

# Retrospective pathology survey of green turtles *Chelonia mydas* with fibropapillomatosis in the Hawaiian Islands, 1993–2003

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**ABSTRACT:** We necropsied 255 stranded green turtles *Chelonia mydas* with fibropapillomatosis (FP) from the Hawaiian Islands, North Pacific, from August 1993 through May 2003. Of these, 214 (84 %) were euthanized due to advanced FP and the remainder were found dead in fresh condition. Turtles were assigned a standardized tumor severity score ranging from 1 (lightly tumored) to 3 (heavily tumored). Tumors were counted and measured and categorized as external, oral, or internal and tissues evaluated by light microscopy. Turtles in tumor score 2 and 3 categories predominated, and tumor score 3 turtles were significantly larger than the other 2 categories. More juveniles stranded than subadults or adults. Total cross-sectional area of tumors increased significantly with straight carapace length (SCL). Frequency distribution of total number of external tumors per turtle was significantly skewed to the right, and there were significantly more tumors at the front than rear of turtles. Eighty percent of turtles had oral tumors, and 51 % of turtles with oral tumors had tumors in the glottis. Thirty-nine percent of turtles had internal tumors, most of them in the lung, kidney and heart. Fibromas predominated in lung, kidney and musculoskeletal system whereas myxofibromas were more common in intestines and spleen. Fibrosarcomas of low-grade malignancy were most frequent in the heart, and heart tumors had a predilection for the right atrium. Turtles with FP had significant additional complications including inflammation with vascular flukes, bacterial infections, poor body condition, and necrosis of salt gland. Turtles with oral tumors were more likely to have secondary complications such as pneumonia. Most turtles came from the island of Oahu (74 %) followed by Maui (20 %), Hawaii, Molokai, and Lanai (<3 % each). On Oahu, significantly more turtles we necropsied stranded along the northwestern and northeastern shores.

**KEY WORDS:** Green turtle · *Chelonia mydas* · Fibropapillomatosis · Pathology · Epizootiology

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## INTRODUCTION

The Hawaiian Islands in the North Pacific have 2 species of marine turtles that live in coastal areas, the hawksbill *Eretmochelys imbricata* and the far more numerous green turtle *Chelonia mydas*. Since green turtles were listed as threatened and protected in 1978, numbers of nesting females at French Frigate Shoals, the primary nesting ground located in the northwestern Hawaiian Islands, have increased steadily (Balazs

& Chaloupka 2004). However, health issues continue to be of concern to the management and recovery of the Hawaiian green turtle population (Balazs & Pooley 1991, Aguirre et al. 1998, Work et al. 2003).

Most research on health of green turtles in Hawaii has focused on fibropapillomatosis (FP), a neoplastic condition first described in green turtles from Florida (Smith & Coates 1938). FP is manifested by external and internal tumors (Herbst 1994), and in more severely affected turtles by anemia, leukopenia and heterophilia

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(Aguirre et al. 1995, Work & Balazs 1999), immunosuppression (Work et al. 2001), and bacteraemia (Work et al. 2003). In Hawaii, FP has had a prevalence of 40 to 60% depending on areas and method of sampling (Balazs & Pooley 1991) and comprises a majority of stranding cases in sea turtles (Murakawa et al. 1999). Like turtles in Florida (Herbst et al. 1998) and Indonesia (Adnyana et al. 1997), turtles with FP in Hawaii have concomitant infections with vascular trematodes (Aguirre et al. 1998). However, green turtles with FP from Hawaii appear unique in that many have oral tumors, particularly in the glottis (Aguirre et al. 2002).

Several factors associated with FP have been investigated, including vascular trematodes (Dailey & Morris 1995), contaminants (Aguirre et al. 1994), marine biotoxins (Landsberg et al. 1999, Arthur et al. in press), and retroviruses (Casey et al. 1997). Herbst et al. (1995) were able to transmit FP experimentally to green turtles using cell-free extracts of tumors, thereby suggesting a viral etiology. Recent evidence strongly implicates a herpesvirus as a probable cause or cofactor of FP (Herbst 1995). Nucleic acid from this virus has been consistently and closely associated with neoplastic tissue of green turtles in Hawaii and Australia (Quackenbush et al. 2001), olive ridley turtles *Lepidochelys olivacea* in Costa Rica (Quackenbush et al. 1998), and green and loggerhead *Caretta caretta* turtles in Florida (Lackovich et al. 1999). However, attempts to culture the FP-associated herpesvirus have been unsuccessful despite the availability and use of green turtle embryo and tumor cell lines (Moore et al. 1997, Lu et al. 2000a). This has stymied efforts to replicate the disease under controlled conditions or to develop serologic tests. Existing serologic tests for the FP-associated herpesvirus are cumbersome and of unknown specificity or sensitivity (Herbst et al. 1998).

Most published necropsy reports on FP in Hawaii and Florida turtles are descriptive and involve small numbers of individuals over short time intervals (Aguirre et al. 1998) or case reports on individual turtles (Norton et al. 1990) although attempts have been made to summarize necropsy findings in larger sets of turtles (Work & Balazs 1998). While such studies are valuable to describe FP in individuals, it is hard to reach conclusions regarding behavior and effects of the disease, particularly over time. There are 2 ways around this. First, individual turtles with disease can be monitored to measure progression of FP. However, this is impractical for free-ranging green turtles where recapture of animals usually involves considerable effort with a low likelihood of success. The other method is to examine large numbers of turtles comprising different size classes in a retrospective study. Our aim was to describe the pathogenesis of FP in different size classes of turtles by analyzing necropsy data collected over a 10 yr period.

## MATERIALS AND METHODS

Stranded turtles were those that crawled or washed ashore moribund or freshly dead. Turtles were examined between August 1993 and May 2003. Moribund animals, and those judged to have poor prognosis for survival based on severity and complexity of lesions, were euthanized by intracardiac injection of barbiturate solution (Beuthanasia D special). Turtles with FP were assigned a tumor score (TS) that quantified the severity of disease. Briefly, the size of tumors on animals was estimated (cm) and placed into 4 groups (<1 cm, 1–4 cm, >4–10 cm, and >10 cm diameter). Based on the number and size of tumors, turtles were assigned a score ranging from 1 (lightly tumored) to 3 (heavily tumored) (Work & Balazs 1999). To obviate inter-observer variation, the same individual scored turtles over the entire period.

