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BIOCHEMICAL RESPONSES TO FIBROPAPILLOMA AND CAPTIVITY IN THE GREEN TURTLE

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ABSTRACT: Blood biochemical parameters were compared for green turtles (*Chelonia mydas*) with and without green turtle fibropapillomatosis (GTFP) from both captive and wild populations in Hawaii (USA) and from a captive population from California (USA), during the period between 1994 and 1996. Statistical analysis did not detect an influence of disease in any of the blood parameters for free-ranging turtles; however, captive turtles in Hawaii with GTFP had significantly higher levels of alkaline phosphatase and significantly lower levels of lactate compared to non-tumored captive turtles. Multivariate analysis found that biochemical profiles could be used to accurately predict if turtles were healthy or afflicted with GTFP. Discriminant function analysis correctly classified turtles as being with or without GTFP in 89% of cases, suggesting that diseased animals had a distinct signature of plasma biochemistries. Measurements of blood parameters identified numerous differences between captive and wild green turtles in Hawaii. Levels of corticosterone, lactate, triglyceride, glucose, and calcium were significantly higher in wild green turtles as compared to captive turtles, while uric acid levels were significantly lower in wild turtles as compared to captive turtles. Additionally, turtles from Sea World of California (San Diego, California, USA), which had been in captivity the longest, had higher levels of alanine aminotransferase and triglycerides as compared to nearly all other groups. Differences in diet, sampling methods, environmental conditions, and turtle size, help to interpret these results.

Key words: Blood biochemical parameters, captive effects, *Chelonia mydas,* fibropapillomatosis, green turtle, immune fraction.

INTRODUCTION

Throughout their tropical and subtropical ranges, green turtles (*Chelonia mydas*) are currently listed as threatened or endangered under the U.S. Endangered Species Act of 1973 (Hirth, 1997). In some areas such as Hawaiian waters, conservation and legislative efforts have helped to increase population sizes (Eckert, 1993). However, a growing number of marine turtle populations are now afflicted with green turtle fibropapillomatosis (GTFP), a debilitating and potentially life-threatening disease. Incidence of green turtles with GTFP is especially high in certain populations in Florida (USA) (33 to 61%, 1986–90; Erhart, 1991) and Hawaii (USA) (49 to 92%, 1989–90; Balazs, 1991).

GTFP is characterized by the growth of benign internal and external fibroepithelial tumors. The etiology of the disease remains unknown, yet a virus is suspected (Herbst et al., 1996). Pollutants and algal blooms have also been hypothesized to play a role in the disease (Hirth, 1997). Because nearly every species of marine turtle has been observed with fibropapilloma tumors (Hirth, 1997), understanding the impacts of this disease at both the individual and population level has been recommended as priorities by the National Marine Fisheries Service (Silver Spring, Maryland, USA; Eckert, 1993).

Measurement of plasma chemistry is commonly used as a diagnostic technique to determine the health status of individual animals, and accumulation of such data enables the assessment of health status of populations. Blood profiles for diseased and non-diseased marine turtles have been reported (Norton et al., 1990; Bolten and Bjorndal, 1992; Aguirre et al., 1995), thereby improving efforts to evaluate potential causes and biochemical consequences of GTFP. Comparisons among studies, however, are somewhat limited due to potential differences among turtle populations as well as variations in analytical methods. This study presents biochemical profiles for turtles with and with-

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out GTFP from the wild and healthy turtles in captivity using identical analytical methods.

The primary objectives of this research were to determine the clinicopathologic effects of GTFP and captivity on green turtles in order to improve the diagnostic capabilities of blood chemistry profiles. All study animals originated from the main Hawaiian Islands, except for the group of adult captive turtles from Sea World of California (SWC; San Diego, California), which originated from various locations in the the Pacific Ocean. Because turtles from SWC have been in captivity for many years, they were especially valuable in order to evaluate the long term effects of time in captivity. Comparisons among these groups offer the opportunity to improve our understanding of the biochemical responses to disease and to captivity on a threatened species of marine turtle faced with a potentially life-threatening disease.

