RESEARCH ARTICLE

Plasma electrophoresis profiles of Blanding's turtles (*Emydoidea blandingii*) and influences of month, age, sex, health status, and location

Kirsten E. Andersson[1]*, **Laura Adamovicz**[1,2], **Lauren E. Mumm**[1], **Samantha E. Bradley**[1], **John M. Winter**[1], **Gary Glowacki**[3], **Carolyn Cray**[4], **Matthew C. Allender**[1]*

1 Wildlife Epidemiology Laboratory, College of Veterinary Medicine, University of Illinois, Urbana, IL, United States of America, **2** Veterinary Diagnostic Laboratory, College of Veterinary Medicine, University of Illinois, Urbana, IL, United States of America, **3** Lake County Forest Preserve District, Libertyville, IL, United States of America, **4** Department of Pathology & Laboratory Medicine, School of Medicine, University of Miami, Miami, FL, United States of America

* kkeandersson@gmail.com (KEA); mcallend@illinois.edu (MCA)

Abstract

Baseline plasma electrophoresis profiles (EPH) are important components of overall health and may aid in the conservation and captive management of species. The aim of this study was to establish plasma protein fractions for free-ranging Blanding's turtles (*Emydoidea blandingii*) and evaluate differences due to age class (adult vs. sub-adult vs. juvenile), sex (male, female, or unknown), year (2018 vs. 2019), month (May vs. June vs. July), health status, and geographical location (managed vs. unmanaged sites). Blood samples were obtained from 156 Blanding's turtles in the summer of 2018 and 129 in 2019 at two adjacent sites in Illinois. Results of the multivariate analysis demonstrated that age class, sex, year, month, health status, and geographical location all contributed to the variation observed in free-ranging populations. Adult females had the highest concentration of many protein fractions, likely associated with reproductive activity. Juveniles had lower protein concentrations. Temperature and rainfall differences between years impacted concentrations between 2018 and 2019, while May and June of both years saw higher levels in some protein fractions likely due to peak breeding and nesting season. Individuals with evidence of trauma or disease also showed increased plasma protein fractions when compared to those that were considered healthy. The two sites showed a wide/large variation over the two years. All of these factors emphasize the importance of considering multiple demographic or environmental factors when interpreting the EPH fractions. Establishing ranges for these analytes will allow investigation into disease prevalence and other environmental factors impacting this endangered species.

Introduction

Climate change, habitat destruction, and disease are potentially affecting the sustainability and conservation of species [1]. Wildlife health surveillance has become a critical component to

maintain imperiled populations [1, 2], but determining health is complex and involves several modalities, including physical examination, clinical pathology, pathogen presence, and contaminant exposure [3–6]. Protein electrophoresis (EPH), a component of clinical pathology investigation, has become commonly used in wildlife studies [7]. Reference interval data in several reptilian species exist and have proven to be valuable in understanding how they handle stress and disease [4, 7–16].

Plasma proteins are key players in the body's innate immune response, and fluctuations in concentrations serve to indicate the presence of inflammation, infection, neoplasia, stress, or trauma [17]. In head-started red-bellied cooters (*Pseudemys rubriventris*) and captive-reared loggerhead sea turtles (*Caretta caretta*), variations in protein fractions were associated with differences in age, diets, immune stimulation, and reproductive stage [18, 19], indicating baseline differences exist and responses to changes in demographic and environmental variation can be measured.

The Blanding's turtle is a semi-aquatic, long-lived species of turtle experiencing population declines over much of its range in southern and central Canada and northern United States. Individual turtles can live up to 80 years of age in the wild [20], but urban development, road mortality events, climate change, illegal poaching, and disease remain the most common threats to sustainability [21]. The Chiwaukee Prairie–Illinois Beach Lake Plain (Lake Plain) contains a population of Blanding's turtles, in which active conservation efforts are aimed at improving the long-term viability in northeastern Illinois and southeastern Wisconsin [22]. In the summer of 2015, conservation efforts incorporated physical exam and health assessment data to aid in a greater understanding of the biological threats these animals face.

The objective of this study was to establish baseline plasma protein fractions for free-ranging Blanding's turtles and to determine differences between age classes (adult vs. sub-adult vs. juvenile), sex (male vs. female), years (2018 vs. 2019), months (May vs. June vs. July), health status, and geographical location. It was hypothesized that total protein levels would be higher in adults than subadults and juveniles, higher in females than males, higher in May than in June and July, and higher in unhealthy turtles than healthy turtles.

Materials and methods

Study sites

Blanding's turtles were sampled from three sites within the Lake Plain including Spring Bluff-Chiwaukee Prairie (SBCP), the managed site, and the North and South units of Illinois Beach State Park (IBSP), the unmanaged sites. SBCP consists of approximately 535 acres of high-quality coastal dune and swale habitat along the coast of Lake Michigan in Illinois and Wisconsin, whereas IBSP consist of 4,160 acres of dune, prairie, oak-savannah, and wetland habitats along 6.5 miles of coastline [22]. Management of mesopredators is also more robust in SBCP than IBSP, with studies focusing on camera trap surveillance for predator presence throughout the year as well as nest predation rates [23].

Capture methods

Turtles were captured with the aid of radiotelemetry, hoop net trap, or incidentally by hand. Radiotelemetry is a three-part system using a radio transmitter, a radio antenna, and a radio receiver. The transmitter is attached to the turtle's shell, which transmits a signal to the antenna and correlates to a beeping produced by the receiver that gets louder as the animal gets closer [23, 24]. Hoop traps were placed in marsh waters and areas that were characteristic of Blanding's habitat or locations near previous Blanding's turtle capture sites. These traps were checked every 24 hours and remained in the same location for up to five days. Telemetry,

traps, and incidental captures were used in both field sites. Many turtles, especially those equipped with a radio transmitter, were sampled up to two times per summer.

Physical examination and sample collection

Each turtle was assigned a permanent ID, marking the shell with a notch code unless previously marked as well as inserting a pit tag (microchip) under the skin, and mass, sex, and age class were recorded. Sex was classified as male, female, or unknown. Sex of head-started turtles, which are clutches deposited in captivity from free-range females, was known due to established incubation temperatures, as males were incubated at 26.5˚C and females at 31.0˚C [23, 25]. The sex of adults was determined based on plastron concavity [26]. The sex of most sub-adults and juveniles was estimated based on position of cloacal opening [27]. Wild-born individuals were classified as unknown sex when a confident determination could not be made. Age class was characterized as juvenile (<250 grams), sub-adult (250–750 grams), or adult (>750 grams). Blanding's turtles were deemed sexually mature at 750 grams and over by the Lake County Forest Preserve District (LCFPD) based on the lightest fertile female noted. This methodology is based on a previously published study by Mumm, et al. [24]. Body fat percentage (FP) was calculated using a published calculation from the relationship of carapace length and mass [28]. Physical examinations were performed noting visual appearance of the eyes, nose, oral cavity, ears, legs, digits, shell, integument, and cloaca. Gravidity was assessed using digital palpation of the prefemoral fossa. For the purpose of statistical analysis, females were classified as gravid if they had palpable eggs or if they had nested within one week of sampling. Nesting was determined either by observation of nesting behavior or the lack of palpable eggs after having previously been confirmed gravid. Turtles were classified as either "apparently healthy" or "unhealthy" based on the presence of clinically significant physical exam abnormalities, including open fractures or wounds; ocular, oral or nasal discharge; depressed mentation; missing nails, digits, or appendages; and evidence carapace/plastron damage.

Whole blood was collected from the sub-carapacial sinus via 22-gauge or 25-gauge needle, subject to the size of the individual. No more than 0.6% of body weight of whole blood was drawn and placed into lithium-heparinized plasma separator tubes. Blood samples were placed on ice in a cooler for one to five hours depending on time of collection until returning to the lab each afternoon. Total protein (TP) was estimated using refractometry. Whole blood samples were centrifuged at 4,185 g for 10 minutes, stored at -20˚C for one to four months, and shipped on dry ice to the University of Miami at the end of the field season. All individuals were released at coordinates of capture. All animal sampling was permitted by the following organizations: Department of Natural Resources (IDNR) (Scientific Collectors Permits (SCP): NH17.5065, NH18.5065, and IDNR Endangered and Threatened permits: SBT-16-062, 1199, 14–046, and 1042), the Wisconsin Department of Natural Resources (WIDNR) (SCP: SCP-SOD-004-2013 and WIDNR Scientific Research License: SRLN-18-026), and the University of Illinois Institutional Animal Care and Use Committee (Protocols: 18000 and 18165).

Protein electrophoresis

Plasma samples were analyzed according to the procedure provided by the Helena SPIFE 3000 system with the use of Split Beta gels (Helena Laboratories, Inc., Beaumont, Texas 77707, USA). Results were produced after gel scanning and analysis by Helena software. Fraction delimits were placed as previously demonstrated for other reptiles [10]. Plasma protein fractions were divided into the following six fractions: a fraction migrating in the prealbumin region ("prealbumin"), albumin, alpha 1 globulins, alpha 2 globulins, beta globulins, and gamma globulins (Fig 1). Percentages for each fraction were determined by this software,
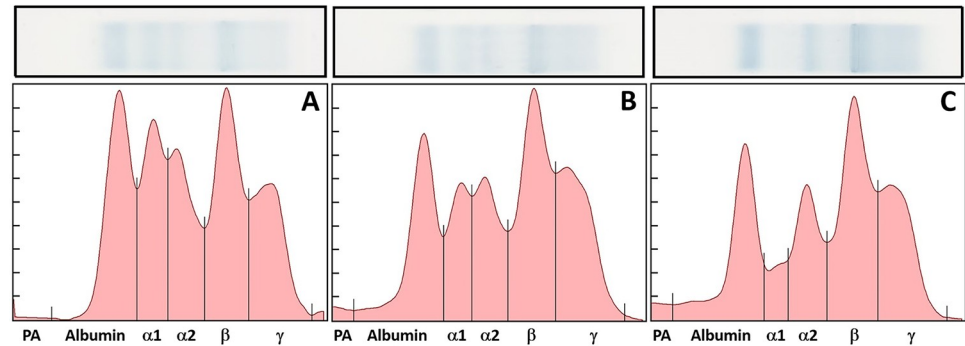
Fig 1. Electrophoretogram comparison of plasma protein fractions. A visual representation of the plasma protein fraction distribution amongst three representatives from each age classes. The top panel shows the protein as they appeared on the gel following electrophoresis, and the bottom panel shows a graphic representation of the concentration differences. Juvenile (A), sub-adult (B), and adult (C).

which gave the relative value, and absolute values (g/L) for each fraction were obtained by multiplying the percentage by the TP concentration. The albumin:globulin ratio (A:G) was calculated by dividing the sum of albumin and prealbumin by the sum of the globulin fractions.

