation. Every turtle was weighed and measured at the site of capture. Capture location was recorded, and then the veterinary staff was contacted to retrieve the turtle for microchip placement and a complete health evaluation.

At the hospital, veterinary staff performed physical examinations and collected blood from all turtles between 1 and 8 hr after capture. Radiographs were taken for all females to check for calcified eggs. Sex was determined based on iris color, tail length and thickness, and plastron concavity.[13] Blood was collected from the jugular vein or subcarapacial sinus and placed in Ca-ethylenediaminetetraacetic acid (EDTA) or heparin (Becton Dickinson, Franklin Lakes, New Jersey 07417, USA). CBC and plasma biochemistry profiles were run within 1 hr of blood collection. Manual CBCs were performed on whole blood smears using Natt and Herricks method (Vetlab Supply, Palmetto Bay, Florida 33157, USA). White blood cells were differentiated into heterophils, eosinophils, lymphocytes, monocytes, and basophils, based on previously described cell types.[20] Azurophils were counted as monocytes due to close morphologic similarity in chelonians.[32] Plasma total solids were determined using a refractometer (American Optical Corporation, Keene, New Hampshire 03431, USA). Biochemistry profiles were run using plasma on an Idexx VetTest 8008 Chemistry Analyzer (Idexx Laboratories, Westbrook, Maine 04092, USA). Plasma biochemistry profiles included albumin, alkaline phosphatase, aspartate aminotransferase (AST), calcium, cholesterol, creatine kinase, globulin, blood glucose, lactate dehydrogenase, phosphorous, total protein (TP), sodium, potassium, chloride, and uric acid. Remaining plasma was stored at −80°C. All newly captured turtles received a microchip subcutaneously in the left inguinal fossa (Biomark, Inc., Boise, Idaho 83702, USA) and were released either the same day or the next morning at the site of capture if healthy. Sick or injured turtles were hospitalized, diagnostics were performed, and turtles were treated, released, or euthanized as indicated. Any turtle hospitalized for a prolonged period was overwintered and released at the original capture site the following spring.

Two hundred and eight records for *T. carolina carolina* captured between 1996 and 2011 were reviewed. These 208 records represent 118 different turtles, many of which were captured and released multiple times. Capture date, location found, physical examination findings, sex, and availability of CBC, plasma biochemistry profile, and banked plasma were recorded for each individual. Results of any additional diagnostic testing, such as radiographic studies, fecal floatation, or serologic examination for infectious diseases were also recorded if available.

Turtles were included in the complete health evaluation portion of the study if they had medical records, including physical examination results, CBC and plasma biochemistry profile data, and banked plasma for *Mycoplasma agassizii* and *Mycoplasma testudineum* testing. A total of 30 adult turtles representing both sick and healthy animals met the inclusion criteria. Six of these turtles were sampled twice, and two were sampled three times, yielding 40 total samples for health evaluation. These samples were divided into sick ($N = 11$) and healthy ($N = 29$) groups, based on clinical findings on physical exam.

CBC and biochemistry profile parameters for the healthy and sick populations were evaluated for distribution using skewness, kurtosis, and an omnibus test for normality. Data variance was evaluated using a modified Levene equal variance test. The mean, standard deviation, median, and range were determined for each parameter. Outliers were identified using Dixon range statistic and excluded.[15] Parameters for healthy and sick populations were compared using equal variance *t*-tests, Aspin-Welch unequal variance *t*-tests, or Mann-Whitney *U*-tests as appropriate for normal and nonnormal data and equal and unequal variance, respectively, with an α value of 0.05. All data analysis was performed using NCSS software (NCSS LLC, Kaysville, Utah 84037, USA).

Samples from healthy animals were subdivided into male ($N = 17$) and female ($N = 12$) groups, and blood parameters were evaluated as above. A chi-square goodness of fit test was used to determine statistically significant differences in sex ratio.

Serologic testing for *M. agassizii* and *M. testudineum* was performed at the University of Florida Diagnostic Laboratory (Gainesville, Florida 32608, USA) using enzyme-linked immunosorbent assay (ELISA) on banked plasma.[34] Titers >32 were considered positive.

