Vol. 111: 61–68, 2014
doi: 10.3354/dao02765

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published August 21

Relating fibropapilloma tumor severity to blood parameters in green turtles *Chelonia mydas*

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ABSTRACT: Fibropapillomatosis is a neoplastic disease that is commonly found in the green turtles *Chelonia mydas* in tropical and subtropical regions of the world. In the current project, juvenile green turtles were captured with large-mesh tangle nets in the Indian River Lagoon and on nearshore reefs of Indian River County, Florida, USA, in 1998 and 1999. The purpose of the study was to determine the relationship between the severity of the disease and the general health of green turtles as indicated by blood parameters. All turtles were measured and examined, and the overall severity of the disease was rated by the size, number, and location of external fibropapilloma tumors. Hematocrit, total protein, and hemoglobin concentration were measured and compared with tumor scores (tumor severity appraisal). As the tumor score increased, the blood parameters of turtles decreased; for instance, the percentage of decrease in hematocrit for mildly afflicted, moderately afflicted, and severely afflicted groups were 2.6, 18.3, and 45.5%, respectively. Severely afflicted turtles suffered from anemia, while individuals with mild affliction did not.

KEY WORDS: *Chelonia mydas* · Fibropapillomatosis · Green turtle · Blood parameter · Hematology · Hematocrit · Total protein · Tumor severity

INTRODUCTION

Various threats, including commercial fisheries, oil spills, ingestion of plastic, disease, and degradation of nesting habitat due to coastal development, affect the recovery of sea turtles from depletion (NMFS & FWS 1991, 1998). Fibropapillomatosis (FP) is a disease of significant concern in wild sea turtles (NMFS & FWS 1991, 1998). As of November 2009, at least 379 peer-reviewed journals, books, newsletter articles, conference proceedings, and reports had been published related to this disease (Murakawa & Balazs 2009). Researchers have confirmed FP in all hard-shelled sea turtles, but the green turtles *Chelonia mydas* show the greatest prevalence in all major ocean basins — in some aggregations as many as 70% of the animals are afflicted (Balazs 1991, Ehrhart 1991, Herbst 1994). Green turtles are classified as 'Endangered' by the International Union for the Conservation of Nature (IUCN 2013).

The preponderance of scientific data supports that FP is caused by an alphaherpesvirus (Casey et al. 1997, Lackovich et al. 1999, Lu et al. 2000, Quackenbush et al. 2001, Herbst et al. 2004, Ene et al. 2005, Greenblatt et al. 2005). Also, there is considerable evidence linking anthropogenic degradation of habitats

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and other factors with disease prevalence (Foley et al. 2005, Van Houtan et al. 2010). Sigua & Tweedale (2003) found that ecological and biological integrity has declined in the past half century in the Indian River Lagoon Florida, USA; they estimated that an average of >832 000 kg of total nitrogen and >94 000 kg of phosphorus, from stormwater discharges and agricultural runoff, enter the Indian River Lagoon annually. Barile (2004) states that the nearshore ocean water, near the current study site, has relatively high nitrate-nitrite, ammonium, and soluble reactive phosphorus concentrations because the water of the 'hypereutrophic' Indian River Lagoon comes out and mixes with ocean water; these results would indicate that the Indian River Lagoon has a lower quality of water than does the nearshore ocean. Aguirre (1991) suggested that comparisons of hematological parameters between healthy and affected sea turtle populations might provide clues to the etiology and natural history of FP. In Molokai, Hawaii, USA, severely afflicted turtles were anemic and hypoproteinemic, but blood parameters in mildly afflicted turtles were the same as in non-diseased individuals (Work & Balazs 1999). In Indonesia, turtles with FP had significantly lower red cell counts than individuals with no tumors (Adnyana et al. 1997). Similar data as antecedents are lacking for green turtles in Florida. One of our study sites, the Indian River Lagoon, had an average annual FP prevalence in green turtles of 49% (range: 28–72%) between 1984 and 2000; 38 of 256 (14.8%) of the green turtles we captured in 1998 and 1999 at the nearshore reef had the disease (Ehrhart et al. 1986, Hirama & Ehrhart 2007). The impact of this disease on the Indian River Lagoon aggregation is unclear but could be significant because it is so prevalent (Ehrhart et al. 1986).

