Plasma Protein Electrophoresis of the Atlantic Loggerhead Sea Turtle, *Caretta caretta*

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ABSTRACT: The objective of this study was to determine reference intervals for plasma protein fractions of normal appearing, wild Atlantic loggerhead sea turtles, *Caretta caretta*. Blood was collected into heparinized vacutainer tubes from the following groups of turtles: 1) ten adult males; 2) eleven adult females; 3) ten juvenile males; and 4) ten juvenile females. Plasma was removed and total protein content of each sample was determined using the biuret method. Plasma proteins were separated using gel electrophoresis and scanned using a laser densitometer. Reference ranges for albumin, alpha, beta, and gamma globulins were established for age and gender classes and statistically analyzed. Significant differences were found between beta globulins of adult and juveniles and between juvenile males and females. A subgroup of turtles had electrophoretograms with beta-gamma bridging and a single adult male loggerhead had a prealbumin fraction; however, these subgroups of turtles were excluded from statistical analysis.

KEY WORDS: loggerhead sea turtle, *Caretta caretta*, protein electrophoresis, plasma.

INTRODUCTION

The loggerhead sea turtle, *Caretta caretta*, is one of six species of marine turtles inhabiting the Atlantic Ocean. It is wide ranging, found from Nova Scotia to Argentina. The largest population in North America (and possibly the world) is found in coastal waters from North Carolina to the Florida Keys. It is the most common species of sea turtle brought into rehabilitation facilities in Florida. Since these turtles are federally listed as threatened in the United States, all turtles brought into rehabilitation facilities are treated with the intent to release them back to the wild. While blood is routinely collected from ill sea turtles and used to determine health status, little information is available regarding reference intervals. The effect of anticoagulants on biochemical values in loggerhead sea turtles has been reported (Bolten, *et al,* 1992).

In human and domestic animal medicine, electrophoresis of plasma proteins can provide information about chronic or acute inflammatory processes in the patient and may help the clinician determine appropriate treatment (Cray and Tatum, 1998). Recently, plasma protein electrophoresis has been advocated for use in pet bird diagnostics, especially when other tests are nondiagnostic (Cray and Tatum, 1998). In the acute stage of chlamydiosis in birds, there are major changes that occur in the electrophoretogram that may support a diagnosis of this disease (Cray and Tatum, 1998). Electrophoresis also has been utilized for diagnosing other diseases such as aspergillosis, hepatitis, and nephritis (Cray and Tatum, 1998). Due to the rapid changes that can occur in an animal's plasma, electrophoresis can also be used for serially evaluating the response to treatment. (Cray, *et al,* 1995, Cray and Tatum, 1998).

Electrophoresis has been proposed for use in reptile and amphibian medicine to aid in the diagnosis of disease (Zaias and Cray, 2002). Plasma protein electrophoresis may be useful as a health assessment tool for evaluating injured sea turtles. In order to assess its utility, normal reference ranges need to be established. Here we report the values for clinically healthy, juvenile and adult loggerhead sea turtles of both sexes captured at a foraging ground in southern Florida.

MATERIALS AND METHODS

Plasma samples were obtained from a loggerhead sea turtle plasma repository located at the Fish and Wildlife Research Institute, Florida Fish and Wildlife Conservation Commission, St. Petersburg, FL. Whole blood was originally collected from forty-one clinically healthy loggerhead sea

turtles from an area of Florida Bay, approximately 16 km by 16 km square centered at latitude 24° 56.0' N and longitude 80° 50.0' W. The blood sampling was conducted as part of an on-going, long-term study of sea turtles in Florida Bay (Schroeder, *et al,* 1998). Blood was collected from ten adult males, eleven adult females, ten juvenile males, and ten juvenile females, May through September, 1993 through 2001. The sex of adult turtles was originally determined using morphological characteristics. The sex of juvenile turtles was determined using plasma testosterone levels, with females having less than 20 pg/ml and males having greater than 30 pg/ml. Five to seven milliliters of blood was collected from the dorsal cervical sinus into lithium heparin coated vacutainer tubes. Tubes were centrifuged; plasma was removed and pipetted into 1.5 mL cryotubes and then the cryotubes were placed in a liquid nitrogen tank. Subsequently, plasma was stored in an ultra freezer at -70°C (-94°F). Plasma was then shipped on dry ice to the University of Florida, College of Veterinary Medicine for total protein determination and plasma protein electrophoresis.