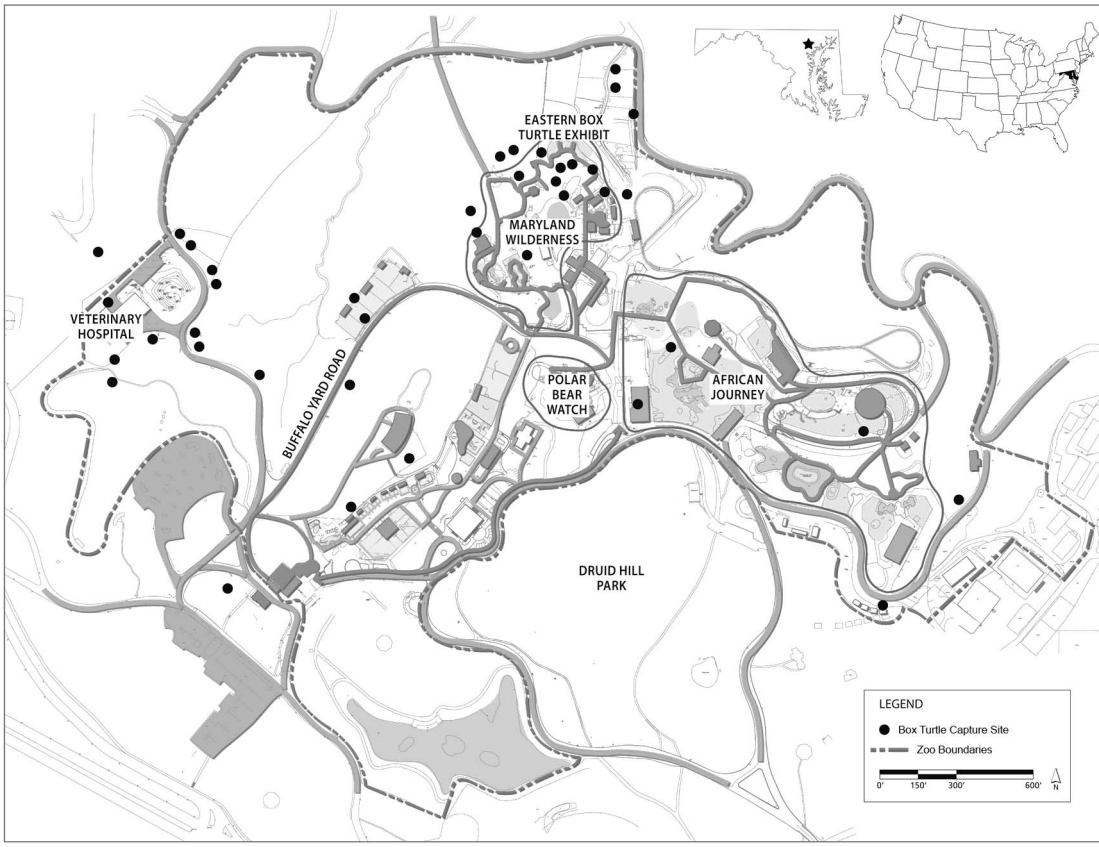

Figure 1. Map of the Maryland Zoo in Baltimore including capture sites for all *T. carolina carolina* included in the health evaluation portion of the study.

RESULTS

Terrapene carolina carolina record review—initial capture

One hundred and eighteen *T. carolina carolina* were captured on or around the grounds of the Maryland Zoo in Baltimore from 1996–2011. Over half of these captures occurred in one of three different areas within the zoo: 29 (24.6%) were in the Maryland Wilderness section of the zoo, 25 (21.2%) turtles were found near the veterinary hospital, and 18 (15.3%) were found on Buffalo Yard Road, a long, heavily traveled section of asphalt path surrounded by deciduous forest that connects the hospital and Maryland Wilderness areas (Fig. 1). No statistically significant differences were observed in sex ratio (61 males, 50 females; $P = 0.296$). Five females were gravid on initial capture, based on radiographic evidence of calcified eggs (one to five eggs confirmed radiographically).

Total *T. carolina carolina* record review—total captures

Twenty-nine *T. carolina carolina* were encountered multiple times, resulting in a total of 208 capture records. Some individuals were captured up to eight times over the course of several years, while others were only encountered once. Ten adult animals were followed over long periods of time, with five having 7 yr between their first and last recapture events, two having 8 yr, and two separated by 9 yr. One turtle was recaptured for the last time 14 yr after its initial capture date.

The total number of turtles captured varied by year, with a general downward trend over time (Fig. 2). No statistically significant differences were observed in sex ratio for the 208 total captures (104 male samples, 97 female samples; $P = 0.621$). Female turtles were found to be gravid at 14 capture events. Of these, five were only evaluated once, six were evaluated multiple times but only determined to be gravid once, and two were gravid at two capture events (one recaptured

