Establishment of reference intervals for plasma protein electrophoresis in Indo-Pacific green sea turtles, *Chelonia mydas*

Mark Flint¹²*, Beren J. Matthews², Colin J. Limpus³ and Paul C. Mills²

¹School of Forest Resources and Conservation, University of Florida, The Florida Aquarium’s Center for Conservation, Apollo Beach, FL 33572, USA
²Veterinary–Marine Animal Research, Teaching and Investigation Unit, School of Veterinary Science, The University of Queensland, Gatton Campus, QLD 4343, Australia
³Department of Environment and Heritage Protection, Dutton Park, QLD 4102, Australia

*Corresponding author: School of Forest Resources and Conservation, University of Florida, The Florida Aquarium’s Center for Conservation, Apollo Beach, FL 33572, USA. Tel: +1 813 419 4917. Email: flintm@ufl.edu

Biochemical and haematological parameters are increasingly used to diagnose disease in green sea turtles. Specific clinical pathology tools, such as plasma protein electrophoresis analysis, are now being used more frequently to improve our ability to diagnose disease in the live animal. Plasma protein reference intervals were calculated from 55 clinically healthy green sea turtles using pulsed field electrophoresis to determine pre-albumin, albumin, α-, β- and γ-globulin concentrations. The estimated reference intervals were then compared with data profiles from clinically unhealthy turtles admitted to a local wildlife hospital to assess the validity of the derived intervals and identify the clinically useful plasma protein fractions. Eighty-six per cent (19 of 22 [95% confidence interval (CI) 65–97]) of clinically unhealthy turtles had values outside the derived reference intervals, including the following: total protein [six of 22 turtles or 27% (95% CI 11–50%)], pre-albumin [two of five, 40% (95% CI 5–85%)], albumin [13 of 22, 59% (95% CI 36–79%)], total albumin [13 of 22, 59% (95% CI 36–79%)], α- [10 of 22, 45% (95% CI 24–68%)], β- [two of 10, 20% (95% CI 3–56%)], γ- [one of 10, 10% (95% CI 0.3–45%)], and β–γ-globulin [one of 12, 8% (95% CI 0.2–38%)] and total globulin [five of 22, 23% (8–45%)]. Plasma protein electrophoresis shows promise as an accurate adjunct tool to identify a disease state in marine turtles. This study presents the first reference interval for plasma protein electrophoresis in the Indo-Pacific green sea turtle.

Key words: *Chelonia mydas*, clinically unhealthy, electrophoretogram, green sea turtle, plasma protein electrophoresis, reference intervals.

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Introduction

The Indo-Pacific green sea turtle (*Chelonia mydas*, Linnaeus 1758) is one of six species of marine turtles inhabiting waters along the Queensland coast. Nationally this species is listed as vulnerable and globally as endangered, with population numbers in a state of flux throughout the world (Chaloupka and Limpus, 2001). The reasons behind such fluctuations are poorly understood, but disease, climate change, bycatch, depredation and habitat loss may all play roles in jeopardizing population stability. For example, each year ~400 green sea turtles are reported dead or stranded along the coastline of Queensland (Greenland and Limpus, 2008a,b), and during periods of environmental catastrophe, such as in 2010–11...
when a cyclone and widespread flooding swamped Queensland, elevated levels of stranding and mortality are reported (Limpu et al., 2012). Some of the stranded animals are rescued and transferred to rehabilitation facilities for treatment. Turtles suspected of infection or disease require clinical diagnostics to identify the disease process accurately, with analysis of blood proving particularly useful for diagnosis (Flint et al., 2010a, 2011; Whiting et al., 2014) and to aid in the selection of appropriate secondary tests (Flint, 2013), including imaging and plasma protein electrophoresis (PPE; Gicking et al., 2004; Valente et al., 2006, 2007a, b).

A number of recent studies have reported reference ranges for haematological and biochemical parameters in marine turtle blood (Deem et al., 2006; Flint et al., 2010a, b), but there has been limited research into the usefulness or validity of other diagnostic tools, such as PPE, as there has been in other diagnostic fields, such as human medicine (Anderson and Anderson, 2002). To date there are no published reference intervals available for normal protein fractions in healthy Indo-Pacific green sea turtles. Plasma protein electrophoresis offers a useful diagnostic tool to clinicians by enabling the expression of protein fractions that can be assessed for patterns known to occur in specific disease syndromes. This is advantageous over standard haematology and blood biochemistry in its ability to do this and to discern chronic and acute inflammatory responses, nutritional disorders and reproductive anomalies.