Statistical analysis

All statistical analyses were conducted in R version 3.6.3 [29] at an alpha level of 0.05, unless otherwise specified. Data distributions were assessed using histograms and the Shapiro-Wilk test and transformation was pursued, if necessary, to meet modeling assumptions.

Protein electrophoresis fractions were modeled using general linear mixed models with the lme4 and lmerTest packages [30, 31]. Fixed effects included spatiotemporal variables (year, month, location), demographic variables (sex, age class, gravidity), and health variables (body condition, presence/absence of physical exam abnormalities), while turtle ID was included as a random effect. Post-hoc testing was performed using the lsmeans package with a Tukey correction to control for multiple statistical tests [32]. Fixed effects with univariable p-values < 0.15 were considered in multivariable models testing specific biological hypotheses about the effects of spatiotemporal, demographic, and health variables on EPH values. Variance inflation factors were evaluated for multivariable models to identify and exclude highly collinear (VIF > 10) predictor variables (function vif, package car) [33]. Candidate model sets were constructed for each EPH fraction and ranked using an information-theoretic approach [34]. Figures were constructed using the ggeffects package [35]. Spatial clustering of EPH fractions was modeled in ArcGIS version 10.6 using hot spot analysis with the Gettis-Ord Gi* statistic from the spatial statistics toolbox. Hot spots are areas with higher plasma protein concentrations, while cold spots are areas with lower plasma protein concentrations.

Coefficients of variation were determined for each EPH fraction using data from apparently healthy turtles evaluated at multiple time points (CV_I) and only a single time point (CV_G). The index of individuality (II) was calculated as CV_I / CV_G and was used to infer the need for subject-based vs. population-based reference intervals [36, 37]. When the II is < 0.6, subject-based reference intervals was used, while an II > 1.4 population-based reference intervals were created (Harris, 1974). When the II was between 0.6 and 1.4, population-based reference intervals were used [37, 38]. Reference change values (RCV) were calculated for each EPH analyte using a published formula [36, 37].

Population-based reference intervals were also determined for each analyte using the nonparametric method (referenceIntervals package) [39], according to American Society for

Veterinary Clinical Pathology guidelines [36]. Outliers were identified and excluded using Horn's method [40]. Ninety percent confidence intervals were generated around the limits of each reference interval using nonparametric bootstrapping with 5000 replicates. The population-based reference interval dataset included only turtles sampled once, and a randomly-selected single time point (https://www.random.org/) from serially-sampled individuals.

Results

Two hundred and eighty-five samples were collected from 215 individual turtles. Fifty animals were sampled two times approximately one year apart, and ten animals were sampled three times–twice within the 2018 active season and a third time in 2019. One hundred fifty-six samples were obtained in 2018, while 129 were collected in 2019. One hundred seventy-eight samples were collected at SBCP, 51 were from IBSP North Unit, 54 were from IBSP South Unit, and two individuals did not have location data recorded. Samples were collected in May (N = 129), June (N = 120), and July (N = 36) from 171 adults, 83 sub-adults, and 31 juveniles. Sex distribution included 174 females, 75 males, and 36 turtles of unknown sex. Twenty-two females were gravid.

Blood samples were collected from the subcarapacial sinus due to its relative ease of access and minimal restraint requirement in a field setting. Grossly hemolyzed or lymph contaminated samples were removed from the study, although microscopic hemolysis cannot be ruled out. The timing of sampling relative to food consumption is unknown, so post-prandial changes, such as lipemia, could impact results. Physical examination was largely unremarkable for most individuals. Clinical signs of upper respiratory disease (URD), including oculonasal discharge, blepharoedema, and/or oral plaques were present in eleven turtles. Integumentary abnormalities including abrasions, lacerations, and/or nodules were present in 28 turtles. Appendicular abnormalities including abnormal nails and/or missing digits, feet, limbs, or tail tips were present in 42 animals. Cloacal abnormalities consisting of erythema, swelling, and/or discharge were present in 15 animals. Shell abnormalities involved the carapace (erosions– 25, predator injury—8) and plastron (erosions– 99, predator injury—12). In total, 30 turtles had active physical exam abnormalities significant enough to compromise health, and these individuals were excluded from the reference interval dataset.

All absolute EPH parameters varied by year, with TP (effect size = 3.70g/L, 95% CI = 1.60–5.90g/L, p = 0.01), albumin (effect size = 1.20g/L, 95% CI = 0.70–1.70g/L, p < 0.01), alpha 1 globulins (effect size = 0.86g/L, 95% CI = 0.60–1.10g/L, p < 0.01) alpha 2 globulins (effect size = 0.63g/L, 95% CI = 0.26–1.00g/L, p = 0.01), and gamma globulins (effect size = 4.30g/L, 95% CI = 3.70–4.80g/L, p < 0.01) higher in 2018 than 2019, and A:G (effect size = 0.02, 95% CI = 0.01–0.03, p = 0.02), prealbumin (effect size = 0.67g/L, 95% CI = 0.58–0.77g/L, p < 0.01), and beta globulins (effect size = 2.60g/L, 95% CI = 1.60–3.60g/L, p = 0.01) higher in 2019 than 2018. The relationships between relative EPH fraction and year were similar, except there was no significant association between relative alpha 2 globulins and year (p = 0.34).

The effects of location on absolute EPH parameters depended on year (significant Year*Location interaction, p < 0.05) for TP, A:G, prealbumin, albumin, and gamma globulins (Fig 2). A significant year*location effect was also identified for relative prealbumin (Fig 3). Location influenced absolute alpha 2 globulins (p = 0.02), absolute beta globulins (p = 0.04), and relative gamma globulins (p = 0.01) independent of year, while it was not a statistically significant predictor of absolute alpha 1 globulins, relative albumin, relative alpha 1 globulins, relative alpha 2 globulins, or relative beta globulins (p > 0.05). In addition to the site-level effects identified using general linear models, finer-scale clusters of high and low absolute EPH values were identified using spatial modeling (Fig 4). The location of these spatial clusters varied

Fig 2. Model predictions for plasma protein electrophoresis values based on year and location. Model predictions with 95% confidence intervals for plasma protein electrophoresis values in free-living Blanding's turtles (*Emydoidea blandingii*) based on year and location. Model estimates were produced by top-ranking general linear mixed models (see Table 2). SB = Spring Bluff-Chiwaukee Prairie, IB-N = Illinois Beach North, IB-S = Illinois Beach South.