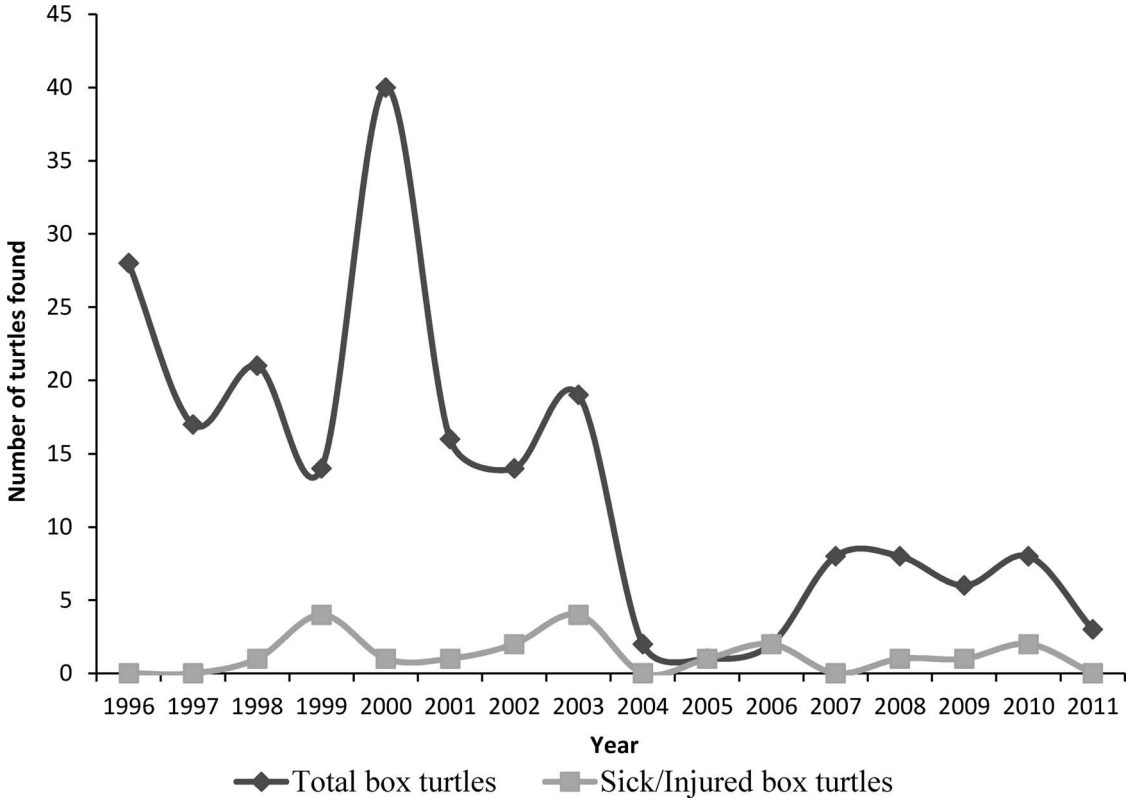

Figure 2. Number of total ($N = 208$) and sick/injured ($N = 20$) *T. carolina carolina* found per year in and around the Maryland Zoo in Baltimore 1996–2011.

1 yr later and one recaptured 8 yr later). Five juvenile turtles and two animals of undetermined sex were also found during the study period.

Over half of the 208 total *T. carolina carolina* captures occurred in one of three different areas within the zoo: 63 (30.3%) turtles were found near the veterinary hospital, 40 (19.2%) were in the Maryland Wilderness section of the zoo, and 25 (12%) were found on Buffalo Yard Road. This is similar to the locations where turtles were frequently encountered at initial capture. Fourteen (6%) turtles were found within Druid Hill Park outside of zoo property, either traveling along the zoo's perimeter fence, sitting near one of the entrances, or in a neighboring Frisbee golf course. The remainder were distributed throughout the zoo. All turtles were found and sampled between April and November, with only one exception: a wounded individual with maggot-infestation found in December 2006. Total turtles captured per month from 1996–2011 included 15 in April, 30 in May, 58 in June, 31 in July, 22 in August, 29 in September, 17 in October, 5 in November, and 1 in December.

Most of the captured turtles were healthy, based on clinical examination. For those turtles evaluated multiple times, the determination of healthy or sick was made each time they were captured based on clinical findings on physical exam. One hundred eighty-eight (90.4%) of the captured turtles were clinically healthy. Many of these animals had limited or no diagnostic testing performed. Twenty-five healthy turtles had plasma banked, but no CBC or plasma biochemistry panel evaluated. Twenty turtles (9.6%) were sick or injured. Fourteen (70%) of the sick turtles were male, while six (30%) were female. Presenting complaints included aural abscesses ($N = 11$), other abscesses ($N = 3$), blepharoedema ($N = 3$), oral plaques ($N = 3$), pneumonia ($N = 3$), lacerations ($N = 2$), shell fractures ($N = 2$), nasal discharge ($N = 2$), and a soft tissue mass ($N = 1$). These animals were hospitalized, treated, and then released. A total of three dead turtles (1.4%) were found on zoo grounds. Autolysis prevented meaningful necropsy in these cases.

Complete health evaluation

Thirty adult turtles were included in the complete health evaluation portion of the study. Eight of these animals were recaptured and resampled, providing a total of 40 samples for analysis. Of these, two females and one male were sampled twice and were clinically healthy each time. Two males were sampled twice and were sick at each evaluation, one with radiographic evidence of pneumonia and one with aural abscesses and oral plaques. One male had an aural abscess at his initial capture date and was healthy on repeat evaluation. One female had an aural abscess at the initial evaluation and was clinically healthy at two later capture dates. Finally, one male was evaluated twice for aural abscessation and was clinically healthy at a later date.

Of the 30 turtles sampled in the complete health evaluation subset, 20 (66.7%) were male and 10 (33.3%) were female. Recapture of three females (two captured twice and one captured three times) and five males (four captured twice and one captured three times) yielded a total of 26 male samples and 14 female samples. There was no statistically significant difference in sex ratio in the 30 turtles ($P = 0.068$) or the 40 samples ($P = 0.058$).

The 40 samples were subdivided into healthy ($N = 29$) and sick ($N = 11$) groups. The healthy group consisted of 17 (56.7%) male samples and 12 (43.3%) female samples. Half of the healthy female samples were from gravid turtles, based on radiographic evidence of calcified eggs. The sick group consisted of nine (81.8%) male samples and two (18.2%) female samples, neither of the latter obviously gravid based on radiographs. There was no statistically significant difference in sex ratio of the healthy group ($P = 0.056$), but there was in the sick group ($P = 0.035$). In decreasing order of frequency, healthy turtles were found in the Maryland Wilderness section of the zoo, around the veterinary hospital, along Buffalo Yard Road, outside of the zoo in Druid Hill Park, near the animal staff offices, in the giraffe yard moat, near the rhino barn, and near the porcupine exhibit. Sick turtles were found primarily near the veterinary hospital or within the Maryland Wilderness section, outside of the zoo in Druid Hill Park, or along the zoo's Blacksmith Road, which is located across the zoo from the veterinary hospital. A sick male turtle was found inside the zoo's eastern box turtle exhibit once in 2010 (Fig. 1).