MATERIALS AND METHODS

Field methods

Nine sub-adult captive turtles were obtained from the wild in Kaneohe Bay (21°30′N, 157°50′W), island of Oahu (Hawaii) between June and September 1994. Turtles were captured by hand, brought onto a boat, and later transported ca. 15 km to Kewalo Research Facility (Honolulu, Hawaii). Five of these turtles had visible signs of GTFP, each with a minimum of six and a maximum of 12 tumors measuring between 1 and 15 cm. Visible tumors were located in the following locations: flippers, cloaca, eyes, mouth, jaw hinges, neck and carapace. Turtles were evaluated for tumor severity based on an index described in Balazs (1991). Based on this index, tumor severity for captive turtles in this study was moderate, with a tumor score range between 2 and 3 (mean: 2.5).

Turtles with GTFP were housed in a separate tank with an independent water supply. Each tank, 8 m diameter, received a constant supply of seawater (ranging from 23 to 27 C). Mean (±SE) size (straight carapace length; SCL) for the GTFP-afflicted and apparently-healthy turtles was 53.6 ± 1.8 cm and 47.9 ± 1.7 cm, respectively. Turtles were each fed two squid per day 6 days a week. Throughout the study period, all turtle weights remained within 1 kg of their original weights. Two captive turtles (one with tumors and one without) were identified as female, and one turtle with tumors was identified as male using standard laparoscopic methods. Sexes for the remaining turtles remain unknown.

At time of capture, blood samples collected from the dorsal cervical sinus, using methods described in Owens and Ruiz (1980), were obtained between 45 to 70 min after turtles were brought onto the boat. All samples taken in the field were collected between 0900 and 1200 hours. Data from this first sampling date were included in calculating means of values while in captivity. This was done so that effects of captivity could be observed using the first available data, as well as to avoid effects of season when interpreting data from wild turtles. Blood samples obtained from captive turtles were drawn within 5 min of being handled in the tank. These blood samples were collected in the late afternoon or early evening on eight separate days from early November to mid December. All samples were collected in heparinzed Vacutainers® and stored on ice for approximately 1–2 hrs before being spun in a centrifuge at 500 g for 10 min. Approximately 5 plasma samples for each turtle were stored at −70 C until analyzed. All hemolyzed samples were discarded. Turtles were maintained in captivity until mid December 1994, at which time they were released back into Kaneohe Bay.

One blood sample from each of 13 wild green turtles from Kaneohe Bay (Hawaii) was obtained between 0900 and 1200 hours on one sampling day in August 1994. Sampling methods were identical to methods described above. Six of these turtles were apparently healthy ("non-tumored"), while seven had visible tumors ("tumored"). Tumor severity for tumored turtles from this sampling effort was also moderate (range between 2 and 3; Balazs, 1991). Seawater temperature (ca. 25 cm depth) was recorded using a thermister probe (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio, USA), and water temperatures remained within 28 to 29 C throughout the 3 hr sampling period. Mean (±SE) SCL for tumored and non-tumored turtles were 55.2 ± 2.1 cm and 50.2 ± 4.5 cm, respectively. Approximately 8 cc of blood was collected from the dorsal cervical sinus using 22-gauge needles and heparinized Vacutainers® from each turtle between 45 to 70 min after removal from the water. Turtles remained on the boat covered with wet towels and placed on their plastrons until blood was collected. Blood was kept on ice for no longer than 2 hr until spun for 10 min in a centrifuge

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at 500 g, and plasma samples were stored at −70 C until analyzed. Hemolyzed samples were discarded. Because Kaneohe Bay is a common feeding ground for green turtles around Oahu, these turtles had most likely been feeding prior to their capture.

Eight captive turtles residing at SWC were removed from the water in the "Turtle Lagoon" between 0830–0930 hours on 19 September 1996. Using a thermistor probe (Yellow Springs Instrument Co., Inc.), ambient water temperature in the lagoon was 23 C. All animals were considered clinically healthy and did not show signs of GTFP. Many of these turtles had been in captivity for over 10 yr, and were originally captured from the Pacific Ocean. Mean (±SE) turtle size (curved carapace length) was 80.3 (±) cm. Approximately 8 cc of blood was collected from the dorsal cervical sinus using 22-gauge needles and heparinized Vacutainers®. Blood was stored on ice for approximately three hours until centrifuged at 500 g for 10 min. Hemolyzed samples were discarded. Plasma samples were stored at −70 C until analyzed. Turtles at SWC had a diet consisting of sardines, smelt, squid and shrimp and were fed three times per week. Turtles had not eaten for 48 hr prior to blood collection.