Turtles were weighed ( $\pm 0.5$  kg) and the straight carapace length (SCL  $\pm 0.1$  cm) was recorded. Turtles with SCL <65 cm were classified as juveniles, subadults (65 to 82 cm), and adults (>82 cm) (Balazs 1980). For each turtle, a numerical body condition index (BCI) was calculated by dividing weight by the cube of the SCL ( $\text{kg}/\text{SCL}^3$ ) (Bjorndal et al. 2000).

All turtles underwent a systematic external and internal examination. Tumors on the eyes (left or right), neck, front or rear flippers (left or right), tail, and scutes (tumors on seams and scutes of the plastron or carapace) were classified as external tumors. External tumors were further categorized as occurring in the front (eyes, neck, front flippers) or rear (rear flippers, tail). Tumors on the oral canthus, glottis, hard or soft palate, pharynx or tongue were classified as oral tumors. Tumors in the lungs, heart, kidney, intestines, spleen, liver, or muscle and bone were classified as internal tumors. Internal tumors were further classified as firm if they were of uniform consistency, or cystic if they contained fluid-filled cysts. Necrosis in tumors was noted and characterized by the presence of laminated fibrin or friable discolored tissue within the tumor. Protein concentration ( $\text{g dl}^{-1}$ ) in fluids from tumor cysts was estimated with a refractometer.

Each external, oral, and internal tumor was measured at its 2 widest cross-sectional dimensions with a ruler. Multi-lobulated external tumors on a single stalk were measured as a single unit. We estimated cross-sectional area ( $\text{cm}^2$ ) of each tumor using the formula for circle ( $\pi r^2$ ) or ellipse ( $\pi \times 0.5a \times 0.5b$  for the major [ $a$ ] and minor [ $b$ ] axis of an ellipse) depending on whether the cross section approximated a circle or ellipse.

Sections of skin, brain, salt gland, thyroid, tongue and glottis, muscle, lung, heart, kidney, spleen, liver, small and large intestines, esophagus, stomach, gonad, urinary bladder, and tumors were fixed in 10% neutral

buffered formalin, embedded in paraffin, sectioned at 5  $\mu\text{m}$ , stained with hematoxylin and eosin, and examined with a light microscope. We used Masson trichrome stain to identify collagen, Alcian blue-Periodic acid Schiff (Alcian blue-PAS) to identify polysulfated proteoglycans, and Gram stain to identify bacteria (Prophet et al. 1992).

Based on histology, external and oral tumors were classified as FP using criteria of Jacobson et al. (1989) and Aguirre et al. (2002). Internal tumors were categorized as myxofibromas, fibromas, or fibrosarcomas of low-grade malignancy. Myxofibromas were tumors consisting of dense to loose bundles of collagen with small to large numbers of pleomorphic fibroblasts where the matrix between collagen fibrils stained with alcian-blue periodic acid-Schiff solution (PAS) (Norton et al. 1990). Fibromas and fibrosarcomas did not stain with alcian-blue-PAS. Fibromas were composed of dense bundles of collagen containing small to large numbers of pleomorphic fibroblasts that were well differentiated from surrounding tissue. Fibrosarcomas of low-grade malignancy were tumors with dense collagen bundles, large numbers of randomly arranged pleomorphic fibroblasts, invasion of surrounding tissue, tissue necrosis or hemorrhage, and mitotic figures (Pulley & Stannard 1990).

Stranding locations of turtles we necropsied were converted to Universal Transverse Mercator-North American Datum 83 coordinates, imported into ArcView 3.2 (ESRI Inc.) and overlaid on coastlines of the main Hawaiian Islands (Hawaii, Oahu, Maui, Molokai, Lanai, Kauai). The Oahu coastline was divided into 4 zones based on discrete geographic contours of the island (NW, NE, S, SW). Spatial patterns of stranding were assessed based on date of stranding, age, sex, tumor score, and total number of turtles stranding  $\text{km}^{-1}$  coastline in each of the 4 areas.

Parametric or non-parametric statistical tests were used depending on whether or not data adhered to assumptions of normality and equal variance. Analysis of variance or Kruskal-Wallis ANOVA (Daniel 1987) was used to compare SCL and BCI between tumor-score categories. We used a G-test (Sokal & Rohlf 1981) to see if sex ratio differed significantly from 1:1 and if there was a significant difference in numbers of turtle strandings on each section of the Oahu coast. Chi-square (Daniel 1987) was used to assess the association between organ and number of internal tumors, the association between morphology of internal tumors and organ where tumor occurred, and the association between pulmonary necrosis and glottal tumors. Student's *t*-test or the Mann-Whitney *U*-test (Daniel 1987) was used to compare tumor burden in front versus rear of turtle. Standard linear regression (Daniel 1987) was used to determine the relationship between total area

and total number of tumors per turtle. Multiple linear regression (Daniel 1987) was used to assess the relationship between total area of external, oral, and internal tumors versus SCL. The level of significance was 0.05.

## RESULTS

Of 255 turtles, we examined 170 (67%) juveniles, 63 (25%) subadults, and 22 (8%) adults. TS-2 and TS-3 turtles comprised a majority of the carcasses examined (Table 1). SCL ranged from 38.8 to 90.3 cm, and TS-3 turtles had significantly ( $F = 4.698$ ,  $p = 0.010$ ,  $df = 253$ ) larger SCL than TS-2 and TS-1 turtles (Table 1). No significant difference was seen in BCI between the three TS categories (Table 1). Sex was determined for 249 turtles, and the sex ratio of males (120) to females (129) did not differ significantly ( $p > 0.05$ ) from 1:1. A total of 214 (84%) stranded turtles were humanely euthanized and the remainder were found freshly dead or died shortly (<12 h) after retrieval. Thirty-nine (15%) carcasses were frozen prior to examination.