Total protein concentration was determined for each sample using a Hitachi 911 auto analyzer (Roche Diagnostics Corporation, Indianapolis, Indiana) by the biuret method (Silverman and Christenson, 1996). Agarose gel electrophoresis was performed on each sample using Beckman Paragon Protein SPE-II Electrophoresis kits (Beckman Coulter Inc. Fullerton, CA). A volume of 0.5 µL of undiluted plasma was applied to the gel through the supplied template. The gel was then subjected to 25 min of pulsing at 100 V. The gel was fixed in acid-alcohol, dried, and stained with paragon blue stain (Beckman Coulter, Inc. Fullerton, CA).

Electrophoretic gels were analyzed using a laser densitometer (Beckman Appraise Densitometer, Beckman Coulter, Inc. Fullerton, CA) at 600 nm. The laser tracings were divided into the followings fractions: pre-albumin (if present), albumin, alpha globulins, beta globulins, and gamma globulins. The relative percentage of each protein fraction was calculated by the densitometer from the area under the curve created by the protein band. The densitometer automatically calculated the absolute value for each fraction by multiplying the total protein of the sample by the corresponding fractional percentage. Albumin to globulin ratios (A:G) were determined according to the following equation: (albumin)/(alpha globulins + beta globulins + gamma globulins). Statistical analyses (mean, standard deviation, minimum, maximum, and student's t-test) were performed using Microsoft Excel (Microsoft Corporation, Seattle, WA). The mean and standard deviation was determined for each protein fraction of each age and gender class. Comparisons were made between each group (males vs. females and adults vs juveniles) and between each subgroup (adult males vs. juvenile males, adult females vs. juvenile females, adult males vs. adult females, juvenile females vs. juvenile males, adult males vs. juvenile females, and juvenile males vs. adult females) using two-tailed students T-test to determine if there was a significant difference. A comparison was made only between those turtles that had distinct albumin, alpha, beta, and gamma fractions. A significant difference was attributed if $p \leq 0.05$.

RESULTS

Electrophoresis revealed the following protein fractions: albumin, alpha globulin, beta globulins, and gamma globulins in plasma samples from 29 of 41 loggerhead sea turtles (Figure 1). In 11 turtles (two adult males, four adult females, two juvenile males, and three juvenile females) a distinction between the beta fraction and gamma fraction could not be made (Figure 2). Finally, in a single adult male loggerhead sea turtle, a prealbumin fraction also was seen (Figure 3).

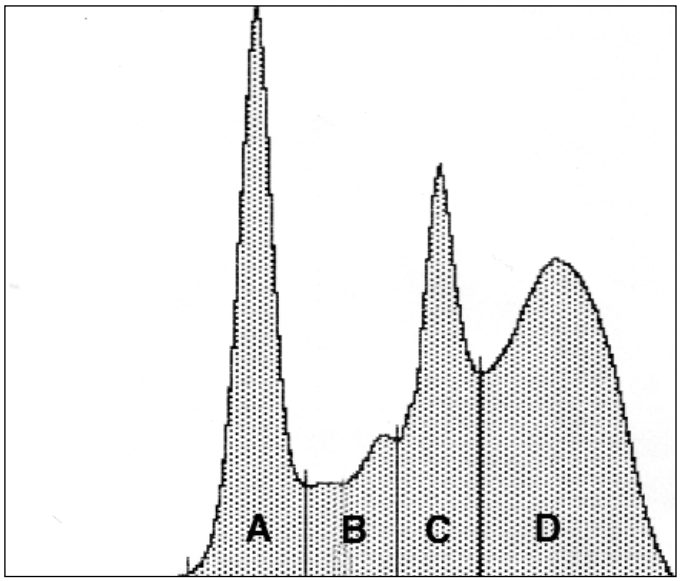

Figure 1. Representative plasma protein electrophoretogram of a loggerhead sea turtle, *Caretta caretta.* The dark horizontal lines from left to right denote the breaks between the albumin (A), Alpha globulins (B), Beta globulins (C), and Gamma globulin (D) fractions.