The most common finding in sick animals in the complete health evaluation subset was aural abscessation. This was the only clinical abnormality in one turtle, while three others had aural abscesses and either nasal discharge, blepharoedema, or oral plaques. One turtle presented with blepharoedema and nasal discharge and had radiographic evidence of pneumonia. Another presented for nasal discharge and poor body condition, and this animal also had radiographic evidence of pneumonia. One presented with an infected tail mass, and one presented with shell fractures and lacerations. All sick turtles were hospitalized for treatment. Hospitalization length ranged from 8 days to 11 mo, with most animals treated for 1 to 3 wk prior to release.

Several statistically significant differences were present in plasma biochemistry profile data between the healthy and sick groups. Samples from sick turtles had lower levels of albumin ($P = 0.02$), globulin ($P = 0.0294$), TP ($P = 0.0163$), calcium ($P = 0.0011$), phosphorous ($P = 0.031$), sodium ($P = 0.0287$), and potassium ($P = 0.00168$) than healthy animal samples. Samples from sick turtles had a significantly higher heterophil-to-lymphocyte ratio ($P = 0.0394$). CBC and plasma biochemistry profile data for sick and healthy groups are provided in Tables 1 and 2.

Statistically significant differences were identified for CBC parameters between healthy male and female samples. Female samples had a higher leukocyte count ($P = 0.000141$), eosinophil count ($P = 0.00609$), total solids ($P = 0.00393$), TP ($P = 0.0478$), globulin ($P = 0.0338$), cholesterol ($P = 0.0311$), calcium ($P = 0.00864$), and phosphorous ($P = 0.00474$). CBC and plasma biochemistry profile data for healthy male and female samples are provided in Tables 3 and 4.

All 40 samples were negative for *M. testudineum* antibodies, and one healthy individual tested positive for *M. agassizii* (titer = 64).

DISCUSSION

Between 1996 and 2011, 118 free-living *T. carolina carolina* were encountered on and around the grounds of the Maryland Zoo in Baltimore. Most turtles were found near the veterinary hospital, the Maryland Wilderness section, and Buffalo Yard Road, which connects the first two areas. These areas all contain deciduous trees, heavy ground cover, and a watershed, representing suitable eastern box turtle habitat.[29,33] Although turtles may frequent these areas due to the presence of preferred habitat, there may also be an aspect of sampling bias because these areas are heavily traveled by veterinary staff and reptile keepers actively looking for turtles. A general

Table 1. Complete blood count data for healthy and sick free-living *T. carolina carolina* at the Maryland Zoo in Baltimore 1996–2011. N = number of samples evaluated, n = number of individual turtles represented.

Parameter	Healthy								Sick							
	N	n	Mean	SD	Median	Minimum	Maximum	Distribution	N	n	Mean	SD	Median	Minimum	Maximum	Distribution
White blood cell count × $10^3/\mu l$	27	23	11.13	5.16	9.46	5.06	28.05	Nonnormal	11	8	11.90	4.58	12.97	1.76	15.2	Normal
Red blood cell count × $10^3/\mu l$	27	24	0.506	0.131	0.480	0.24	0.725	Normal	11	8	0.482	0.189	0.5	0.12	0.80	Normal
Hematocrit (%)	28	24	21.39	6.01	21.5	8	34	Normal	11	8	19.36	6.92	18	10	32	Normal
Total solids (g/dl)	20	19	5.85	1.93	6.1	3	10.6	Normal	4	3	4.33	1.52	4.35	2.6	6	Normal
Heterophils × 10^3	28	24	2.61	1.93	2.01	0.684	8.42	Nonnormal	11	8	3.98	3.27	2.59	0.563	10.8	Normal
Lymphocytes × 10^3	27	23	5.17	3.45	4.35	1.001	13.675	Nonnormal	11	8	4.01	2.21	3.89	0.757	7.65	Normal
Eosinophils × 10^3	28	24	1.72	1.79	1.02	0	6.14	Nonnormal	11	8	1.54	1.69	1.05	0.106	5.12	Normal
Basophils × 10^3	27	23	0.962	0.443	0.911	0.284	1.68	Nonnormal	11	8	1.57	0.986	1.65	0	2.86	Normal
Monocytes × 10^3	26	23	0.524	0.712	0.240	0	2.43	Nonnormal	11	8	0	0	0	0	0	—
Heterophil/lymphocyte[a]	27	23	0.685	0.719	0.391	0.103	3.19	Nonnormal	11	8	1.22	0.965	0.844	0.129	2.71	Normal

[a] Statistically significant difference between healthy and sick groups; Mann-Whitney U-test ($P < 0.05$).

decline was noted in turtle numbers found over time. This may be due to population decline, or more likely due to a decrease in active searching for turtles, because at the beginning of the project, students from a local university actively searched for turtles.

Sick turtles were frequently found in the same areas as healthy turtles. There was no evidence of increased illness or injury in areas of the zoo with less ideal box turtle habitat; however, low sample size prohibits rigorous evaluation of location as a factor in the development of injury or illness. The turtles found outside zoo grounds were encountered along the fence line or near zoo entrances, and 92.8% of these were categorized as healthy.

