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Stress Response of Juvenile Green Sea Turtles (*Chelonia mydas*) with Different Fibropapillomatosis Scores

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ABSTRACT: Green sea turtles (*Chelonia mydas*) with cutaneous fibropapillomatosis (FP) occur in some populations worldwide, and the survivability of these individuals may be compromised depending on the disease severity score. Furthermore, populations may be negatively affected in areas with a high prevalence of the disease. The stress responses (corticosterone, glucose, lactate, and hematocrit) to capture and handling were assessed in animals with different FP severity scores. From 18 January 2013 to 31 July 2014, 33 juvenile (sex was not determined) *C. mydas* were collected from the effluent of a steel mill in the state of Espírito Santo, Brazil, by casting a net from the bank of a canal in the morning. The body conditions of animals with FP scores of FP2 and FP3 were poor, which suggests that these turtles were unable to adequately feed themselves, and animals with an FP score of FP3 exhibited an impaired corticosterone response. This may compromise the fitness of animals in populations with a high incidence of advanced-stage FP.

Key words: Body condition, disease, green turtle, virus.

Fibropapillomatosis (FP) is a common disease in the green sea turtle, *Chelonia mydas*, with prevalence rates up to 92% in some wild populations (Aguirre and Lutz 2004). As the disease progresses, the tumor masses, located internally and externally on the soft skin regions, typically compromise metabolism and hinder swimming, feeding, and sight. Additionally, green turtles with severe FP are immunosuppressed (Work et al. 2001). This condition may result in chronically declining health and predispose turtles to infectious and parasitic diseases, which can be concomitant to neoplasm and worsening FP, thereby decreasing their chances of survival.

Work and Balazs (1999) determined that FP severity might be defined using a scale related to tumor number and size, and Aguirre et al. (1995) and Swimmer (2000) studied the stress responses of green turtles with and without FP that were subjected to capture, and they characterized the stress response dynamics over time. Aguirre et al. (1995) demonstrated that corticosterone was a good indicator of stress in green sea turtles and that its levels peaked from 1 to 3 h after capture. However, the relationship between the FP severity score and stress responsiveness is unknown, so the fitness of animals with a higher FP severity score, and ultimately that of the population, may be compromised. We assessed the effect of FP severity score on the corticosterone, glucose, lactate, and hematocrit responses in juvenile *C. mydas* subjected to capture.

The study area included a canal carrying the effluent from a steel mill in the state of Espírito Santo, Brazil (20°15′50″S, 40°13′44″W), where the local FP prevalence in green sea turtles is approximately 34% (Torezani et al. 2010). From 18 January 2013 to 31 July 2014, a total of 33 animals were collected by casting a net from the bank of the canal in the morning. All of the turtles were considered juveniles because each was less than 65 cm in size (Patricio et al. 2011); sex was not determined. The animals were classified by FP severity score, which ranged from FP0, no visible external tumors, to FP3, severely affected by external tumors (Work and Balazs 1999); the presence or absence and the severity of FP was determined based on an external evaluation only. Blood was collected less than 5 min after capture (hour 0), and the animals were then placed in the prone position in individual plastic boxes and kept for 1 h before a second blood sample was taken (hour 1). At each sampling, a 1 mL blood sample was withdrawn from the cervical

venous sinus into tubes containing sodium heparin using a vacuum collection system. Whole blood was used for hematocrit determination, and the remaining samples were centrifuged within 30 min of collection to separate the plasma and then stored in coolers at 8 C for transport to the laboratory, where they were stored at –80 C until analysis.

Biometric measurements were taken following the blood collections following Torezani et al. (2010). The body condition index (BCI) was calculated as the mass (kg)/curved carapace length (m) along the back (Santos et al. 2015).