Grossly, external tumors were sessile, pedunculated, unlobulated to multilobulated, smooth to rough, with a cross-sectional area ranging from 0.5 to 379  $\text{cm}^2$  (Fig. 1A). The frequency distribution of external tumors per turtle was significantly ( $p < 0.001$ ) skewed to the right with fewer turtles having the highest number of tumors (Fig. 2). There was a significant ( $F = 48.252$ ,  $r^2 = 0.16$ ,  $p < 0.001$ ,  $df = 253$ ) linear relationship between total tumor area per turtle and total number of tumors per turtle. Total area of external, oral, and internal tumors increased significantly ( $F = 16.535$ ,  $p < 0.001$ ,  $df = 253$ ) with increasing SCL; however, there was no significant increase when total number of tumors was compared to SCL (Fig. 3). The number of tumors per turtle (median) at the front (11) was significantly ( $t = 89624.000$ ,  $p < 0.001$ ) greater than the number of tumors at the rear (3). The regression slope of tumors at the front of turtles versus total tumor burden was significantly steeper ( $z = 6.54$ ,  $p < 0.001$ ) than the slope of tumors at the rear versus total tumor burden

Table 1. *Chelonia mydas*. Mean  $\pm$  SD for straight carapace length (SCL) and body condition index (BCI) for stranded turtles with fibropapillomatosis (FP) in tumor score (TS) categories 1 ( $n = 14$ ), 2 ( $n = 120$ ), and 3 ( $n = 121$ ). \*Values significantly different at  $p < 0.05$

	TS-1	TS-2	TS-3
SCL	59.3 $\pm$ 15.2	58.7 $\pm$ 11.0	63.4 $\pm$ 12.5*
BCI	0.100 $\pm$ 0.039	0.107 $\pm$ 0.019	0.113 $\pm$ 0.045

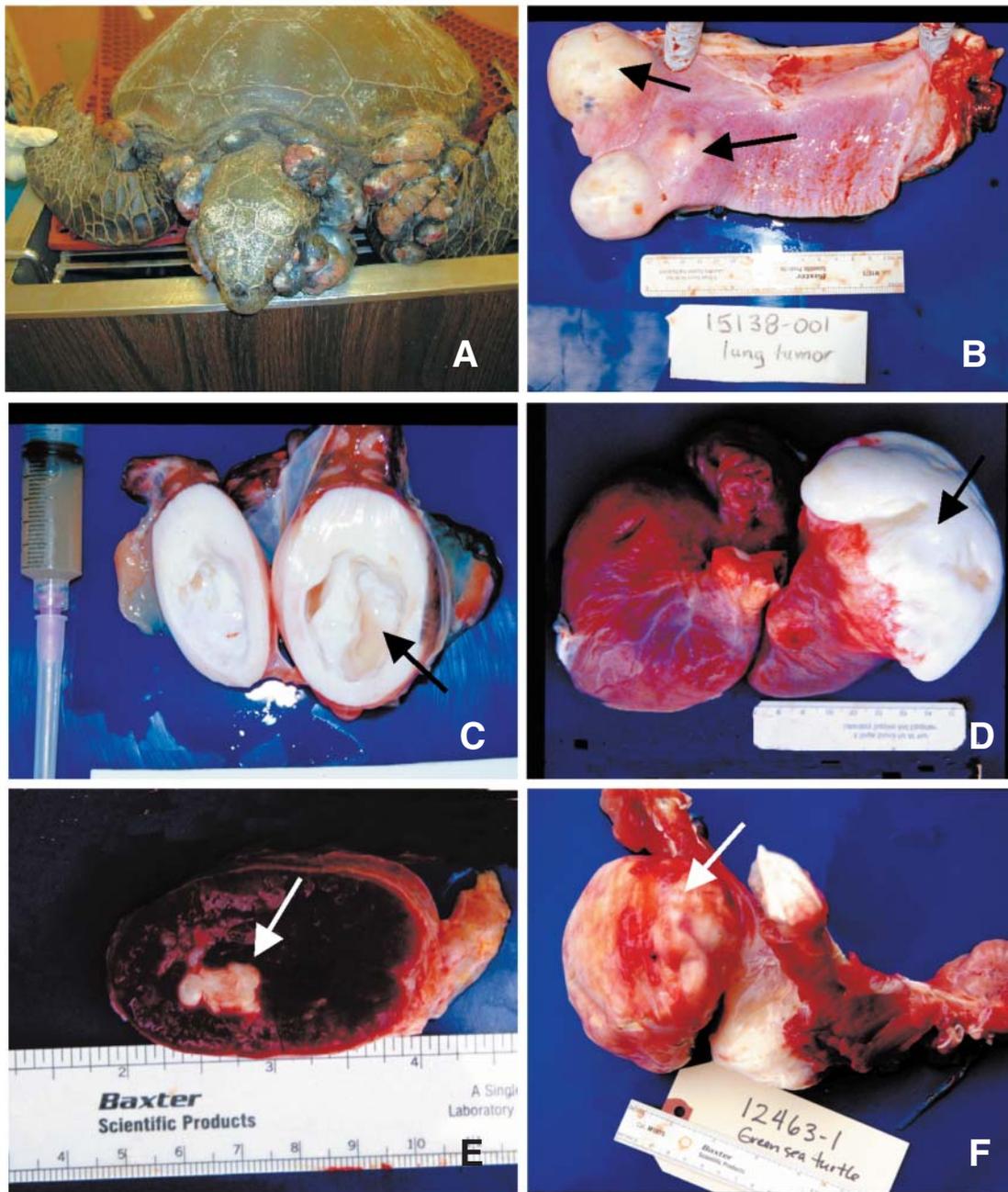


Fig. 1. *Chelonia mydas*. (A) Turtle with epithelial tumors on front flipper, eyes, and oral canthus. (B) Tumors (arrows) in the caudal lung. (C) Cross section of renal tumor; note cyst (arrow) in tumor and fluid in syringe that was obtained from the cyst. (D) Tumor (arrow) in right atrium of heart. (E) Splenic tumor (arrow). (F) Tumor on scapula (arrow)

(Fig. 4). There was no significant difference in number of tumors per turtle on the right versus left side. Ninety two percent of turtles (234/254) had tumors in one or both eyes or eyelids. Twenty-seven of 255 (11%) turtles had tumors on the scutes and seams.

Data for oral tumors were available for 254 turtles (the head of 1 turtle was missing and not available for examination), and 203 (80%) turtles had oral tumors.

Grossly, oral tumors in the angle of the jaws that projected externally were variably pigmented; oral tumors inside the mouth were not. Of 203 turtles with oral tumors, 104 (51%) had tumors in the glottis. Glottal tumors were sessile to pedunculated and had a smooth to rugose cauliflower-like appearance. In some cases, glottal tumors formed a ball-valve that protruded into the glottal inlet and thus into the trachea, preventing

closure of and impeding air flow through the glottis. Of 104 turtles with glottal tumors, 35 (34%) had tumors in other locations in the mouth, including hard and soft palate, choanae, and tongue.