The purpose of this study was to compare and contrast the severity of tumors in green turtles with general health as indicated by hematocrit, hemoglobin concentration, and total protein content in blood serum. By understanding these relationships, we can estimate what percentage of the green turtles are in poor general health in a green turtle developmental habitat in Florida (Ehrhart et al. 2007).

MATERIALS AND METHODS

Study sites and capture methods

Details of the study sites (lagoon site: 27° 50′ N, 80° 27′ W; nearshore reef study site (hereafter, ocean site): 27° 50′ N, 80° 25′ W), including maps, the duration of the study (1998–1999), and capture methods have been described in Hirama & Ehrhart (2007).

Turtle size and fibropapillomatosis severity

Standard straight carapace lengths were measured to the nearest millimeter using calipers for all green turtles *Chelonia mydas* captured. Each turtle was subjectively assigned a tumor severity score based on tumor size and number: Score 0 = non-FP, Score 1 = mildly afflicted, Score 2 = moderately afflicted, Score 3 = severely afflicted (Hirama & Ehrhart 2007; following the categories of Work & Balazs 1999 and Work et al. 2001). For the lagoon sample of 309 captured individuals, 119 were categorized as Score 0, 100 as Score 1, 61 as Score 2, and 29 as Score 3; for the ocean sample of 136 captured individuals, 109 were categorized as Score 0, 25 as Score 1, 2 as Score 2, and 0 as Score 3.

Blood parameters

We evaluated a turtle's general health condition using hematocrit, hemoglobin, and total protein. Work & Balazs (1999) showed that hematocrit and estimated total solids significantly decrease as FP tumor score increases. Shortly after a turtle had been brought to the boat (5.2 m, skiff), approximately 6 ml of blood was drawn into a heparinized tube from its cervical sinus (Owen & Ruiz 1980). The blood was kept chilled during transport to the laboratory.

The sample sizes for 3 blood parameters were inconsistent because of the availability of equipment and grants (Table 1). For hematocrit, microtubes were filled with blood and spun for 3 min in a microcentrifuge (Compur M1100); the percentages of red blood cell volumes were read from a scale on the rotor of the instrument. Cyanomethemoglobin methods with Drabkin's hemoglobin reagent solution (Sigma Chemicals Kit: 525-A) were used to determine hemoglobin concentration. For understanding protein concentration in plasma, we collected 2 sets of blood parameters: plasma refractive index (low cost, fast process, less accurate) using a hand refractometer and total protein concentration (high cost, slow process, more accurate) using the biuret method (Sigma Tech. Bull. No. 540; Sigma 1973). A linear regression equation was determined between these 2 parameters. A plasma refractive index, which provides an estimate of total solids in plasma, was estimated for 171 turtles captured in the lagoon and for all 136 tur-

Table 1. Sample sizes for 3 blood parameters from the Indian River Lagoon and nearshore ocean reef (Indian River County, Florida, USA) with fibropapilloma (FP) tumor scores

	Non-diseased (FP Score 0)	Mildly afflicted (FP Score 1)	Moderately afflicted (FP Score 2)	Severely afflicted (FP Score 3)
Lagoon				
Hematocrit (n = 192)	83	62	30	17
Hemoglobin concentration (n = 179)	81	55	28	15
Total protein (n = 171)	78	54	26	13
Ocean				
Hematocrit (n = 136)	109	25	2	0
Hemoglobin concentration (n = 76)	61	13	2	0
Total protein (n = 136)	109	25	2	0

Table 2. Coefficients of the predictive model for (a) total protein, (b) hematocrit, and (c) hemoglobin as a response. The intercept corresponds to fibropapilloma (FP) Score 0 and to the lagoon site. For the model overall: adjusted R^2 = (a) 0.38, (b) 0.41, (c) 0.31; F = (a) 24.89, (b) 29.49, (c) 15.45; df = (a) 8,297, (b) 8,318, (c) 8,245; p < 0.001. CL: carapace length

