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COMMUNICATION

# Serum Protein Analysis of Nurse Sharks

**Leila AtallahBenson***
*Department of Marine Ecosystems and Society, Rosenstiel School of Atmospheric Science, University of Miami, 4600 Rickenbacker Causeway, Miami, Florida 33149, USA; and Shark Research and Conservation Program, University of Miami, 4600 Rickenbacker Causeway, Miami, Florida 33149, USA*

**Liza Merly**
*Department of Marine Biology and Ecology, Rosenstiel School of Atmospheric Science, University of Miami, 4600 Rickenbacker Causeway, Miami, Florida 33149, USA; and Shark Research and Conservation Program, University of Miami, 4600 Rickenbacker Causeway, Miami, Florida 33149, USA*

**Carolyn Cray**
*Division of Comparative Pathology, Department of Pathology and Laboratory Medicine, Miller School of Medicine, University of Miami, Post Office Box 016960 R46, Miami, Florida 33101, USA*

**Neil Hammerschlag**
*Department of Marine Ecosystems and Society, Rosenstiel School of Atmospheric Science, University of Miami, 4600 Rickenbacker Causeway, Miami, Florida 33149, USA; and Shark Research and Conservation Program, University of Miami, 4600 Rickenbacker Causeway, Miami, Florida 33149, USA*

*Abstract*

**Serum protein electrophoresis (EPH) is used to assess relative concentrations of blood proteins in clinical and biological studies. Serum EPH fractions have been determined for elasmobranchs using mammalian albumin, alpha 1-, alpha 2-, beta-, and gamma-globulin fractions, and have been deemed fractions 1 through 5, respectively. However, serum EPH fraction concentration reference intervals (RIs) have not been widely established for different elasmobranch species. In this study, RIs for fractions 1 through 5 were determined from 45 wild-caught Nurse Sharks *Ginglymostoma cirratum* (27 females and 23 males) in South Florida. Serum samples were isolated from whole blood following caudal venipuncture. Body condition was also measured in the field to assess the relative health of the individuals sampled. There was no relationship between body condition and serum EPH fraction concentrations. In addition, there was no difference in body condition or serum EPH fraction concentrations between females and males. Total solids and total protein values were significantly different ($P < 0.001$). Nurse Shark serum EPH fraction 1 was found within the mammalian albumin migrating band distance and was negligible. Fraction 2 showed no peak in the mammalian alpha 1-globulin range. A thin, medium peak in the mammalian alpha 2-globulin range represented fraction 3. In the mammalian beta-globulin range, fraction 4 consisted of the majority of protein observed. It was represented by a smooth, broad peak. A short, medium broad peak in the mammalian gamma-globulin range represented fraction 5. The Nurse Shark serum EPH fraction RIs provided in this study may be utilized to clinically evaluate the health of Nurse Sharks in captivity and in the wild, and to compare the health of their populations around the world experiencing various anthropogenic stressors and other environmental impacts.**

Peripheral blood proteins carry out many critical biological functions for vertebrates. The relative concentrations of these proteins can be used as proxies for health parameters in clinical settings and as a research tool (Jain et al. 2011). Serum protein electrophoresis (EPH) is one tool that can be used to evaluate proteins based on their migration characteristics (Eckersall 2008). Previous studies focusing on elasmobranch proteins using agarose gel electrophoresis platforms have shown that five fractions of blood proteins are present. These fractions are based on

*Corresponding author: latallahbenson@gmail.com
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mammalian protein migration characteristics: albumin, alpha 1-, alpha 2-, beta-, and gamma-globulins, respectively (Haman et al. 2012; Cray et al. 2015).

Species-specific serum EPH fraction concentration reference intervals (RIs) are important for understanding protein parameters and how electrophoretic patterns correspond to healthy and abnormal individuals (Haman et al. 2012). Healthy individuals are expected to have EPH values comparable to an established RI; therefore, individual sharks whose health may be compromised can be identified in captive settings (e.g., exhibits and research facilities) and in the wild. Reference intervals can facilitate the monitoring of individual and population health that could be affected by different biotic and abiotic factors, such as population density, prey abundance, water temperature, overfishing, pollution in urban areas, or other anthropogenic stressors (Morgan and Burgess 2007; Schlaff et al. 2014). Population health evaluations can provide insight about habitat quality, which in turn may highlight areas of concern (Deem et al. 2001).