The total corticosterone levels were determined by a competitive enzyme linked immunoassay (ELISA) with an anticorticosterone polyclonal antibody and a corticosterone-alkaline phosphatase conjugate specific to reptiles (ADI-901-097, Enzo Life Sciences, Farmingdale, New York, USA). A four-parameter logistic calibration curve with five standard dilutions was designed using Softmax Pro 6.2.1 software (Molecular Devices, Sunnyvale, California, USA) and a microplate reader (Spectra Max 190, Molecular Devices) at 405 nm; the correlation coefficient of the standard curve was 1. The minimum detectable concentration was $\geq$0.032 ng/mL, and all samples were diluted with assay buffer to a volume ratio of 1:2 and analyzed in duplicate. The samples were not subjected to an extraction step but were pretreated with steroid displacement buffer to remove binding proteins, according to the manufacturer's protocol, so the resulting values were for total corticosterone. The bound mean (SD) of the samples was 76±16%, and samples whose absorbance values remained above the limits of the standard curve were diluted with assay buffer and retested. The intraassay coefficients were estimated for each sample, and the average variation was 1.58±1.55% (the correlation coefficient from all samples was lower than 10%). According to the manufacturer, the cross-reactivity of the corticosterone antibody was corticosterone (100%), 11-dehydrocorticosterone (<0.03%), deoxycorticosterone (28.6%), and other steroids (<1.7%). The use of heparinized plasma samples for corticosterone determination by ELISA in green sea turtles was validated by Ikonomopoulou et al. (2014), but we constructed a parallelism curve with serially diluted samples to validate the use of the corticosterone ELISA kit with green sea turtle heparinized plasma. Plasma glucose and lactate concentrations were analyzed with Beckman Coulter kits in a Beckman Coulter AU2700 chemistry analyzer (Beckman Coulter Inc., Brea, California, USA). The hematocrit values were determined after 10 min of centrifugation in micro-hematocrit tubes.

The results were expressed as the mean standard error, and the normality of the data was assessed using a Kolmogorov-Smirnov test. Raw data that were not normally distributed (corticosterone and lactate) were $\log_{10}$ transformed. Comparisons between the tested stress parameters were performed by two-factor (FP and time) analysis of variance followed by Tukey's test (α=0.05), while the curved carapace length, body mass, and BCI were analyzed by one-way analysis of variance followed by Tukey's test (α=0.05). Additionally, the relationship between animal size (all study animals) and basal corticosterone (hour 0) was tested by linear regression. All statistical analyses were performed using the Sigma Plot 12.5 software (Systat Software Inc, San Jose, California, USA).

The mean body mass (P=0.049) and curved carapace length (P=0.021) of the FP1 animals were significantly greater than those of the FP3 animals, but the FP0 and FP2 turtles did not differ from the other groups (Table 1). There were no significant differences (P=0.11) in the BCI between groups (Table 1).

No relationship was found between animal size and the basal corticosterone concentration (y=–0.0002x+10.974; r^2=0.0749; P=0.106), indicating that the size of the animals was not related to the assessed stress responses. At the hour 0 and hour 1 time points, the corticosterone concentrations were similar between the analyzed groups, but it significantly increased from hour 0 to hour 1 in the FP0 (P=0.014), FP1 (P=0.023), and FP2 (P=0.025) animals (Table 2). There were no significant differences (P=0.096) in the FP3 animals between the tested time points. At hour 0, the plasma glucose concentration

TABLE 1. Biometric values (mean±SE) of wild *Chelonia mydas* juveniles according to the degree of fibropapillomatosis (FP). Animals were collected in the mornings from 18 January 2013 to 31 July 2014 by casting a net from the bank of a canal carrying the effluent from a steel mill in the state of Espírito Santo, Brazil. Different letters in the same column indicate a significant difference ($P<0.05$) among FP score groups.