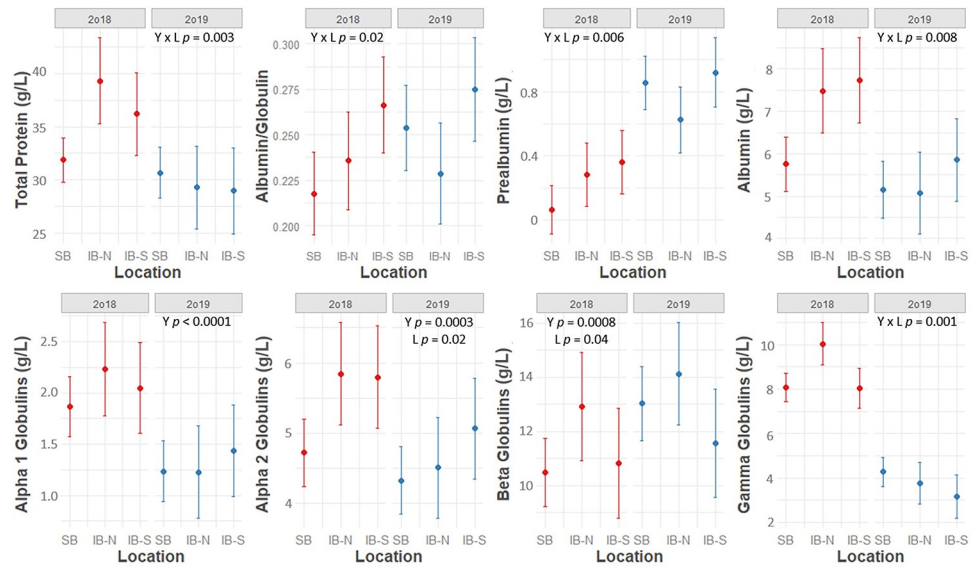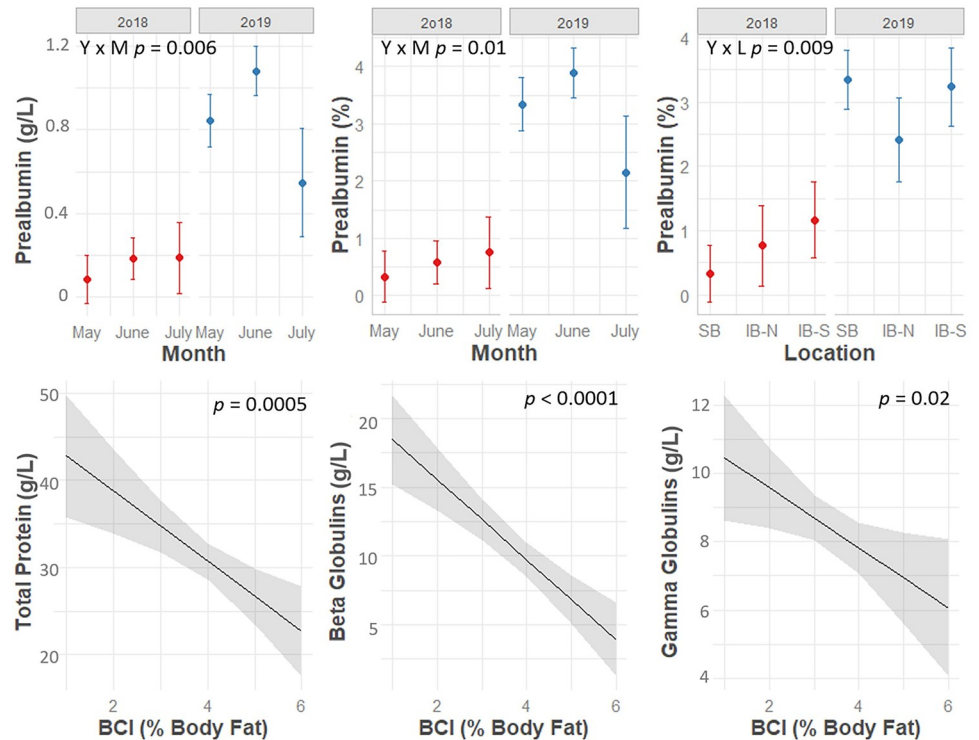**Fig 3. Model predictions for plasma protein electrophoresis values based on year, location, month, and BCI.** Model predictions with 95% confidence intervals for plasma protein electrophoresis values in free-living Blanding's turtles (*Emydoidea blandingii*) based on year, location, month, and body condition index (BCI). Model estimates were produced by top-ranking general linear mixed models (see Table 2).
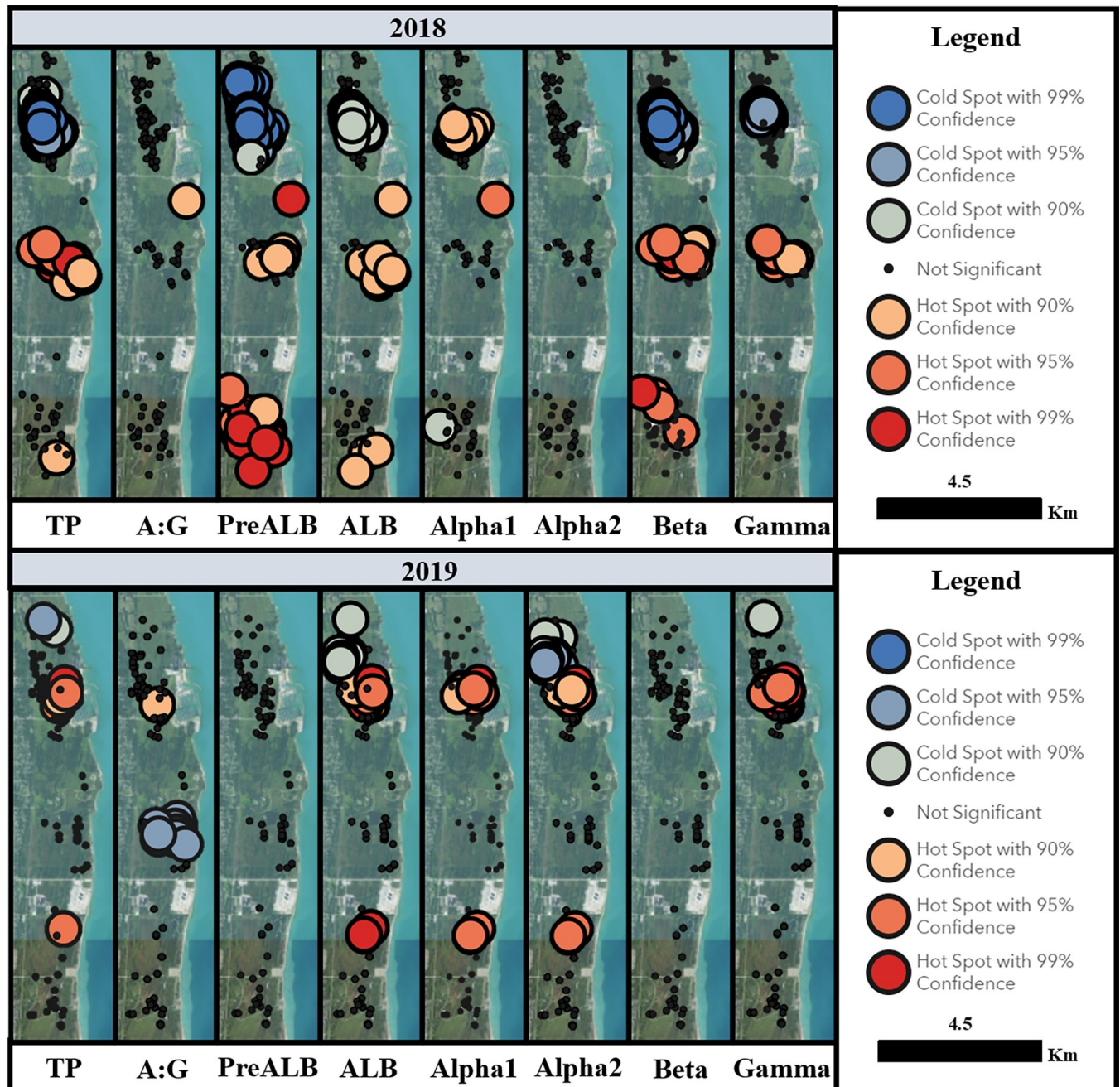
Fig 4. 2018 and 2019 spatial clusters of plasma protein electrophoresis values in free-living Blanding's turtles (*Emydoidea blandingii*). Study site map displaying hot and cold spots for each analyte in 2018 and 2019. Hot spots are areas with higher plasma protein concentrations, while cold spots are areas with lower plasma protein concentrations. SBCP, the managed site, is the northernmost territory on the map followed by the North unit of IBSP as the central territory and the South unit of IBSP as the southernmost territory. Evaluated using Gettis-Ord Gi* models. Map obtained from USGS National Map Viewer. TP = total protein (g/L), PreALB = prealbumin (g/L), ALB = albumin (g/L), Alpha1 = alpha 1 globulins (g/L), Alpha2 = alpha 2 globulins (g/L), Beta = beta globulins (g/L), Gamma = gamma globulins (g/L), A:G = albumin / globulin.

https://doi.org/10.1371/journal.pone.0258397.g004

between years, and differences occurred both between and within study sites, especially in turtles sampled at SBCP in 2019 (Fig 4).

A:G (p < 0.01), absolute albumin (p = 0.01), relative albumin (p < 0.01), and relative gamma globulins (p = 0.02) differed by month, while the effects of month on absolute and

Table 1. Protein electrophoresis values that vary by month and the presence of plastron abnormalities in free-living Blanding's turtles (*Emydoidea blandingii*). Model estimates were produced by top-ranking general linear mixed models (see Table 2).

Analyte	Level	Model Estimate	SE	Contrast	Difference	95% CI	P—value
Month							
Albumin/Globulin	May	0.27	0.01	May vs. June	-0.04	-0.06, -0.02	< 0.01
	June	0.31	0.01	June vs. July	0.04	0.01, 0.06	0.01
	July	0.27	0.01	May vs. July	-0.01	-0.03, 0.02	1.00
Albumin (g/L)	May	6.00	0.40	May vs. June	-1.40	-2.20, -0.60	0.01
	June	7.40	0.40	June vs. July	1.33	0.20, 2.30	0.01
	July	6.10	0.50	May vs. July	-0.10	-1.10, 0.90	1.00
Albumin (%)	May	19.00	0.64	May vs. June	-2.10	-3.30, -0.95	0.01
	June	21.10	0.44	June vs. July	1.80	0.33, 3.20	0.01
	July	19.40	0.68	May vs. July	-0.36	-1.70, 0.98	0.80
Gamma Globulins (%)	May	17.20	0.41	May vs. June	1.31	0.20, 2.40	0.02
	June	15.90	0.39	June vs. July	-0.81	-2.30, 0.70	0.40
	July	16.70	0.58	May vs. July	0.51	-0.97, 2.00	0.70
Plastron							
Beta Globulins (g/L)	Normal	11.30	0.70	N vs. E	-1.60	-2.90, -0.40	0.01
	Erosions	12.90	0.70	E vs. I	-2.10	-4.90, 0.70	0.15
	Injury	15.00	1.40	N vs. I	-3.70	-6.60, -0.90	0.01
Beta Globulins (%)	Normal	35.60	0.80	N vs. E	-0.27	-1.64, 1.10	0.70
	Erosions	35.90	0.90	E vs. I	-3.33	-6.40, -0.28	0.03
	Injury	39.20	1.50	N vs. I	-3.60	-6.60, -0.61	0.02

SE = standard error. N = normal. E = erosions. I = injury.

relative prealbumin concentration depended on year (significant Month*Year interaction) (Fig 2, Tables 1 and 2). Specifically, relative gamma globulins were significantly higher in May compared to June while A:G, absolute albumin, and relative albumin were significantly higher

Table 2. Top models for predicting protein electrophoresis values in free-living Blanding's turtles (*Emydoidea blandingii*) based on Akaike's information criterion, corrected for sample size (AIC$_c$).

Analyte	Model	N	K	AIC$_c$	w$_i$
Total Protein (g/L)	Y * L + BCI + Integ + App	265	11	748.00	0.98
Albumin / Globulin	Y * L + M + Sex + Age + Cloaca	279	15	-825.40	0.98
Prealbumin (g/L)	Y * L + Y * M + URD	279	13	-995.90	0.80
Prealbumin (%)	Y * L + Y * M + URD	279	13	-1534.60	0.69
Albumin (g/L)	Y * L + M + App + Gravid	278	12	-7.70	0.57
Albumin (%)	Y + M + Age + Sex + Gravid	279	11	-1154.10	0.94
Alpha 1 Globulins (g/L)	Y + Age + Carapace	279	10	-423.10	0.58
Alpha 1 Globulins (%)	Y + Age + Carapace	279	10	-1232.30	0.80
Alpha 2 Globulins (g/L)	Y + L + Age	283	8	-157.30	0.76
Alpha 2 Globulins (%)	Sex + Age + Gravid	280	8	-1207.10	0.95
Beta Globulins (g/L)	Y + L + Sex + BCI + Integ + Plastron + App	265	15	307.80	0.99
Beta Globulins (%)	Y + Age + Sex + Plastron + Gravid	280	13	-891.00	0.99
Gamma Globulins (g/L)	Y * L + Age + BCI + App	266	12	-34.80	0.99
Gamma Globulins (%)	Y + L + M + Age	283	10	-1129.80	0.86

Y = year, L = location, M = month, BCI = body condition index, Integ = integument, App = appendages, URD = upper respiratory disease.

Table 3. Protein electrophoresis values that vary by age class in free-living Blanding's turtles (*Emydoidea blandingii*). Model estimates were produced by top-ranking general linear mixed models (see Table 2).