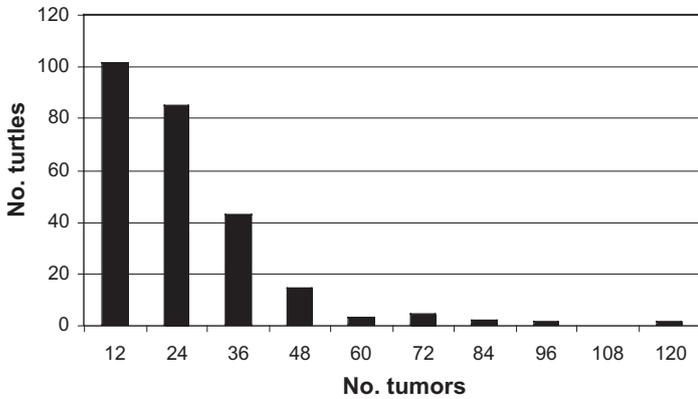


Fig. 2. *Chelonia mydas*. Frequency distribution of total number of tumors per turtle

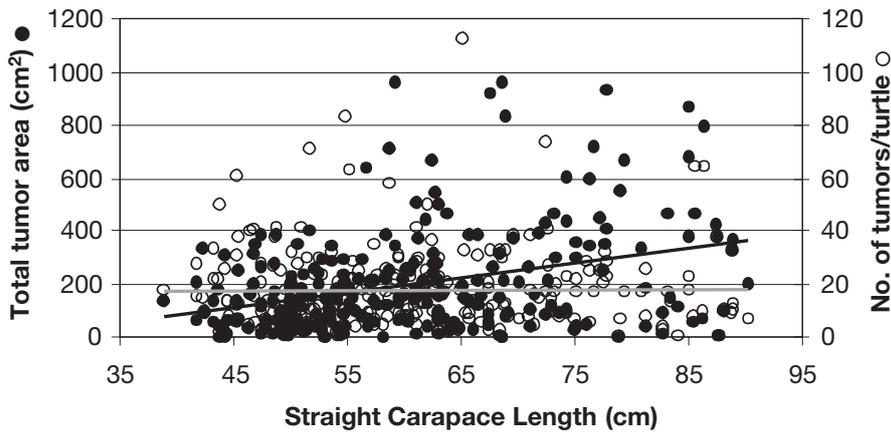


Fig. 3. Regression of total tumor area per turtle (●, —) and total number of tumors per turtle (○, - -) versus SCL (cm)

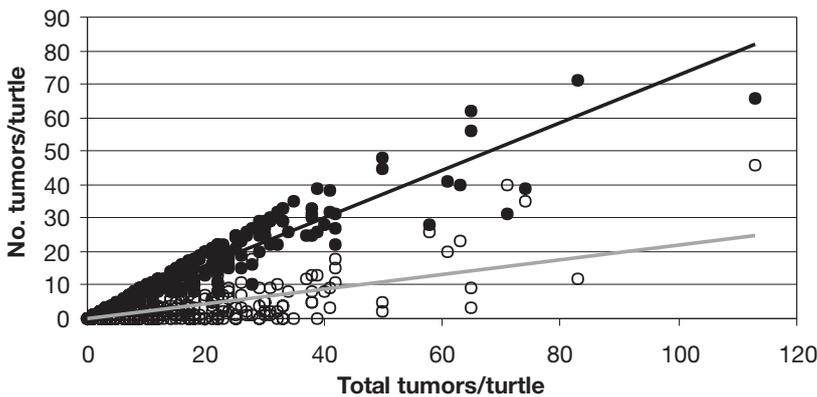


Fig. 4. *Chelonia mydas*. Regression of number of tumors per turtle on the front (●, —) and rear (○, - -) versus total tumor burden

Table 2. *Chelonia mydas*. Number of instances where internal tumors were found in a specific organ in turtles having 1, 2, 3, or 4 different organs affected by tumors

Organ	Number of organs affected				Total
	1	2	3	4	
Lung	35	22	11	4	72
Heart	5	13	8	2	28
Kidney	7	11	4	1	23
Muscle	4	4	2	2	12
Instestines	3	5	6	3	17
Spleen	1	0	5	4	10
Liver	0	1	0	0	1

Thirty-nine percent (99/255) of turtles had internal tumors. Of 99 turtles with internal tumors, 55, 28, 12, and 4 turtles had tumors in 1, 2, 3, or 4 different organs, respectively. There was a significant ( $\chi^2 = 38.496$ ,  $df = 18$ ,  $p = 0.003$ ) association between the type of organ and total number of organs affected by tumors with the lungs, heart, and kidney most often affected (Table 2). The spleen was more often affected when 3 or more organs were tumored, and tumors were rarely found in the liver. There was a significant ( $\chi^2 = 28.98$ ,  $df = 5$ ,  $p < 0.001$ ) association between gross morphology of tumors and type of organ (Table 3). Tumors of the lungs and kidneys were well-circumscribed, firm, white, and often contained variably sized cysts filled with clear protein-rich fluid ( $>4 \text{ g dl}^{-1}$ ). Intestinal tumors were pedunculated to sessile, projected from the serosal surface, and also contained variably sized thin-walled fluid-filled sacs. In 2 turtles, tumors originated from the mucosa of the duodenum and stomach. Distribution of lung tumors did not appear to follow the bronchiolar tree (Fig. 1B). In some cases, kidney tumors completely engulfed the organ leaving little to no renal tissue (Fig. 1C). Necrosis within tumor tissue was infrequent but, when present, was most often in the lung (7%) and generally limited to larger tumors with cross sectional area  $>100 \text{ cm}^2$ . There was no significant association between number of external and internal tumors.

Tumors in the heart (Fig. 1D), liver, spleen (Fig. 1E), muscle, and bone (Fig. 1F) were generally firm, white, and circumscribed; fluid-filled cysts in those organs were less common than in the kidneys (Table 3). In 28 turtles with heart

Table 3. *Chelonia mydas*. Number of turtles with internal tumors manifesting particular gross and microscopic characteristics partitioned by type of organ. See text for explanation. Myxo: myxofibroma; FLGM: fibrosarcoma of low-grade malignancy

Organ	Gross morphology		Microscopic morphology		
	Firm	Cystic	Fibroma	Myxo	FLGM
Heart	26	2	11	2	15
Lung	60	7	43	20	0
Kidney	11	11	7	5	0
Spleen	10	0	4	6	0
Muscle/bone	10	1	6	2	0
Intestines <sup>a</sup>	10	7	4	10	1

<sup>a</sup>Two intestinal tumors manifested as mucosal fibropapillomas

Table 4. *Chelonia mydas*. Association between turtles with glottal tumors and gross evidence of pulmonary necrosis (number in each category). Significant association at  $p < 0.001$

Status	With lung necrosis	Without lung necrosis	Total
With glottal tumor	18	86	94
Without glottal tumor	3	146	149
Total	21	232	253

tumors, exact location of the tumor in the wall of the chamber was recorded in 23 animals. In those 23 cases, the right atrium was affected 89% of the time; 12 turtles had tumors in the right atrium only, 8 had tumors in the right atrium and ventricle, 2 had tumors in the ventricle only, and 1 had tumors in all 3 chambers. In general, tumors were limited to the wall and rarely projected into the chambers.

