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Evaluation of hematological and plasma biochemical parameters in green sea turtle (*Chelonia mydas* Linnaeus, 1758) from nesting colonies of the northern coast the Sea of Oman

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Abstract

The green turtle (*Chelonia mydas*) has been a species of global concern for decades. As an attempt to study health status of nesting green turtles, Blood biochemistry and hematological values were obtained from 18 clinically normal, nesting female green sea turtles on the northern coast of the sea of Oman. Mean curved carapace length (CCL) was 111±4 cm with a range of 103—122 cm. Barnacles were recorded on three turtles. No fibropapillomas were observed on any of the 18 turtle. The mean PCV was 0.41 (proportion of 1) with a range of 0.30–0.58. No basophils or hemoparasites were detected in any of the 18 turtles tested. All biochemistry and hematological values of green sea turtles were within published reference ranges of healthy sea turtle population. No statistically significant differences were noted between the two anticoagulants for Plasma biochemistry values. Plasma alpha–tocopherol concentrations in the 18 turtles tested was 7.8±2.8 g ml^{-1} with a range of 0.5-11.8 g ml^{-1}. Plasma retinol was evaluated in 18 turtles and the concentration was 0.4 ±0.1 g ml^{-1} with a range of 0.2-0.6 g ml^{-1}. The present study provides baseline data of the green sea turtle characterized by biological and non-biological factors, which may provide the basis for future studies.

Keywords: Blood, Biochemistry, Hematological, *Chelonia mydas*, Sea of Oman

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Introduction

Marine turtle species are an integral part of oceanic ecosystems throughout the world (Chaloupka *et al.*, 2004). Green turtle is the largest of the hard shelled sea turtles and is the second largest (after *Dermochelys*) of the seven species. Green turtle is a circumglobal species found in tropical and sub-tropical waters (Aguirre and Lutz, 2004; Chaloupka and Limpus, 2005).

Major challenges for conservation of widely distributed, long lived taxa are assessing conservation status at biologically appropriate scales and establishing conservation priorities based on those assessments. In some regions of the world, local extinction of green turtles are likely to occur as a result of factors such as by-catch, directed harvest and habitat loss. In contrast, many green turtles nesting populations are actually on the increase as a result of direct conservation action and are not under threat of extinction. Conventional conservation programs have revolved about direct threats to populations (e.g. over-harvesting of both eggs and adults and accidental mortality in the nets and long-lines of fishing fleets) with little consideration given to sub lethal risks (Keller *et al.*, 2004; Day *et al.*, 2007).

The Coast of Oman Sea has some of the most important nesting sites for green sea turtles in the world. At present, there is limited information on the biomarkers of health status of the population of green turtle (*Chelonia mydas*), in Oman sea. Consequently, evaluating health status parameter is now a top research priority for green turtle conservation in this region. In this regard, biomarkers reflecting the health status of turtles at different biological levels (such as biochemical and cellular) respond almost more rapidly to stress. Understanding the blood biochemistry is a useful diagnostic tool for evaluating and monitoring health and physical status of wildlife (Aguirre and Balazs, 2000).

The purpose of the present study was to determine biochemical and hematological parameters in the blood of nesting green turtles as a health biomarker; to evaluate and compare sodium heparin and lithium heparin as anticoagulants for measuring selected biochemical values.

Materials and methods

Sampling and analyses

Field trips were made to the sampling sites during the 2014–2015. Nesting females green turtles were captured from their main nesting sites on the northern coast of the sea of Oman (see Table 1 and Fig. 1).