Analyte	Level	Model Estimate	SE	Contrast	Difference	95% CI	P—value
Albumin/Globulin	Adult	0.25	0.01	Ad vs. Juv	-0.07	-0.11, -0.04	< 0.01
	Sub-Adult	0.28	0.01	Ad vs. SA	-0.04	-0.06, -0.01	0.01
	Juvenile	0.32	0.02	SA vs. J	-0.04	-0.07, -0.01	0.03
Albumin (%)	Adult	17.90	0.41	Ad vs. Juv	-3.80	-5.80, -1.80	<0.01
	Sub-Adult	19.80	0.63	Ad vs. SA	-1.80	-3.10, -0.56	0.01
	Juvenile	21.80	0.87	SA vs. J	-2.00	-4.00, 0.02	0.05
Alpha 1 Globulins (g/L)	Adult	1.60	0.10	Ad vs. Juv	-2.40	-2.90, -1.80	< 0.01
	Sub-Adult	2.70	0.10	Ad vs. SA	-1.00	-1.40, -0.70	< 0.01
	Juvenile	4.00	0.20	SA vs. J	-1.30	-1.90, -0.80	< 0.01
Alpha 1 Globulins (%)	Adult	5.20	0.30	Ad vs. Juv	-9.30	-10.60, -8.00	< 0.01
	Sub-Adult	8.70	0.40	Ad vs. SA	-3.50	-4.40, -2.60	< 0.01
	Juvenile	14.50	0.60	SA vs. J	-5.80	-7.20, -4.40	< 0.01
Alpha 2 Globulins (g/L)	Adult	5.10	0.20	Ad vs. Juv	0.10	-0.70, 0.90	1.00
	Sub-Adult	5.80	0.30	Ad vs. SA	-0.70	-1.30, -0.20	0.01
	Juvenile	5.00	0.40	SA vs. J	0.80	0.04, 1.70	0.04
Alpha 2 Globulins (%)	Adult	14.30	0.37	Ad vs. Juv	-1.10	-2.90, 0.60	0.30
	Sub-Adult	16.40	0.50	Ad vs. SA	-2.10	-3.20, -1.00	< 0.01
	Juvenile	15.50	0.78	SA vs. J	1.00	-0.90, 2.80	0.40
Beta Globulins (%)	Adult	41.70	0.79	Ad vs. Juv	9.61	6.40, -12.83	< 0.01
	Sub-Adult	34.40	0.97	Ad vs. SA	7.28	5.40, -9.16	< 0.01
	Juvenile	32.10	1.50	SA vs. J	2.33	-0.98, 5.65	0.20
Gamma Globulins (g/L)	Adult	7.00	0.30	Ad vs. Juv	2.00	0.60, 3.40	0.01
	Sub-Adult	6.60	0.40	Ad vs. SA	0.30	-0.80, 1.40	0.85
	Juvenile	5.00	0.50	SA vs. J	1.70	0.60–3.00	0.01
Gamma Globulins (%)	Adult	19.00	0.31	Ad vs. Juv	6.00	4.37, 7.63	< 0.01
	Sub-Adult	18.00	0.45	Ad vs. SA	0.98	-0.22, 2.20	0.13
	Juvenile	13.00	0.65	SA vs. J	5.00	3.36, 6.68	< 0.01

SE = standard error. Ad = adult. SA = sub-adult. Juv = juvenile.

in June compared to both May and July. Relative and absolute prealbumin followed a similar trend to A:G and albumin in 2019, but values were not significantly different by month in 2018.

Age class influenced A:G and all relative EPH fractions except prealbumin; it was also found to be an important predictor of absolute alpha 1 globulins, alpha 2 globulins, and gamma globulins (Table 3). Juvenile turtles had the highest values for A:G, relative albumin, relative alpha 1 globulins, and absolute alpha 1 globulins. Subadults had the highest values for relative and absolute alpha 2 globulins. Adults had the highest values for relative beta globulins, relative gamma globulins, and absolute gamma globulins.

Sex influenced the A:G, relative albumin, relative alpha 2 globulins, relative beta globulins, and absolute beta globulins (Table 4). Specifically, male turtles had higher A:G, relative albumin, and relative alpha 2 globulins, while females had higher absolute and relative beta globulin values. Gravid females had higher relative beta globulins and lower A:G, absolute and relative albumin, and alpha 2 globulins.

Multiple EPH fractions were associated with health predictors. Body condition was negatively associated with TP, absolute beta globulins, and absolute gamma globulins (Fig 2).

Table 4. Protein electrophoresis values that vary by sex and gravidity in free-living Blanding's turtles (*Emydoidea blandingii*). Model estimates were produced by top-ranking general linear mixed models (see Table 2).

Predictor	Analyte	Level	Model Estimate	SE	Difference	95% CI	P—value
Sex	**Albumin (%)**	Male	20.40	0.60	1.21	0.31–2.11	0.01
		Female	19.20	0.50			
	Albumin/Globulin	Male	0.30	0.01	0.02	0.01–0.04	0.01
		Female	0.27	0.01			
	Alpha 2 Globulins (%)	Male	16.20	0.50	1.60	0.72–2.40	0.01
		Female	14.60	0.43			
	Beta Globulins (g/L)	Male	12.30	0.90	1.80	0.50–3.20	0.01
		Female	14.10	0.80			
	Beta Globulins (%)	Male	34.50	0.97	3.04	1.56–4.53	< 0.01
		Female	37.60	0.92			
Gravidity	**Albumin/Globulin**[a]	Non-Gravid	0.31	0.01	0.03	0.02–0.06	0.04
		Gravid	0.27	0.02			
	Albumin (g/L)	Non-Gravid	7.10	0.30	1.20	0.03–2.30	0.04
		Gravid	6.00	0.60			
	Albumin (%)	Non-Gravid	21.00	0.32	2.42	0.88–3.95	0.01
		Gravid	18.60	0.84			
	Alpha 2 Globulins (%)	Non-Gravid	16.40	0.28	1.90	0.63–3.24	0.01
		Gravid	14.40	0.70			
	Beta Globulins (%)	Non-Gravid	34.40	0.73	3.40	1.07–5.71	0.01
		Gravid	37.80	1.30			

SE = standard error.

[a] Gravidity was not included in the top-ranking model for Albumin/Globulin due to confounding with the "cloaca" variable, but considered separately it is significantly associated with Albumin/Globulin.

Turtles with predator injuries or erosions of the plastron had higher relative and absolute beta globulins than those with normal plastrons (Table 1). Abnormalities of the appendicular system were associated with higher TP and absolute albumin, beta globulins, and gamma globulins (Table 5). Integumentary abnormalities were associated with lower TP and absolute beta globulins (Table 5). Turtles with upper respiratory disease had lower absolute and relative prealbumin than those without (Table 5). Finally, cloacal abnormalities were associated with a lower A:G (Table 5). Top models for each EPH fraction tended to include spatiotemporal, demographic, and health predictors, highlighting the influence that each of these components has on the distribution of blood proteins in Blanding's turtles (Table 2).

Reference intervals were constructed in both a subject-based and population-based manner for each EPH analyte (Table 6). Based on the index of individuality, subject-based reference intervals are superior for absolute and relative alpha 1 globulins, absolute beta globulins, and relative albumin in Blanding's turtles. The remainder of the II values fell between 0.6 and 1.4, indicating that population-based reference intervals were employed and interpreted with caution for all other fractions.

Discussion

We set out to describe baseline plasma protein fractions in a well-studied population of Blanding's turtles in northeastern Illinois and southeastern Wisconsin and observed EPH fractions varied significantly based on spatiotemporal, demographic, and health factors. Understanding

Table 5. Protein electrophoresis values that vary based on the presence of physical examination abnormalities in free-living Blanding's turtles (*Emydoidea blandingii*). Model estimates were produced by top-ranking general linear mixed models (see Table 2). Top models for relative and absolute alpha 1 globulins contained "carapace", however no contrasts for this predictor were significant therefore not reported.

Predictor	Analyte	Level	Model Estimate	SE	Difference	95% CI	P—value
Appendages	**Total Protein (g/L)**	Normal	31.90	1.10	5.30	1.90–8.06	0.01
		Abnormal	37.10	1.80			
	Albumin (g/L)	Normal	6.10	0.30	9.00	0.10–1.70	0.03
		Abnormal	7.00	0.50			
	Beta Globulins (g/L)	Normal	12.10	0.70	2.20	0.50–3.80	0.01
		Abnormal	14.20	1.00			
	Gamma Globulins (g/L)	Normal	5.50	0.30	1.40	0.60–2.10	0.01
		Abnormal	6.80	0.40			
Integument	**Total Protein (g/L)**	Normal	31.90	1.10	4.60	0.70–8.50	0.02
		Abnormal	27.20	2.00			
	Beta Globulins (g/L)	Normal	14.10	0.70	2.00	0.20–3.80	0.03
		Abnormal	12.20	1.10			
Upper Respiratory Disease	**Prealbumin (g/L)**	Absent	0.58	0.03	0.27	0.03–0.52	0.03
		Present	0.31	0.12			
	Prealbumin (%)	Absent	1.88	0.13	1.04	0.11–1.97	0.03
		Present	0.84	0.48			
Cloaca	**Albumin/Globulin**	Normal	0.31	0.01	0.04	0.01–0.07	0.01
		Abnormal	0.26	0.02			

SE = standard error.

how reptile clinical pathology values correlate to landscape changes is important for contextualizing health assessments and evaluating ecosystem wellness [1, 2, 5, 6].

We documented several statistically significant inter-annual differences in Blanding's turtle total protein and EPH fractions. These changes may be attributable to fluctuation in climactic variables that influence turtle metabolism and resource availability. Temperature is a key determinant of metabolic rates in ectotherms, including reptiles [41, 42], and previous studies in loggerheads (*Caretta caretta*) and green turtles (*Chelonia mydas*) observed a negative correlation with the A:G and environmental temperature [16]. In Lake County, the average air temperatures in May (60.4˚F/15.8˚C) and June (66.4˚F/19.1˚C) of 2018 were warmer than those in May (54.7˚F/12.6˚C) and June (64.2˚F/17.9˚C) of 2019 [43]. Similar to the temperature-associated protein changes in sea turtles, Blanding's turtle TP, albumin, alpha 1 and alpha 2 globulins, and gamma globulin concentrations were greater in 2018, while prealbumin, beta globulin, and A:G were greater in 2019. It is plausible that turtles would be more active, consume more food items, initiate reproductive activity, and mount immune responses more efficiently at higher temperatures, all of which may increase circulating protein concentrations and contribute to the observed inter-annual variability in EPH fractions [41].