Elasmobranch serum EPH fraction concentration RIs have yet to be widely determined, as this taxonomic group is underrepresented in clinical literature (Harms et al. 2002; Cain et al. 2004; Ferreira et al. 2010; Haman et al. 2012; Persky et al. 2012; Krol et al. 2014; Cray et al. 2015; Cusack et al. 2016; Hyatt et al. 2016). To establish RIs, blood must be collected from 120 individuals within a species as recommended by the American Society for Veterinary Clinical Pathology. Alternatively, when sample sizes are smaller, reference ranges can be calculated (Friedrichs et al. 2011).

Total protein (TP) and total solids (TS) are two metrics in serum that can be used to determine EPH fractions. Total protein is a measurement of the total concentration of protein, whereas total solids measures the refractive index of the sample. Proteins are responsible for much of the refractive index when compared with other blood constituents, so TS is often used as an estimate of TP. However, since TS includes other nonprotein solids (e.g., urea, triglycerides, and cholesterol), its values are expected to be higher than TP (by approximately 2 g/dL) so modern refractometers are calibrated to reflect this difference (Hunsaker et al. 2016). In elasmobranchs, nonprotein solids in serum (especially urea: Hammerschlag 2006) may be significantly higher than in other vertebrate groups. Urea has been shown to significantly increase the measure of total protein by refractometry in other groups (Legendre et al. 2017). Here, TP and TS were compared to determine what impact their differences may have on serum EPH fraction concentration RIs.

When investigating an individual animal's EPH fraction concentration, the sample should be compared with an RI that has been determined from a healthy, representative population, either in a captive or wild setting. Health assessments can be performed by examining individuals for any lesions and assessing their body condition score via morphometrics, which typically reflect their energy stores and can influence their success in life-history events (Gallagher et al. 2014b; Irschick and Hammerschlag 2015). In this study, physical exams were conducted and body conditions were assessed to ensure that the baseline EPH RI provided was representative of a healthy population.

The Nurse Shark *Ginglymostoma cirratum* is a hardy species that is common to Florida and the Caribbean, and it also has a wide distribution across the Atlantic Ocean. Nurse Sharks are found in tropical to subtropical waters and feed on benthic fish and crustaceans. This species is listed as "data deficient" on the International Union for Conservation of Nature's Red List, due to a lack of knowledge about their migratory behaviors and gene flow between different populations (Rosa et al. 2006). In South Florida, Nurse Sharks are subjected to many anthropogenic stressors including hurricanes and an increasingly urbanized environment, which could impact resident populations. The objective of the current study was to provide baseline serum EPH fraction concentration RIs for Nurse Sharks within the Miami area of South Florida to assist in population health monitoring.

## METHODS

A total of 45 mature Nurse Sharks (21 males and 24 females) were caught off the coast of Miami, Florida. Sampling occurred from 2014 to 2017, across both wet and dry seasons. Individuals were targeted using a circle-hook drumline system (Gallagher et al. 2014a). Once caught, Nurse Sharks were brought onto a slightly submerged platform and manually restrained for the duration of data collection. No ventilation pump system was used, as Nurse Sharks are capable of buccal pumping. Only individuals in good health were used in this study, as determined by a visual assessment for lesions, body condition, and behavior considered typical for this species.

The following morphometrics were taken from 38 individuals to calculate body condition (C): precaudal length (PCL), fork length (FL), total length (TL), lateral span (LS), frontal span (FS), proximal span (PS), and caudal keel circumference (CKC) (Gallagher et al. 2014b). Body condition was defined as follows: $C = \Sigma(LS + FS + PS + CKC)/(PCL)$ (Gallagher et al. 2014b).

Blood was sampled from the caudal vein via an 18-gauge, 3-in hypodermic needle. A total of 10 mL of whole blood was collected. A sterile, 15-mL Falcon tube of approximately 5 mL whole blood was placed directly on ice and allowed to clot. Most samples were processed within 3–5 h, with some samples extending to 8 h depending on boat field logistics. The clotted samples were centrifuged at $400 \times g$ for

10 min. The serum was then aspirated to a new tube and the pellet was discarded. This process was then repeated. The remaining serum was kept at −20°C until electrophoresis was performed, typically within 3 months of collection.

Samples were analyzed using a SPIFE 3000 electrophoresis analysis system following the manufacturer's instructions (Helena Laboratories, Beaumont, Texas; Cray et al. 2011). Fraction delimits, referred to as fractions 1–5 and based on mammalian albumin, alpha 1-, alpha 2-, beta-, and gamma-globulin migration characteristics, were determined (Cray et al. 2015). The TP was determined by the biuret method (Pierce BCA assay; Gornall et al. 1949). The TS was determined with a nontemperature compensated refractometer (Schuco, Japan; Cray et al. 2015).

