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Hematologic and biochemical characteristics of stranded green sea turtles

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Abstract. To improve understanding of pathophysiologic processes occurring in green sea turtles (*Chelonia mydas*) stranded along the east coast of Australia, we retrospectively examined the hematologic and biochemical blood parameters of 127 green turtles admitted to 2 rehabilitation facilities, Dolphin Marine Magic (DMM) and Taronga Zoo (TZ), between 2002 and 2016. The predominant size class presented was small immature animals (SIM), comprising 88% and 69% of admissions to DMM and TZ, respectively. Significant differences in blood profiles were noted between facility, size, and outcome. Elevated levels of aspartate aminotransferase (AST) and heterophils were poor prognostic indicators in animals from TZ, but not DMM. SIM animals at both institutions had lower protein levels than large older (LO) animals. SIM animals at DMM also had lower hematocrit and monocyte concentration; SIM animals at TZ had lower heterophil counts. Urea was measured for 27 SIM animals from TZ, but the urea-to-uric acid ratio was not prognostically useful. Strong correlations were seen between AST and glutamate dehydrogenase (GDH; $r = 0.68$) and uric acid and bile acids ($r = 0.72$) in the 45 SIM animals from DMM in which additional analytes were measured. χ^2 contingency tests showed that the most recently published reference intervals were not prognostically useful. A paired *t*-test showed that protein levels rose and heterophil numbers fell in the 15 SIM animals from TZ during the rehabilitation process. Our results indicate that further work is required to identify reliable prognostic biomarkers for green turtles.

Key words: Anemia; biochemistry; cachexia; green sea turtles; hematology; immunosuppression; kidney; liver.

Introduction

The green sea turtle (*Chelonia mydas*) is 1 of 7 species of marine turtles found in circumtropical regions.[8] Hunting these animals for their meat and harvesting their eggs has seen green turtle populations decline drastically.[18] More recent anthropogenic threats include entanglement in fishing gear,[9] marine debris,[30,31] and ocean climate change.[20] In response to declining populations, green turtles and the beaches on which they nest are protected in many countries, and global conservation priorities have been established.[35] Following the introduction of these protections, some populations have demonstrated promising signs of recovery.[6]

Despite protection from key threats, there has been an increase in the reporting of disease in green sea turtles.[36] These diseases include a range of infectious and non-infectious conditions such as fibropapillomatosis,[11] spirorchidiasis,[17] coccidiosis,[22] gastrointestinal disorders,[17] cachexia, and a condition described as "hepatorenal insufficiency."[14] Many of the infectious pathogens involved in these diseases have been present in the marine environment for millions of years.[23] Environmental factors may be contributory in places where the incidence of disease is higher (e.g., polluted environments).[3,34]

To optimize green sea turtle rehabilitation and characterize disease, there is a need to accurately assess the health of individual stranded animals and ascertain the causal factors associated with these stranding events. To date, health assessment techniques are subjective, given that there is poor correlation between clinical signs and histologic disease,[17] and that quantitative assessment of body condition appears to be inconsistent with the subjective appearances of health.[16] To

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address the shortcomings associated with clinical examination, numerous studies have examined hematologic and biochemical analytes in free-ranging green turtles, resulting in the creation of reference intervals (RIs) to define healthy individuals; however, the resulting RIs can differ in different parts of the world.[2,7,16,28] Other modalities such as plasma protein electrophoresis[13,15,24] and assessment of the correlation between survival and biochemical analytes in sea turtles undergoing rehabilitation are being investigated.[25]

We examined archived hematologic and biochemical data from 2 rehabilitation hospitals over 14 y to investigate 1) the prognostic reliability of the most recently published local RIs for this species; 2) pathophysiologic changes within stranded animal blood profiles, and 3) changes that occurred within these blood profiles during rehabilitation.