Temperature, however, is not the only environmental factor that differed between years. Rainfall was also greater during May and June of 2018 (30.2 inches total) compared to the same time period in 2019 (18.76 inches total), while humidity was a bit more consistent between years (77.2% average relative humidity in May and June of 2018 and 80.75% average relative humidity in May and June of 2019) [43]. Water availability and humidity both influence behavioral thermoregulation in ectotherms and can modify their activity levels in complex ways [44]. Increased activity secondary to rainfall may create more opportunities for antigenic stimulation and contribute to changes in food consumption, reproductive behaviors,

Table 6. Summary data including data distribution, measure of central tendency (mean for normally distributed variables, median for non-normally distributed variables), measure of dispersion (standard deviation for normally distributed variables, 10th– 90th percentiles for non-normally distributed variables), and reference intervals for plasma protein electrophoresis in free-living, apparently healthy Blanding's turtles (*Emydoidea blandingii*).

Analyte	N	Dist	CT	Disp	Min	Max	Reference Interval	90% CI LB	90% CI UB	II	RCV (%)	Sub vs. Pop
Total Protein (g/L)	193[a]	NG	31.00	18.00–47.50	13.00	56.00	15.70–52.30	15.40–17.60	48.60–53.60	0.68	62.70	Pop
Albumin/Globulin	195[b]	G	0.29	0.07	0.14	0.46	0.17–0.43	0.16–0.19	0.40–0.46	0.66	41.20	Pop
Prealbumin (g/L)	196	NG	0.40	0.05–1.20	0.00	2.30	0.00–2.00	0.00–0.10	1.80–2.30	0.90	237.00	Pop
Albumin (g/L)	196	NG	6.30	3.40–9.90	1.90	15.60	2.50–13.40	2.10–3.00	12.90–15.00	0.66	74.40	Pop
Alpha 1 Globulins (g/L)	196	NG	1.90	1.00–4.30	0.70	11.60	0.80–5.70	0.70–0.80	4.10–6.10	0.54	88.40	Sub
Alpha 2 Globulins (g/L)	196	NG	4.70	2.70–7.40	1.70	11.20	2.10–10.00	1.90–2.50	9.60–10.70	0.67	70.80	Pop
Beta Globulins (g/L)	196	NG	10.80	5.04–17.90	3.40	30.00	4.00–25.70	3.30–4.40	21.80–29.90	0.56	66.70	Sub
Gamma Globulins (g/L)	195[c]	NG	5.60	2.80–10.70	1.40	16.50	1.70–14.10	1.30–1.90	12.30–15.70	0.99	144.00	Pop
Prealbumin (%)	196	NG	1.00	0.00–4.20	0.00	10.10	0.00–6.70	0.00–1.00	4.90–7.80	0.93	266.00	Pop
Albumin (%)	195[d]	G	20.30	3.80	13.00	31.00	14.00–29.00	13.00–15.00	27.00–31.00	0.59	29.90	Sub
Alpha 1 Globulins (%)	196	NG	6.00	4.00–14.00	2.70	28.00	3.00–23.00	2.90–3.30	19.00–28.90	0.34	51.80	Sub
Alpha 2 Globulins (%)	195[e]	NG	16.00	11.00–19.70	9.00	24.00	10.00–21.70	9.70–11.00	20.50–23.30	0.62	31.20	Pop
Beta Globulins (%)	196	NG	35.80	25.00–49.10	18.00	59.10	20.00–55.20	19.00–21.00	51.90–57.70	0.82	59.20	Pop
Gamma Globulins (%)	196	NG	19.00	11.30–28.00	8.00	37.00	9.700–32.10	9.40–10.00	31.20–34.10	1.20	114.00	Pop

Dist = distribution, NG = Non-Gaussian, G = Gaussian, CT = measure of central tendency, Disp = measure of dispersion, CI = confidence interval, LB = lower bound of reference interval, UB = upper bound of reference interval, II = index of individuality, RCV = reference change value, Sub = subject-based refence interval recommended, Pop = population-based reference interval recommended.

[a] Outliers removed: 10.00, 71.00, 74.00 g/L.

[b] Outliers removed: 0.51.

[c] Outliers removed: 22.6 g/L.

[d] Outliers removed: 11%.

[e] Outliers removed: 26%.

and other physiologic processes with a resultant increase in plasma protein concentrations [44]. While the effects of some climactic variables on ectotherm physiology have been at least partially characterized, many other environmental variables may also impact resource availability and overall wellness. Additional research is needed to determine the underlying environmental causes of temporal variability in reptile protein electrophoresis values. It is likely that inter-annual differences in protein electrophoresis values also exist for other reptiles [45]. Unfortunately, direct comparison to existing literature is difficult, because although some multi-year chelonian EPH studies exist [4, 10, 16, 18, 46–52], the possibility of inter-annual variation in EPH values is infrequently assessed. Our findings indicate that future EPH studies in reptiles should consider the potential for significant inter-annual effects.

TP and absolute EPH fractions differed by location in Blanding's turtles, similar to eastern box turtles (*Terrapene carolina carolina*) [4], alligator snapping turtles (*Macrochelys temminckii*) [46] and green turtles [45]. SBCP and IBSP vary significantly in their habitats despite their close proximity. SBCP offers a coastal dune and swale habitat that has been managed and preserved since 2004, whereas IBSP includes adjacent north (N) and south (S) units containing dune, prairie, oak-savannah and wetland habitats, with limited management prior to 2017 [22]. The effect of location on EPH parameters depended on the year; and the differences in EPH values between the study sites and years were rarely consistent. The only consistent location-related differences between 2018 and 2019 were the cold spots at the northern end of SBCP, potentially indicating a more consistent health status for turtles occupying this area. Inter-annual fluctuation in TP and EPH fractions between and within the different study sites

may indicate transient, localized changes in health status with unclear management implications. Focused, longitudinal assessment of telemetered individuals within these areas will be useful to identify biotic and abiotic factors associated with acute changes in EPH parameters. Our findings indicate that it is important to consider both location and time at multiple different scales in order to obtain a nuanced understanding of the drivers of turtle health status.

Several Blanding's turtle plasma protein values were affected by month; specifically, A:G, absolute albumin, relative albumin, and both absolute and relative prealbumin values peaked in June, while relative gamma globulins were highest in May. Similar patterns have been documented in other chelonians including Hermann's tortoises (*Testudo hermanni*) [53], alligator snapping turtles [46], and eastern box turtles [4]. These changes may be attributed to increased interactions with other turtles during the mating season and/or reproductive physiology. In the Lake Plain, Blanding's turtle mating season occurs from March to May, while nesting season typically begins in June. Turtles are more likely to interact with each other and potentially transmit pathogens such as *Emydoidea* herpesvirus 1 during mating season [54], which may contribute to an increase in gamma globulins (i.e. immunoglobulins). Alternatively, the increased gamma globulin concentration in May could be secondary to vitellogenesis, as the release of estrogen stimulates hyperglobulinemia in chelonians [55, 56]. Elevated albumin concentrations in June may be attributable to dehydration associated with prolonged overland trips and nesting-associated exertion, similar to previous reports in sea turtle species [48, 57–59]. Studies in other chelonian and lizard species have documented elevations in albumin and total proteins during the summer months and have correlated these elevations to the increased food consumption and reproductive activity in this time period [4, 60, 61].

Gravid females had higher relative beta globulins and lower A:G, absolute and relative albumin, and alpha 2 globulins. As reviewed above, gravid reptiles can develop hyperglobulinemia during vitellogenesis in response to estrogen [62]. A study conducted in pond sliders (*Trachemys scripta*) demonstrated that estrogen also downregulates albumin, which may be a factor in the lower albumin concentrations observed in gravid female Blanding's turtles [63]. Higher globulins and lower albumin secondary to estrogen production would also support the lower A:G in gravid females. In birds, elevated beta globulins are attributed to egg production [64]. In leatherbacks, alpha 2 globulins decrease over the nesting season due to inanition [65]. Our findings are important to provide context for future studies on EPH in gravid chelonians, as several of the changes associated with gravidity (elevated beta globulins, lower albumin and A:G) can also be interpreted as indicative of inflammation and poor health [17].

Male turtles had higher A:G, relative albumin, and relative alpha 2 globulins while females had higher absolute and relative beta globulin values. Many of these findings differ from those in other chelonians. Relative albumin was higher in female red-eared sliders (*Trachemys scripta elegans*) and map turtles (*Graptemys geographica*) [66], and absolute albumin was higher in female eastern box turtles [4]. Male loggerhead sea turtles had higher absolute and relative beta globulin concentrations, although there was a great deal of beta-gamma bridging indicating possible underlying disease processes in those individuals [10]. Male radiated tortoises had higher relative alpha 2 globulins during winter sampling, and female eastern box turtles and radiated tortoises had higher absolute and relative beta globulin concentrations [4, 67]. Consistent with a previous study in this population of Blanding's, but contrary to several other studies in chelonians, there was no difference in total protein between the sexes [24]. Many of these findings may be confounded by the timing of our sampling, since it was concentrated during the breeding and nesting season. It is possible that if these turtles were sampled later in the year we would find that sex-based differences in Blanding's turtles are more in line with what is reported for other species. Blanding's turtles have some unique sex-associated

EPH patterns compared to other chelonians, underscoring the need for studies like this to understand species differences in clinical pathology values.

Protein fraction concentrations varied across age groups, with juvenile turtles having the highest values for A:G, relative albumin, relative alpha 1 globulins, and absolute alpha 1 globulins. Similar trends are seen in juvenile loggerheads [16], juvenile Kemp's ridley sea turtles (*Lepidochelys kempii*) [68, 69], and juvenile gopher tortoises (*Gopherus polyphemus*) [70]. Adults had the highest values for relative beta globulins, relative gamma globulins, and absolute gamma globulins, which are similar to findings in other chelonians. Adult loggerheads [10], eastern box [4], and green turtles [49] all had higher absolute beta globulin levels, and adult Kemp's ridley sea turtles [69] had higher absolute beta and gamma globulins compared to their juvenile counterparts. In general, the changes found can be attributed to increased antigenic challenge as turtles age and become reproductively mature, a consistent finding in other chelonian studies [16, 19, 46].