Data were analyzed for normality using the Shapiro-Wilk test. Since the majority of the fractions were not normally distributed, nonparametric tests were used throughout the analysis. The Spearman test was used to analyze correlations between parameters. A paired two-tailed *t*-test was used to compare TS and TP. Reference limits were established ($n = 45$) by non-Gaussian methods with 90% CI by current American Society for Veterinary Clinical Pathology guidelines (Friedrichs et al. 2012). All statistical analyses were conducted using R (version 3.4.1), MATLAB (version R2017a), and Microsoft Excel (version 16.23).

## RESULTS

The TL of the sampled sharks ranged from 154 to 289 cm, with a mean ± SD of 230 ± 28.8 cm (Table 1). Body condition indices ranged from 0.78 to 1.38, with a mean of 1.11 (Table 1). The seven sharks whose body condition could not be calculated (due to a lack of span measurements) appeared to have similar body conditions and behaviors to the other individuals sampled.

Serum samples from 45 sharks were subjected to electrophoresis. Mild hemolysis occurred in a few of the serum samples. Protein electrophoresis fraction concentrations were defined, as shown in Table 2 (Cray et al. 2015; Hyatt et al. 2016). A minor band was identified within the mammalian albumin fraction migrating distance (fraction 1). A small, sloped increase in protein concentration was observed in the mammalian alpha 1-globulin range (fraction 2) across all samples. Serum EPH fraction 3 exhibited a medium, thin peak within the mammalian alpha 2-globulin range, which was also consistent across all individuals sampled. Fraction 4, determined within proximity to mammalian beta-globulin fractions, contained the majority of protein and was represented by a wide, broad peak (Figure 1). Fraction 5 was established in the mammalian gamma-globulin range and was present as a broad, small peak. The fraction 3 : fraction 4 ratio, as described by Hyatt et al. (2016), ranged from 0.38 to 0.09, with a mean of 0.23. There was no significant difference between the sexes regarding length, body condition, or serum EPH fraction concentrations. Body condition did not correlate with any of the serum EPH fractions. The TS mean ± SE was 4.9 ± 0.2, which was significantly higher ($P < 0.001$) than that of the TP (3.5 ± 0.1; Table 3).

## DISCUSSION

Serum EPH RIs were established in Nurse Sharks. Generally, the EPH fraction concentrations from sera were similar to those reported in the Atlantic Sharpnose Shark *Rhizoprionodon terraenovae*, Bonnethead *Sphyrna tiburo*, Spiny Dogfish *Squalus acanthias*, and Cownose Ray *Rhinoptera bonasus* (Haman et al. 2012; Cray et al. 2015; Hyatt et al. 2016). The minor fraction 1 band (rather than albumin) may contain high-density lipoproteins as described in other elasmobranchs, as the location and concentration of fraction 1 in this study is consistent with the location of fraction 1 in those studies (Metcalf and Gemmell 2005; Cray et al. 2015). The low levels of albumin fractions compared with mammals are in accordance with previous reports in the literature for elasmobranchs, as elasmobranchs do not possess albumin (Ballantyne 1997; Metcalf and Gemmell 2005;

TABLE 1. Reference intervals for morphometric data and body conditions of Nurse Sharks caught in South Florida. The PCL, FL, TL, and CKC were not normally distributed, and no outliers were removed. Values in parentheses represent the 90% CI; LRL = lower reference limit; URL = upper reference limit; and SF = serum fraction.

| Parameter | *n* | Mean ± SD | Median | Minimum | Maximum | LRL | URL |
|---|---|---|---|---|---|---|---|
| LS (cm) | 38 | 56.9 ± 12.3 | 59 | 33 | 81 | 53.6 (43.2–64.0) | 60.1 (45.0–75.2) |
| FS (cm) | 38 | 55.5 ± 10.4 | 58.0 | 36.0 | 75.5 | 52.7 (44.0–61.5) | 58.2 (45.5–70.9) |
| PS (cm) | 38 | 39.30 ± 9.48 | 40 | 20 | 64 | 36.8 (28.8–44.9) | 41.8 (30.2–53.5) |
| CKC (cm) | 38 | 26.10 ± 5.19 | 28 | 10 | 39 | 24.8 (20.4–29.2) | 27.5 (21.1–33.9) |
| PCL (cm) | 45 | 161.0 ± 20.1 | 165 | 110 | 188 | 157 (139–166) | 166 (142–190) |
| FL (cm) | 45 | 183.0 ± 21.5 | 188 | 131 | 220 | 178 (159–196) | 188 (162–214) |
| TL (cm) | 45 | 230.0 ± 28.8 | 236 | 154 | 289 | 224 (199–248) | 237 (203–272) |
| C | 38 | 1.11 ± 0.12 | 1.1 | 0.8 | 1.4 | 1.05 (0.94–1.15) | 1.14 (0.99–1.29) |