Other than tumors, atrophy of pectoral muscles and serous atrophy of coelomic fat was the most common (67%) gross finding in turtles with FP. Thirty-five percent of turtles had trematode-induced granulomata or egg packets in the intestinal serosa or nodules in the wall of the aorta caused by vascular trematodes. Pulmonary necrosis was common in turtles with glottal tumors; turtles with glottal tumors were 9 times more likely ( $\chi^2 = 16.867$ ,  $df = 1$ ,  $p < 0.001$ ) to have pulmonary necrosis (Table 4). Necrosis of the pectoral muscle was seen when the acromion process of the pectoral girdle eroded through the softened plastron in emaciated turtles. Focal necrosis associated with mineralization was occasionally seen in salt glands. Other gross lesions included accumulation of clear fluid in the coelomic cavity, variable numbers of trematodes in the lumen of the urinary bladder, and firm compacted feces in the large intestines. Infrequent findings included monofilament fishing line entanglement in the front flippers (2 turtles), and ingestion of fishing line associated with

intussusception of intestines, mucosal erosion, coelomitis, or abscessation of the liver (14 turtles).

On microscopy, oral and skin tumors consisted of an arborizing to sessile matrix of densely packed collagen mixed with pleomorphic, plump, streaming to variably disorganized fibroblasts overlaid by markedly orthokeratotic acanthotic epidermis with prominent rete pegs. Occasional tumors had keratin pearls within the collagenous matrix lined by acanthotic epidermis.

There was a significant ( $\chi^2 = 13.261$ ,  $df = 5$ ,  $p < 0.05$ ) association between microscopic appearance of tumors and organ location of internal tumors. Fibromas predominated in the lungs, kidneys, and musculoskeletal tissues whereas myxofibromas were more common in the spleen and intestines. Fibrosarcomas of low-grade malignancy were mainly found in the heart (Table 3). Putative early lung tumors appeared to arise from subepithelial connective tissue and were characterized by small aggregates of dense collagen mixed with numerous fibroblasts overlaid by hypertrophied ciliated columnar epithelium. In larger tumors, lung epithelia became more hyperplastic, some tumors contained variably sized cysts lined by ciliated columnar epithelium, and the core of the tumor developed a more collagenous and less cellular appearance. In many cases, tumors displaced adjacent pulmonary smooth muscle walls (Fig. 5A). Cardiac fibromas were similar to lung fibromas with well-defined edges; however, cysts lined by columnar epithelium were not seen (Fig. 5B). Fibrosarcomas of low-grade malignancy consisted of collagen mixed with numerous fibroblasts in random sheets that infiltrated adjacent myocardium and caused atrophy or necrosis of enveloped myofibers (Fig. 5C,D). Fibroblasts in these tumors had small to large, ovoid to round to pleomorphic nuclei with prominent nucleoli and occasional binucleate cells (Fig. 5E). Mitotic figures were occasionally seen (less than 2 per 10 high powered [400 $\times$ ] field), and there were varying severities of necrosis and thrombosis (Fig. 5F). Renal tumors appeared to displace the interstitium causing atrophy of kidney tubules (Fig. 5G,H).

Fibromas of the small intestines had numerous fibroblasts in a dense collagenous matrix whereas myxofibromas had a looser matrix with fewer cells mixed with proteoglycan (Fig. 6A,B). Fibropapillomas of the stomach or intestinal mucosa consisted of a pedunculated mass of collagen and fibroblasts overlaid by squamous mucosa projecting prominent rete pegs with occasional keratin pearls in the collagenous matrix (Fig. 6C,D). Myxofibromas of the spleen, and fibromas of the spleen, liver, and bone had similar characteristics as those seen in other organs (Fig. 6E,H).

Additional common microscopic findings included hepatocellular atrophy, focal necrosis and chronic inflammation of the salt gland, liver, lung, and kidney.