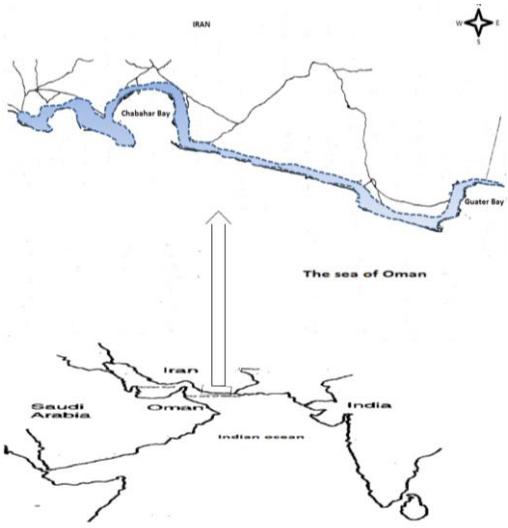


Figure 1: Study area.

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Table 1: Sampling sites in the northern side of the sSea of Oman.

Sampling sites	Coordinates	
	Longitude	Latitude
Ramin	25 15 45.75N	60 46 8.60E
Kachoo	25 14 5.09N	60 53 30.50E
Lipar	25 15 03.46N	60 49 54.75E

Nesting females were approached approximately 10 min after egg laying activity ceased. A complete visual physical examination was performed and curved carapace length (CCL) was measured. Health status of turtles was rated based on nest-building behavior and general body condition (Labrada-Martagon *et al.*, 2010; Harris *et al.*, 2011). Blood was collected from the inter-digital vein of the hind flipper via a dorsal approach with the use of an 18-gauge, 3.7-cm needle and a 15-ml syringe Pre-coated with sodium heparin (heparin sodium injection, USP, PPC, Inc., C504730, Canada) (n=18) or a non-heparin-coated syringe (n=18). Immediately following collection, the heparinized blood was placed in serum separator tubes (BD Microtainer®, 365956, Becton, Dickinson and Company, USA) and the blood in plain syringes was placed in lithium heparin tubes (BD Microtainer®, 365971, Becton, Dickinson and Company, USA) and buffered citrate sodium tubes (BD Vacutainer® CPT™, 362760, Becton, Dickinson and Company, USA). Blood tubes were kept on ice during the remaining time researchers were on the beach collecting samples (range 20 min to 2 hrs.).

Whole blood has been collected in the buffered citrate sodium was placed in cryotubes (Nunc® CryoTube®, 66021-992, VWR, USA). Thin blood smears were fixed with 99% methanol. Packed cell volumes (PCV) were determined using a portable 12-V centrifuge (Model Biofuge stratus, heraeus, Germany), and plasma total solids were measured with the use of a handheld refractometer (Master-53α, 2351, USA). Red blood cell (RBC) counts were performed using the BD Unopette Brand Test for Manual RBC Counts (Catalog Nos. 365854/365855, USA).

Blood films fixed in methanol were stained with Wright-Giemsa for evaluation of circulating cell morphology, estimation of leukocyte numbers and differential leukocyte counts. A minimum of 300 leukocytes were counted for differential leukocyte determinations. Leukocytes were categorized into one of five groups: monocytes, heterophils, lymphocytes, eosinophils, and basophils. Identification of blood cells types was based on previously described nomenclature (Deem *et al.*, 2006). Red blood cells were rated for haemoparasite recognition. Furthermore, total white blood cells counts were guessed from blood films by multiplying the average number of leukocytes detected per microscopic field times the objective power squared (Harvey, 2001).

For biochemical analysis, samples were centrifuged (Model Biofuge

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stratus, heraeus, Germany) at 400 g for 3 min then up to 5 mL of the plasma were frozen at -20 °C for up to 4 weeks before analysis. Plasma samples were analyzed, in duplicate, using spectrophotometric techniques by using a semi-automated clinical chemistry analyzer Pictus B (Diatron, Hungary) and commercial kits (Randox Laboratories Ltd., Ardmore, United Kingdom). These kits have been used to obtain blood chemistry reference values of the same species, *C. mydas*, in Australia, the Indian Ocean and the Pacific Ocean (Whiting *et al*., 2007; Labrada-Martago *et al.,* 2010). Triglycerides were measured using a commercial kit supplied by Sigma-Aldrich (Catalog Number TR0100; St. Louis, USA) and modified to be used with 5 µl of serum (Guglielmo *et al.*, 2005) and read using a microplate spectrophotometer (Epoch, BioTek, USA). Free glycerol was determined first; then after lipoprotein lipase hydrolysis, triglycerides were determined by subtracting free glycerol values. The biochemical panel included alanine aminotransferase (ALT), amylase, aspartate aminotransferase (AST), blood urea nitrogen (BUN), calcium, cholesterol, creatine kinase (CK), creatinine, gamma glutamyl transferase (GGT), glucose, lactate dehydrogenase (LD), lipase, phosphorous, potassium, sodium, total protein, triglyceride, and uric acid (UA). In order to measuring Alpha-tocopherol (vitamin E) and retinol (vitamin A) concentrations, the same method proposed earlier by Bechert *et al*. (2007) was followed.