The overall health status of individuals also contributed to variations in plasma protein concentrations. Higher absolute beta and gamma globulins were found in turtles with lower body fat percentage, which both increase in the presence of acute and chronic inflammation. BCS is a reliable measure of health status in other reptiles, with a good body condition score equating to better immune function and capability to fight periodic bouts of disease [71]. The elevated relative and absolute beta globulins in turtles with plastron injuries and erosions might be due to the plastron constantly being in contact with either unclean water or the ground, increasing the potential for chronic antigenic stimulation when injuries or abnormalities are present.

Appendage abnormalities were associated with higher TP and absolute beta and gamma globulins, indicating the presence of possible chronic inflammation. The loss of nails or digits from a variety of causes results in open wounds where infections may develop. Missing nails and digits might also have an impact on the turtle's overall health, making tasks like swimming and foraging more difficult [72]. In eastern box turtles, microvascular problems and primary microbial infections can cause the loss of digits and nails, indicating that even apparently minor anatomical abnormalities may have physiologically significant impacts on these turtles [73]. Integument abnormalities were associated with lower TP and absolute beta globulin concentrations. A study conducted in green and loggerhead sea turtles with traumatic wounds to their carapace, head, and/or flippers showed similar trends, with those experiencing trauma having lower beta globulin concentrations than their healthy counterparts [16].

Turtles with evidence of upper respiratory disease had lower relative prealbumin concentrations. Like albumin, prealbumin is a negative acute phase protein and decreases in the presence of inflammation [74]. Prealbumin concentrations can also be lower in cases of protein malnutrition [74]. These turtles may have lower concentrations because they are ill with upper respiratory infection, and this illness could be preventing them from taking in an adequate amount of protein. It is important to note, however, that prealbumin has not yet been validated in chelonian species, so it is unclear if prealbumin is truly what is represented in the prealbumin region of the electrophoretogram. The lower A:G in turtles with cloacal abnormalities likely consistent with inflammation associated with infection, inflammation, or stress, with a lower ratio usually indicating hyperglobulinemia [75].

The population of Blanding's turtles that was studied showed a high degree of within-individual variability in EPH parameters at different points in time. This was also identified for hematologic and plasma biochemical parameters in the same population [24]. Reptile clinical pathology parameters are widely variable, and it is important to understand the many factors come together to influence the absolute value of each analyte. Our findings in Blanding's turtles indicate that the index of individuality and subsequent need for subject-based reference

intervals should be investigated in other reptile species in order to improve the interpretation of clinical pathology testing.

There are a few limitations in this study that could be addressed in future research. Due to radiotelemetry strategy in the Lake County location, there was a bias towards adult female turtles, which are followed closely to identify nest location. Additionally, a limited number of overtly unhealthy turtles were identified, with only a few individuals having physical examination abnormalities. While this is recognized as a positive finding considering that it indicates the population is doing well, it does limit the ability to determine how EPH values change in states of poor health. Furthermore, turtles that could be identified more than once over the course of the sampling period were sampled every three to six weeks, which could indicate the fluctuation of an inflammatory response or antigenic stimulation over time.

All blood samples were obtained from the subcarapacial sinus. Blood and lymphatic vessels are very closely associated with one another at this site, making it possible that blood samples could become contaminated with lymphatic fluid during venipuncture [76]. While samples with obvious lymph contamination were discarded, undetectable lymph contamination could have negatively impacted results. Lymph contamination has been known to falsely decrease PCV and hemoglobin concentrations and may have similar affects on plasma protein concentrations [77]. For those turtles that were collect and sampled from traps, the stress of trapping could ultimately play a role in affecting plasma protein fractions [50], but the significance has not been studied in Blanding's turtles. Timing samples to be collected pre- or post-prandial to account for lipemia is difficult to control in wildlife research, but a study conducted in Kemp's ridley and green sea turtles revealed that feeding had very minimal effects on plasma biochemical values and are therefore unlikely to alter clinical interpretation [78].

There is a discrepancy between the use of TS verses total protein (TP). A refractometer is an efficient way to measure TS in a field research setting, but TS includes both plasma proteins as well as additional plasma solutes [79]. While there have been multiple studies conducted in other chelonian species that show a significant correlation between TS and TP [19, 80], a direct relationship in Blanding's turtles has not been previously identified. Following separation, the collected plasma was frozen until protein electrophoresis could be run. Studies conducted in other reptile species have identified EPH differences in fresh plasma compared to frozen/thawed samples, and some of those studies recommended using fresh samples for best results [13, 81]. In our circumstances, it would have been impractical and cost-prohibitive to ship over 200 fresh plasma samples for analysis separately, underscoring the need for us to freeze and batch-run our samples. Hemolysis also has the potential to affect EPH values, but no grossly hemolyzed samples were use in this study [82].

The baseline plasma protein reference intervals generated in this study will be useful in defining the health status of this population. There is some overlap in the top models for predicting relative and absolute EPH fractions; however there also instances where the values vary, with absolute values being high while relative values are low for the same variable. This variation demonstrates the importance of considering both relative and absolute fractions when interpreting EPH values because they might be driven by different processes. Results of this study validate that month, location, sex, age class, and health status should be considered when interpreting EPH fractions. Although EPH does not provide details on specific diseases or stressors, it is a helpful tool that can aid in identifying when intervention and treatment might be needed. With the increased use of protein electrophoresis to evaluate the health status of animals in the veterinary medical field, the application of this tool in conservation of wild populations is becoming more widely accepted and studied. With baseline concentrations established and evaluated for variation, future studies can aim to validate and expand upon the normal reference intervals in this species.

Supporting information

S1 File. Combined data. All project data collected upon turtle capture, which includes identifying information, temporal data, capture method, weather data, measurements, physical exam findings, and release information.
(XLSX)

Author Contributions

Conceptualization: Kirsten E. Andersson, Laura Adamovicz, Gary Glowacki, Matthew C. Allender.

Data curation: Kirsten E. Andersson, Laura Adamovicz, Lauren E. Mumm, Samantha E. Bradley, Gary Glowacki, Matthew C. Allender.

Formal analysis: Laura Adamovicz, Matthew C. Allender.

Funding acquisition: Gary Glowacki.

Investigation: Kirsten E. Andersson, Laura Adamovicz, Lauren E. Mumm, Samantha E. Bradley, John M. Winter, Carolyn Cray, Matthew C. Allender.

Methodology: Kirsten E. Andersson, Laura Adamovicz, Lauren E. Mumm, Carolyn Cray, Matthew C. Allender.

Project administration: Kirsten E. Andersson, Laura Adamovicz, Lauren E. Mumm, Samantha E. Bradley, Matthew C. Allender.

Resources: Laura Adamovicz, Carolyn Cray, Matthew C. Allender.

Software: Laura Adamovicz, Matthew C. Allender.

Supervision: Kirsten E. Andersson, Laura Adamovicz, Matthew C. Allender.

Validation: Laura Adamovicz, Matthew C. Allender.

Visualization: Laura Adamovicz, Matthew C. Allender.

Writing – original draft: Kirsten E. Andersson, Laura Adamovicz.

Writing – review & editing: Kirsten E. Andersson, Laura Adamovicz, Lauren E. Mumm, Samantha E. Bradley, Gary Glowacki, Carolyn Cray, Matthew C. Allender.

References

1. Deem SL, Karesh WB, Weisman W. Putting theory into practice: Wildlife health in conservation. Conserv Biol. 2008; 15(5): 1224–1233. https://doi.org/10.1111/j.1523-1739.2001.00336.x

2. Ryser-Degiorgis MP. Wildlife health investigations: needs, challenges and recommendations. BMC Vet Res. 2013; 9(1): 223. https://doi.org/10.1186/1746-6148-9-223 PMID: 24188616

3. Mörner T, Obendorf DL, Artois M, Woodford MH. Surveillance monitoring of wildlife diseases. Rev Sci Tech. 2002; 21(1): 67–76. https://doi.org/10.20506/rst.21.1.1321 PMID: 11974631

4. Flower JE, Byrd J, Cray C, Allender MC. Plasma electrophoretic profiles and hemoglobin binding protein reference intervals in the eastern box turtle (*Terrapene carolina carolina*) and influences of age, sex, season, and location. J Zoo Wildl Med. 2014; 45(4): 836–842. https://doi.org/10.1638/2014-0035.1 PMID: 25632671

5. Aguirre AA, Lutz PL. Marine turtles as sentinels of ecosystem health: Is fibropapillomatosis an indicator? Ecohealth. 2004; 1: 275–283. https://doi.org/10.1007/s10393-004-0097-3

6. Page-Karijian A, Perrault JR. Sea turtle health assessments: Maximizing turtle encounters to better understand health. In: Nahill B, editor. Sea turtle research and conservation: Lessons from Working in the Field. Elsevier, Academic Press; 2021. pp. 31–44.

