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Venipuncture site and population affect blood analytes in head-started and free-ranging eastern box turtles (*Terrapene carolina*)

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OBJECTIVE
To compare head-started and free-ranging eastern box turtle (*Terrapene carolina)* blood analytes to evaluate head-starting practices and venipuncture site suitability.

METHODS
Eastern box turtles were head-started by Jekyll Island State Park Authority's Georgia Sea Turtle Center. Free-ranging resident box turtles on Jekyll Island State Park were captured. A physical examination and CBC were performed. Blood collection was performed from a jugular vein and the subcarapacial venous sinus. Passive integrated transponder tags were placed prior to release. Recapture and repeat assessments were attempted annually. Blood analyte data were analyzed for observational study of effects of group (head-started or free-ranging) and venipuncture site.

RESULTS
Jugular blood samples from 15 head-started turtles (mostly immature; 88 samples) had lower total solids, lower heterophil-to-lymphocyte ratio, and higher lymphocytes than jugular samples from 24 free-ranging turtles (mostly adult; 64 samples). In head-started individuals, PCV, total solids, WBC count, and heterophils were lower in subcarapacial than jugular samples.

CONCLUSIONS
Disparities between head-started and free-ranging turtles could indicate variance in stress, age, sex, or season. The jugular vein is the most suitable venipuncture site in comparison to the subcarapacial venous sinus, which exhibited lymphatic and/or CSF dilution.

CLINICAL RELEVANCE
Results of the present study provide insight into variances in head-started and free-ranging box turtle populations, which must be considered for accurate hemogram interpretation. The importance of standardized protocol and serial sampling from individuals is emphasized, as values may vary by venipuncture site, population, and intrinsic factors (eg, age, sex, season) within species.

Keywords: blood, hematology, eastern box turtle, *Terrapene carolina*, chelonian

Eastern box turtles (*Terrapene carolina*) are a terrestrial chelonian found in the eastern and midwestern US.[1,2] They inhabit moist woodlands, pastures, and marshy meadows and play important ecological roles, participating in seed dispersal, nutrient cycling, and trophic balance.[3–5] Box turtles are listed as "vulnerable" by the International Union for the Conservation of Nature and Natural Resources.[6] The causes for the observed widespread population declines are primarily anthropogenic, such as habitat destruction and fragmentation, environmental pollution, and global climate change.[3,5,7] As urbanization has led to fragmentation of the eastern box turtle's habitat, encounters with predators, illegal collection, and road mortality have increased.[5,8] These factors place a heavy impact on the future viability of this species due to its slow life history. Box turtles are a long-lived species, living 50 to 80 years.[5,9] They do

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not reach sexual maturity until 5 to 10 years of age, producing 1 clutch/y, with clutch size ranging from 1 to 11 eggs.[5,10,11] Therefore, adult populations significantly impacted by major mortality events are unable to rapidly recover.[4,5] In this way, wildlife rehabilitation plays an important role in species survival through population reinforcement, such as the rehabilitation and release of adult turtles and the implementation of head-start programs. Head-starting is the process of breeding and/or rearing captive animals for a period of time in which they are allowed to grow to a threshold size that significantly reduces predation susceptibility.[4]

Veterinarians routinely use blood analysis as an important tool for evaluating the health of wildlife species[12]. For captive-reared animals, a CBC can aid in determining whether the animal is a good candidate for release[12]. Alternatively, comparing blood analytes of head-started individuals to free-ranging individuals may be a way to evaluate husbandry practices and further elucidate patterns of natural biology. The first objective of this study was to evaluate head-start practices by comparing blood analytes between head-started and free-ranging box turtles. We anticipated that these differences, if present, would be insignificant. The subcarapacial venous sinus is a commonly used venipuncture site, especially in small chelonians.[12] However, it is hypothesized that the subcarapacial venous sinus is more likely to exhibit dilution due to lymphatic and/or CSF contamination.[12] Therefore, the second objective of this study was to compare blood analyte data from the jugular vein and subcarapacial venous sinus venipuncture sites in all turtles.