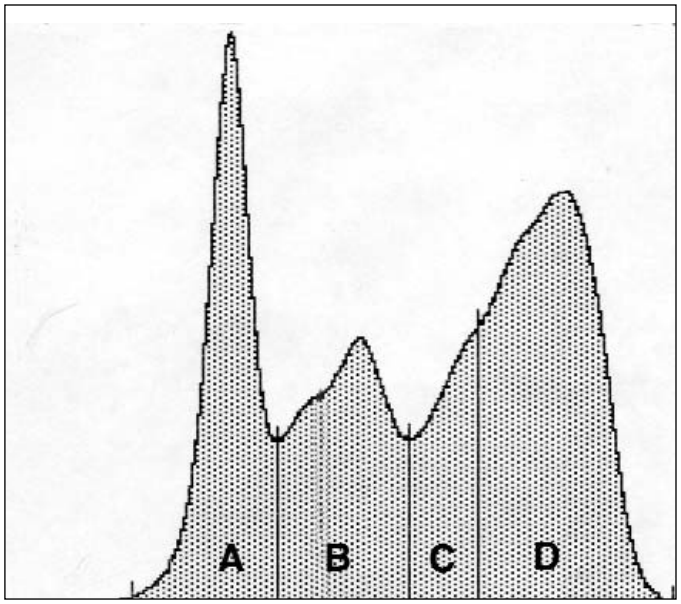

Figure 2. Plasma protein electrophoretogram with beta-gamma bridging. Albumin (A) and alpha globulin (B) fractions are present. Note the absence of a spike in the beta globulin region and the lack of a break between the beta and gamma globulins (C).

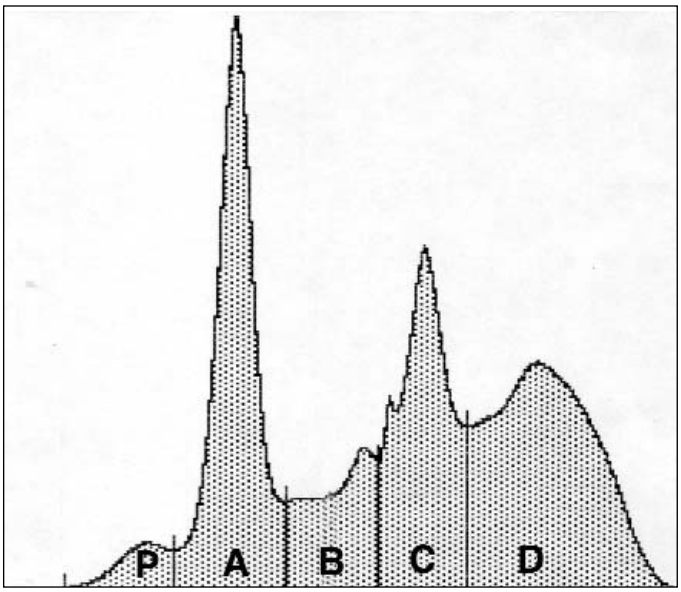

Figure 3. Plasma protein electrophoretogram of the loggerhead sea turtle, *Caretta caretta* (adult male) with a pre-albumin (P) band. The albumin (A), alpha globulin (B), beta globulin (C), and gamma globulin (D) fractions are present.

The values for mean [± standard deviation (SD)], maximum and minimum plasma protein fractions for the 29 loggerhead sea turtles with distinct fractions and for the 11 loggerhead sea turtles showing beta-gamma bridging are presented in tables one and two respectively. Significant differences were found in the beta globulin fraction of adult turtles compared to juvenile turtles ($p = 0.0028$), male turtles compared to female ($p = 0.014$), juvenile males compared to juvenile females ($p = 0.014$), adult females compared to juvenile females ($p = 0.0078$), and adult males vs. juvenile females ($p < 0.0003$). No significant differences were found in the remaining comparisons.