Term	Estimate	SE	*t*-value	p (>|*t*|)
(a) Total protein				
Intercept	2.93	0.23	13.00	<0.001
Ocean site	–0.40	0.08	–5.30	<0.001
CL	0.04	0.00	8.33	<0.001
FP Score 1	0.72	0.43	1.67	0.096
FP Score 2	0.23	0.66	0.35	0.725
FP Score 3	–5.34	1.45	–3.67	<0.001
CL × FP Score 1	–0.01	0.01	–1.29	0.199
CL × FP Score 2	–0.01	0.02	–0.66	0.508
CL × FP Score 3	0.11	0.04	3.20	0.002
(b) Hematocrit				
Intercept	28.83	1.54	18.70	<0.001
Ocean site	–4.00	0.52	–7.75	<0.001
CL	0.07	0.03	2.50	0.013
FP Score 1	–0.65	2.92	–0.22	0.825
FP Score 2	–0.85	4.56	–0.19	0.853
FP Score 3	–36.62	6.99	–5.24	<0.001
CL × FP Score 1	0.01	0.06	–0.08	0.933
CL × FP Score 2	–0.11	0.11	–1.05	0.293
CL × FP Score 3	0.59	0.17	3.57	<0.001
(c) Hemoglobin				
Intercept	8.48	0.71	11.96	<0.001
Ocean site	–1.65	0.24	–6.76	<0.001
CL	0.03	0.01	2.18	0.030
FP Score 1	0.22	1.34	0.16	0.870
FP Score 2	–5.26	1.88	–2.81	0.005
FP Score 3	–9.65	2.85	–3.39	<0.001
CL × FP Score 1	–0.01	0.03	–0.19	0.852
CL × FP Score 2	0.09	0.04	2.10	0.037
CL × FP Score 3	0.16	0.07	2.39	0.017

Table 3. Least-squares means, their standard errors, and 95% confidence limits by capture site (lagoon or ocean) and by fibropapilloma (FP) disease score (FP0 to FP3) for the models in Table 2. Because of the interaction of FP score class with carapace length, each FP mean is estimated at the average carapace length for that disease score class

	Least-squares mean	SE	95% confidence limits Lower	Upper
Total protein (g dl^{-1})				
Lagoon	4.53	0.07	4.39	4.68
Ocean	4.13	0.09	3.95	4.31
FP0	4.42	0.04	4.34	4.51
FP1	4.54	0.07	4.41	4.67
FP2	4.03	0.11	3.81	4.26
FP3	3.45	0.16	3.13	3.78
Hematocrit (%)				
Lagoon	28.27	0.41	27.47	29.07
Ocean	24.28	0.55	23.20	25.36
FP0	30.33	0.29	29.76	30.91
FP1	29.77	0.44	28.90	30.64
FP2	24.45	0.74	23.00	25.91
FP3	17.74	1.00	15.76	19.71
Hemoglobin (g dl^{-1})				
Lagoon	9.04	0.18	8.69	9.40
Ocean	7.40	0.26	6.88	7.91
FP0	9.10	0.14	8.83	9.37
FP1	8.96	0.21	8.55	9.37
FP2	7.40	0.31	6.79	8.02
FP3	5.89	0.43	5.04	6.75

tles captured in the ocean (Table 1). Among those, 41 (19 from the lagoon sample and 22 from the ocean sample) were randomly chosen and analyzed for total protein concentration using the biuret method. Of these individuals, all had an FP score of 0, except 1 turtle with Score 1 and 1 with Score 2, both from the lagoon. For turtles for which total protein value was not measured directly (152 from the lagoon, 114 from the ocean), the refractive index was converted to total protein by the linear regression equation described in the following subsection.