Plasma proteins are an important molecular group of circulating proteins necessary for maintaining normal physiological function. They are responsible for controlling osmotic pressure across membranes, facilitating transport of ions, hormones and compounds around the body and initiating immunological responses. Abnormalities in these proteins, namely albumin, α-, β- and γ-globulins, are indicative of disease processes in both human and veterinary medicine (Bolten and Bjorndal, 1992; Cray et al., 1995; Cray and Tatum, 1998; Tatum et al., 2000; Anderson and Anderson, 2002; Zaias and Cray, 2002; Gicking et al., 2004; Deem et al., 2006), particularly those associated with acute and chronic inflammatory responses.

Plasma protein electrophoresis reference ranges have been reported for three sea turtle species: the loggerhead (Caretta caretta), green and leatherback (Dermochelys coriacea; Cray et al., 2001; Gicking et al., 2004; Deem et al., 2006, 2009). The methodologies used for fraction differentiation in these studies were reported as means and standard deviations and did not necessarily use an a priori or partitioning approach, which have limitations in producing robust reference ranges when dealing with wildlife populations of unknown selection criteria when compared with human approaches (Kjelgaard-Hansen, 2010).

The aim of the present study was to develop robust reference intervals for PPE in three age classes (small immature, large immature and mature) for both sexes (male and female) of Indo-Pacific green sea turtles, resident in the foraging grounds of Moreton and Shoalwater Bays, Queensland. These reference ranges were then used to identify protein fraction abnormalities in unhealthy turtles, in conjunction with other clinical diagnostic tests, for future use to diagnose clinical ill health as a primary tool.

Materials and methods

Sample size, capture technique and turtle selection

Sixty plasma samples were randomly selected as a subset from 211 healthy green sea turtles sampled as part of an ongoing disease surveillance programme in Moreton (27°20′S, 153°23′E) and Shoalwater Bays (22°20′S, 150°12′E) between June 2007 and July 2008. For both study sites, the previously documented ‘rodeo technique’ (Limpus, 1978) was used to capture turtles, which were then transported to the research vessel or shore, respectively, for each study site, for assessment and sampling. All turtles sampled were clinically assessed as ‘healthy’ based on previously determined criteria (Flint et al., 2010a). Biological and morphometric data were collected, including species, size (curved carapace length), body weight, sex, age class and reproductive status (Flint et al., 2010a). Ten plasma samples from each age class (small immature, large immature and mature) of both sexes (male and female) were analysed as described below.

Blood collection and sample preparation

Ten millilitres of whole blood was collected via the dorsal cervical sinus (Owens and Ruiz, 1980) into lithium heparin-coated vacutainer tubes (BD Vacutainer®), centrifuged (Hettich EBA-20, Hettich Zentrifugen) at 400g for 3 min, the plasma separated and frozen at −20°C prior to storage at −80°C, as per previously described methods (Flint et al., 2010a). The duration of storage at −70°C varied from 6 to 18 months from the time of collection. Samples were slowly thawed to room temperature before analysis was performed.

Sample analysis

Total plasma protein concentration was determined as previously described (Gicking et al., 2004). In brief, each sample was assessed by the biuret method using a calibrated auto-analyser (Olympus AU400, Olympus). Agarose gel electrophoresis was performed on each sample using commercial Helena Laboratories TITAN GEL Serum Protein System kits (Helena Laboratories, Beaumont, TX, USA). A 3.6 μl sample was diluted 1:4 in buffer, loaded onto the gel using the manufacturer-supplied template and the gel run at 120 V for 15 min. The gel was then fixed, stained and dried ready for scanning. The gels were scanned using a laser densitometer (Helena Laboratories Electrophoresis Data Center, Beaumont, TX, USA) at a wavelength of 595 nm to determine plasma protein fractions.

Traces were digitized and analysed to determine where appropriate fraction demarcation should occur. The method...
Reference interval calculations were conducted on 55 of 60 samples analysed. Five samples were identified as outliers on examination of protein data and were excluded from subsequent analyses because they were found to have total protein values outside relevant published reference intervals (Flint et al., 2010a). These excluded animals included one small immature female, one small immature male, two mature females and one mature male. Their removal was not considered to skew the sex or age distribution of the analysed data set.

Previous studies on blood parameters of green sea turtles in Australian waters, including plasma albumin and globulin level reference values, showed that geographical location did not influence the resultant interval (Flint et al., 2010a). Data sets for both sites (Moreton Bay and Shoalwater Bay) were combined for statistical analysis and reference interval calculations.