Methods

Study population, release, and recapture

Methods and procedures were approved by the University of Georgia (Athens, GA) IACUC (AUP#A2016 03-022-Y3-A0). This study was carried out between May 2011 and August 2015 on Jekyll Island State Park, a 2,338-hectare barrier island off the coast of Georgia. Approximately two-thirds of the island is protected from development and is habitat to a native population of box turtles. Eastern box turtles were head-started by the Jekyll Island State Park Authority's Georgia Sea Turtle Center (GSTC) on Jekyll Island, GA. The GSTC accepts displaced, injured, or diseased box turtles for evaluation of health status and potential rehabilitation. Box turtles that were deemed nonreleasable (due to initial injuries that were rehabilitated) were housed at the GSTC in an outdoor enclosure. The turtles were allowed to reproduce with other captive adults and the eggs were allowed to incubate in situ in the display. Additionally, eggs were collected and artificially incubated from freshly dead or injured turtles. When necessary, the injured turtles were induced to lay eggs with an SC injection of 5 to 10 IU of oxytocin/kg in the axillary region. These eggs were incubated at the GSTC at 29.4 to 30.6 °C. Once hatched, all hatchlings were reared together in group housing at the GSTC until 2 to 3 years of age in a 3.47 X 1.24-m outdoor education enclosure with predominantly soil substrate with native forbs, grasses, shrubs, and trees. The turtles were allowed to hibernate naturally during the cooler months. The head-started turtles were given constant water access and fed a diet of frozen mixed vegetables and berries (thawed before feeding), earthworms, and canned dog food (various flavors of Cesar wet food). The turtles likely foraged on native insects found in the enclosure as well. Three to four plates of food were placed in the enclosure daily (5 g of dog food, 2 g of vegetables, 2 g of fruit, and 1 earthworm when available). The frequency of feedings was tapered for brumation purposes as outdoor temperatures began to drop.

Prior to release, health assessments were performed on all individuals to confirm suitability for release. Each health assessment included a physical examination, CBC, biochemical panel, and *Mycoplasma*, herpesvirus, intranuclear coccidiosis, ranavirus, and adenovirus PCR. Passive integrated transponder tags were injected SC in the right inguinal fossa via sterile technique. The needle entry points were sealed with cyanoacrylic tissue glue (Vetbond; 3M). A radio transmitter (model R1860, 15 g; Advanced Telemetry Systems Inc) was attached externally to individual costal scutes with epoxy to prevent growth restriction. Transmitter antennas were fed into 3-mm-diameter coffee stirrers, and acrylic paint was used to match the carapace color. Transmitters were no heavier than 10% of the individual's body weight. Sex was not determined prior to release due to the inability to externally verify sex in immature animals. Two- to three-year-old head-started box turtles were released on Jekyll Island intact maritime forest with close proximity to a freshwater source. The most prevalent vegetation in this area included herbs, grasses, saw palmettos (*Serenoa repens*), slash pines (*Pinus elliottii*), Darlington oaks (*Quercus hemispherica*), and southern live oaks (*Quercus virginiana*). A maximum of 8 hatchlings were released each year in 1 location within 1 hectare of each other. Attempts were made to recapture head-started turtles annually. Each turtle's location was documented via handheld GPS (Trimble Juno 3B; Duncan Parnell). At each recapture event, physical examination and blood collection (for CBC and biochemistry panel) were performed under manual restraint, and transmitters were replaced.

Concurrently, free-ranging resident box turtles within Jekyll Island State Park were captured by hand during opportunistic visual encounters with surface-active animals. Some individuals were reported via public and residents calling in to the GSTC. Health assessments performed on each captured turtle included a physical examination, oral examination, sexing, CBC, and biochemistry panel. Physical and oral examinations were performed by the same person throughout the study. Each captured free-ranging box turtle was equipped with an internal passive integrated transponder tag and external radio transmitter via the same techniques as the head-started turtles.