Statistical analyses

Statistical analyses were performed with R Version 3.0.1 functions (R Core Team 2013). Additional func-

Table 4. Pairwise comparison matrix (p-values) between least-squares means of blood parameters for fibropapilloma (FP) score groups after adjustment by Holms sequential Bonferroni method (Holm 1979). Each pairwise test was evaluated at the average carapace length of the higher FP score class. All least-squares mean comparisons between lagoon and ocean samples were significant at $p < 0.001$ so are not shown here. Significant p-values ($p < 0.05$) are in **bold**

FP score	FP score 1	FP score 2	FP score 3
Total protein			
0	0.099	0.449	**<0.001**
1		**0.008**	**<0.001**
2			**0.023**
Hematocrit			
0	0.874	**<0.001**	**<0.001**
1		**<0.001**	**<0.001**
2			**<0.001**
Hemoglobin			
0	1.0	**<0.001**	**<0.001**
1		**<0.001**	**<0.001**
2			**0.027**

tions were used from the following R packages: AICcmodavg (Mazerolle 2013), car (Fox & Weisberg 2011), Hmisc (Harrell et al. 2013), lsmeans (Lenth 2013a), MASS (Venables & Ripley 2002), moments (Komsta & Novomestky 2012), and multcomp (Hothorn et al. 2008). Linear models for predicting total blood protein, hematocrit, and hemoglobin concentration initially included all possible interactions among predictors. Interactions were progressively removed from highest to lowest p-values until only those for which $p \leq 0.05$ (if any) remained. This approach favored the same final models as those obtained by comparing with the corrected Akaike's information criterion (AIC_c) of the models considered (Burnham & Anderson 2002).

Models were evaluated with refractive index, carapace length, and capture site as predictors of the total protein measured. Because no interactions were significant, and because the main effects of capture site and carapace length were not significant, a regression model with refractive index alone as its predictor was used to estimate total protein for all captured individuals whose total blood protein was not measured by the biuret method. The relationship was assumed to be the same for all FP score categories, although the regression was based almost entirely on individuals with an FP score of 0. The regression equation used was total protein = –197.58 + 196.28 × refractive index (adjusted $R^2 = 0.875$, $F = 281$, df = 1, 39, $p < 0.001$).

Total protein, hematocrit, and hemoglobin concentration were modeled as functions of capture site, FP score, and straight carapace length. Residuals from each model fit were examined for normality and influential cases (Fox 2008). Least-squares means (SAS Institute 2012, Lenth 2013a,b) were computed and compared for the effects of capture site and FP score class. For total protein, models using inverse variance weighting for the point estimates predicted