Materials and methods

All data examined in our study were analyzed retrospectively, following the collection of samples by veterinarians, acting under the authority of the Veterinary Practice Act 2003 (https://goo.gl/k54Shx). The collection of samples was part of routine clinical investigation to assess the health of stranded green sea turtles admitted to rehabilitation hospitals at Dolphin Marine Magic (DMM; Coffs Harbour, NSW, Australia) and Taronga Zoo (TZ; Sydney, NSW), between 2002 and 2016.

All of the turtles in our study were considered clinically "unhealthy" at the time of presentation, based on their appearance and behavior. Blood samples were collected from the dorsal occipital sinus of each turtle within 7 d of presentation, following previously described methods using a 5-mL syringe with a 21-gauge, 2.5-cm needle.[27] Blood samples were transferred into sterile vacutainers containing lithium heparin and refrigerated until analysis. All analyses occurred within 24 h of sample collection. Each sample was assessed during collection for any evidence of lymphatic contamination, and if such contamination was suspected, the sample was discarded and recollected.

Blood samples collected from DMM were submitted to Laverty Vetnostics Pathology (North Ryde, NSW), a National Association Testing Authorities–accredited commercial pathology laboratory. Samples collected from TZ were analyzed on-site at the Taronga Wildlife Hospital. The same staff was present at both laboratories for the duration of the study.

Both facilities used a microhematocrit centrifuge to measure hematocrit (Hct) following centrifugation (5 min, 6,000 × *g*). Blood smears were made from the heparinized blood within 1 h of collection, and the total white blood cell (TWBC) and differential WBC counts were calculated.[33] For samples collected at DMM, this was achieved via a leukocyte estimate and differential from the blood smear,[19] and for samples collected at TZ, via an improved Neubauer hemocytometer.[26] Where anemia was noted, red blood cell morphology and reticulocyte counts were performed to classify the anemia as regenerative or nonregenerative. The instrumentation used for the biochemical analysis varied between laboratories. To measure uric acid, glucose, aspartate aminotransferase (AST), protein, and creatine kinase (CK), samples from DMM were analyzed by 1 of 2 analyzers (Modular [P modules], Modular Evo [P modules], Roche Diagnostics, Basel, Switzerland; Cobas 8000 [c502 and c702 modules], Roche Diagnostics) as equipment was upgraded partway through the study. Samples collected from TZ were analyzed in-house (VetScanVS2 analyzer, REM Systems, Sydney, Australia). Urea was only measured from TZ samples (Reflotron, DTS Diagnostics, Sydney, Australia).

Permutational analysis of variance (PERMANOVA)[4] across blood profiles demonstrated significant mean differences between laboratories ($p < 0.01$; Fig. 1). All further analysis was conducted separately on data from DMM and data from TZ. Based on the distribution of size, data were divided into previously described age classes based on curved carapace length (CCL). Animals with CCL <65 cm were considered small immature (SIM) animals, and animals with a CCL >65 cm were considered large older (LO) animals for further analysis.[17]

PERMANOVA multivariate analyses using 2 orthogonal factors (size class: SIM vs. LO; outcome: released vs. deceased) were used to compare the similarity of blood profiles between factors for each facility. PERMANOVA univariate analyses were also undertaken to investigate the significance of specific hematologic and biochemical response variables. Further PERMANOVA analyzes were carried out on 27 SIM turtles from TZ in which urea was measured to test whether the urea-to-uric acid ratio differed with outcome. All PERMANOVA analyses were performed using the software PRIMER 7.0.11 (http://www.primer-e.com/) and used Euclidean distances derived from data that were normalized using the *Normalize Variable* function in the software package.

Hypotheses about the relationships among the analytes that have the potential to arise from more than one tissue were tested with a Pearson correlation coefficient (r) for 45 SIM animals from DMM. This included the analysis of archived results for the additional analytes (alanine aminotransferase, ALT; alkaline phosphatase, ALP; bile acids; cholesterol; and glutamate dehydrogenase, GDH), which were measured on either the Roche Modular Evo or the Roche Cobas 8000 depending on the time period within the study that the samples were analyzed.