Discussion

In this study, the biuret method was used to determine plasma protein concentration of loggerhead sea turtles. In avian medicine, this has been found to be more accurate and precise than refractometry for the measurement of plasma total protein (Lumeij and McClean, 1996). The mean total protein content of loggerhead sea turtle plasma (± SD) was 4.3 ± 0.71 g/dL. This is similar to total protein concentration found in various species of birds including the brown pelican, *Pelecanus occidentalis*, (5.0 ± 1.2 g/dL) and numerous species of raptors (Tatum, *et al,* 2000, Zaias, *et al,* 2000). Total protein was also comparable to that previously reported for loggerhead sea turtles (4.1 ± 1.3g/dL) (Bolten, *et al,* 1992).

In one loggerhead sea turtle, an adult male, a prealbumin band was seen. In other animals, the prealbumin band is com-

Table 1. Plasma protein fractions identified in loggerhead sea turtles, *Caretta caretta*.

Group	Total Protein (g/dL ± SD)	Albumin (g/dL ± SD)	Alpha (g/dL ± SD)	Beta (g/dL ± SD)	Gamma (g/dL ± SD)	A:G
All (N=29)	**4.3 ± 0.72**	**1.0 ± 0.17**	**0.48 ± 0.10**	**0.80 ± 0.20**	**1.94 ± 0.62**	**0.33 ± 0.10**
Minimum	**2.9**	**0.7**	**0.3**	**0.51**	**0.77**	**0.17**
Maximum	**5.4**	**1.3**	**0.81**	**1.3**	**3.0**	**0.63**
Adult Males						
(N=7)	**4.6 ± 0.33**	**1.1 ± 0.11**	**0.54 ± 0.12**	**0.99 ± 0.17**	**1.97 ± 0.27**	**0.32 ± 0.05**
Minimum	**4.2**	**0.92**	**0.43**	**0.74**	**1.58**	**0.23**
Maximum	**5.2**	**1.25**	**0.81**	**1.31**	**2.48**	**0.**
Adult females						
(N=7)	**4.4 ± 0.75**	**0.97 ± 0.13**	**0.49 ± 0.05**	**0.81 ± 0.14**	**2.1 ± 0.64**	**0.30 ± 0.62**
Minimum	**3.3**	**0.79**	**0.42**	**0.56**	**1.1**	**0.18**
Maximum	**5.4**	**1.2**	**0.56**	**0.98**	**2.98**	**0.38**
Juvenile males						
(N=8)	**4.1 ± 0.66**	**0.96 ± 0.19**	**0.46 ± 0.11**	**0.78 ± 0.13**	**1.8 ± 0.61**	**0.33 ± 0.10**
Minimum	**3.5**	**0.71**	**0.34**	**0.59**	**1.27**	**0.17**
Maximum	**5.4**	**1.23**	**0.66**	**1.05**	**2.93**	**0.45**
Juvenile females						
(N=7)	**3.9 ± 0.78**	**1.0 ± 0.17**	**0.44 ± 0.06**	**0.60 ± 0.07**	**1.9 ± 0.76**	**0.38 ± 0.15**
Minimum	**2.9**	**0.77**	**0.36**	**0.51**	**0.77**	**0.25**
Maximum	**5.2**	**1.28**	**0.54**	**0.73**	**2.88**	**0.63**

Table 2. Plasma protein fractions identified in loggerhead sea turtles, *Caretta caretta,* with beta-gamma bridging.

	Total Protein (g/dL ± SD)	Albumin (g/dL ± SD)	Alpha (g/dL ± SD)	Beta — Gamma (g/dL ± SD)	A:G
All (n=11)	4.8±0.50	1.0±0.18	0.53±0.22	3.4±0.42	0.30 ±0.06
Min	4.0	0.76	0.28	2.3	0.17
Max	5.8	1.4	0.95	4.4	0.41

posed of transthyretin, a protein that binds the thyroid hormones, L-thyroxin and L-3.5.3' triiodothyronine (Chang, *et al,* 1999, Harr, 2002). This carrier protein is highly conserved and is similar across most species of birds, herbivorous marsupials and other mammals. It also has been reported in sea bream fish, *Sparus aurata,* (Santos and Power, 1999). In order to validate the nature of this fraction in loggerhead sea turtles, the protein would need to be extracted and the amino acid composition determined for comparison to other transthyretins. This was beyond the scope of this study.