Statistical analysis

Descriptive statistics (mean± SD) were performed with the use of Microsoft Excel (Microsoft Corporation, Redmond, Washington 98052, USA). Nonparametric statistical tests were used for comparisons because of the abnormal distribution of some data. Kruskal-Wallis one-way analysis of variance (KWANOVA) was used to determine differences in blood haematological values. Plasma biochemistry values for samples collected in sodium heparin versus those collected in lithium heparin were analyzed by the Mann–Whitney *U*-test ($p<0.05$).

Results

A total of 18 females green sea turtles received physical examinations. Carapace length was measured for 18 turtles. Mean curved carapace length (CCL) was 111±4 cm with a range of 103-122 cm. Barnacles were recorded on three turtles. No fibropapillomas were observed on any of the 18 turtles.

Hematology

Hematological data as well as the results of the related statistical analyses are shown in Table 2. The mean PCV was 0.41 (proportion of 1) with a range of 0.30–0.58. No basophils or haemoparasites were detected in any of the 18 turtles tested.

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Table 2: Hematologic values in nesting green turtles (*Chelonia Mydas*) nesting on Chabahar Beach, Islamic Republic of Iran.

Parameters [a]	N	Mean	SD	Range
PCV (l/l [%])	18	0.36 [36]	0.037 [3.7]	0.28–0.42 [28–42]
TS (g L^{-1} [g dl^{-1}])	18	43 [4.3]	8 [0.8]	26–58 [2.6–5.8]
RBC ($\times 10^3$ µ L^{-1})	18	374	187	166–777
WBC($\times 10^3$ µ L^{-1})	18	4.6	1.5	2.7–7.6
Heterophils ($\times 10^3$ µ L^{-1})	18	2.5	1.3	0.03–4.9
Lymphocytes($\times 10^3$ µ L^{-1})	18	1.5	0.8	0.0–3.0
Monocytes ($\times 10^3$ µ L^{-1})	18	0.3	0.1	0.1–0.7
Eosinophils ($\times 10^3$ µ L^{-1})	18	0.1	0.1	0–0.4

[a] PCV, packed cell volume; TS, total solids; RBC, red blood cells; WBC, white blood cells. No basophils were detected in any of the 18 green sea turtles evaluated. Samples were collected in sodium heparin

Plasma biochemistry

Plasma biochemistry data as well as the results of the related statistical analyses are shown in Table 3. No statistically significant differences were noted between the two anticoagulants for any of the measures.

Table 3: Plasma biochemistry values from blood collected in sodium and lithium heparin from nesting green turtles (*Chelonia mydas*)