Laboratory methods

Plasma corticosterone levels of all turtle groups except SWC turtles were determined during the winter of 1996 using standard radioimmunoassay techniques at the University of Florida, Gainesville, where assays specific to green turtle plasma have been developed and methods previously described (Gregory, 1994; Aguirre et al., 1995).

Plasma samples from all turtle groups were analyzed for biochemical profiles using an Analyst® Benchtop Chemistry System (Hemagen Diagnostics, Inc., Waltham, Massachusetts, USA) at the Hubbs-Sea World Research Institute (San Diego, California) during the summer of 1996. Parameters analyzed included: various blood enzymes, calcium, cholesterol, triglyceride, glucose, uric acid, and total protein. Plasma lactate levels were determined using a 2300 STAT YSI Glucose-L-Lactate Analyzer (Yellow Springs Instrument Co., Inc.) at Scripps Institute of Oceanography, University of California, San Diego. Lactate samples were analyzed in duplicate, and the mean was recorded.

Statistical analyses were performed using SAS software V. 6.08 (SAS Institute, Inc., Cary, North Carolina, USA). To determine differences in mean values of biochemical parameters among turtle groups while accounting for repeated sampling of individual turtles, a repeated measures analysis of variance followed by a Tukey multiple comparison post-hoc test was used. While this analysis does not resolve the lack of independence among samples, it does account for differences in dates of blood collection. Discriminant function analysis was used to determine if there was a biochemical signature that could identify if turtles were with or without tumors. Complete data sets were available for 18 non-tumored and 26 tumored turtles from both captive and wild populations of green turtles from Hawaii. Sample size differences were accounted for by weighing parameters by number of cases per group.

RESULTS AND DISCUSSION

General

Plasma biochemistry findings on 30 green turtles are summarized in Table 1. Statistical analysis did not detect an influence of disease in any of the blood parameters for free-ranging turtles, however captive turtles with GTFP had higher levels of ALP and lower levels of lactate compared to apparently-healthy captive turtles in Hawaii. While univariate analysis revealed differences in only two parameters, a multivariate approach found distinct differences between diseased and healthy turtles. Predictions of turtle group (with or without tumors) based on discriminant function analysis classification were correct 89% for turtles with and without tumors, suggesting that diseased animals had a distinct signature of plasma biochemistries. Measurements of blood parameters indicate numerous differences between free-ranging and captive green turtles in Hawaii. Levels of corticosterone, lactate, triglyceride, glucose, and calcium were higher in free-ranging green turtles as compared to captive turtles, while uric acid levels were lower in free-ranging turtles as compared to captive turtles. Additionally, turtles from SWC, which had been in captivity the longest, had relatively high levels of ALT and triglycerides. Differences in diet, sampling methods, environmental conditions, and turtle size help to interpret these results.

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TABLE 1. Plasma biochemical profiles (mean, standard error) for captive and free-ranging subadult green turtles with and without fibropapillomatosis (GTFP).

	Captive, without GTFP[a] Mean (SE)*	Captive, with GTFP Mean (SE)*	Free-ranging, without GTFP Mean (SE)*	Free-ranging, with GTFP Mean (SE)*	Captive (SWC), without GTFP Mean (SE)*
Corticosterone (ng/ml)	1.4 (0.48)^{A}	2.1 (0.37)^{A}	6.1 (0.74)^{B}	5.3 (0.68)^{B}	N/a
Lactate (mmol/L)	9.5 (1.07)^{A}	5.5 (0.75)^{A}	17.4 (1.79)^{B}	16.2 (1.66)^{C}	1.8 (1.55)^{D}
Glucose (mg/dl)	103.9 (4.99)^{B}	100.5 (3.96)^{BC}	106.7 (6.91)^{B}	117.7 (6.40)^{AB}	111.9 (5.99)^{B}
AST (GOT) (U/L)	176.6 (26.50)^{AC}	238.4 (21.92)^{AD}	297.0 (32.95)^{A}	230.1 (30.50)^{A}	418.4 (28.53)^{B}
ALT (GPT) (U/L)	11.1 (7.95)^{A}	13.9 (7.07)^{A}	9.7 (6.64)^{A}	9.8 (7.27)^{A}	41.3 (6.64)^{A}
Alkaline phosphatase (U/L)	38.1 (7.68)^{C}	65.1 (6.81)^{A}	53.8 (6.78)^{AB}	61.0 (6.28)^{A}	64.2 (6.78)^{A}
Total protein (g/dl)	4.1 (0.33)^{A}	3.9 (0.29)^{A}	4.3 (0.34)^{AB}	5.0 (0.31)^{B}	5.4 (0.29)^{B}
Calcium (mg/dl)	6.8 (0.62)^{B}	6.0 (0.53)^{B}	8.2 (0.57)^{AB}	9.4 (0.53)^{A}	7.3 (0.49)^{B}
Cholesterol (mg/dl)	163.4 (21.59)^{A}	180.4 (19.11)^{A}	147.7 (19.14)^{A}	157.9 (17.72)^{A}	290.1 (16.57)^{B}
Triglyceride (mg/dl)	134.9 (29.92)^{A}	117.5 (25.70)^{A}	175.3 (31.66)^{A}	222.4 (29.31)^{A}	486.7 (31.66)^{B}
Uric acid (mg/dl)	2.2 (0.19)^{A}	2.4 (0.14)^{A}	1.3 (0.29)^{B}	1.4 (0.2)^{B}	2.0 (0.25)^{AB}