We used χ^2 contingency tests to assess the prognostic capacity of the RIs currently accepted as describing healthy green sea turtles in Australian waters[16] by evaluating whether the proportion of surviving animals was greater for those within the existing local RIs[16] compared to those outside these RIs for DMM and TZ animals separately. Paired *t*-tests were used to evaluate the hematologic and biochemical changes that occurred during rehabilitation. These analyses included response variables from 15 SIM animals from TZ,

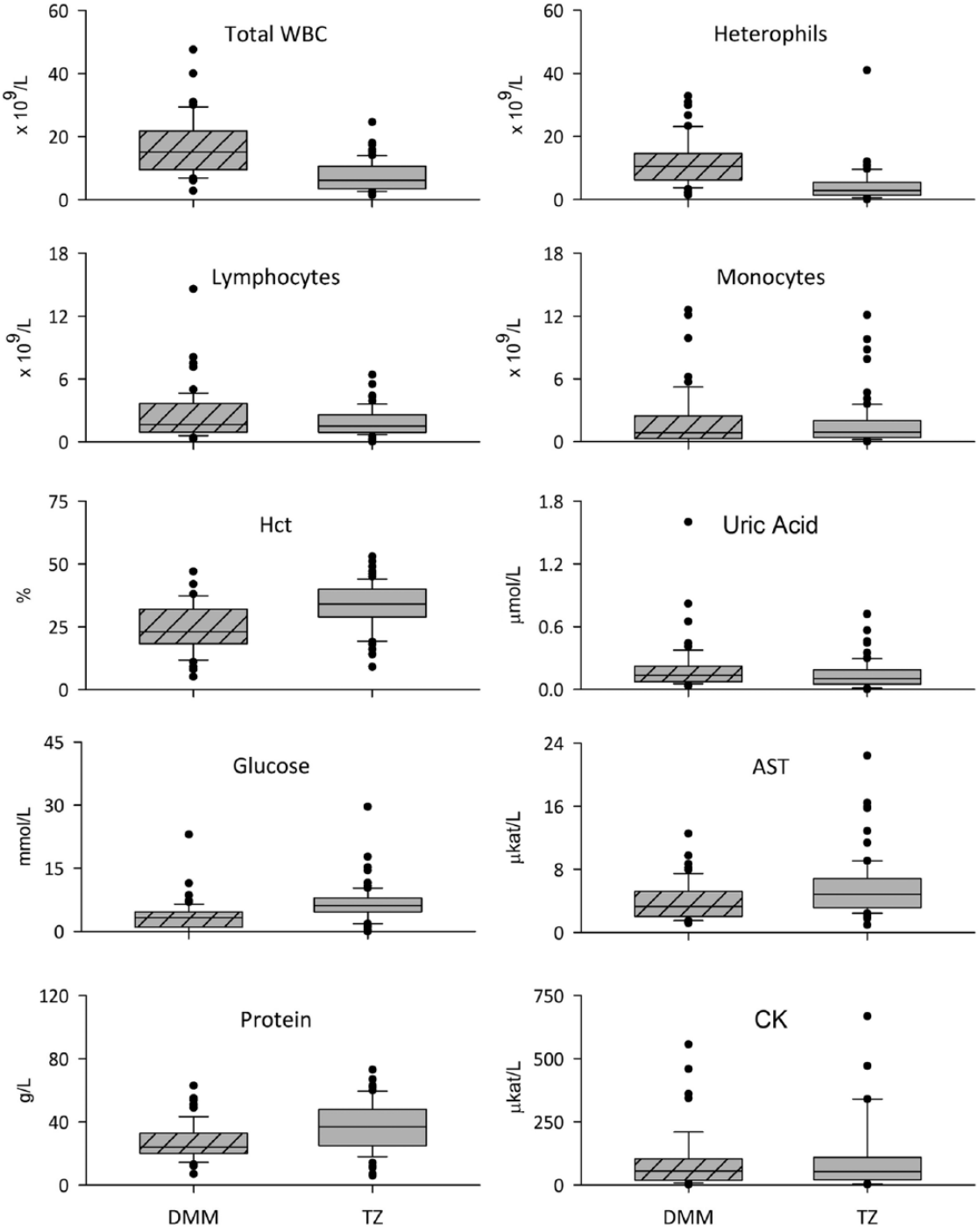

Figure 1. Box and whisker plots of the interquartile range and outliers for each analyte measured at Dolphin Marine Magic (DMM) and Taronga Zoo (TZ).