All turtles were encountered during the species' typical active season from March through November, except for the previously mentioned wounded turtle, which was found in December 2006 near the veterinary hospital. This animal was treated with antimicrobials, overwintered in the hospital, and released at the site of capture in May 2007. In response to warm temperatures, box turtles are known to move between different overwintering sites during brumation, and it was likely that this animal was in the midst of such a move when it was discovered in 2006.[8] The turtle may also have been removed from its burrow by a predator, sustaining wounds in the process. This individual had previously been encountered in 1997 near the veterinary hospital and in 2000 along Buffalo Yard Road. It was deemed healthy based on physical exam both times, and no diagnostic tests were performed. In 2011, it was encountered again on Buffalo Yard Road and was deemed healthy based on physical exam, CBC, and biochemistry profile evaluation. Eastern box turtles typically restrict their movement patterns within home ranges in their natural habitats, so it is unsurprising to encounter the same individual within the same area over the course of many years.[33]

The types of illness and injury for eastern box turtles at the Maryland Zoo are consistent with those presented to the Wildlife Center of Virginia and the University of Tennessee, College of Veterinary Medicine Wildlife Clinic. At both of these institutions, trauma and infectious presentations, including aural abscessation, were two of the most common presenting complaints for free-ranging eastern box turtles.[4,30]

CBC and plasma biochemistry profile parameters were similar to previous published concentrations in *T. carolina carolina,* with some

Table 2. Plasma biochemistry profile concentrations for healthy and sick free-living *T. carolina carolina* at the Maryland Zoo in Baltimore 1996–2011. *N* = number of samples evaluated, *n* = number of individual turtles represented.

	Healthy								Sick							
Parameter	*N*	*n*	Mean	SD	Median	Minimum	Maximum	Distribution	*N*	*n*	Mean	SD	Median	Minimum	Maximum	Distribution
Albumin (g/dl)[a]	28	24	1.57	0.760	1.6	0	2.9	Normal	11	8	0.937	0.671	0.91	0	2.1	Normal
Alkaline phosphatase (U/L)	28	24	95.86	56.43	84	25	237	Nonnormal	11	8	79.27	33.98	69	40	158	Normal
Aspartate aminotransferase (U/L)	28	24	119.5	111.13	77	28	519	Nonnormal	11	8	79.73	53.37	67	23	202	Normal
Calcium (mg/dl)[b]	27	23	12.77	4.31	11.7	9.1	27.4	Nonnormal	11	8	9.47	1.41	9.3	7.3	12.3	Normal
Cholesterol (mg/dl)	28	24	290.14	140.21	293.5	62	601	Normal	10	8	202.1	182.6	164.5	13	520	Normal
Creatine kinase (U/L)	27	23	204.48	133.7	164	25	462	Normal	10	7	462.9	395.2	344.5	20	1157	Normal
Globulin (g/dl)[b]	26	24	3.18	0.807	3.1	1.9	4.6	Normal	11	8	2.69	0.417	2.6	2.1	3.3	Normal
Blood glucose (mg/dl)	27	23	68.78	30.96	61	30	141	Normal	11	8	93.8	46.9	94	39.9	192	Normal
Lactate dehydrogenase (U/L)	23	21	1272.52	1086.1	921	329	4518	Nonnormal	7	5	1477.3	775.4	1105	631	1951	Normal
Phosphorous (mg/dl)[b]	27	23	3.76	1.32	3.4	2.2	6.9	Nonnormal	11	8	2.79	0.970	2.7	1.4	4.3	Normal
Total protein (g/dl)[c]	28	24	4.74	1.88	4.85	0.7	9.5	Normal	11	8	3.59	0.952	3.2	2.4	5.2	Normal
Sodium (meq/L)[a]	22	19	140.82	7.31	138	132	156	Normal	7	5	128	12.6	123	116	150	Normal
Potassium (meq/L)[b]	23	20	4.13	1.01	4	2.9	7	Nonnormal	7	5	2.97	0.489	2.8	2.5	3.9	Normal
Chloride (meq/L)	22	19	107.59	5.30	108.5	100	120	Normal	7	5	99.4	11.3	99	82	117	Normal
Uric acid (mg/dl)	28	24	0.66	0.9	0.3	0.09	3.8	Nonnormal	11	8	0.66	0.649	0.7	0.1	2.1	Normal

[a] Statistically significant difference between healthy and sick groups; Equal variance *t*-test ($P < 0.05$).
[b] Statistically significant difference between healthy and sick groups; Mann-Whitney *U*-test ($P < 0.05$).
[c] Statistically significant difference between healthy and sick groups; Aspin-Welch unequal variance *t*-test ($P < 0.05$).

Table 3. Complete blood count data for healthy, free-living male and female *T. carolina carolina* at the Maryland Zoo in Baltimore 1996–2011. N = number of samples evaluated, n = number of individual turtles represented.