	Sodium heparin			Lithium heparin		
Measure [a]	N	Mean ±SD	Range	N	Mean ±SD	Range
Sodium (mmol L^{-1})	18	143 ± 8	128—154	18	136 ± 9	123—147
Potassium (mmol L^{-1})	18	4.2 ± 0.8	2.7—5.3	18	4.2 ± 0.9	3.1—5.1
BUN(mmol L^{-1})	18	1.43 ± 1.03	0.91—4.93	18	1.01 ± 0.11	0.71—3.93
Uric acid(mol L^{-1})	18	12.1 ± 0.0	12.1	18	12.1 ± 0.0	12.1
TP (g L^{-1})	18	44 ± 9	31—58	18	40 ± 10	30—50
Cholesterol	18	9.14 ± 2.38	6.81—13.07	18	8.08 ± 1.98	3.92–10.76
Triglyceride (mmol L^{-1})	18	4.09 ± 0.74	2.83—4.94	18	4.96 ± 0.66	4.82—5.58
Calcium(mmol L^{-1})	18	2.93 ± 0.35	1.1—2.1	18	3.24 ± 0.3	1.15—2.3
Phosphorous(mmol L^{-1})	18	3.73 ± 0.51	3.07—4.01	18	3.72 ± 0.65	3.11—4.77
Creatinine(mol L^{-1})	18	22.22 ± 6.73	15.32—40.1	18	17.52 ± 5.34	8.44—44.32
ALT (U L^{-1})	18	4 ± 1	4—9	18	4 ± 1	2—6
AST (U L^{-1})	18	167 ± 51	101—234	18	174 ± 39	135—231
LD (U L^{-1})	18	1,636 ± 683	1,030—3,352	18	1,401 ±443	733—2,011
Glucose (mmol L^{-1})	18	4.01±0.62	3.05—4.61	18	4.32 ±0.50	3.31—4.86
Amylase (U L^{-1})	18	608 ± 51	518—758	18	631 ±94	466—843
Lipase (U L^{-1})	18	3 ± 1	2—5	18	2 ± 0	2
GGT (U L^{-1})	18	14 ± 3	11—16	18	12 ± 2	9—12

[a] BUN, blood urea nitrogen; TP, total protein; ALT, alanine aminotranferase; AST, aspartate aminotransferase; LD, lactate dehydrogenase; GGT, gamma glutamyl transferase. No measures were statistically different between samples collected in lithium versus sodium heparin.

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Vitamin E and A

Plasma alpha–tocopherol concentrations in the 18 turtles tested was 7.8±2.8 g ml^{-1} with a range of 0.5—11.8 g ml^{-1}. Plasma retinol was evaluated in 18 turtles and the concentration was 0.4±0.1 g ml^{-1} with a range of 0.2-0.6 g ml^{-1}.

Discussion

Although anticoagulants have been suggested as influencing plasma biochemistry values for reptilian species, (Murray, 2000) values for the turtles in this study were not statistically different for any of the variables. Bolten *et al.* 1992 (loggerhead sea turtles) and Deem *et al*. 2006 (leatherback sea turtles) also demonstrated that there were no significant differences in the use of these two anticoagulants for the blood analysis (Deem *et al.*, 2006).

The green turtles in our study had lower eosinophils than those reported in the green and loggerhead turtles (Deem *et al.*, 2006). High circulating eosinophils in green and loggerhead turtles may be related to spirorchids and other parasites commonly found in those species (Deem *et al.*, 2006). Results of the present study represent a slightly higher percentage of heterophils than lymphocytes. As a general rule, the primary white blood cell type found in marine turtles is the heterophil, but this may be dependent on age and species of turtle. Heterophils are consistently the primary cell type in Loggerheads, Green sea turtles, and Kemps' Ridley sea turtles, while lymphocytes appear to be the predominant cell type in immature Green sea turtles (Al Kindi, 2002). Much of the discrepancies over predominant cell type have been due to difficulty in identifying characteristics of the cells and their "official" classification over the last two decades. The PCV values reported for the nesting females are within the normal range for this species and indicate that anemia is not present (Jacobson *et al*., 2004; Deem *et al.,* 2006; Harris *et al.*, 2011). Results of the present study show that nesting females have high total erythrocyte counts. This finding is significant, as a lower erythrocyte count may be indicative of anemia of chronic disease. In chelonian, red blood cells indices may be interpreted as a comparative index of condition, nutrition, or general health (Peterson, 2002; Oliveira-Junior *et al.*, 2009) because anemia is the common effect of chronically poor nutrition, particularly with respect to protein intake (Peterson, 2002). If nesting females are mildly anemic due to a chronic condition, conceivably the fate of their nests could be adversely affected. Therefore, they may provision their eggs with insufficient nutrient or mineral concentrations needed for the embryos to survive. The differential count varied slightly from those reported in other sea turtle species (Work and Balazs, 1999; Smith *et al.*, 2000; Jacobson *et al.*, 2004). The PCV and mean RBC counts of the green turtles in our study were similar to the reports from other studies of nesting females green turtles from the sea of Oman (Alkindi, 2002).