FP score	*n*	Curved carapace length (cm)	Body mass (kg)	Body condition index
FP0	8	38.7+13.8 ab	8.2+1.1 ab	0.96+0.04 a
FP1	8	44.4+15.8 a	13.0+1.2 a	1.08+0.01 a
FP2	10	41.0+14.7 ab	10.7+1.4 ab	1.07+0.04 a
FP3	7	37.3+13.7 b	7.6+1.0 b	1.06+0.03 a

between the analyzed groups was similar; at hour 1, the FP1 animals had plasma glucose concentrations similar to those of the FP0 animals but significantly higher than those of the FP2 ($P=0.011$) and FP3 ($P<0.001$) animals. The plasma glucose concentrations significantly increased from hour 0 to hour 1 in the FP0 ($P=0.008$) and FP1 ($P<0.001$) animals (Table 2). No intergroup or intragroup differences in lactate were found at hours 0 or 1, and the hematocrit was significantly greater in the FP1 animals compared to the FP3 animals at both sampling times: hour 0 ($P=0.016$) and 1 ($P=0.013$). There were no significant differences in hematocrit between the FP0 and FP2 animals and any other group (Table 2).

The activity of the hypothalamic-pituitary-adrenal axis (HPA) in green sea turtles is similar to that of other vertebrates (Hamann and Jessop 2005); corticosterone concentration increases in response to a stressor, which triggers a cascading effect that leads to increased glucose use (Moore and Jessop 2003). This is consistent with the findings of our study, wherein the corticosterone and glucose concentrations significantly increased at the hour 1 time point in the FP0 and FP1 animals, and corticosterone also significantly increased in the FP2 animals at hour 1. In contrast, there was no significant difference between the hour 0 and hour 1 time points in the FP3 animals, which demonstrates that these animals could no longer modulate the HPA in response to a stressor. This result may be related to the anemic state of the FP3 animals as indicated by the lower hematocrit values and the presence of large tumors near the mouth and eyes, which can interfere with feeding. These factors can deplete the energy reserves of a turtle so that the HPA no longer functions normally.

TABLE 2. Corticosterone, glucose, lactate, and hematocrit levels (mean±SE) in wild *Chelonia mydas* juveniles with different degrees of fibropapillomatosis (FP) at different times after capture (hour 0 and hour 1). Animals were collected in the morning from 18 January 2013 to 31 July 2014 by casting a net from the bank of a canal carrying the effluent from a steel mill in the state of Espírito Santo, Brazil. * indicates significant differences ($P<0.05$) within the same FP score group for the different time points (hour 0 and hour 1). Different letters indicate significant differences ($P<0.05$) within the same time point for the different FP score groups.

Sampling	FP score	Total corticosterone (ng/mL)	Glucose (mg/dL)	Lactate (g/dL)	Hematocrit (%)
Hour 0	0	6.4±2.2 a	83.3±4.4 a	6.3±1.4 a	29.3±29.80 ab
	1	3.7±1.9 a	76.8±2.9 a	4.9±0.7 a	36.0±3.13 a
	2	3.6±0.8 a	85.0±3.7 a	6.8±0.7 a	31.1±2.80 ab
	3	2.3±0.2 a	81.3±5.8 a	4.9±1.3 a	26.9±3.34 b
Hour 1	0	12.1±3.4 a*	102.9±6.9 ab*	8.6±1.7 a	32.4±2.80 ab
	1	7.5±3.1 a*	116.9±5.1 a*	8.6±1.3 a	35.1±3.13 a
	2	9.0±1.7 a*	95.5±5.0 b	8.8±1.5 a	31.5±2.80 ab
	3	5.7±0.8 a	86.9±4.1 b	5.9±0.8 a	24.3±3.34 b

Aguirre and Lutz (2004) observed that the occurrence of FP (primarily advanced-stage FP) might decrease the swimming, diving, location, predation, deglutition, and escape responses of green turtles. Our study showed that advanced-stage FP also suppresses the release of corticosterone from the HPA, thereby inhibiting the stress response. The combination of these behavioral factors and the inability to respond physiologically indicates that the fitness of turtles with advanced-stage FB is compromised, which may affect population-level fitness and threaten the future of such green turtle populations.

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