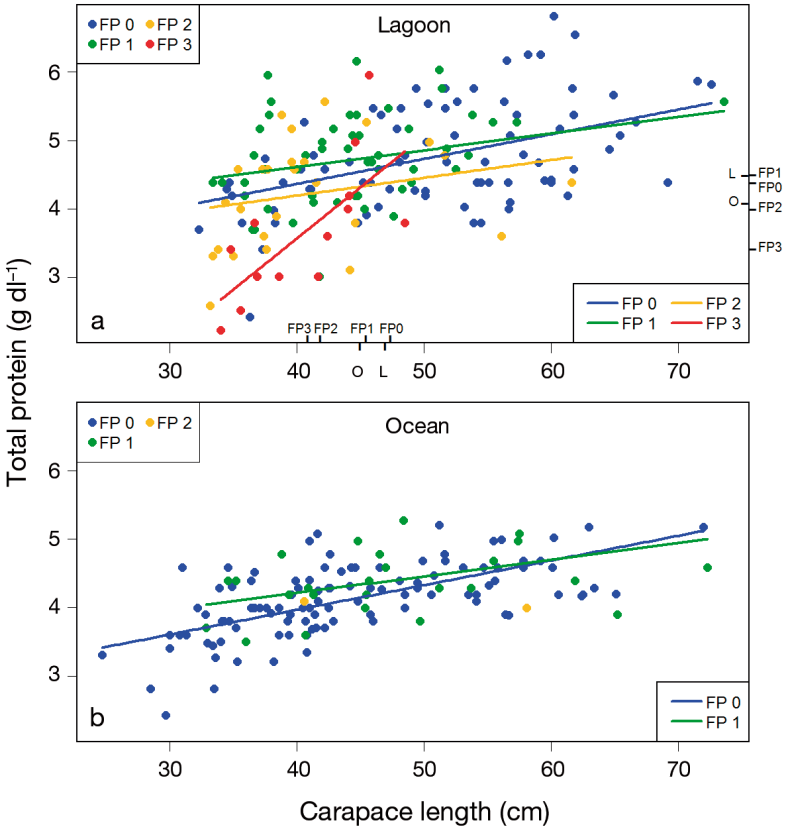

Fig. 1. Total protein versus carapace length for individuals from the (a) lagoon and (b) ocean. Colors differentiate individuals by fibropapilloma (FP) score. Colored lines indicate the average relationship fitted by the model in Table 2 over the range of carapace lengths observed for the FP score group. Fits are shown only for FP 0 and FP 1 classes for the ocean sample because few FP2 individuals and no FP3 individuals were captured at the ocean site. Symbols on the right axis of (a) indicate the least-squares means for the lagoon (L), ocean (O), and the 4 FP score classes. Because of the significant interaction between FP score and carapace length, the least-squares means for FP score class were computed at the average carapace length of individuals for which total protein was measured or estimated in each class. Symbols on the carapace length axis of (a) indicate mean carapace lengths for the L sample, the O sample, and the 4 FP score classes

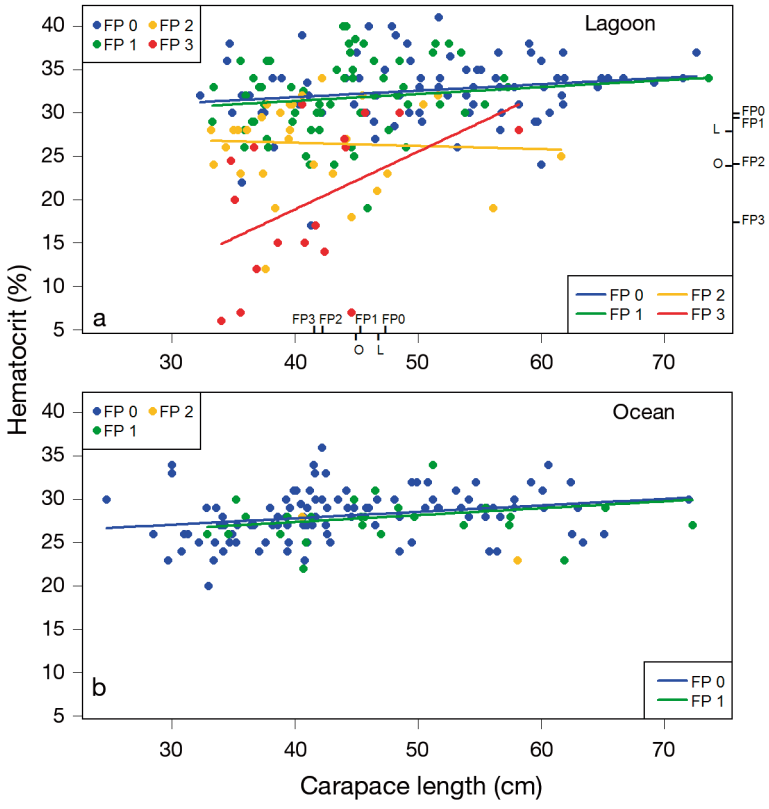

Fig. 2. Hematocrit versus carapace length for individuals from the (a) lagoon and (b) ocean. Abbreviations and symbols are as in Fig. 1; the model represented is from Table 2b. Least-squares means for fibropapilloma (FP) score class were computed at the average carapace length of individuals for which hematocrit was measured in each class

from the refractive index were also checked to ensure that the main results were not heavily influenced by protein values estimated with low precision. Because almost all individuals with high FP scores were captured at the lagoon site, models were also checked after deleting individuals with FP scores of 2 or 3.