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The results of Plasma alpha-tocopherol (vitamin E) and retinol (vitamin A) acquired in the present study were similar to those reported for the nesting green sea turtle by Frutchey (2004). Vitamin A and E are one of the fat-soluble vitamins. There are an antioxidant, helps in the hatchling growth and repair of tissues, and ~~is~~ they are important for proper functioning of the eyes, skin, mucous membranes, and ducts. Hypovitaminosis A and E is a totally preventable disease. Feeding the appropriate diet will assure turtles is going to receive the daily requirements of vitamins. In studies that have been conducted on sea turtles, there was no effect of vitamin E concentration on either clutch size or hatching success, and concentrations remained stable throughout the nesting season (Frutchey, 2004). There appears to be some seasonality in circulating retinol concentrations, particularly for the herbivorous species, which may link with vegetation variability.

The results indicated that BUN and UA values were similar to those reported for reptiles by Campbell (1996); however, the results acquired in the present study were lower than those previously reported in females green sea turtles by Bolten and Bjorndal (1992) and Whiting *et al.* (2007). Potential explanations for this disparity include the possible fasting state, normal physiological variation, or an evolutionary adaptation to a low-protein diet. Low values for BUN and UA in chelonians can be also associated with low protein diets or hepatic insufficiency (Whiting *et al.*, 2007). The decreases in the levels of uric acid in the present study may also be indicative of lesser consumption of proteins in the diets of nesting green turtles during this period. However, uric acid, the major nitrogen-containing excretion product, can be an indicator of increased catabolism of proteins, of the breakdown of tissues resulting from an inadequate diet (Whiting *et al.,* 2007), or of metabolic changes during the reproductive period of the green turtle.

Cholesterol and triglyceride values were high in this nesting green turtles, when compared to those found in wild green turtles (Bolten *et al.,* 1992). Cholesterol and triglyceride values are often elevated during vitellogenesis. The higher cholesterol plasma levels in the turtles during the summer months (peak period) may be related to the requirement for a large amount of cholesterol for the growth and maturation of the ovarian follicles. During the falls, the cholesterol levels dropped off significantly because the follicles had already attained the mature size (Deem *et al.,* 2006; Koch *et al.,* 2007). Abdulaziz *et al.* (2001), Deem *et al*. (2006) and Labrado-Martago *et al*. (2010**)** also found higher cholesterol levels in nesting green turtles in comparison to non-nesting green turtles. Also, the composition of the marine flora and the composition of the diet of the turtles may be effect on cholesterol and triglyceride values (Labrado-Martago *et al.,* 2010).

The elevated plasma calcium measured in nesting green turtles is

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most likely a result of calcium mobilization during vitellogenesis (Deem *et al.,* 2006). A similar pattern was also supported by several researchers (AlKindi *et al.,* 2000; Abdulaziz *et al.,* 2001; Jacobson *et al.,* 2004; Deem *et al*., 2006; Labrado-Martago *et al*., 2010). The mean calcium values were in the low to middle range reported in nesting leatherback turtles in Costa Rica (Bolten *et al*., 1992; George, 1997; Smith *et al.,* 2000) The differences in values may be related to stress which has been known to change the electrolyte levels or the differences could be related to individual variations at the time of sampling**.** Nesting turtles are also under tremendous pressure because of crowding stress, which may contribute to the rise in electrolyte levels.