The distributions of the values for each fraction were assessed using Stata version 10 (StataCorp, College Station, TX, USA). Not all variables were normally distributed; therefore inter-quartile ranges were used to determine whether separate reference intervals were required for different subgroups (sex and age class) for each variable. For each pairwise comparison, the differences between the medians and the width of the inter-quartile ranges were expressed as a proportion of the smaller value. Previously described rules (Flint et al., 2010a) were used to determine whether separate reference intervals were required. Samples were pooled if the difference between the medians was <50% and difference between the inter-quartile ranges was <100% or if the difference between the medians was between 50 and 100% and the inter-quartile range difference was <50%. All other subgroups were analysed separately.

Reference intervals were calculated using previous defined methods (Flint et al., 2010a). In brief, 95% reference intervals and associated 90% confidence intervals (CIs) for the limits of each interval were estimated using two software programs, Reference Interval software (RIS; Pesce et al., 2005) and RefVal 4.11 software (Solberg, 2006). When using RIS, the 95% reference intervals were calculated using an Excel spreadsheet (Microsoft, Redmond, WA, USA) for each variable. Data were transformed to approximate a normal distribution using the Box–Cox transformation and outliers identified for exclusion from subsequent calculations (Horn et al., 2001; Horn and Pesce, 2005). Associated 90% CIs for the upper and lower limits of the reference intervals were estimated using bootstrapping, with 1000 replications for RIS and 500 for RefVal. This approach to data analysis eliminates the inherent uncertainty of resultant reference intervals by creating a consistent chain of decisions (Kjelgaard-Hansen, 2010).

Reference intervals reported are from RIS unless the lower or upper limit differed between the two methods by >10%, in which case both RIS and RefVal intervals are reported.

Comparison of values from unhealthy turtles with derived reference intervals

To determine whether the calculated reference intervals were applicable for diagnostic purposes, PPE results from 21 clinically unhealthy green turtles were compared with the derived ranges. The Australian Wildlife Hospital routinely undertakes electrophoresis analysis of samples collected from green sea turtles presented for rehabilitation. Clinical histories, including complete blood biochemical and haematological results, from 21 individual turtles with PPE analysis carried out between January 2008 and February 2009 were provided. One turtle had consecutive samples analysed 2 months apart, giving a total of 22 samples for comparison.

Two methods of comparing the derived reference intervals with the clinically unhealthy turtle results were used. The first involved tabulating the data provided for each protein fraction and directly comparing the proportions of animals with values outside the reference intervals developed in this study. The second involved visually re-evaluating the electrophoreogram to ensure that protein fractionation was consistent with

![Figure 1: Template developed to determine where protein fractions transpire; values are given as a percentage of the horizontal axis of the electrophoreogram. The differentiation between β and γ fractions is often not determined and is thus represented by a dotted line.](https://academic.oup.com/conphys/article-abstract/3/1/cov037/2571258/3)
The methods described in this article. The same assessor interpreted all traces in an attempt to reduce operator error. Proportions of clinically unhealthy turtles with values outside the presented reference intervals were calculated for both methods. Exact binomial 95% CIs were then calculated for each proportion using Stata version 10 (StataCorp, 2007).

**Results**

The analysis of sea turtle plasma by electrophoresis identified pre-albumin, albumin, α-, β- and γ-globulin protein fractions. The most frequently identified fractions were pre-albumin \((n = 54)\), albumin \((n = 55)\) and α \((n = 55)\). Distinction of the β and γ fractions was not possible in 71% of samples (Fig. 2a and b).

The reference intervals calculated are reported in Table 1. The rules identified that separate reference intervals be calculated for each age class for pre-albumin and β fractions. The small number of samples with a distinguishable β fraction in each age class [small immature \((n = 6)\), large immature \((n = 6)\) and mature \((n = 4)\)] did not provide sufficient data for bootstrapping; therefore, the reference intervals

![Figure 2](https://academic.oup.com/conphys/article-abstract/3/1/cov037/2571258)
without an associated 95% CI are presented for this fraction. Otherwise, data sets were grouped.