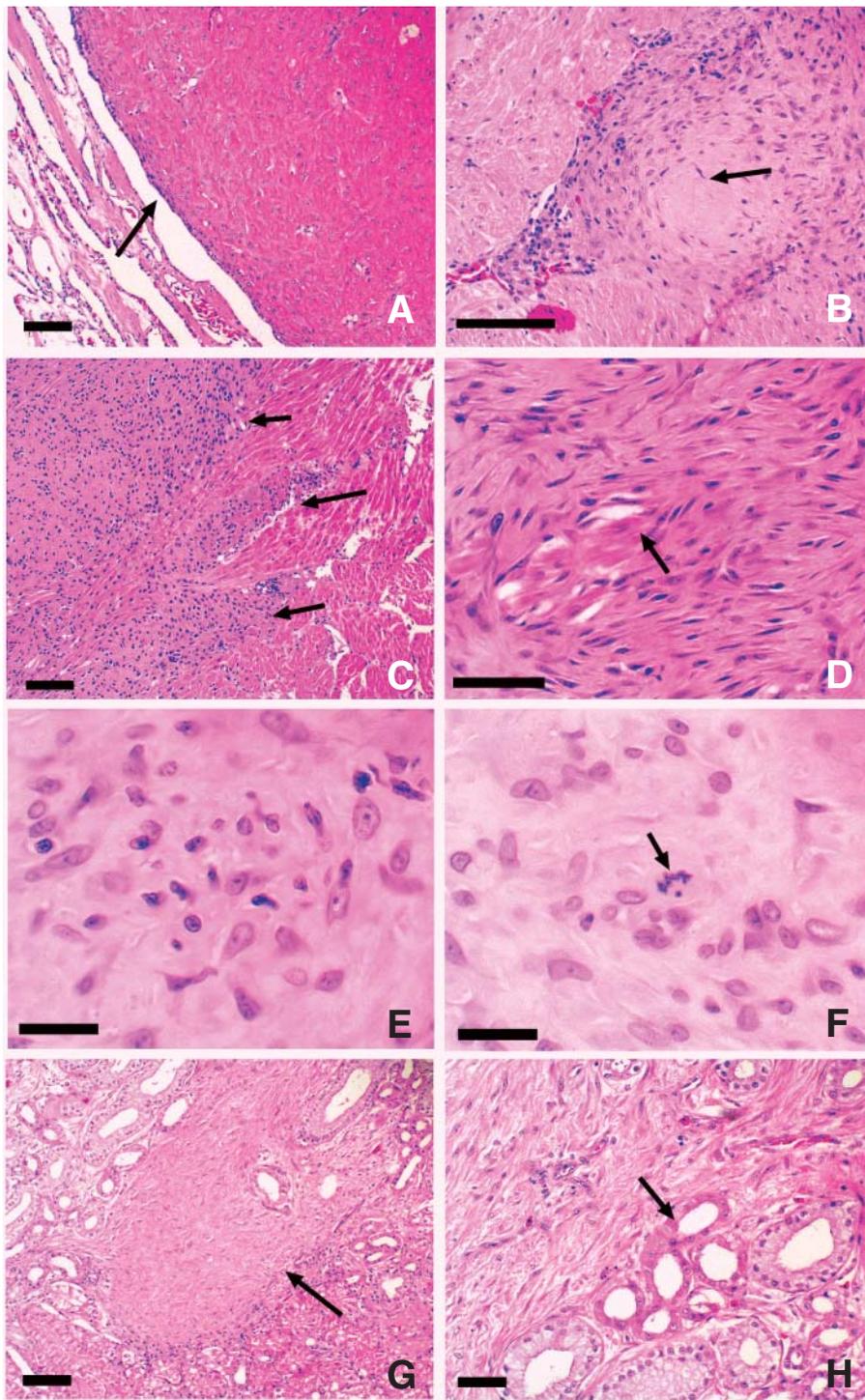


Fig. 5. *Chelonia mydas*. (A) Pulmonary fibroma. Note how mass of fibroblasts (arrow) is well defined from surrounding compressed smooth muscle walls of lung; scale bar = 100  $\mu$ m. (B) Presumed incipient cardiac fibroma. Note nidus of collagen (arrow) surrounded by whorls of fibroblasts that are well differentiated from myocardium (left); scale bar = 100  $\mu$ m. (C) Cardiac fibrosarcoma of low-grade malignancy. Note mass (left) with rami of collagen and fibroblasts (arrows) infiltrating myocardium (right); scale bar = 100  $\mu$ m. (D) Same as (C). Note numerous fibroblasts and connective tissue encompassing atrophied myofibers (arrow); scale bar = 50  $\mu$ m. (E) Cardiac fibrosarcoma of low-grade malignancy. Note nuclear polymorphism; scale bar = 50  $\mu$ m. (F) Same as (E). Note mitotic figure (arrow); scale bar = 50  $\mu$ m. (G) Renal fibroma. Note projection of connective tissue and fibroblasts (arrow) encompassing adjacent renal collecting ducts; scale bar = 100  $\mu$ m. (H) Same as (G). Note fibroblast/collagen matrix (upper left) infiltrating around atrophied renal tubules (arrow); scale bar = 50  $\mu$ m