After biometrics were obtained, the free-ranging turtles were released in close proximity to where they were captured. Attempts were made to recapture the free-ranging box turtles annually; a physical examination, CBC, and biochemical panel were performed under manual restraint and transmitters were replaced.

Blood collection and analyses

At each health evaluation event for an individual turtle, blood was collected from the jugular vein and in some cases the subcarapacial venous sinus. In most cases, the jugular vein could be visualized when the head of the turtle was extended, and pressure was applied to the distal cervical region to raise the jugular vein. After cleaning the site with alternating betadine scrub and alcohol 3 times, blood was obtained from the jugular vein with a 1-inch, 27-gauge needle and a heparinized 3-mL syringe from which the heparin was thoroughly expelled prior to sampling. Pressure was placed on the venipuncture site for 3 minutes to prevent hematoma formation. The subcarapacial venipuncture collection site was prepped in the same way as the jugular vein site, and a 1-inch, 25-gauge needle on a 3-mL heparinized syringe was utilized to obtain the blood. Before processing, each box turtle was weighed. A maximum of 0.5 mL/100 g of body weight was drawn from the jugular vein. Additionally, 0.25 mL/100 g of body weight was drawn from the subcarapacial venous sinus.[12]

Three blood films were prepared from well-mixed whole blood collected from each site within 10 minutes. The blood films were air dried, fixed in methanol, and stained with Wright-Giemsa stain (Harleco; EMD Millipore). The stained slides were examined microscopically for hemoparasites, RBC morphology, WBC estimates, and WBC differential counts (200 WBCs counted).[13] A small amount of heparinized whole blood was transferred to a capillary tube and centrifuged to measure PCV. A refractometer was used to measure plasma total solids (TS).

Statistical analysis

Turtles with physical examination abnormalities or positive viral, coccidiosis, or *Mycoplasma* testing were excluded from the study. The remaining blood sample was centrifuged for plasma harvest and archiving. The biochemical data are the subject of a separate publication and have not been included here. All analyses were performed with JMP Pro, version 16.0.0 (SAS Institute Inc). The data were separated by both group (free-ranging vs head-started) and venipuncture site (jugular vein vs subcarapacial venous sinus). To reduce the potential for interobserver variation, statistical analysis of WBC differentials was performed only on samples reviewed by 1 evaluator (NIS). Furthermore, only jugular samples were used to compare head-started and free-ranging turtles to avoid varying degrees of dilution in subcarapacial samples. Linear mixed models were used to test the effects of group and venipuncture site on all blood analytes (PCV, TS, WBC count, heterophils, lymphocytes, eosinophils, basophils, monocytes, heterophil-to-lymphocyte [H:L] ratio) due to repeated measurements within turtle. The model included fixed factors of group, venipuncture site, and a group-by-venipuncture site interaction effect and a random intercept for each individual turtle. Normality of model residuals were evaluated via inspection of QQ-plots and histograms. Multiple comparisons were adjusted for with the Tukey test. Range, mean, and SD were obtained for each blood analyte by group and venipuncture site.

Results

At the time of release of head-started turtles, the average carapace length was 97 ± 6.3 mm (range, 88 to 108 mm). The mean mass was 185 ± 44.5 g (range, 132 to 264 g; **Table 1**). The head-started population comprised mostly juvenile turtles (4 adults, 11 juveniles), while the free-ranging population comprised mostly adult turtles (22 adults, 2 juveniles; **Table 2**). Each head-started turtle was too young to be sexed by external observation. Out of the 24 free-ranging turtles, 13 were male, and 11 were female. Thirty-eight samplings were eliminated from the study due to positive infectious disease testing **(Table 3)**. Based on clinical examination, all study turtles were deemed clinically normal (including normal behaviors) and had no external injuries or other abnormalities.