Parameter	Female								Male							
	N	n	Mean	SD	Median	Minimum	Maximum	Distribution	N	n	Mean	SD	Median	Minimum	Maximum	Distribution
White blood cell count × 10^3/µl[a]	12	9	15.04	5.414	13.64	9.46	28.05	Nonnormal	15	14	8.27	2.596	7.7	5.06	16.28	Nonnormal
Red blood cell count × 10^3/µl	11	9	0.543	0.123	0.565	0.375	0.725	Normal	16	15	0.480	0.134	0.47	0.24	0.70	Normal
Hematocrit (%)	12	9	22.25	6.73	21	8	34	Normal	16	15	20.75	5.556	22	10	28	Normal
Total solids (g/dl)[b]	8	7	7.263	1.761	7.1	4.7	10.6	Normal	12	12	4.910	1.418	4.35	3	7.3	Normal
Heterophils × 10^3	12	9	3.384	2.513	2.260	0.684	8.415	Normal	16	15	2.023	1.111	1.689	0.84	5.012	Nonnormal
Lymphocytes × 10^3	12	9	6.402	3.716	5.890	1.705	13.46	Normal	15	14	4.190	2.978	4.081	0	13.68	Nonnormal
Eosinophils × 10^3[a]	12	9	2.634	1.817	2.406	0.33	6.138	Normal	16	15	1.027	1.465	0.7182	0	5.88	Nonnormal
Basophils × 10^3	11	8	1.047	0.435	1.091	0.293	1.683	Normal	16	15	0.903	0.452	0.853	0.284	1.568	Normal
Monocytes × 10^3	11	9	0.542	0.655	0.273	0	2.217	Nonnormal	16	15	0.478	0.758	0.214	0	3.72	Nonnormal
Heterophil/lymphocyte	12	9	0.6756	0.634	0.4179	0.1030	2.3	Nonnormal	15	14	0.5256	0.4535	0.3538	0.1071	1.857	Nonnormal

[a] Statistically significant difference between male and female groups; Mann-Whitney U-test ($P < 0.05$).
[b] Statistically significant difference between male and female groups; Equal variance t-test ($P < 0.05$).

exceptions.[23,28] Healthy turtles in this study had higher total solids, TP, globulin, and albumin and lower uric acid levels than those in Tennessee and Indiana.[23,28] Elevations in protein levels may be due to differences in diet, temperature, antigenic stimulation, or season.[7] The relatively lower uric acid in this study may reflect a lower protein diet, lymph contamination of samples, or differences between analyzers.[35] Healthy turtles in this study had lower total leukocyte counts, eosinophils, lymphocytes, and basophils compared with turtles in Tennessee.[28] Leukogram differences may be due to extrinsic factors (temperature, season, and stress), lymph contamination of samples, or overall difference in health status between the Maryland and Tennessee populations.[7,28] Despite differences in leukocyte numbers, lymphocytes were the predominant leukocyte in both populations, which is consistent with previous reports.[17] Variations in blood parameters between the Maryland and Indiana populations may be due to small sample sizes (<40 animals in each study).

Sick turtles had lower levels of albumin, globulin, TP, calcium, phosphorous, sodium, and potassium than healthy turtles. Hypoproteinemia in sick animals is likely multifactorial. Potential etiologies include anorexia, malnutrition, malabsorption, maldigestion, protein-losing enteropathy, parasitism, blood loss, hepatic or renal disease.[7,35] Electrolyte disturbances are common in sick and injured animals and may be due to decreased intake, increased gastrointestinal or renal losses, acid-base disturbances, and losses through wounds or effusions.[7]

Sick turtles had a higher heterophil-to-lymphocyte ratio than healthy turtles. Elevation of heterophils and/or lowering of lymphocytes are typically associated with stress, inflammation, and/or infection; an elevated heterophil-to-lymphocyte ratio is not necessarily unexpected in sick and injured turtles compared with healthy ones. The heterophil-to-lymphocyte ratio has long been used as an indicator of stress in birds.[11,19] It has also been recognized as an indicator of stress in reptiles, including green turtles (*Chelonia mydas*) and eastern box turtles (*T. carolina carolina*).[1,9] The stress of illness is likely enough to produce changes in the heterophil and lymphocyte populations in sick and injured *T. carolina carolina*. Thus, the heterophil-to-lymphocyte ratio may be a useful indicator of stress and/or illness in this species, though additional studies are indicated to clarify the clinical utility of this parameter.

Table 4. Plasma biochemistry profile concentrations for male and female free-living *T. carolina carolina* at the Maryland Zoo in Baltimore 1996–2011. *N* = number of samples evaluated, *n* = number of individual turtles represented.

	Female								Male							
Parameter	*N*	*n*	Mean	SD	Median	Minimum	Maximum	Distribution	*N*	*n*	Mean	SD	Median	Minimum	Maximum	Distribution
Albumin (g/dl)	11	8	1.863	0.7862	2	0	2.9	Normal	17	16	1.388	0.7026	1.3	0	2.6	Normal
Alkaline phosphatase (U/L)	11	8	108.6	52.70	90	47	213	Normal	17	16	87.59	58.77	59	25	237	Nonnormal
Aspartate aminotransferase (U/L)	11	8	129.9	93.89	79	59	372	Nonnormal	17	16	112.8	123.3	75	28	519	Nonnormal
Calcium (mg/dl)[a]	10	7	16.35	5.376	13.95	10.6	27.4	Normal	17	16	10.67	1.118	10.67	9.1	13	Normal
Cholesterol (mg/dl)[b]	11	8	360	142.5	367	88	601	Normal	17	16	244.9	122.3	221	62	464	Normal
Creatine kinase (U/L)	11	8	223	138.4	177	65	462	Normal	16	15	191.8	133.4	162.5	25	456	Normal
Globulin (g/dl)[c]	10	8	3.92	1.250	3.7	2.8	7	Nonnormal	17	16	2.98	0.8151	2.8	1.9	4.5	Normal
Blood glucose (mg/dl)	11	8	68.82	31.20	52	40	135	Normal	16	15	68.75	31.83	64	30	141	Normal
Lactate dehydrogenase (U/L)	8	6	1425.4	1337.4	1246	353	4518	Nonnormal	15	14	1191	968.9	842	329	4073	Nonnormal
Phosphorous (mg/dl)[b]	10	7	4.65	1.504	4.2	2.7	6.9	Normal	17	16	3.235	0.8817	2.9	2.2	5.2	Normal
Total protein (g/dl)[b]	11	8	5.609	1.892	5.6	1.9	9.5	Normal	17	16	4.182	1.698	4	0.7	7	Normal
Sodium (meq/L)	8	6	140.5	6.525	138.5	135	155	Nonnormal	14	13	141	7.951	137.5	132	156	Normal
Potassium (meq/L)	9	7	4.433	1.220	4.1	2.9	7	Normal	14	13	3.943	0.835	3.95	2.9	5.7	Normal
Chloride (meq/L)	8	6	105.5	4.751	104.5	100	111	Nonnormal	14	13	108.8	5.395	109	100	120	Normal
Uric acid (mg/dl)	11	8	0.678	0.877	0.3	0.09	3.1	Nonnormal	17	16	0.649	0.942	0.3	0.09	3.8	Nonnormal