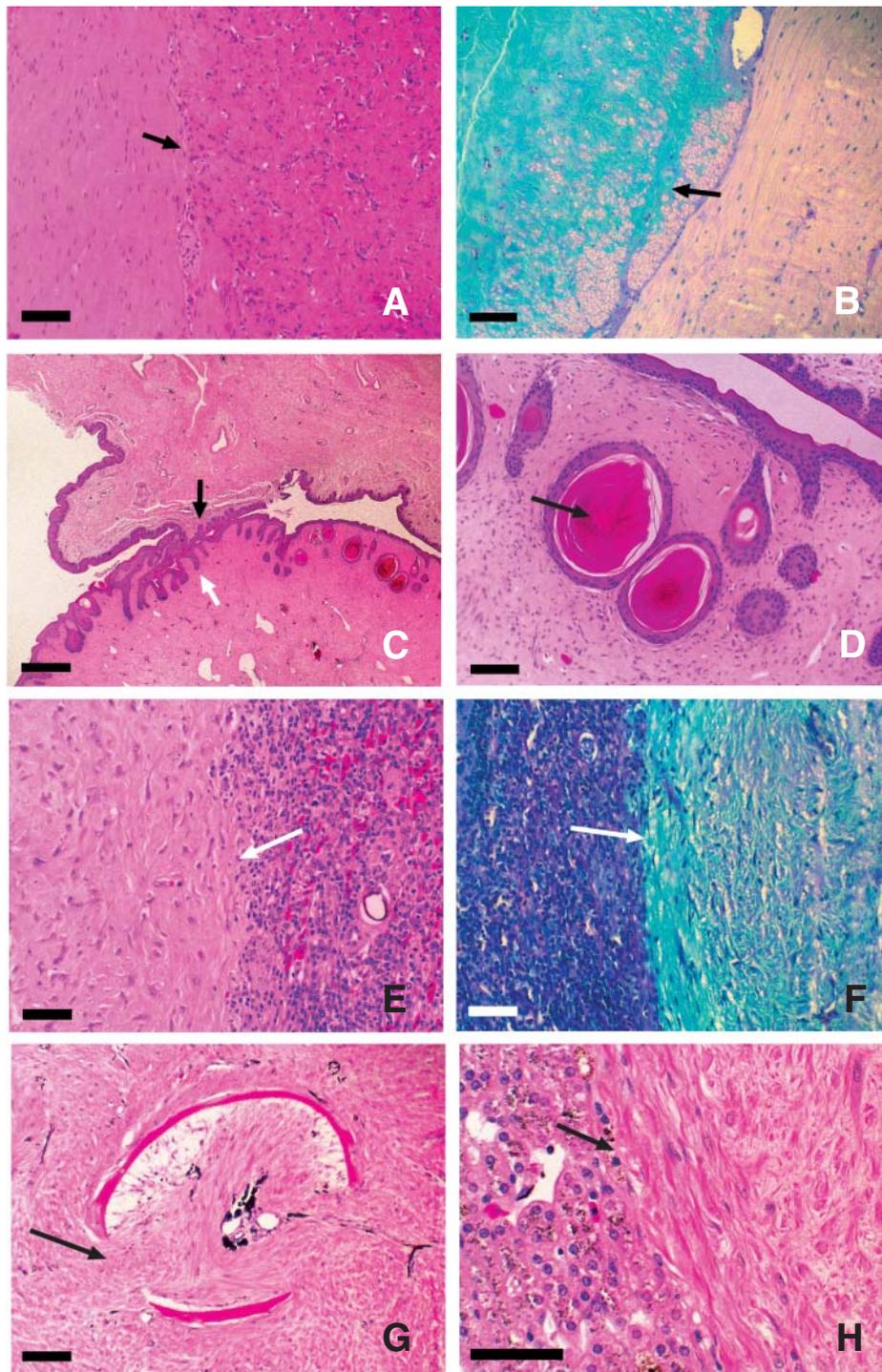


Fig. 6. *Chelonia mydas*. Tissues stained with alcian blue-PAS (B,F) or hematoxylin and eosin (all others). (A) Fibroma in small intestinal wall. Note mass of collagen and fibroblasts (right) with well-defined border (arrow) separating it from smooth muscle; scale bar = 100  $\mu$ m. (B) Myxofibroma small intestinal wall. Note mass (arrow) composed of loosely arranged collagen fibers and sparse fibroblasts within a matrix of proteoglycan (blue material) and normal smooth muscle on right; scale bar = 100  $\mu$ m. (C) Stomach mucosa fibropapilloma. Note large tumor (bottom) with pedunculated stalk (black arrow) and prominent rete pegs (white arrow) arising out of mucosa (top); scale bar = 500  $\mu$ m. (D) Same as (C). Note keratin cysts lined by epidermal tissue (arrow); scale bar = 50  $\mu$ m. (E) Splenic fibroma. Note connective tissue and fibroblasts with distinct border from spleen tissue (arrow); scale bar = 50  $\mu$ m. (F) Splenic myxofibroma. Note loosely arranged collagen fibers and small fibroblasts in a proteoglycan matrix (blue material); scale bar = 50  $\mu$ m. (G) Fibroma in cancellous bone of carapace, note resorption associated with invasion of trabecula by whorls of connective tissue and fibroblasts (arrow) mixed with occasional melanophores; scale bar = 100  $\mu$ m. (H) Hepatic fibroma. Note connective tissue and fibroblasts (right) well separated (arrow) from adjoining atrophied hepatocytes; scale bar = 50  $\mu$ m.

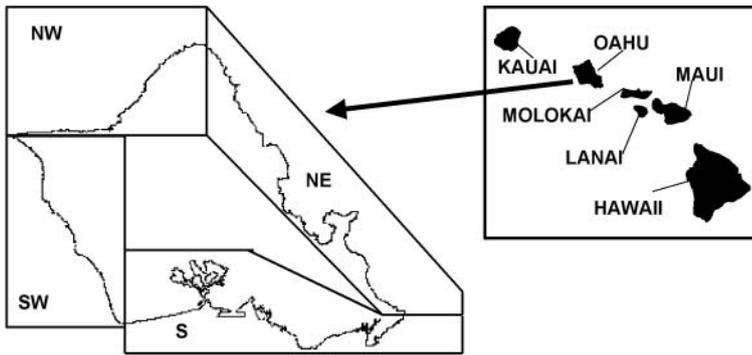


Fig. 7. Main Hawaiian islands (inset) and 4 coastal stranding zones of FP-afflicted turtles on Oahu based on discrete geographic contours

All turtles also had mild to severe vascular trematodiasis characterized by presence of intravascular adults or eggs in masses of eosinophilic debris, chronic inflammation in the aortic wall, or foci of mixed granulocytic or mononuclear inflammation associated with trematode eggs in multiple organs. Necrosis of the salt gland was often accompanied by hyperplasia of the salt gland epithelium and mineralization. Focal necrosis in lungs was exemplified by accumulations of eosinophilic debris in airways and smooth muscle walls often associated with foreign bodies (probable ingesta) and gram-negative rods. In some cases, necrosis in the liver, skeletal muscle, lung and spleen was accompanied by aggregates of gram-negative rods. Acute renal tubular necrosis, characterized by intracytoplasmic aggregates of eosinophilic granules, cytoplasmic fragmentation, and nuclear pyknosis of proximal tubules was also seen. Rare lesions included focal myocardial necrosis, myocarditis, and necrosis of the spleen.

Of the 255 turtles, most (189) came from the island of Oahu, followed by Maui (51), Hawaii (7), Molokai (4), Kauai (3) and Lanai (1). For Oahu, when compared to the proportion of available coastline upon which turtles could strand, there were significantly ( $G = 102$ ,  $df = 1$ ,  $p < 0.001$ ) more turtles with FP stranded on the NW and

Table 5. Proportion of the coastline on Oahu that is NE, NW, S and SW, number of turtles stranding in each area, and expected number based on proportion of coastline. Expected n calculated from  $G$ -test. \*Significantly different between shores ( $p < 0.05$ )

Shore	Proportion coastline	Actual n	Expected n
NE	0.26	85*	50
NW	0.13	54*	24
S	0.51	48	97
SW	0.10	2	18

NE coasts (Fig. 7, Table 5). For Oahu, there was no geographic pattern seen when animals were mapped by sex, age, tumor score, or seasonal pattern. We also did not detect temporal patterns in prevalence of FP across seasons (all islands) in our sample.

## DISCUSSION

In free-ranging non-stranded green turtles in Hawaii, FP affects mainly juvenile turtles (Balazs & Pooley 1991). A similar pattern was seen in affected turtles near death (stranded turtles) in this study, suggesting that FP detrimentally affects survival in juveniles. This is corroborated by physiologic findings in non-stranded juvenile and subadult turtles that have revealed immunosuppression and bacteraemia in turtles that are tumor score 2 and above (Work et al. 2001, 2003).

Populations of nesting females at French Frigate Shoals have been increasing (Balazs & Chaloupka 2004), and prevalence and case severity of FP in nesting female adult turtles is much lower than that in juveniles (Balazs & Pooley 1991). Lower prevalence of FP in adults can be explained by mortality prior to adulthood or ability of adults and subadults to recover from disease. Bennett et al. (2000) used underwater photography and recognition of facial scute patterns to identify individual turtles with FP over time. They observed regression of tumors in 32% of non-stranded turtles in waters off west Maui, and regression of tumors was seen mostly in adults and rarely in juveniles.