RESULTS

If point estimates from the regression of total protein versus refractive index were weighted equally to direct measures by the biuret method, then similar predictive models (in terms of main effects and interactions retained) were favored for all 3 blood parameters of the green turtles *Chelonia mydas* (Table 2). On average, blood parameter values were lower in individuals captured at the ocean site than for those captured in the lagoon after effects of carapace length and FP score group had been taken into account (Tables 3 & 4). There were significant interactions between FP score and the effect of carapace length. Blood parameter values tended to increase mildly with carapace length of turtles with FP Scores 0 to 2, but increased more steeply with carapace length of turtles with FP Score 3 in the lagoon (Figs. 1–3). There was little difference in blood parameter values between individuals with FP Score 0 and 1, but those with FP Score 2 in the lagoon had significantly lower values than individuals with FP Scores 0 or 1 for hematocrit and hemoglobin, and those with FP Score 3 had significantly lower values than all other FP score groups for all 3 blood parameters. When point estimates from the regression of total protein versus refractive index were weighted by the standard errors of the predicted values, the model favored for total protein (not shown) was simpler in that the interaction between FP score and carapace length was not significant, but otherwise gave similar trends. If individuals with FP Scores 2 and 3 were omitted, the effects of capture site and of carapace length on all 3 blood parameters remained highly significant and indicated similar effects to those of the models that included individuals with FP Scores 2 and 3.

DISCUSSION

Hematocrit, hemoglobin concentration, and total protein were inversely related (the higher the FP score, the lower the blood parameters) to tumor severity and, thus, greater FP tumor severity may be detrimental to green turtles' health. Causes of hemorrhagic (blood loss) anemia include traumatic injuries, bloodsucking parasites, a coagulopathy, or an ulcerative lesion; in most reptiles, hematocrit <20% is considered anemia, while normal individuals have about 30% (Campbell 2006). Moderately and severely afflicted individuals were, in general, hypoproteinemic and anemic; turtles in those categories comprised 19.7% (Score 2) and 9.4% (Score 3), respectively, of the animals at the lagoon study site, but only 1.5% (Score 2) and no (Score 3) animals at the ocean study site. Although trend analyses of each FP tumor score at the lagoon and reef study sites have not been performed in recent years, they seem to remain relatively constant (authors' pers. obs.). The 3 blood parameters from the lagoon site

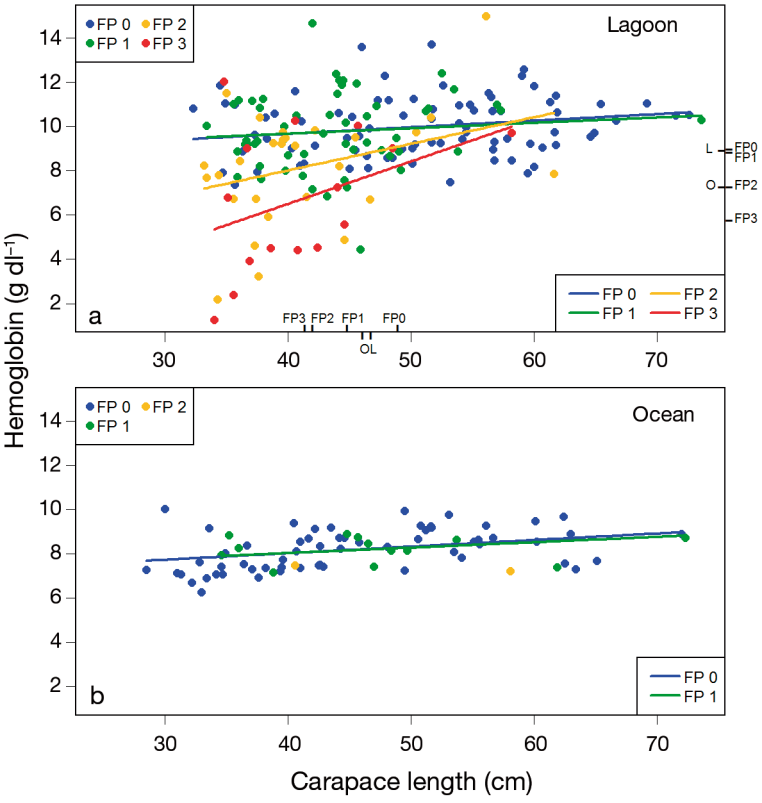

Fig. 3. Hemoglobin versus carapace length for individuals from the (a) lagoon and (b) ocean. Abbreviations and symbols are as in Fig. 1; the model represented is from Table 2c. Least-squares means for fibriopapilloma (FP) score class were computed at the average carapace length of individuals for which hemoglobin was measured in each class