In the comparison of jugular head-started and jugular free-ranging samples for each blood analyte, several statistically significant differences were observed. Total solids and H:L ratio were significantly lower in head-started turtles (N = 17) than free-ranging turtles (N = 7) by on average 1.32 g/dL (95% CI, 0.35 to 2.29 g/dL; P = .004) and 2.96 g/dL (95% CI, 0.44 to 5.48 g/dL; P = .016), respectively **(Table 4;**

Table 1—Morphometrics of head-started eastern box turtles (*Terrapene carolina*) at time of release.

	Mean ± SD	Range
Carapace length (mm)	97 ± 6.3	88–108
Mass (g)	185 ± 44.5	132–264

Table 3—Infectious disease testing results of all eastern box turtles combined, including repeated samples of some individuals (when recaptured annually).

	Positive (N)	Negative (N)
Adenovirus PCR	8	77
Terrapene HV1 qPCR	20	110
Ranavirus FV3 qPCR	4	126
Mycoplasma PCR	6	62
Total	**38**	**375**

Table 2—Age and sex classification of head-started and free-ranging eastern box turtles included in statistical analysis.

	Juvenile (N)	Adult (N)	Male (N)	Female (N)	Sex-Undetermined (N)
Head-started	11	4	—	—	15
Free-ranging	2	22	13	11	0

Table 4—Descriptive statistics for hemogram data in head-started and free-ranging eastern box turtles by venipuncture site, including repeated samples of some individuals (when recaptured annually).

Analyte	Group	Jugular (N)	Mean ± SD (range)	Subcarapacial (N)	Mean ± SD (range)	*P* value
PCV (%)	Head-started	17	24.1 ± 5.4 (10.0–31.0)	14	14.4 ± 5.4 (6.0–23.0)	< .001*
	Free-ranging	7	26.6 ± 3.6 (22.0–32.0)	3	18.3 ± 5.5 (13.0–24.0)	.111
	P value	—	.693	—	—	—
Total solids (g/dl)	Head-started	17	4.32 ± 0.60 (3.20–5.20)	14	3.50 ± 0.66 (2.10–4.60)	.009*
	Free-ranging	7	5.73 ± 0.99 (4.4–7.2)	3	5.30 ± 1.18 (4.3–6.6)	.736
	P value	—	.004*	—	—	—
WBCs (K/µL)	Head-started	17	15.14 ± 3.30 (9.2–21.0)	14	12.06 ± 3.08 (6.3–16.5)	< .001*
	Free-ranging	7	12.90 ± 4.03 (8.0–17.0)	3	8.30 ± 5.20 (2.4–12.2)	.249
	P value	—	.469	—	—	—
Heterophils (K/µL)	Head-started	17	5.83 ± 3.21 (2.12–14.70)	14	3.44 ± 2.49 (0.51–9.76)	.022*
	Free-ranging	7	5.79 ± 1.75 (3.52–8.48)	3	4.07 ± 2.22 (1.56–5.77)	.736
	P value	—	1.000	—	—	—
Lymphocytes (K/µL)	Head-started	17	4.75 ± 1.35 (2.73–7.48)	14	5.31 ± 1.28 (3.20–7.68)	.487
	Free-ranging	7	1.99 ± 1.18 (0.40–3.52)	3	1.69 ± 1.29 (0.86–3.17)	.988
	P value	—	< .001*	—	—	—
Eosinophils (K/µL)	Head-started	17	2.79 ± 1.37 (0.38–5.51)	14	2.01 ± 1.56 (0.25–5.61)	.435
	Free-ranging	7	3.55 ± 2.53 (0.00–7.14)	3	1.26 ± 1.11 (0.02–2.16)	.29
	P value	—	.776	—	—	—
Basophils (K/µL)	Head-started	17	0.82 ± 0.55 (0.00–2.32)	14	0.50 ± 0.31 (0.00–1.20)	.215
	Free-ranging	7	1.05 ± 1.11 (0.24–3.30)	3	1.01 ± 1.19 (0.00–2.32)	.818
	P value	—	.957	—	—	—
Monocytes (K/µL)	Head-started	17	0.96 ± 0.70 (0.11–2.00)	14	0.85 ± 0.56 (0.00–1.76)	.93
	Free-ranging	7	0.46 ± 0.52 (0.00–1.28)	3	0.29 ± 0.31 (0.00–0.62)	.906
	P value	—	.301	—	—	—
H:L ratio	Head-started	17	1.46 ± 1.22 (0.4–5.4)	14	0.77 ± 0.77 (0.10–3.10)	.148
	Free-ranging	7	4.27 ± 2.90 (1.70–8.80)	3	2.97 ± 2.29 (1.50–5.60)	.657
	P value	—	.016*	—	—	—