TABLE 2. Reference intervals for absolute values (g/dL) and relative percentages (%) of serum fractions determined by serum protein electrophoresis of Nurse Sharks caught in South Florida ($n = 45$). Total serum protein (biuret) was used to calculate the absolute values. Fractions 1, 3, and 5 were not normally distributed. No outliers were removed. Values in parentheses represent the 90% CI; LRL = lower reference limit; URL = upper reference limit; and SF = serum fraction.

| Fraction | Mean ± SD | Median | Minimum | Maximum | LRL | URL |
|---|---|---|---|---|---|---|
| 1 (g/dL) | 0.08 ± 0.05 | 0.1 | 0.0 | 0.2 | 0.07 (0.02–0.11) | 0.09 (0.03–0.16) |
| 1 (%) | 2.18 ± 1.43 | | | | | |
| 2 (g/dL) | 0.06 ± 0.02 | 0.1 | 0.1 | 0.1 | 0.05 (0.04–0.07) | 0.07 (0.04–0.09) |
| 2 (%) | 1.77 ± 0.58 | | | | | |
| 3 (g/dL) | 0.58 ± 0.18 | 0.5 | 0.3 | 1.2 | 0.54 (0.39–0.69) | 0.63 (0.41–0.84) |
| 3 (%) | 17.05 ± 3.57 | | | | | |
| 4 (g/dL) | 2.51 ± 0.50 | 2.6 | 1.1 | 3.8 | 2.39 (0.43–1.81) | 2.64 (2.03–3.24) |
| 4 (%) | 737.00 ± 4.13 | | | | | |
| 5 (g/dL) | 0.19 ± 0.06 | 0.2 | 0.1 | 0.4 | 3.52 (2.97–4.07) | 3.57 (2.79–4.35) |
| 5 (%) | 5.49 ± 1.46 | | | | | |

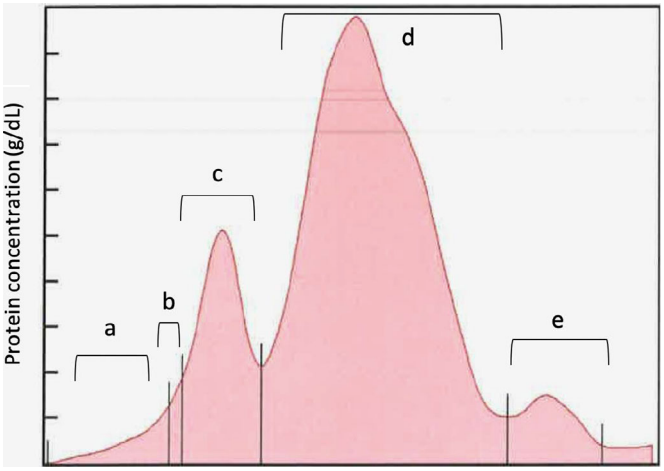


FIGURE 1. A representative serum protein electrophoretogram of wild-caught Nurse Sharks. The following five fractions were identified in this study: (a) fraction 1 (albumin), (b) fraction 2 (alpha 1-globubin), (c) fraction 3 (alpha 2-globulin), (d) fraction 4 (gamma-globulin), and (e) fraction 5 (beta-globulin).

Cray et al. 2015). Fractions 2 and 3 adhered to an ill-defined and small fraction 2 that increased in protein concentration throughout the fraction, as well as a medium, thin peak in fraction 3 that has been observed in other elasmobranch species (Cray et al. 2015). In birds, reptiles, and mammals, fraction 3 is thought to contain alpha-2 macroglobulin, hapto-globulin, and ceruloplasmin; however, the proteins found in fraction 3 in elasmobranchs have yet to be determined (Hyatt et al. 2016). In Hyatt et al. 2016, fraction 3 decreased in Bonnetheads exhibiting inflammation. A change in the fraction 3 protein concentration in Nurse Sharks may be clinically relevant. The sharks sampled in this study were observed to have a consistent medium, high peak in fraction 3. Future studies on Nurse Sharks should include researching whether changes in fraction 3 occur in animals that are either ill or injured or whether differences in this fraction are observed in animals living in different habitats where poor water quality or other stressors are present.