[a] Maintained in Honolulu, Hawaii.

* Indicates statistical significance. Similar subscript letters (A, B, C, D) indicate no statistical difference ($P \geq 0.05$) between turtle groups. Groups with any combination of different subscript letters indicate statistically different means ($P \leq 0.05$).

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Corticosterone and lactate

Adrenal glucocorticoids, such as cortisol and corticosterone, are often used as indices of stress among the vertebrate classes (Harvey et al., 1984). Activity of the hypothalamo-pituitary-adrenal axis in marine turtles is typical of most vertebrates (Morris, 1982), whereby the organisms' response to stress influences glucose utilization and other metabolic activities stimulated by the adrenal or inter-renal gland (Norris, 1980). Recent studies with loggerhead (*Caretta caretta*) and green turtles report a positive relationship between corticosterone levels and stress (Wibbels et al., 1990; Gregory, 1994). The sole use of glucocorticoids as an indicator of stress, however, is not recommended due to the array of effects that a stressor may evoke (Gregory, 1994; Valverde et al., 1996).

Elevated corticosterone levels have been associated with GTFP in green turtles from Hawaii, which the authors attributed to chronic stress (Aguirre et al., 1995). They found turtles sampled at 1 hr post-capture had mean plasma corticosterone levels significantly higher in turtles with GTFP (5.5 ng/ml) as compared to healthy turtles (2.29 ng/ml). In this study, however, corticosterone levels were similar between diseased and non-diseased turtles both in captivity and in the wild.

Another unexpected finding in this study was the higher corticosterone values for free-ranging turtles than for turtles maintained in captivity. This might be explained by the time delay from when animals were captured in the field until blood was collected (range: 45 to 70 min) compared to the immediate collection of blood from captive animals. Aguirre et al. (1995) reported a two-fold increase in corticosterone levels 1 hr after capture as compared to levels determined immediately upon capture. Gregory (1994) found that levels were highest at 3 hr post-capture, and suggested that basal corticosterone levels be determined only with plasma samples obtained within 10 min of capture.

Furthermore, circadian and seasonal variation in corticosterone levels also may limit accurate interpretation of the observed data. In free-ranging olive Ridley sea turtles (*Lepidochelys olivacea*), corticosterone levels did not vary throughout a 24 hr period (Valverde et al., 1996). However, in captive loggerhead sea turtles, levels peaked in the early morning (Schwantes, 1986), which may have been in response to scheduled feeding regimes. Daily corticosterone cyclicity is not known specifically for free-ranging or captive green turtles. Seasonal variation in corticosterone levels has been shown in other reptiles, often as a result of reproductive or temperature changes. Employing similar sampling and laboratory methods to those reported in this paper, Aguirre et al. (1995) report a nearly two-fold increase in corticosterone levels for tumor-free green turtles from Hawaii sampled in fall as compared to values from this study where free-ranging turtles were sampled during summer. Similarly, free-ranging loggerhead turtles sampled in summer had significantly higher levels of corticosterone than those sampled in winter (Gregory, 1994), which was explained by elevated cloacal temperatures and the subsequent increase in metabolic demands. Differences in reproductive condition were not likely to have influenced data since sampled turtles were all subadults. Because of the numerous potential influences upon corticosterone levels in marine turtles, interpreting data from this study is limited until more is understood on the effects of diurnal and seasonal variation, disease, captivity, and acute stress.