P values are from linear mixed model tests comparing group and venipuncture site.
*Value is significant.
H:L ratio = Heterophil-to-lymphocyte ratio.

Supplementary Figures S1 and S2). Lymphocytes were significantly higher in head-started turtles (N = 17) than free-ranging turtles (N = 7) by on average 2.85 K/µL (95% CI, 1.23 to 4.48 K/µL; *P* = < .001; **Supplementary Figure S3**). No significant differences were observed in WBC count, heterophils, eosinophils, basophils, or monocytes between head-started and free-ranging turtles.

Packed cell volume, TS, WBC count, and heterophils from the subcarapacial sinus (N = 14) were significantly lower in comparison to those from the jugular vein (N = 17) in head-started turtles by on average 9.76% (95% CI, 4.68% to 14.85%; *P* < .001), 0.73 g/dL (95% CI, 0.15 to 1.31 g/dL; *P* = .009), 3.37 K/µL (95% CI, 1.13 to 5.61 K/µL; *P* = < .001), and 2.49 K/µL (95% CI, 0.28 to 4.71 K/µL; *P* = .022), respectively (Table 4; **Supplementary Figures S4–S7**). No significant differences were observed in lymphocytes, eosinophils, basophils, monocytes, or H:L ratio between venipuncture sites in head-started turtles. No significant differences were observed between venipuncture sites of any analyte among free-ranging turtles.

Discussion

Three blood analytes were found to be significantly different in jugular samples between head-started and free-ranging turtles. The lower TS in head-started individuals may indicate differences in life stage or some degree of nutritional or dietary differences within head-started turtles that may be corrected through husbandry practices.[14] Total solids may be higher in free-ranging turtles as a reflection of dehydration, stress, or differences in life stage.[14] As all turtles were deemed healthy through physical examination and viral testing, it is unlikely that impaired liver function, malabsorption, or protein-losing conditions were present, though subclinical disease cannot be excluded.[15] The lack of differences in PCV between groups may indicate basic nutritional needs were met and the absence of severe chronic disease, which is consistent with each turtle presenting as apparently healthy upon physical examination.[14]

Differences between head-started and free-ranging turtles in lymphocytes and H:L ratio may be explained by the groups' varying response to handling. The free-ranging turtles may exhibit an increased stress response to human interaction compared to the head-started animals. Therefore, the free-ranging turtles may exhibit a stronger stress response upon capture and recapture that influences their blood analytes, typically exhibited by a lymphopenia, which could explain why lymphocytes were observed to be lower and the H:L ratio observed to be higher in free-ranging compared to head-started turtles.[14]

Age, sex, and season are most likely significant confounding factors that must be addressed. The head-started population mostly comprised juvenile turtles (73%; N = 11), while the free-ranging population mostly comprised adult turtles (91%; N = 24).