7. Knotek Z, Musilova A, Pinterova K, Knotkova Z. Plasma protein electrophoresis as a diagnostic tool for endangered Asian reptiles. World Small Animal Veterinary Association World Congress Proceedings. 2015. Available from https://www.vin.com/apputil/content/defaultadv1.aspx?id=7259164&pid=14365&print=1

8. Allender MC, Junge RE, Baker-Wylie S, Hileman ET, Faust LJ, Cray C. Plasma electrophoretic profiles in the eastern massasauga (*Sistrurus catenatus*) and influences of age, sex, year, location, and snake fungal disease. J Zoo Wildl Med. 2015; 46(4): 767–773. https://doi.org/10.1638/2015-0034.1 PMID: 26667532

9. Cooper-Bailey K, Smith SA, Zimmerman K, Lane R, Raskin RE, Denardo D. Hematology, leukocyte cytochemical analysis, plasma biochemistry, and plasma electrophoresis of wild-caught and captive-bred Gila monsters (*Heloderma suspectum*). Vet Clin Pathol. 2011; 40(3): 316–323. https://doi.org/10.1111/j.1939-165X.2011.00337.x PMID: 21827515

10. Gicking JC, Foley AM, Harr KE, Raskin RE, Jacobson E. Plasma protein electrophoresis of the Atlantic loggerhead sea turtle, *Carretta carretta*. J Herpetol Med Surg. 2004; 14(3): 13–18. https://doi.org/10.5818/1529-9651.14.3.13

11. Giménez M, Saco Y, Pato R, Busquets A, Martorell JM, Bassols A. Plasma protein electrophoresis of *Trachemys scripta* and *Iguana iguana*. Vet Clin Pathol. 2010; 39(2): 227–235. https://doi.org/10.1111/j.1939-165X.2009.00204.x PMID: 20059755

12. Machado CC, Silva LFN, Ramos PRR, Takahira RK. (2006). Seasonal influence on hematologic values and hemoglobin electrophoresis in Brazilian *Boa constrictor amarali*. J Zoo Wildl Med. 2006; 37(4): 487–491. https://doi.org/10.1638/05-124.1 PMID: 17315433

13. Proverbio D, Bagnagatti De Giorgi G, Della Pepa A, Baggiani L, Spada E, Perego R, et al. Preliminary evaluation of total protein concentration and electrophoretic protein fractions in fresh and frozen serum from wild Horned Vipers (*Vipera ammodytes ammodytes*). Veterinary Clinical Pathology. 2012; 41(4): 582–586. https://doi.org/10.1111/j.1939-165x.2012.00486.x PMID: 23078521

14. Silva LFN, Riani-Costa CCM, Ramos PRR, Takahira RK. Seasonal influence on biochemical profile and serum protein electrophoresis for *Boa constrictor amarali* in captivity. Braz J Biol. 2011; 71(2): 517–520. https://doi.org/10.1590/s1519-69842011000300023 PMID: 21755171

15. Work TM, Rameyer RA, Balazs GH, Cray C, Chang SP. Immune status of free-ranging green turtles with fibropapillomatosis from Hawaii. J Wildl Dis. 2001; 37(3): 574–581. https://doi.org/10.7589/0090-3558-37.3.574 PMID: 11504232

16. Osborne AG, Jacobson ER, Bresette MJ, Singewald DA, Scarpino RA, Bolten AB. Reference intervals and relationships between health status, carapace length, body mass, and water temperature and concentrations of plasma total protein and protein electrophoretogram fractions in Atlantic loggerhead sea turtles and green turtles. J Am Vet Med Assoc. 2010; 237(5): 561–567. https://doi.org/10.2460/javma.237.5.561 PMID: 20807135

17. Cray C. Acute phase proteins in animals. Prog Mol Biol Transl Sci. 2012; 105: 113–150. https://doi.org/10.1016/B978-0-12-394596-9.00005-6 PMID: 22137431

18. Innis CJ, Tlusty M, Wunn D. Hematologic and plasma biochemical analysis of juvenile head-started northern red-bellied cooters (*Pseudemys rubriventris*). J Zoo Wildl Med. 2007; 38(3): 425–432. https://doi.org/10.1638/1042-7260(2007)38[425:HAPBAO]2.0.CO;2 PMID: 17939352

19. Rousselet E, Stacy NI, Lavictoire K, Higgins BM, Tocidlowski ME, Flanagan JP, et al. Hematology and plasma biochemistry analytes in five age groups of immature, captive-reared loggerhead sea turtles (*Caretta caretta*). J Zoo Wildl Med. 2013; 44(4): 859–874. https://doi.org/10.1638/2012-0162R1.1 PMID: 24450044

20. Congdon JD, Sels RC. Relationships of reproductive traits and body size with attainment of sexual maturity and age in Blanding's turtles (*Emydoidea blandingii*). J Evol Biol. 1993; 6(4): 547–557. https://doi.org/10.1046/j.1420-9101.1993.6040547.x

21. van Dijk PP, Rhodin AGJ. *Emydoidea blandingii*. The IUCN Red List of Threatened Species. 2011. Available from https://doi.org/https%3A//dx.doi.org/10.2305/IUCN.UK.2011-1.RLTS.T7709A155088836.en

22. Glowacki G. Blanding's Turtle recovery program: 2017 Summary Report. Lake County Forest Preserve District, Blanding's Turtle Recovery Program. 2017.

23. Kuhns AR. Recovery of the Blanding's turtle (Emydoidea blandingii) at Spring Bluff Nature Preserve, Lake Count Forest Preserves. Lake County 552 Forest Preserve District, Blanding's Turtle Recovery Program Federal Aid Project T-39-D-1. 2010. Available from: https://www2.illinois.gov/dnr/conservation/iwap/documents/swgreports/t-39%20d-1%20final%20recovery%20of%20the%20blandings%20turtle.pdf

24. Mumm LE, Winter JM, Andersson KE, Glowacki GA, Adamovicz LA, Allender MC. Hematology and plasma biochemistries in the Blanding's turtle (*Emydoidea blandingii*) in Lake County, Illinois. PloS One. 2019; 14(11). https://doi.org/10.1371/journal.pone.0225130 PMID: 31730637

25. Gutzke WH, Packard GC. The influence of temperature on eggs and hatchlings of Blanding's turtles, *Emydoidea blandingii*. J Herpetol. 1987; 21(2): 161. https://doi.org/10.2307/1564476

26. Ernst CH, Lovich JE. *Emydoidea blandingii*, Blanding's Turtle. In: Turtles of the United States and Canada. 2nd ed. Baltimore: The John Hopkins University Press; 2009. pp. 233–249.

27. Mosimann JE, Bider JR. Variation, sexual dimorphism, and maturity in a Quebec population of the common snapping turtle, *Chelydra serpentina*. Can J Zool. 1960; 38:19–38.

28. Newman E, Allender M, Thompson D, Glowacki G, Ivančić M, Adkesson M, et al. Measuring fat content using computed tomography to establish a body condition index in free-ranging Blanding's turtles (*Emydoidea blandingii*) in Illinois. J Zoo Wildl Med. 2019; 50(3): 594–603. https://doi.org/10.1638/2018-0154 PMID: 33517628

29. R Core Team. 2020. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

30. Bates D, Maechler M, Bolker B, Walker S. Fitting linear mixed-effects models using lme4. J Stat Softw. 2015; 67(1): 1–48. https://doi.org/10.18637/jss.v067.i01

31. Kuznetsova A, Brockhoff PB, Christensen RHB. lmerTest package: Tests in linear mixed effects models. J Stat Softw. 2017; 82(13): 1–26. https://doi.org/10.18637/jss.v082.i13

32. Lenth RV. Least-squares means: The R package lsmeans. J Stat Softw. 2016; 69(1): 1–33. https://doi.org/10.18637/jss.v069.i01

33. Fox J, Weisberg S. An {R} Companion to Applied Regression. Sage Publications, USA. 2011. Available from http://socserv.socsci.mcmaster.ca/jfox/Books/Companion.

34. Barton K. MuMIn: Multi-Model Inference. R package version 1.43.15. 2019. Available from https://CRAN.R-project.org/package=MuMIn.

35. Lüdecke D. ggeffects: Tidy data frames of marginal effects from regression models. J Open Source Softw. 2018; 3(26): 772. https://doi.org/10.21105/joss.00772

36. Friedrichs KR, Harr KE, Freeman KP, Szladovits B, Walton RM, Barnhart KF, et al. ASVCP reference interval guidelines: determination of de novo reference intervals in veterinary species and other related topics. Vet Clin Pathol. 2012; 41: 441–453. https://doi.org/10.1111/vcp.12006 PMID: 23240820

37. Walton RM. Subject-based reference values: biological variation, individuality, and reference change values. Vet Clin Pathol. 2012; 41:175–181. https://doi.org/10.1111/j.1939-165X.2012.00414.x PMID: 22390475

38. Harris EK. Effects of intra- and interindividual variation on the appropriate use of normal ranges. Clin Chem. 1974; 20:1535–1542. PMID: 4430131

39. Finnegan D. referenceIntervals: Reference Intervals. R package version 1.1.1. 2004. Available from https://CRAN.R-project.org/package=referenceIntervals.

40. Horn PS, Feng L, Li Y, Pesce AJ. Effect of outliers and nonhealthy individuals on reference interval estimation. Clin Chem. 2001; 47: 2137–2145. PMID: 11719478

41. Shine R. Life-history evolution in reptiles. Annu Rev Ecol Ecol Syst. 2005; 36(1): 23–46. https://doi.org/10.1146/annurev.ecolsys.36.102003.152631

42. Huey R, Slatkin M. 1976. Costs and benefits of lizard thermoregulation. Q. Rev. Biol. 51:363–84. https://doi.org/10.1086/409470 PMID: 981504

43. NOAA. National Centers for Environmental Information; 2020. US Local Climatological Data. NCEI DSI 3505. Available from https://www.ncdc.noaa.gov/cdo-web/datasets/LCD/stations/WBAN:04845/detail.

44. Rozen-Rechels D, Dupoué A, Lourdais O, Chamaillé-Jammes S, Meylan S, Clobert J, et al. When water interacts with temperature: Ecological and evolutionary implications of thermo-hydroregulation in terrestrial ectotherms. Ecol Evol. 2019; 9(17): 10029–10043. https://doi.org/10.1002/ece3.5440 PMID: 31534711

45. Flint M, Brand A, Bell IP, Hof CA. Monitoring the health of green turtles in northern Queensland post catastrophic events. Sci Total Environ. 2019; 660: 586–592. https://doi.org/10.1016/j.scitotenv.2019.01.065 PMID: 30641386

46. Chaffin K, Norton TM, Gilardi K, Poppenga R, Jensen JB, Moler P, et al. Health assessment of free-ranging alligator snapping turtles (*Macrochelys temminckii*) in Georgia and Florida. J Wildl Dis. 2008; 44(3): 670–686. https://doi.org/10.7589/0090-3558-44.3.670 PMID: 18689653

47. Deem SL, Dierenfeld ES, Sounguet GP, Alleman AR, Cray C, Poppenga RH, et al. Blood values in free-ranging nesting leatherback sea turtles (*Dermochelys coriacea*) on the coast of the Republic of Gabon. J Zoo Wildl Med. 2006; 37(4): 464–471. https://doi.org/10.1638/05-102.1 PMID: 17315430

48. Deem SL, Norton TM, Mitchell M, Segars A, Alleman AR, Cray C, et al. Comparison of blood values in foraging, nesting, and stranded loggerhead turtles (*Caretta caretta*) along the coast of Georgia, USA. J Wildl Dis. 2009; 45(1): 41–56. https://doi.org/10.7589/0090-3558-45.1.41 PMID: 19204334