in which blood was collected and analyzed within the first 7 d of presentation and again after a minimum of 28 d of rehabilitation.

Results

Included in our study were 56 animals from DMM and 71 animals from TZ, with SIM animals representing 88% and 69% of admissions, respectively. The survival rate at DMM and TZ was 65% and 27%, respectively, for SIM animals, and 29% and 18%, respectively, for LO animals. A significant difference ($p < 0.05$) was seen between the blood profiles of released versus deceased animals at TZ (Table 1). Animals that were released initially had lower heterophil counts ($p = 0.02$) and levels of AST ($p = 0.03$). No significant differences regarding outcome were seen at DMM for

Table 1. Hematologic and biochemical results, by green sea turtle size class, outcome, and facility.

	SIM				LO			
	Released		Deceased		Released		Deceased	
	DMM	TZ	DMM	TZ	DMM	TZ	DMM	TZ
n	32	13	17	36	2	4	5	18
TWBC ($\times 10^9$/L)	17.9	6.1	13.7	8.1	19.0	8.0	12.4	8.6
	1.7	*0.9*	*1.7*	*1.0*	*8.0*	*1.7*	*3.4*	*0.9*
Heterophils ($\times 10^9$/L)	13.2	2.8	9.8	4.2	10.2	14.0	5.0	3.9
	1.4	*0.5*	*1.5*	*0.6*	*1.9*	*9.2*	*1.7*	*0.6*
Lymphocytes ($\times 10^9$/L)	2.8	1.3	2.2	1.8	1.5	2.1	2.2	2.3
	0.5	*0.3*	*0.3*	*0.2*	*0.9*	*0.6*	*0.6*	*0.4*
Monocytes ($\times 10^9$/L)	1.4	1.9	1.3	1.7	6.6	0.9	5.3	2.0
	0.3	*1.0*	*0.4*	*0.4*	*5.6*	*0.2*	*2.0*	*0.5*
Hct (L/L)	0.23	0.33	0.24	0.34	0.31	0.31	0.34	0.32
	0.0	*0.0*	*0.0*	*0.0*	*0.1*	*0.0*	*0.0*	*0.0*
Uric acid (μmol/L)	0.2	0.1	0.3	0.2	0.2	0.2	0.2	0.1
	0.1	*0.0*	*0.0*	*0.0*	*0.1*	*0.0*	*0.1*	*0.0*
Glucose (mmol/L)	4.2	5.8	2.2	6.2	5.8	5.9	3.7	7.8
	0.7	*0.8*	*0.5*	*0.9*	*2.9*	*1.4*	*1.4*	*1.1*
AST (μkat/L)	3.2	4.2	5.1	6.6	3.8	2.2	*5.0*	5.1
	0.3	0.7	*0.9*	0.8	*0.4*	*0.7*	*1.3*	0.5
Protein (g/L)	23.9	30.0	24.2	33.3	47.0	40.8	50.8	52.7
	1.5	*1.7*	*1.7*	*2.6*	*7.0*	*6.8*	*4.6*	*3.1*
CK (μkat/L)	74	77	107	156	111	83	168	119
	19	*26*	*24*	*46*	*35*	*34*	*133*	*45*

AST = aspartate aminotransferase; CK = creatine kinase; DMM = Dolphin Marine Magic; Hct = hematocrit; LO = large older turtles; SIM = small immature turtles; TWBC = total white blood cells; TZ = Taronga Zoo. Numbers in italics are 1 standard error of each mean.

multivariate or univariate analyses (Table 2). At both DMM and TZ, SIM animals had lower levels of protein ($p < 0.01$) and ($p < 0.01$), respectively. At DMM, SIM animals also had lower levels of monocytes ($p < 0.01$) and Hct ($p = 0.04$), whereas at TZ, SIM animals had lower heterophil counts ($p = 0.01$). There were no significant interactions between the factors at either facility.