[a] Statistically significant difference between male and female groups; Aspin-Welch unequal variance *t*-test ($P < 0.05$).
[b] Statistically significant difference between male and female groups; Equal variance *t*-test ($P < 0.05$).
[c] Statistically significant difference between male and female groups; Mann-Whitney *U*-test ($P < 0.05$).

Female turtles had a higher leukocyte count, eosinophil count, total solids, TP, globulin, cholesterol, calcium, and phosphorous compared with male turtles. Elevations in calcium, phosphorous, plasma proteins (especially globulins), and cholesterol have been previously documented in chelonians undergoing active vitellogenesis.[7,28,35] Female eastern box turtles in the Tennessee study had elevated AST, calcium, albumin, TP, and phosphorous compared with males.[28] Female eastern box turtles in Indiana did not have any significantly different biochemistry profile concentrations compared with males, though trends, such as higher calcium, globulin, and albumin were observed in gravid females.[23] Low sample size likely impacted the lack of statistically significant differences in the Indiana study. Elevations in total leukocyte count and eosinophils are typically associated with variation in season, temperature, and reproductive activity, infection or inflammation, and parasitism.[7] No significant differences were observed in any leukocyte counts between male and female eastern box turtles in Tennessee, except for basophils, which were significantly higher in females.[28] The exact cause and clinical significance of the leukocyte elevations in the female turtles of this study is unknown. Low sample size (six healthy gravid and six healthy nongravid females) prohibits vigorous evaluation of the differences in hematology and biochemistry concentrations between gravid and nongravid females in this study.

Some variability in CBC and plasma biochemistry profile parameters may be due to differences in venipuncture site and anticoagulant. Four blood samples representing two healthy male turtles, one sick male, and one sick female were collected from the jugular vein; seven samples, representing two healthy females, three healthy males, and two sick males, had unrecorded venipuncture sites; and the remaining 29 blood samples were collected from the subcarapacial sinus. Significant differences in CBC and plasma biochemistry profiles have been reported due to differences in venipuncture site for desert tortoises (*Gopherus agassizii*) and marginated tortoises (*Testudo marginata*), including changes in red blood cell count, hemoglobin, TP, uric acid, AST, alanine aminotransferase, calcium, phosphorous, and others.[18,24] Blood samples collected from the subcarapacial sinus have a higher risk for lymph contamination than samples from the jugular vein due to the anatomy of the sinus. However, the CBC and biochemistry profile changes in eastern box turtles that would be expected with different venipuncture sites have not been elucidated yet, and additional research is indicated to fully understand the nature of these differences. The majority of the 40 blood samples were collected in lithium heparin; however, three were collected in EDTA during the course of the 16-yr study. EDTA has been reported to cause hemolysis in Herman's tortoises (*Testudo hermanni*), although some clinicians prefer EDTA as an anticoagulant for hematology in chelonian species and believe this benefit outweighs the risk of potential hemolysis.[25,26] Hematocrit in two of these animals was at the lower end of the data range (10 and 14%) while it was at the higher end of the range in the third (26%). A preferred anticoagulant for *T. carolina carolina* has not yet been experimentally determined.

The single sick male box turtle found within the exhibit in 2010 is from an unknown source. The Maryland Zoo's eastern box turtle exhibit consists of a c-shaped stone wall with a 4-foot glass panel in the front. The wall is built into the side of a wooded hill, and there is no fencing at the top of the hill. Due to exhibit design, the turtle may have fallen in from the top, or it may have been dropped in at the front of the exhibit as an unwanted pet or a suspected escaped collection animal. This turtle displayed oral plaques, aural abscesses, generalized edema, polyuria, and diarrhea. Oral swabs tested negative for ranavirus via polymerase chain reaction (PCR) at the University of Florida Diagnostic Laboratory, and it slowly improved with gavage feeding, injectable and topical antimicrobials, diuretic therapy, and lancing of its aural abscesses. This turtle was released after approximately 2 mo of hospitalization in the Maryland Wilderness section near the eastern box turtle exhibit and was never recaptured.