49. Flint M, Matthews BJ, Limpus CJ, Mills PC. Establishment of reference intervals for plasma protein electrophoresis in Indo-Pacific green sea turtles, *Chelonia mydas*. Conserv Physiol. 2015; 3(1). https://doi.org/10.1093/conphys/cov037 PMID: 27293722

50. Innis C, Merigo C, Dodge K, Tlusty M, Dodge M, Sharp B, et al. Health evaluation of leatherback turtles (*Dermochelys coriacea*) in the northwestern Atlantic during direct capture and fisheries gear disentanglement. Chelonian Conserv Biol. 2010; 9(2): 205–222. https://doi.org/10.2744/ccb-0838.1

51. Page-Karjian A, Rivera S, Torres F, Diez C, Moore D, Dam RV, et al. Baseline blood values for healthy free-ranging green sea turtles (*Chelonia mydas*) in Puerto Rico. Comp Clin Path. 2014; 24(3): 567–573. https://doi.org/10.1007/s00580-014-1947-1

52. Perrault JR, Miller DL, Eads E, Johnson C, Merrill A, Thompson LJ, et al. Maternal health status correlates with nest success of leatherback sea turtles (*Dermochelys coriacea*) from Florida. PLoS One. 2012; 7(2). https://doi.org/10.1371/journal.pone.0031841 PMID: 22359635

53. Andreani G, Carpenè E, Cannavacciuolo A, Girolamo ND, Ferlizza E, Isani G. Reference values for hematology and plasma biochemistry variables, and protein electrophoresis of healthy Hermann's tortoises (*Testudo hermanni* ssp). Vet Clin Pathol. 2014; 43(4): 573–583. https://doi.org/10.1111/vcp.12203 PMID: 25285592

54. Lindemann D, Allender M, Thompson D, Glowacki G, Newman E, Adamovicz L, et al. Epidemiology of Emydoidea herpesvirus 1 in free-ranging Blanding's turtles (*Emydoidea blandingii*) from Illinois. J Zoo Wildl Med. 2019; 50(3): 547–556. https://doi.org/10.1638/2018-0074 PMID: 33517623

55. Campbell TW. Clinical pathology of reptiles. In: Mader DR, editor. Reptile Medicine and Surgery. St. Louis: Saunders Elsevier; 2006. pp 453–470.

56. Dessauer HC. (1970). Blood chemistry of reptiles: Physiological and evolutionary aspects. In: Gans C, Parsons TS, editors. Biology of the Reptilia, Vol. 3. New York: Academic Press; 1970. pp. 1–72.

57. Dowling Z, Hartwig T, Kiviat E, Keesing F. Experimental management of nesting habitat for the Blanding's turtle (Emydoidea blandingii). Ecological Rest. 2010; 28(2): 154–159. https://doi.org/10.3368/er.28.2.154

58. Congdon J, Kinney O, Nagle R. Spatial ecology and core-area protection of Blanding's Turtle (*Emydoidea blandingii*). Can J Zool. 2011; 89: 1098–1106. https://doi.org/10.1139/z11-091

59. Price ER, Sotherland PR, Wallace BP, Spotila JR, Dzialowski EM. Physiological determinants of the interesting interval in sea turtles: a novel 'water-limitation' hypothesis. Biol Lett. 2019; 12(20190248). https://doi.org/10.1098/rsbl.2019.0248 PMID: 31164061

60. Yang PY, Yu PH, Wu SH, Chie CH. Seasonal hematology and plasma biochemistry references range values of the yellow-marginated box turtle (*Cuora flavomarginata*). J Zoo Wildl Med. 2014; 45:278–286. https://doi.org/10.1638/2013-0125R1.1 PMID: 25000688

61. Musilová A, Knotková Z, Pinterová K, Knotek Z. Variations of plasma protein electrophoresis in healthy captive green iguanas (*Iguana iguana*). Vet Clin Pathol. 2015; 44(2): 243–248. https://doi.org/10.1111/vcp.12238 PMID: 25639702

62. Mader DR, Divers SJ. Current therapy in reptile medicine and surgery. St. Louis: Saunders Elsevier; 2004.

63. Selcer KW, Palmer BD. Estrogen downregulation of albumin and a 170-kDa serum protein in the turtle, *Trachemys scripta*. Gen Comp Endocrinol. 1995; 97(3): 340–352. https://doi.org/10.1006/gcen.1995.1034 PMID: 7789749

64. Harris DJ. Clinical Tests. In: Tully T, Dorrestien G, Jones A, editors. Handbook of Avian Medicine, 2nd ed. Philadelphia: Elsevier; 2009. pp. 77–84.

65. Perrault JR, Wyneken J, Page-Karjian A, Merrill A, Miller DL. Seasonal trends in nesting leatherback turtle (*Dermochelys coriacea*) serum proteins further verify capital breeding hypothesis. Conserv Physiol. 2014; 2(1). https://doi.org/10.1093/conphys/cou002 PMID: 27293623

66. Leineweber C, Öfner S, Stöhr AC, Marschang RE, Mathes K. A comparison of thyroid hormone levels and plasma capillary zone electrophoresis in red-eared sliders (*Trachemys scripta* elegans) and map turtles (*Graptemys* spp.) depending on season and sex. Vet Clin Pathol. 2019; 49(1): 78–90. https://doi.org/10.1111/vcp.12838 PMID: 32237094

67. Zaias J, Norton T, Fickel A, Spratt J, Altman NH, Cray C. Biochemical and hematologic values for 18 clinically healthy radiated tortoises (*Geochelone radiata*) on St Catherines Island, Georgia. Vet Clin Pathol. 2006; 35(3): 321–325. https://doi.org/10.1111/j.1939-165x.2006.tb00139.x PMID: 16967417

68. Perrault JR, Schmidt JR, Walsh CJ, Yordy JE, Tucker AD. Brevetoxin exposure, superoxide dismutase activity and plasma protein electrophoretic profiles in wild-caught Kemp's ridley sea turtles

(*Lepidochelys kempii*) in southwest Florida. Harmful Algae. 2014; 37: 194–202. https://doi.org/10.1016/j.hal.2014.06.007

69. Perrault JR, Arendt MD, Schwenter JA, Byrd JL, Harms CA, Cray C, et al. Blood analytes of immature Kemp's ridley sea turtles (*Lepidochelys kempii*) from Georgia, USA: reference intervals and body size correlations. Conserv Physiol. 2020; 8(1). https://doi.org/10.1093/conphys/coaa091 PMID: 33304585

70. Page-Karjian A, Rafferty K, Clerson X, Stacy NI, Moore JA, Hirsch SE, et al. Comprehensive health assessment and blood analyte reference intervals of gopher tortoises (*Gopherus polyphemus*) in southeastern FL, USA. Conserv Physiol. 2021; 9(1). https://doi.org/10.1093/conphys/coab015 PMID: 33815802

71. Lamberski N, Braun J, Witte C, Christopher M, Field K, Averill-Murray R, et al. Identifying key clinical signs and validating body condition scores to minimize disease spread and maximize individual survival during desert tortoise translocations. Joint Annual Meeting for Wildlife Disease Association and European Association of Wildlife Diseases. 2012. Available at: https://www.researchgate.net/publication/271519082_Identifying_key_clinical_signs_and_validating_body_condition_scores_to_minimize_disease_spread_and_maximize_individual_survival_during_desert_tortoise_translocations.

72. Fujii JA, McLeish D, Brooks AJ, Gaskell J, Van Houtan KS. Limb-use foraging marine turtles, an evolutionary perspective. PeerJ. 2018; 6. https://doi.org/10.7717/peerj.4565 PMID: 29610708

73. Adamovicz L, Allender MC, Archer G, Rzadkowska M, Boers K, Phillips C, et al. Investigation of multiple mortality events in eastern box turtles (*Terrapene carolina carolina*). PloS One. 2018; 13(4). https://doi.org/10.1371/journal.pone.0195617 PMID: 29621347

74. Beck FK, Rosenthal TC. Prealbumin: a marker for nutritional evaluation. Am Fam Physician. 2002; 65: 1575–1578. PMID: 11989633

75. Zaias J, Cray C. Protein electrophoresis: A tool for the reptilian and amphibian practitioner. J Herpetol Med Surg. 2002; 12(1): 30–32.

76. Rohilla MS, Tiwari PK. Simple method of blood sampling from Indian freshwater turtles for genetic studies. Acta Herpetol. 2020; 3(4): 65–69.

77. Stacy NI, Alleman AR, Sayler KA. Diagnostic hematology of reptiles. Clin Lab Med. 2011; 31(1): 87–108. https://doi.org/10.1016/j.cll.2010.10.006 PMID: 21295724

78. Anderson ET, Minter LJ, Clarke EO, Mroch RM, Beasley JF, Harms CA. The effects of feeding on hematological and plasma biochemical profiles in green (*Chelonia mydas*) and Kemp's ridley (*Lepidochelys kempii*) sea turtles. Vet Med Int. 2011; 2011. https://doi.org/10.4061/2011/890829 PMID: 21776356

79. Stewart K, Mitchell MA, Norton T, Krececk RC. Measuring the level of agreement in hematologic and biochemical values between blood sampling sites in leatherback sea turtles (*Dermochelys coriacea*). J Zoo Wildl Med. 2021; 43(4): 719–725. https://doi.org/10.1638/2011-0045R.1 PMID: 23272336

80. Bolten AB, Bjorndal KA. Blood profiles for a wild population of green turtles (*Chelonia mydas*) in the southern Bahamas: Size-specific and sex-specific relationships. J Wildl Dis. 1992; 28(3): 407–413. https://doi.org/10.7589/0090-3558-28.3.407 PMID: 1512872

81. Alberghina D, Marafioti S, Spadola F, Panzera M, Piccione G. Influence of short-term storage conditions on the stability of total protein concentrations and electrophoretic fractions in plasma samples from loggerhead sea turtles, *Caretta caretta*. Comp Clin Path. 2014; 24(5): 1091–1095. https://doi.org/10.1007/s00580-014-2038-z

82. Stacy NI, Chabot RM, Innis CJ, Cray C, Fraser KM, Rigano KS, et al. Plasma chemistry in nesting leatherback sea turtles (*Dermochelys coriacea*) from Florida: Understanding the importance of sample hemolysis effects on blood analytes. PloS One. 2019; 14(9). https://doi.org/10.1371/journal.pone.0222426 PMID: 31504062