For the 27 SIM animals from TZ in which the urea-to-uric acid ratio was measured, there was no significant difference between animals that lived and animals that died ($p = 0.96$). For the 45 SIM animals from DMM in which additional biochemical analytes were measured, strong relationships where seen between AST and GDH ($r = 0.68$), and uric acid and bile acids ($r = 0.72$; Supplementary Table 1).

χ^2 contingency tests comparing the survival rate of LO animals in and outside of the RIs was not possible because the survival rate of these animals was very low. For SIM animals, there was no significant difference for any analyte in the proportion of animals that died between the turtles within and outside of the RIs currently accepted as describing healthy animals (Supplementary Table 2). During the rehabilitation period, significant increases were demonstrated in protein ($p < 0.01$) and decreases in heterophils ($p = 0.03$) using a paired *t*-test (Table 3) for 15 SIM animals at TZ.

Discussion

The most recently published biochemical and hematologic RIs for green sea turtles in Australia[16] did not provide prognostic information for clinicians. This may be because of the inclusion of animals with subclinical disease in the collection of data from seemingly healthy free-ranging groups, given that previous studies have shown little correlation between the clinical presentation of an animal and the histologic findings at postmortem.[14] Although rigorous statistical techniques have been used to address this problem and eliminate outliers prior to calculating RIs,[16,28] the presence of widespread subclinical disease in a sampled population may not generate outliers. This is supported by the postmortem detection of spirorchidiasis in the majority of Australian green sea turtles,[17] including a prevalence rate of 98% from the site from which the data for the current RIs were collected.[21] Alternatively, the poor prognostic capabilities of the current RIs seen in our study may be the result of secondary disease processes such as immunosuppression and dehydration occurring in parallel, and effectively masking primary disease processes such as infection and/or inflammation and hypoproteinemia and/or anemia, respectively.

Table 2. PERMANOVA multivariate and univariate results for facilities, green turtle size class, and outcome.

		Size		Outcome	
	Facility	DMM	TZ	DMM	TZ
Blood profiles (multivariate)	<0.01	<0.01	0.01	0.49	<0.05
TWBC	<0.01	0.95	0.41	0.33	0.53
Heterophils	<0.01	0.20	0.01	0.25	0.02
Lymphocytes	0.04	0.58	0.15	0.91	0.48
Monocytes	0.57	<0.01	0.75	0.91	0.53
Hct	<0.01	0.04	0.45	0.73	0.73
Uric acid	0.06	0.78	0.46	0.32	0.06
Glucose	<0.01	0.36	0.51	0.08	0.39
AST	<0.01	0.61	0.10	0.23	0.03
Protein	<0.01	<0.01	<0.01	0.69	0.05
CK	0.42	0.44	0.87	0.38	0.46

AST = aspartate aminotransferase, CK = creatine kinase; DMM = Dolphin Marine Magic; Hct = hematocrit; TWBC = total white blood cells; TZ = Taronga Zoo.

Table 3. Hematologic and biochemical results for 15 SIM animals at the Taronga Zoo collected at 7 and 28 d after admission.