showed higher values than those from the ocean site. The growth rate study regarding green turtles from the same study sites used here showed that individuals in the lagoon had significantly faster growth rates than those at the ocean site; the authors speculated that the lagoon's higher biomass of drift algae and slower water movements (lower energy consumption to swim and feed) could be the reasons for the difference (Kubis et al. 2009).

Hematocrit analyses indicated that moderately and severely afflicted turtles had lower red blood cell volumes than did non-diseased or mildly afflicted individuals. Studies of green turtles in Hawaii (Aguirre et al. 1995, Work & Balazs 1999) found similar trends, although mean hematocrit values for all tumor scores were higher in Hawaii than at our study sites.

The results of hematocrit and hemoglobin concentration analyses suggest that moderately and severely afflicted turtles have a lower oxygen-carrying capacity than do non-diseased or mildly afflicted individuals; this could cause the moderately to severely afflicted turtles to have abbreviated diving times. If severely afflicted turtles have shorter dive durations per breath — resulting in less foraging time per dive — than non-FP or mildly afflicted turtles, they would have to expend more energy than apparently healthy individuals to obtain the same amount of food. If this hypothesized relationship between low hematocrit and hemoglobin concentration and shorter dives is correct, then the quantity of food that turtles obtain during foraging dives may be affected by the severity of disease, i.e. the number and size of tumors. The marked decrease in total protein in most of the severely afflicted turtles suggests that they were nutritionally deficient compared with non-diseased, mildly afflicted, and moderately afflicted turtles. Moderately to severely afflicted individuals either have insufficient food intake or fail to assimilate protein from the ingested food, or possibly a combination of both.

At both lagoon and ocean sites the general health of green turtles that were mildly afflicted by FP appeared to be similar to that of non-FP turtles. Similar results were found in a study carried out in Indonesia that compared blood parameters of mildly afflicted FP and non-FP turtles (Adnyana et al. 1997). The average (±SD) number of tumors per turtle in Indonesia was 5.0 ± 4.1, similar to that at our ocean site (4.7 ± 12.6; Hirama & Ehrhart 2007).

Although it seems likely that the hematologic trends that we have documented result from FP infection, it is also possible that FP infection is more likely for individuals that are already experiencing poorer general health or that the disease and depressed blood parameters reflect an undetected common cause. The absence of moderate and severe FP infections in the largest turtles might be a result of earlier mortality from nutritional or locomotory impairment of infected individuals. The swimming speed of a moderately to severely afflicted FP turtle is slower (and the animals are therefore easier to capture by hand from a boat) than that of a non-diseased turtle (authors' pers. obs.). A future correlative study of swimming speed and tumor severity might be informative. In any case, the similarity in the forms of predictive models favored for the 3 blood parameters suggests that they may be varying in response to the same underlying cause.

Acknowledgements. This research was funded by the National Marine Fisheries Service (Grant Number 40AANF903414), the Bernice Barbour Foundation, and the University of Central Florida Biology Department. Authors thank the University of Central Florida Marine Turtle Research Group for assistance in capturing turtles. The authors are grateful to J. Boyett, B. Crowder, D. Bagley, A. Foley, R. Hardy, L. Herren, E. Leone, A. Meylan, P. Pritchard, F. Snelson, B. Stacy, N. Stacy, J. Weishampel, and B. Witherington for their advice and comments.

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Submitted: December 17, 2013; Accepted: May 12, 2014
Proofs received from author(s): August 3, 2014