Female green sea turtles had calcium: phosphorus ratio <1, with a mean phosphorus values of 3.72 mmol L^{-1} for soduime and 3.73 mmol L^{-1} lithium heparin samples. The mean phosphorus value appeared elevated in comparison with findings from other sea turtles by Bolten and Bjorndal (1992), Campbell (1996), George (1997) and Deem *et al*. (2006). It is possible that the phosphorus value were elevated in the green turtles as a result of phosphorus mobilization in association with egg production. Ratios of calcium to phosphorus were less than one for nesting females in this study, similar to nesting leatherbacks from Gabon (Deem *et al.,* 2006) and Trinidad (Harms *et al*., 2007). Alternatively, phosphorus values may have been elevated as a result of phosphorus release from the erythrocytes, because samples were not processed immediately (Deem *et al.,* 2009). Inverse calcium: phosphorus ratio may be normal for nesting green turtles or for the species in general. Further studies are necessary to determine the calcium: phosphorus ratio in nesting and non-nesting green turtles.

The mean AST and ALT activity were in the middle to high range, when compared to AST and ALT activity findings in green turtles (Flint *et al.,* 2010). Many field studies have demonstrated the interest in measuring AST and ALT activity as an exposure biomarker in sea turtles (Hasbfnl *et al*., 1998; Whiting, 2002; Jacobson *et al.,* 2004; Keller *et al.,* 2004; Bischoff and Ramaiah, 2007; Flint *et al.,* 2010). Increase in transaminase activity in the blood is an indicator of hepatic damage (Flint *et al.,* 2010) even when no symptoms are present. The liver protects the individual by removing compounds, such as xenobiotics, from the blood (Keller *et al.,* 2004; Bischoff and Ramaiah, 2007). It is necessary to carry out studies to examine the activity and distribution of the transaminases in the organs of the sea turtles.

For the green turtles captured in this study, high levels of glucose, lactate dehydrogenase, GGT were observed. This pattern also has been reported by several researchers (AlKindi *et al.,* 2000; Horowitz and Klein, 2000; Abdulaziz *et al.,* 2001; Campbell, 2004; Jessop and Hamann, 2004; Deem

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et al., 2006; Hamann *et al.,* 2007; Flint *et al.,* 2010). Reptiles are unable to sustain vigorous activity without utilizing anaerobic metabolism, and the nesting process in marine turtles is therefore likely to be sustained through glycolysis and lactate accumulation (Jessop and Hamann, 2004). Based on the findings of this research, it could be concluded that intense nesting activity exceeds the limit on the female turtle's capacity to use aerobic metabolism. A possible explanation for this increase in blood lactate concentration of female green sea turtles may be that lactate is being released from the muscles to the blood, which will then be transported to the liver and converted back into glucose (gluconeogenesis), either for immediate use or to be stored as glycogen. Both aerobic and anaerobic muscle metabolisms are fuelled by glucose and during heavy exercise glucose is mobilized from glycogen stores in the liver. During prolonged exercise liver glycogen stores become depleted and other sources of fuel such as lipid and proteins are gradually mobilized (Horowitz and Klein, 2000; Hamann *et al.*, 2007). Hence in female green sea turtles elevated concentrations of glucose being released from the liver and a consequent increase in blood glucose concentration.

There is little information to aid for interpretation of the observed changes in relation to the increase in the activity of GGT in blood. Increases in the activity of GGT enzyme were previously reported for green turtle (*C. mydas*) Along the Pacific Coast (Labrada-Martago *et al.,* 2010). Increase in the activity of this enzyme is an indication of liver damage in mammals (Bossart *et al.,* 2001).

Our study is the first report to provide blood haematology and biochemical assessment of female green turtles *(C. mydas)* in the northern coast of the Sea of Oman and it will provide an important dataset for assessing future impacts on marine turtle health. The physical state and the health of the green sea turtles was found to be good, in that they did not present any obvious signs of disease or any external lesions or injuries. Further studies are also needed to increase the knowledge the way in which seasonal, annual, and geographic variations affect the biochemical parameters of the sea turtle.

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