Presenting pathologies for the clinically unhealthy turtles included anaemia (six of 22), disease (three of 22), emaciation (three of 22), fishing interaction (linear foreign body; three of 22), impaction (four of 22), positive buoyancy (nine of 22), respiratory (one of 22) and trauma (four of 22). A large proportion (86%) of the unhealthy turtles had values outside the reference intervals presented, as follows: total protein [six of 22 turtles or 27% (95% CI 11–50%)], pre-albumin [two of five, 40% (95% CI 5–85%)], albumin [13 of 22, 59% (95% CI 5–85%)], total albumin [13 of 22, 59% (95% CI 5–85%)], α [10 of 22, 45% (95% CI 24–68%); Fig. 2c], β [two of 10, 20% (95% CI 3–56%)], γ [one of 10, 10% (95% CI 0.3–45%)], β–γ-globulin [one of 12, 8% (95% CI 0.2–38%)] and total globulin [five of 22, 23% (95% CI 8–45%)]. From this data set, there appeared to be an association with presenting presumptive diagnosis and abnormal protein fractions for half of all categorized syndromes with elevated α protein, and more than two-thirds of cases of disease with elevated γ protein and linear foreign body cases with elevated albumin protein fractions (Table 2).

**Discussion**

This article presents the first reference interval for plasma protein fractions in green sea turtles in Australian waters using robust analysis techniques not previously applied to turtle PPE. It demonstrated that sex and age classes may be combined for most examined fractions and compared favourably with studies of samples from other regions analysed in a similar manner (Page-Karjian et al., 2014). Previously reported PPE values were limited to mean and standard deviation, which does not necessarily express a robust clinically relevant reference interval (Cray et al., 2001; Gicking et al., 2004; Deem et al., 2006; Kjelgaard-Hansen, 2010). The present study demonstrated that the derived reference intervals may be used successfully to identify changes in protein fractions in clinically unhealthy turtles and thus aid accurate diagnosis.

This study had limitations; for instance, the electrophoresis process may have benefited from refined methodologies (e.g. serial dilutions and different pulsations) specifically designed for reptiles, improved resolution, and samples were thawed, refrozen and stored for several months prior to analysis. Resultant ranges did not appear to be affected and were

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**Table 1:** Plasma protein reference intervals for healthy green sea turtles (*Chelonia mydas*) of Moreton and Shoalwater Bays sampled between 2007 and 2008

| Variable | Class | Number of turtles | 95% Reference interval (90% confidence interval) |
|----------|-------|-------------------|-----------------------------------------------|
|          |       |                   | Lower limit | Upper limit |
| Total protein | 194   | 20.8 (18.5, 23.5) | 62.1 (60.7, 63.7) |
| Pre-albumin | Small immature | 18 | 0.89 (0.7, 1.0) | 4.26 (2.8, 5.3) |
|            | Large immature | 20 | 0.36 (0.0, 0.6) | 4.23 (3.5, 4.7) |
|            | Mature | 16 | 0.75 (0.0, 1.1) | 2.64 (2.4, 2.9) |
| Albumin   | 55    | 10.78 (9.6, 11.9) | 23.29 (22.2, 24.2) |
| Total albumin | 55 | 12.58 (11.6, 13.6) | 25.24 (24.2, 26.2) |
| α         | 55    | 3.97 (3.6, 4.6) | 11.05 (10.2, 11.8) |
| β         | 16    | 1.38 (0.0, 3.8) | 10.98 (9.6, 12.3) |
| β–γ       | Small immature | 6 | 0.00 | 5.17 |
|           | Large immature | 6 | 0.00 | 9.05 |
|           | Mature | 4 | 0.00 | 13.15 |
| γ         | 16    | 2.80 (0.0, 6.9) | 17.90 (15.5, 19.8) |
| β–γ       | 55    | 8.31 (5.9, 10.8) | 26.07 (24.9, 27.3) |
|           |       | 7.11 (7.01, 11.38)* | 26.32 (23.93, 27.49)* |
| Total globulin | 55 | 10.74 (6.4, 13.8) | 35.51 (33.6, 37.1) |
|           |       | 11.90 (10.80, 18.30)* | 33.20 (31.80, 34.00)* |

*a*All reference intervals are given in grams per litre. 
*b*All reference intervals are from Reference Interval software other than those indicated by an asterisk (*), which are from RefVal. 
*d*Derived from larger data set results (Flint et al., 2010a). 
*Separate β reference interval calculations are reported without bootstrapping or confidence intervals because of limited numbers.*
The technique described in this study whereby band fractions were identified using the template developed provided a clearer, more repeatable method of separating different fractions than the densitometer method used for previous studies (Cray et al., 2001; Gicking et al., 2004; Deem et al., 2006, 2009). Overlaying normal profiles demonstrated that similar bands existed on the majority of examined gels. The position, shape and size of these fractions were consistent with fractions reported in the literature (Gicking et al., 2004). The presence of a pre-albumin fraction was observed in the majority of our traces, in contrast to a previous study in which only one of 41 turtles exhibited this fraction (Gicking et al., 2004). However, it is still a subjective interpretation, and quantification of the exact protein composition of each identified band would require amino acid sequencing.