	7 d	28 d	*p* value
TWBC ($\times 10^9$/L)	7.7	6.3	0.19
	0.9	*1.1*	
Heterophils ($\times 10^9$/L)	3.3	1.8	0.03
	0.5	*0.3*	
Lymphocytes ($\times 10^9$/L)	2.4	3.4	0.23
	0.5	*0.7*	
Monocytes ($\times 10^9$/L)	1.8	1.1	0.39
	0.8	*0.3*	
Hct (L/L)	0.27	0.29	0.26
	0.0	*0.0*	
Uric acid (μmol/L)	0.1	0.0	0.07
	0.0	*0.0*	
Glucose (mmol/L)	5.7	7.1	0.19
	0.9	*0.4*	
AST (μkat/L)	5.6	6.0	0.82
	0.8	*1.1*	
Protein (g/L)	23.7	37.6	<0.01
	1.8	*2.7*	
CK (μkat/L)	107	52	0.19
	29	*26*	

AST = aspartate aminotransferase; CK = creatine kinase; Hct = hematocrit; TWBC = total white blood cells. Numbers in italics are 1 standard error of each mean.

The observed differences between laboratories may be the result of different analytical techniques or may reflect differing disease processes in green sea turtles regionally. The elevated glucose in TZ animals compared to DMM animals is likely to reflect the increased time between sample collection and sample analysis that would occur in samples from DMM, as they were not analyzed on-site and the lithium heparin vacutainer would not conserve glucose.[12] Given that different techniques were used to assess the TWBC and differential white cell count between facilities, the origin of the observed differences is unclear. However, the significant difference between Hct at each facility, despite the use of the same methodology, may indicate that regional differences do occur. TZ is located adjacent to more temperate waters, and environmental factors such as water temperature may play an important role.

Overall, the occurrence of disease predominantly in SIM animals was consistent with previous studies.[11,17] The reason for the over-representation of this demographic is unknown; previous studies have suggested that it could be the result of increased exposure to parasites because of habitat use or immunological naivety.[17] However, it is also possible that the size structure of the turtles presenting is representative of the local population.[11] SIM animals recruit to the neritic feeding grounds[5] following a pelagic period known as the "lost years,"[29] and it is possible that environmental components could play a role in the development of disease.

Despite the observation of both regenerative and non-regenerative anemias in animals undergoing rehabilitation, the Hct of stranded animals was not significantly associated with survival and did not change significantly during rehabilitation. This finding contradicts previous work that reported lower Hct values in stranded animals compared with that of healthy free-ranging animals (Work T, et al. Causes of green turtle (*Chelonia mydas*) morbidity and mortality in Hawaii. Proc Ann Sea Turtle Symp; March 1997; Orlando, FL). However, there is a marked difference between Hct levels accepted as healthy in Australia[16,37] compared to other regions.[2,7,28] This may be a reflection of the prevalence of spirorchidiasis in Australian waters,[17] given that infection with this parasite in loggerhead turtles (*Carretta caretta*) has been shown to induce lesions histologically similar[38] to the inflammation-associated anemia found in humans infected with schistosomes.[10]

The observed monocytosis in LO animals at DMM, relative to SIM animals, may reflect a different disease process

affecting these animals or differing immunocompetence in the older animals. The bulk of the LO animals in the DMM data set were presented to care during a regionally significant coccidiosis outbreak, and the relative monocytosis in this age class may represent the cell-mediated inflammation that is associated with these acute infections.

The correlation between uric acid and bile acids may represent the presence of concurrent renal insufficiency and compromised hepatic function. This is an observation that had been made and described as hepatorenal insufficiency in a previous study.[14] Compromised cardiac output, as can occur secondary to systemic disease processes such as cachexia, may result in generalized hypoperfusion. This would lead to hepatorenal insufficiency and potentially generate the observed changes. The strong correlation between AST and GDH supports the hypothesis that elevated AST observed in deceased TZ animals is related to hepatic compromise as opposed to skeletal muscle damage. However, care should be taken with this interpretation, given that a temporal lag in the AST elevation after muscle damage could lead to a loss of correlation with CK.

Given the potential for dehydration, the interpretation of protein levels varies in relation to an animal's clinical condition. Hypoproteinemia has been demonstrated in animals that are in poor health.[1,16] This finding is supported by our study, given the significant increase in protein levels that occurred during rehabilitation, which ultimately rose to normal levels.[1,2,7,16,28,32,37]

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Declaration of conflicting interests

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