Given the high aerobic capacity of marine turtles (Seymour, 1982), levels of lactate may not accurately reflect physical exertion, but rather may be indicative of the stress involved in breath-holding, or apnea. Berkson (1966) found that blood lactate in green turtles increased in response to dives and forced submergence. Similarly, Lutz and Dunbar-Cooper (1981) found a ten to forty times increase in blood lac-

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tate for trawled turtles compared to quiescent turtles, and Wood and Ebanks (1984) observed a nearly 20-fold increase in blood lactate level for a green turtle forcibly submerged underwater for 15 min. The authors attributed this rise in lactate to severe stress experienced by the turtle. However, similar to corticosterone, levels of lactate were highly labile, and caution should be used when assessing an animal's level of stress based on one or even multiple plasma chemistry variables. Rather, data from this study can be used to conclude that captive turtles without GTFP experience a higher adrenocortical response than their tumor-free captive counterparts, and that relatively high levels of corticosterone observed in free-ranging turtles was positively correlated with levels of lactate. More research should be conducted to clarify the relationships among lactate, corticosterone, and stress levels in green turtles.

Blood enzymes

In numerous species, damage to the cell membrane in skeletal muscle, cardiac muscle, or in the liver causes release of enzymes from the cell into the blood (Meyer et al., 1992). In captivity and in the wild, AST levels were similar for turtles with and without tumors. This finding was unexpected due to elevated levels of AST reported for green turtles afflicted with GTFP both in captivity (Norton et al., 1990; Varela, 1997), and in the wild (Aguirre et al., 1995; Aguirre, 1996). Furthermore, desert tortoises (*Xerobates agassizii*) afflicted with chronic upper respiratory tract disease (URTD) also had elevated AST, which the authors attributed to tissue damage (Jacobson et al., 1991). However, high variation among levels of AST reported in the above-mentioned studies suggest more research is needed before accurate assessment of the association between disease and AST can be made.

Levels of ALT in green turtles apparently were not affected by the presence of tumors, as supported in this study and by reports of Aguirre et al. (1995). Elevated levels of ALT in a debilitated turtle (Norton et al., 1990) may have resulted from the animal's prolonged maintenance in captivity rather than to disease. This is supported by results from this study that found ALT levels highest in SWC turtles, which have been in captivity for many years. Prolonged unnatural diets and confinement in captivity likely affect liver functioning, eventually resulting in elevated ALT.

Alkaline phosphatase (AKP) is a membrane-associated enzyme found in numerous tissues. Increased AKP levels in reptiles have been linked to hyperparathyroidism, numerous bone diseases (e.g., Paget's disease, rickets), and renal insufficiency (Frye, 1981). AKP levels were not associated with GTFP in free-ranging turtles in this study, nor in captive turtles from Florida (Varela, 1997). However, captive turtles in Hawaii had higher levels of AKP than turtles without tumors. The reverse effects were observed for free-ranging green turtles from Hawaii reported earlier (Aguirre et al., 1995; Aguirre, 1996).

Protein, calcium, lipid metabolism

In the present study, the synergistic effects of disease and captivity resulted in significantly depressed levels of plasma protein, though the effect of disease alone did not appear to influence protein levels. Hypoproteinemia has been reported for diseased green turtles in other studies (Norton et al., 1990; Aguirre et al., 1995; Aguirre, 1996), and the condition results when the body is unable to produce enough protein or when there is an increased loss of proteins. For captive, healthy turtles from SWC and the Miami Seaquarium (Varela, 1997), protein levels were relatively high, likely resulting from a prolonged diet high in protein.

Results from this study did not show an effect of disease on calcium levels, which was similar to previous findings for green turtles in Hawaii (Aguirre et al., 1995;

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Aguirre, 1996) and desert tortoises (Jacobsen et al., 1991). However, levels of calcium were lower in captive turtles compared to free-ranging animals, presumably as a result of changes in diet. Disorders likely to cause hypocalcemia in diseased or captive animals include conditions such as necrotizing pancreatitis and dietary imbalance (insufficient Vitamin D, excess phosphorous; Meyer et al., 1992). Previously reported hypocalcemia for diseased, captive green turtles (Norton et al., 1990; Varela, 1997) may result from the synergistic effects of captivity and disease. Managers of captive green turtle populations can reverse symptoms of hypocalcemia with dietary supplements or by maintaining green turtles on their natural, herbivorous diet.