Of the 55 samples analysed from clinically healthy turtles in the present study, visual examination of the electrophoreograms revealed that a number \( n = 20 \) differed from what would be expected in a healthy specimen trace. Healthy animals had values outside of range but within acceptable limits when the results were assessed statistically for type I and type II error detection using the reference calculation tools. Arguably, the visual method may be elucidating subtle sub-clinical changes that are washed out using statistical methods.

We conducted a comparison between our reference interval, derived from clinically healthy turtles, with 21 unhealthy animals (producing 22 examined samples) with conditions ranging from trauma to disease syndromes presented to Australian Wildlife Hospital between 2007 and 2009. Visual examination of the electrophoreogram traces from the unhealthy animals suggested that 86% (19 of 22) were outside our reference intervals in at least one protein fraction. Re-evaluation of the electrophoreogram using the statistical method showed an area of uncertainty between distinctly healthy and unhealthy animals (Table 1). This suggested that the interpretation method used in the present study to describe where fractions bands occur may better defined as having a band of uncertainty with respect to whether a turtle should be classified as healthy or unhealthy, and may represent subclinically diseased animals within the population. This finding is in accordance with other blood parameter studies in marine turtles (Flint et al., 2010b). Elevations in the \( \alpha \) fraction were the most common for any pathology. Albumin and \( \gamma \) fraction anomalies were associated with linear foreign bodies and disease, respectively, and may be useful in diagnosing these conditions if the correlation between fraction and disease is proved to be medically relevant (Table 2).

Little is known about sea turtle physiology and molecular biochemistry. Consequently, much of the understanding of how proteins function in sea turtles is extrapolated from avian and mammalian species (Gicking et al., 2004). While we have demonstrated that there is likely to be an association between clinical ill health and altered protein profiles, a more comprehensive understanding of how various disease states manifest in clinical diseased turtles is required to improve PPE interpretation and see its diagnostic value. A study of unhealthy turtles that have been definitively diagnosed with a singular disease syndrome is necessary to gain a more complete understanding of how plasma protein fractions respond to disease physiology in marine turtles. Furthermore, it may also be useful to investigate use of this tool to characterize inflammatory proteins in green sea turtles and develop monoclonal reagents against the turtle proteins to help chart the inflammatory response in this species better.

This study produced the first comprehensive reference intervals for plasma protein fractions in Indo-Pacific green sea turtles. This testing modality has great potential for aiding the diagnostic process when used in conjunction with other routine tests, such as biochemical and haematological profiles; however, further research is needed to explore the association between protein fractions in health and disease using electrophoresis in sea turtles to reduce potential confounding factors.

### Table 2: Counts of plasma protein fractions outside of determined reference intervals for unhealthy green sea turtles (Chelonia mydas) used in this study for each presumptive diagnosis

| Presumptive diagnosis | Number of cases | Number of cases (%) outside of determined reference range for each protein fraction |
|-----------------------|----------------|---------------------------------------------------------------------------------|
|                       |                | Pre-albumin | Albumin | \( \alpha \) | \( \beta \) | \( \gamma \) |
| Anaemia               | 6              | 1 (17)     | 3 (50)  | 2 (33)     | 1 (17)     | 0            |
| Disease               | 3              | 0          | 1 (33)  | 3 (100)    | 1 (33)     | 2 (67)       |
| Emaciation            | 3              | 0          | 1 (33)  | 2 (67)     | 1 (33)     | 1 (33)       |
| Impaction             | 4              | 0          | 1 (25)  | 1 (25)     | 2 (50)     | 1 (25)       |
| Linear foreign body   | 3              | 0          | 3 (100) | 2 (67)     | 0          | 0            |
| Positive buoyancy     | 9              | 1 (11)     | 2 (22)  | 6 (67)     | 3 (33)     | 2 (22)       |
| Respiratory           | 1              | 1 (100)    | 1 (100) | 0          | 0          | 0            |
| Trauma                | 4              | 0          | 2 (50)  | 1 (25)     | 0          | 1 (25)       |

* A turtle may have more than one presumptive diagnosis, and fractions may occur more than once.
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