In previous studies on elasmobranchs, fraction 4 has been defined with one or two peaks associated with low-density lipoproteins and very low-density lipoproteins (Metcalf and Gemmell 2005). In this study, Nurse Shark beta-globulin fraction concentrations consisted of one broad peak. In mammals, the majority of the beta-globulin fraction is composed of transferrin and beta-lipoproteins, but can also hold immunoglobulins, complement proteins, and acute phase proteins, such as C-reactive protein (O'Connell et al. 2005). This fraction may be beneficial to analyze as a biomarker of wild shark health. In Whitespotted Bamboo Sharks *Chiloscyllium plagiosum*, beta-globuin protein fractions were found to be higher in females than in males (Krol et al. 2014), but this was not observed for Nurse Sharks in this study. In abnormal Bonnetheads, the ratio of fraction 3 to fraction 4 was significantly lower with a mean of 0.50 than that of normal Bonnetheads with a mean of 0.78 (Hyatt et al. 2016). In comparison, the mean Nurse Shark fraction 3 to fraction 4 ratio (0.23) was much lower (range: 0.09–0.38). The ratio between fractions 3 and 4 may prove to be a clinical marker in Nurse Sharks as it is with Bonnetheads, but further research is required within species and across elasmobranchs. The clinical evaluation of changes in both fractions 3 and 4 should be investigated in future studies.

In other elasmobranch species, fraction 5 in the mammalian gamma-globulin fraction has been hypothesized to contain the immunoglobulin M response (Cray et al. 2015). In Hyatt et al. 2016, the abnormal Bonnetheads

TABLE 3. Serum TP and TS of Nurse Sharks caught in South Florida ($n = 45$). The TP and TS were not normally distributed, and no outliers were removed. Values in parentheses represent the 90% CI; LRL = lower reference limit; URL = upper reference limit; and SF = serum fraction.

| Parameter (g/dL) | Mean ± SD | Median | Minimum | Maximum | LRL | URL |
|---|---|---|---|---|---|---|
| Total serum protein | 3.41 ± 0.64 | 3.4 | 1.6 | 5.5 | 2.25 (1.70–2.80) | 3.57 (2.79–4.35) |
| Total serum solids | 4.68 ± 1.29 | 5.0 | 1.1 | 6.8 | 4.38 (3.27–5.48) | 4.99 (3.44–6.53) |

exhibited an increased fraction 5, potentially due to an active immune response. The fraction 5 RI that was determined from the Nurse Sharks in this study was slightly higher than that observed in free-range Atlantic Sharpnose Sharks, Bonnetheads, and Spiny Dogfish (Haman et al. 2012). This may suggest that Nurse Sharks in this study had a slightly elevated level of immune reactivity. This may also be due to differences in species, resource consumption, or environmental conditions (Martin 2009). Differences within the same species for populations that are free ranging versus captive may exist for fraction 5 concentrations based on their relative health and immune activity, given various levels of exposure to different biotic and abiotic factors (Percin and Konyalioglu 2008).

The TS and TP were significantly different, which has been shown in other elasmobranch species and reaffirms that method-specific intervals should be used in each instance (Haman et al. 2012). It is likely that concentrations of urea and other metabolites in the blood may increase the measurement of TS in elasmobranchs (Harms et al. 2002). For this reason, TP may be a more accurate measure for assessing blood proteins and should be utilized when determining serum EPH fraction concentrations in elasmobranch studies in the future.

Differences between the sexes were nonsignificant across length, body condition, and serum EPH fraction concentrations. All individuals sampled were mature and sampled across 3 years. Reproductive cyclicity may cause significant differences in the sexes including higher levels of beta-globulins in females than in males (Krol et al. 2014), but females in this study did not have elevated levels of beta-globulins. The reproductive statuses of females in this study were not known.

This study provides important baseline serum EPH fraction concentration RIs that can aid in the monitoring of Nurse Shark populations in South Florida and elsewhere. Establishing RIs for various clinical parameters in wild sharks may provide an opportunity to effectively assess these populations. Future studies should assess deviations from the serum EPH fraction concentration RI baselines represented here to evaluate sick or abnormal Nurse Sharks. It will also be beneficial to examine Nurse Sharks from other locations to determine if these intervals are consistent across populations and differing habitat qualities, the latter of which may arise from various anthropogenic factors such as pollution and poor water quality. Studies on wild sharks may also provide valuable information for clinical investigations of Nurse Sharks under human care. With increased sampling and collaboration, it is possible to improve our understanding of healthy shark populations and establish standardized ways to monitor them effectively.

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