Different methods have been used for determining plasma albumin concentrations. Nonspecific dye binding methods are commonly used but suffer from variability when used in different species due to differences in dye affinity (Harr, 2002). The bromcresol green dye binding method for determining albumin concentration has been shown to be an inaccurate method for measuring the albumin level of nonmammalian plasma (Spano, *et al,* 1988, Cray and Tatum, 1998, Harr, 2002). Since electrophoresis directly measures albumin present in a plasma sample, the albumin levels obtained by gel electrophoresis and densitometry are a more accurate than dye methods and is the recommended method for measurement of albumin (Harr, 2002).

The mean plasma albumin concentration (1.0 ± 0.17 g/dL) for loggerhead sea turtles was lower than the reference ranges established for domestic mammals (dogs, 2.5 – 3.6 g/dL and horses, 2.7 – 4.2 g/dL) (Meyer and Harvey, 1998) and for certain species of birds including the screech owl, *Otus asio,* (2.2 ± 0.5 g/dL), the red-tailed hawk, *Buteo jamaicensis*, (2.2 ± 0.5 g/dL), the great horned owl, *Bubo virginanus*, (1.9 ± 0.2 g/dL), the turkey vulture, *Cathates aura,* (2.3 ± 0.4 g/dL), the barn owl, *Tyto alba*, (2.1 ± 0.4 g/dL), and the black vulture, *Coragyps atratus*, (1.9 ± 0.2 g/dL) (Rosenthal, 2000, Zaias, *et al,* 2000, Tatum, *et al,* 2000). However, plasma albumin concentrations were higher then those reported for loggerhead sea turtles in another report (0.6 ± 0.8 g/dL) (Bolten, *et al,* 1992). The difference may be due to the different methods used to measure albumin levels.

There may be a physiologic basis for the apparent hypoalbuminemia in aquatic turtles. Massat and Dessauer (1968) hypothesized that the hypoalbuminemia was an adaptation to deep water diving and subsequent lactic acidosis. Low plasma albumin would result in a physiologic ascites and pericardial effusion. The high bicarbonate levels in these fluids would serve as a buffer for anaerobic metabolism. A turtle could then remain underwater for longer periods of time without the need to surface.

In general, alpha globulins of mammals include acute phase proteins consisting of alpha-lipoprotein, alpha-1-antitrypsin, haptoglobin, ceruloplasmin, amyloid-A, and alpha-2-macroglobulin (Kaneko, 1997). Ceruloplasmin and haptoglobin have been positively identified in the plasma of *C. caretta* (Masquera, *et al,* 1976). In this study, alpha globulins could not be separated into alpha 1 and alpha 2 fractions. The alpha globulin values (mean ± SD) of the loggerhead sea turtles were very low (0.5 ± 0.1 g/dL) when compared to other species (Meyer and Harvey, 1998, Tatum, *et al,* 2000, Zaias, *et al,* 2000). Since the levels of acute phase proteins may have diagnostic utility, further analyses of the alpha globulins bands are warranted to assess their presence. Similar to the alpha globulins, the beta globulins of the loggerhead sea turtle could not be separated into beta-1 and beta-2 fractions. In domestic animals, the beta globulins are also acute phase proteins that include fibrinogen, transferrin, beta-lipoprotein, and complement (Kaneko, 1997). Fibrinogen is an important indicator of inflammation and is present in plasma. Plasma is preferable to serum for nonmammalian blood for electrophoresis because it contains fibrinogen, an important indicator of inflammation (Rosenthal, 2000, Zaias and Cray, 2002). However, because there are many other proteins in the beta-globulin region, plasma gel electrophoresis is not likely to accurately measure fibrinogen alone. While transferrin is found in the beta fraction of mammals, in a previous study in turtles (including the loggerhead sea turtle) it was identified in the gamma-globulin fraction (Masquera, *et al,* 1976).