White blood cell counts and heterophils reportedly decrease with age in other species of chelonians, such as loggerhead sea turtles (*Caretta caretta*).[16] Therefore, the differences observed between head-started and free-ranging turtles could have been influenced by age. Each head-started turtle was too young to be sexed by external observation. However, each free-ranging turtle was able to be sexed. Out of the 24 free-ranging turtles, 13 were male and 11 were female. In other species of chelonians, such as the Asian box turtle (*Cuora flavomarginata*), males are known to have higher eosinophil counts than females.[17] Therefore, sex is a potential confounding factor also in this study that cannot be accounted for since all individuals in both treatment groups could not be sexed.

Adult eastern box turtles will exhibit breeding behaviors in the spring/summer.[5,10] During this time, they interact with other turtles more often than during the fall/winter and therefore may be more frequently exposed to pathogens via direct contact. During the fall/winter, the eastern box turtle overwinters below the soil surface or under leaf litter in a period of brumation.[3,5,18] In other chelonian species, brumation is known to induce a decrease in immune function due to temporary lymphoid organ atrophy, lymphopenia, decreased food intake, and decreased metabolic activity.[14,19,20] The individuals in this study were not all sampled within the same season. Therefore, the WBC differential could have been affected by seasonal behavior patterns and therefore contributed to this study as a confounding factor.

Additionally, not all individuals were able to be sampled each year (2011 to 2015) because of their ability to avoid capture due to their frequently covert behaviors. While the transmitters allowed the location of the turtles, individuals that were buried or under woody debris were not extracted to avoid stress or damage to microhabitat. Therefore, each individual turtle was sampled between 1 and 5 times, and other turtles were only recaptured during the same season each year. An increased resampling of head-started individuals (N = 15) led to a larger capture-event sample size (N = 88), while free-ranging individuals (N = 24) were less commonly recaptured, resulting in a smaller capture-event sample size (N = 64). This led to a smaller sample size among free-ranging turtles (N = 7), in contrast to head-started turtles (N = 17), when groups were compared using only jugular vein samples.

This study provided new insight into venipuncture site variations in box turtles. Based on the results of this study, the jugular vein is the most suitable venipuncture location. The subcarapacial venous sinus samples were significantly diluted, most likely by lymph and/or CSF contamination, and consequently this technique should be avoided.[21,22] As PCV and TS are common table-side measurements for both companion and wildlife species, it is important to recognize the potential variation as a result of venipuncture site in chelonians when serially monitoring or comparing to references. The same is true for other blood analytes that were found to have significant differences, such as WBC and heterophil counts. When determining an appropriate venipuncture site, it is also important to consider the stress of the animal, the strength of the turtle and therefore its ability to evade restraint, and the level of skill of the person obtaining the sample. With an inexperienced person, the jugular venipuncture site may be more stressful for a conscious animal, as it is more technically challenging.[23]

Although the free-ranging samples did not show any significant differences between blood data from jugular vein and subcarapacial sinus, this may be due to the smaller sample size within free-ranging turtles in comparison to head-started turtles. Additionally, the use of preheparinized syringes is contraindicated when collecting small volumes of blood due to potential hemodilution.[24] Preheparinized syringes should be used only if clotting during collection is a documented challenge in a given species. However, all sample collections in our study utilized preheparinized syringes (after thorough expelling of heparin before blood sampling), and over 1 mL of blood was collected at each sampling. Therefore, all samples most likely exhibited similar degrees of potential heparin-induced hemodilution.

Results of the present study indicated significant variations between head-started and free-ranging box turtle populations and emphasized the need for standardized methodology for blood sampling and analysis. Additionally, the jugular vein is the most suitable venipuncture site in comparison to the subcarapacial venous sinus due to lymph and/or CSF dilution. These data will be beneficial to establish a standard in box turtle venipuncture site selection and to provide insight into differences that may occur between head-started and free-ranging eastern box turtles. Additionally, the study highlighted the importance of reviewing serial samples from individuals, rather than reliance on published blood analyte data, as values may vary by population and due to various intrinsic factors (eg, age, sex, season) within the same species.

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Supplementary Materials

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