Disturbances in lipid metabolism can be measured via changes in serum cholesterol, which is secreted from the liver in the form of bile acids. Some causes of increased plasma cholesterol include high fat diets, severe trauma, liver damage, renal loss of protein, and starvation (Meyer et al., 1992). Cholesterol levels were not correlated with disease for turtles in captivity or in the wild in this study. The relatively high levels of cholesterol for captive turtles from SWC, as well as from Miami Seaquarium (Varela, 1997), may be due to a prolonged fatty diet. Also, Bolten and Bjorndal (1992) found a positive correlation between cholesterol levels and body size, as well as higher cholesterol levels in female green turtles than males. Although the sex ratio of all captive turtles was approximately equal, high levels of cholesterol in SWC turtles may be at least partly attributed to their larger adult body sizes as compared to sub-adults study animals in Hawaii.

In captive reptiles, the most common cause for elevated plasma triglyceride is a metabolic defect in lipid transport or protein synthesis, resulting in obesity caused by overfeeding (Frye, 1981). Elevated levels of triglyceride in captive turtles from SWC and Miami Seaquarium (Varela, 1997) were likely due to a prolonged fatty diet, insufficient exercise, or both.

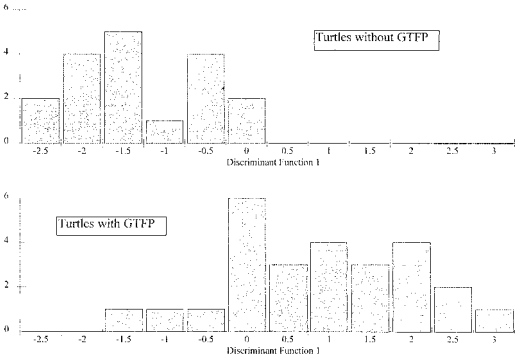

FIGURE 1. Distribution of green turtles with (bottom) and without (top) GTFP on the first axis of a discriminant function based on all biochemical parameters. (Turtles without GTFP: mean = −1.3, SD = 0.75, n = 18; Turtles with GTFP: mean = 0.92, SD = 1.1, n = 26).

When levels of uric acid rise, crystal deposition of the acid occurs in the tissues, leading to a condition similar to gout. Visceral gout and renal retention are commonly observed in captive reptiles, often as a result of a diet high in protein or organ meat. Chelonians in particular appear to be susceptible to articular gout, which has clinical signs similar to humans and other mammals—swollen, firm, and painful joints (Frye, 1981). Although GTFP did not influence levels of uric acid in this study, maintenance in captivity resulted in significantly elevated levels. The exclusive protein diet (squid) fed to captive turtles in Hawaii may be responsible for the twofold increase over values for free-ranging turtles. Similar values of uric acid in free-ranging and SWC turtles may be due to a mixed diet of protein and fat fed to turtles at SWC, suggesting that a more natural, mixed diet can be used to avoid gout-like conditions in captive animals.

Discriminant function analysis

Results from discriminant function analysis suggest that turtles could be classified into groups (tumored and non-tumored) by a multivariate analysis of the plasma biochemical parameters tested (Fig. 1). In

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both groups, turtles were correctly identified as tumored or non-tumored in 89% of the cases ($P \leq 0.005$), indicating that turtles affected with GTFP had distinct combinations of plasma biochemistries from non-tumored turtles. While the nature and cause of these differences remain uncertain, the high predictive ability of multivariate analysis suggests that GTFP results in significant alterations in an animals' biochemistry.

CONCLUSIONS

Results from this study contribute to the growing data-base on blood profiles for wild, captive, healthy, and diseased green turtles that can be used to identify biochemical responses to disease and captivity. The many inconsistencies among study findings support the need to improve study designs so that effects of disease, captivity, and sampling techniques can be isolated. Further research in this area will improve efforts to assess consequences of GTFP at both the individual and population level, and may also lead to a diagnostic tool that would facilitate efforts to monitor and eventually retard the spread of this potentially life-threatening epizootic.

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