In 2011, the Maryland Zoo's collection of eastern box turtles suffered a concurrent outbreak of ranavirus, herpesvirus, and mycoplasmosis with a 48% mortality rate, despite aggressive treatment.[31] This outbreak began simultaneously with the discovery of another unknown animal in the box turtle exhibit that had not been previously microchipped or identified as part of the collection. These cases of noncollection animals appearing inside exhibits, and the close proximity of collection and free-living eastern box turtles, justify the continued monitoring of the health status of the free-living eastern box turtle population at the Maryland Zoo to keep the collection chelonians healthy.

Only 1 of 40 box turtle samples tested positive for *M. agassizii* in this study. The positive animal

was a gravid adult female with a normal physical exam and unremarkable bloodwork. This turtle was only captured once during the study period, so further monitoring for development of clinical mycoplasmosis and convalescent titers was not available. The *M. agassizii* ELISA has not been validated for eastern box turtles but appears accurate from use in prior studies and clinical cases.[6] *Mycoplasma testudineum* has never been reported in this species. The low number of positive animals on these tests may indicate a low level of exposure to mycoplasmosis in box turtles at the Maryland Zoo, though only 40 (19%) of the captured animals were tested, so true population prevalence cannot be estimated. This population may also harbor a species of *Mycoplasma* spp. that does not cross-react on either test. Poylmerase chain reaction (PCR), which has previously identified a novel, box turtle-specific mycoplasma organism, could be considered.[14] Unfortunately, samples for PCR (oropharyngeal swabs and/or nasal flushes) were not available, and thus the test could not be used. The number of animals with *Mycoplasma* spp. exposure in the turtle subset tested in this study is lower than other studies focusing on eastern box turtles in New York and Virginia.[6,14] This may be due to the more isolated nature of the box turtle population in Druid Hill Park compared with the animals sampled in the aforementioned studies. It may also reflect a lower prevalence of mycoplasmosis in Maryland eastern box turtle populations. Finally, sampling in the Virginia study population was directed against animals showing clinical signs of mycoplasmosis and likely represents an inflated prevalence compared with that in the surrounding population. Despite this, mycoplasmosis is a disease of conservation concern for other chelonians, such as desert tortoises (*G. agassizii*) and gopher tortoises (*Gopherus polyphemus*), and with increasing compromise to *T. carolina carolina* populations due to progressive anthropogenic factors, infectious disease will likely become increasingly important for box turtle conservation.[21,34] During the concurrent ranavirus, herpes virus, and mycoplasmosis outbreak that occurred in the collection turtles at the Maryland Zoo, 68.2% of collection turtles were PCR positive for *Mycoplasma*.[31]

Other infectious diseases may pose more of an immediate threat to *T. carolina carolina* populations. Ranavirus has been associated with mortality events in both captive and free-living eastern box turtles in several U.S. states.[12,22] Current recommendations for antemortem testing for this disease include PCR or quantitative PCR (qPCR) on oropharyngeal swabs and/or whole blood.[2,3] Appropriate samples were not available for ranavirus testing, so disease prevalence is unknown. Furthermore, standard PCR for ranavirus testing is infrequently positive for asymptomatic animals, and its effectiveness as a screening tool for ranavirus infection is questionable.[2] qPCR has been shown to be significantly more sensitive and may be more useful as a tool for early detection and disease monitoring in the future; however, qPCR was not yet available at the time of biomaterial testing for this study.[3] This screening will become increasingly important as subclinical ranavirus infections have recently been identified in box turtles and amphibians.[10]

Chelonian herpes virus and adenovirus are other important causes of morbidity in wild turtle and tortoise populations.[21] Similar to ranavirus, samples for herpesvirus and adenovirus testing were not available during this study, so no comments can be made on the prevalence of these diseases in the study population. However, during the 2011 ranavirus outbreak, 54.5% of collection animals were PCR positive for herpes virus, and this virus may be present in the free-living population as well.[31]

Limitations of this study are largely related to its retrospective nature. Procedural variability included different venipuncture sites, anticoagulants, and the lack of CBC and biochemistry profile evaluation for every turtle captured.[18,24,26] Complete infectious disease testing was not pursued due to lack of diagnostic tests and scientific knowledge of these diseases in box turtles at the time of many of the examinations. The final sample size was small because complete bloodwork and banked plasma were only available for 30 animals (40 total captures).

Additional limitations stem from the statistical methodology selected. Ideally, and with a larger sample size, a repeated measures two-way analysis of variance would have been chosen to determine differences in CBC and plasma biochemistry concentrations between healthy and sick turtles and between healthy male and healthy female turtles. This test allows for analysis of data, including some independent samples and some repeated measures, using individual turtles as blocking factors. However, the sample size was too small to run this test with adequate power. Similarly, mixed model logistic regression could have been considered in this situation, but the small sample size would have prohibited a robust analysis. Data restriction in the form of removing

CBC and biochemistry panel concentrations for repeated individuals was also considered; however, this too would reduce the sample size and decrease the power of the statistical methods. Utilizing the chosen statistical methods increases the chance for Type I error in interpreting the results. As such, interpretation of the statistically significant differences described in this paper should be considered carefully, and application of these results to other populations may not be appropriate.

Despite these limitations, this study provides unique insight into a box turtle population from a single site over many years. Most captured animals were healthy, and there was low exposure to mycoplasmosis in the subset of turtles tested, an infectious disease of chelonian conservation concern.[21,34] The CBC and plasma biochemistry profile parameters will be useful for future evaluation of animals within this study population. The overall health data collected has implications for zoo collection management, including possible exhibit revision and directed and ongoing infectious disease screening.

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