The beta globulins (mean ± SD) of this group of turtles (0.79 ± 0.2 g/dL) were similar to the values (mean ± SD) found for the *brown pelican, Pelecanus occidentalis,* (0.92 ± 0.3 g/dL) (Zaias, *et al,* 2000), the red-tailed hawk, *Buteo jamaicensis,* (0.77 ± 0.3 g/dl), barn owl, *Tyto alba*, (0.72 ± 0.2 g/dL), and the black vulture, *Coragyps atratus,* (0.68 ± 0.3 g/dL) (Tatum, *et al,* 2000). When compared, significant differences ($p \leq 0.05$) were found between the beta globulin levels of adult and juvenile, male and female, juvenile male and juvenile female, adult male and juvenile female, and adult female and juvenile female loggerhead sea turtles. Reasons for the differences between the adult and juvenile animals and between male and female turtles are unknown and further study is necessary to determine the basis for these differences. In psittascine birds, monoclonal increases in beta globulins will occur during prolonged periods of egg laying (Cray and Tatum, 1998). A monoclonal increase is identified as a sharp, narrow spike similar in shape to albumin while polyclonal increases consist of a single broad-based or multiple small peaks (Meyer and Harvey, 1998). The beta globulin fractions of the loggerhead sea turtles were polyclonal in appearance and adult male turtles had generally higher beta globulin levels (0.99 ± 0.17 g/dL) in comparison to adult and juvenile females and juvenile

male loggerhead sea turtles (0.81 ± 0.14 g/dL, 0.60 ± 0.07 g/dL, 0.78 ± 0.13 g/dL, respectively).

Gamma globulins are primarily composed of the various classes of immunoglobulins. While it is unknown what immunoglobulins the loggerhead sea turtle possesses, Benedict and Pollard (1972) found that the green turtle, *Chelonia mydas*, possessed three classes of immunoglobulins. There was a 17S immunoglobulin that resembled mammalian IgM. Additionally, a 7S immunoglobulin and a 5.7S immunoglobulin were identified. As noted above, it has been found that transferrin migrates in the gamma globulin region in the loggerhead sea turtle (Masquera, *et al,* 1976).

Gamma globulin levels on average were four to ten times greater in the loggerhead sea turtles compared to a variety of avian species. The bird with a gamma globulin fraction closest to that of loggerhead sea turtles is the brown pelican; (0.78 ± 0.4 g/dL; Zaias, *et al,* 2000). When compared to established mammal reference ranges, the gamma globulin levels for the loggerhead sea turtle were similar to but generally higher to than in dogs (0.9 – 2.1 g/dL), horses (0.55 – 1.9 g/dL), and cows (1.69 – 2.25 g/dL) (Kaneko, 1997). The reason for loggerhead sea turtles having such high levels of gamma globulins is unknown. While all turtles sampled in this study were wild and at the time of capture and blood collection all appeared to be in good health, it was likely that they were infected with digenetic trematodes of the family Spirorchiidae, a common parasite of wild loggerhead sea turtles (Jacobson, *et al,* 2003). Possibly, parasitism stimulates the production of specific antibodies in loggerhead sea turtles. Presence of such antibodies has been reported in the green turtle (Graczyk, *et al,* 1995). It is reasonable to assume that similar antibodies exist in loggerhead sea turtles exposed to these parasites.

In the current report, eleven turtles with beta-gamma globulin bridging were seen. Chronic disease can lead to increases in both the beta and gamma globulin fraction such as in birds with mycobacteriosis (Cray and Tatum, 1998). Bacterial hepatitis has also been identified as a cause of beta-gamma bridging in a cockatoo (Cray and Tatum, 1998). In mammals, beta-gamma bridging is pathognomonic for chronic active hepatitis, which results in increased IgA, IgM, or both (Kaneko, 1997). Parasitic infection may account for the bridging. The spirorchiids cause wide-spread granulomatous inflammation in many different tissues (Wolke, *et al,* 1982, Gordon, *et al,* 1998). The presence of spirorchiidae infection is common and it has been suggested that they may be responsible for a recent die-off of juvenile loggerhead sea turtles in Florida waters (Jacobson, *et al,* 2003). Possibly, chronic spirorchiid infection could be an explanation for the beta and gamma globulin bridging seen in the 11 loggerhead sea turtles in this report.

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