In our study, TS-2 and TS-3 categories predominated, and TS-3 animals comprised larger size classes, a phenomenon also seen in free-ranging non-stranded turtles (Work et al. 2003). Increasing size with increasing TS suggests that FP is probably lethal in turtles; otherwise, we would expect a decrease in TS categories in larger size classes as turtles recover and tumors regress. How decreased survival due to FP in juveniles and subadults impacts turtle demographics in Hawaii cannot be assessed from these data; however, this topic merits further investigation. The lack of difference in the ratio of males to females indicated that FP is not a sex-specific disease. Koga & Balazs (1995) also found a 1:1 male female ratio in 421 stranded turtles from Hawaii except in larger size classes (>91 cm). Wibbels et al. (1993), using serum hormone assays, found a sex ratio of 1:1 in immature turtles from French Frigate Shoals.

The frequency distribution of total number and surface area of tumors in turtles (Fig. 2) was similar to

frequency distributions of intensity of parasitic infections in wildlife (Anderson & Gordon 1982). Such right-skewed distribution is indicative of heterogeneity between hosts in either the exposure or susceptibility to infection with the etiologic agent, or the defensive capacities of the host against the causative agent(s) of FP (Anderson & Gordon 1982). Adnyana et al. (1997) also found that the frequency distribution of the number of tumors per animal in green turtles from Indonesia was skewed to the right. Given this skewed distribution, if tumor intensity is treated similarly to parasite infection, tumor intensity may be used to model the basic reproduction ratio ( $R_0$ ) of FP using techniques applicable for other infectious diseases (McCallum et al. 2001).

Total area of external, oral, and internal tumors increased with the size of the turtles. That larger turtles had larger tumors suggests that FP is a chronic disease because tumors initiating later in life would presumably be smaller than those which had initiated earlier. Adnyana et al. (1997), looking at commercially harvested Indonesian green turtles, found that numbers of tumors per turtle increased with size whereas we did not see this in stranded turtles from Hawaii. The lack of difference in the BCI between TS indicates either that stranded turtles at end-stage disease are in equally poor body condition or that the BCI is insufficiently sensitive to quantify differences in body condition in turtles with late stage disease. We found no significant difference in numbers of tumors per turtles between juveniles, subadults and adults. In contrast, Aguirre et al. (1998), using a smaller data set, found that numbers of tumors in adults were lower than for the other 2 groups.

More tumors were seen at the front of turtles than the rear regardless of total tumor burden. In contrast, Adnyana et al. (1997) found more tumors at the rear of green turtles with FP from Indonesia. Adnyana et al. (1997) examined turtles at slaughter, which were probably not near end-stage disease like the turtles in our study, and this may account for the difference. In Hawaii, predilection for more tumors in the front, along with a prevalence of tumors involving the eyes or conjunctiva of >90%, could be due to behavior of turtles or factors in the etiology or transmission of FP that promotes tumor growth in the front. For example, cleaner fish (wrasses) seem to favor cleaning the front of turtles, and genomic material from the FP-associated herpesvirus has been documented in these fish (Lu et al. 2000b). The greater number of tumors at the front could also be a function of skin surface area available for colonization; however, this would not explain more tumors at the rear of Indonesia green turtles that do not differ significantly in external morphology from Hawaiian green turtles (Adnyana et al. 1997). Deter-

mining exactly how tumors form in turtles may shed more light on this issue.

In 8 turtles with a single tumor, the tumor was present on the eye ( $n = 2$ ), front flipper ( $n = 3$ ), mouth ( $n = 2$ ) or rear flipper ( $n = 1$ ). Of these 8 turtles, 3 were juveniles, 2 were subadults, and 3 were adults; all but 1 was emaciated. Based on these data, we suspect there is probably not a particular site predilection for genesis of external tumors. These individuals had no other lesions than vascular fluke infection. The poor body condition of these turtles concomitant with small numbers of external tumors suggests that flukes or other factors in addition to FP caused the emaciation. While tumor regression may explain the single tumor and emaciation in larger individuals, such an explanation is less likely for immature turtles given the lower likelihood of regression in that age group (Bennett et al. 2000).

Prevalence of glottal tumors (51%) in stranded turtles in this study was significantly ( $z = 2.132$ ,  $p = 0.033$ ) higher than that observed (40%) in 236 stranded turtles with FP (Aguirre et al. 2002) perhaps due to differences in time of sampling or size classes of individuals examined, although these data were not presented. A case of oral FP was recently reported from green turtles in Florida (Bresette et al. 2003). Oral tumors have not been documented in Indonesia (Adnyana et al. 1997). Gross and microscopic appearance of skin and oral tumors was similar to that described elsewhere (Aguirre et al. 1998, 2002, Herbst et al. 1999). Turtles with glottal tumors were more likely to have pulmonary necrosis. We believe that prevention of closure of the glottis due to tumor obstruction led to aspiration of foreign material. We would suspect that turtles with pneumonia would have a lower likelihood of survival; however, our data could not confirm this. Longer-term studies using mark-recapture techniques may tease out the effects of glottal tumors on turtle survival.

Prevalence of internal tumors (39%) in this study was not significantly different from the 34% seen by Aguirre et al. (1998) in 15 stranded turtles from Hawaii and the 41% of 17 Puerto Rico green turtles reported by Williams et al. (1994). However, prevalence of internal tumors in these studies was significantly ( $z = 2.867$ ;  $p = 0.004$ ) higher than the 17% of 52 stranded Florida green turtles reported by Herbst (1994). The lower prevalence of internal tumors in stranded Florida turtles could be due to examination of younger turtles; however, the size of turtles examined was not reported (Herbst 1994). Alternatively, lower prevalence of internal tumors in Florida turtles compared to turtles from other areas could be due to the strain of FP-associated virus, physiology or anatomy of turtles. For example, turtles from Hawaii have a crop whereas turtles from Florida do not (Balazs et al. 1998), and there are genetic differences in FP-associated herpesviruses

between Hawaii, Costa Rica, Australia, and Florida (Quackenbush et al. 2001). Adnyana et al. (1997) did not report internal tumors in slaughtered green turtles with FP from Indonesia; we suspect that lack of internal tumors in Indonesian turtles can be attributed to their examining slaughtered turtles that were not near end-stage disease.

Herbst (1994) found that 69% of turtles from Florida with internal tumors had tumors in more than one organ in contrast to turtles from Hawaii where the percentage (44%) was significantly ( $z = 3.136$ ,  $p = 0.002$ ) lower. In a sample of 15 stranded turtles from Hawaii, Aguirre et al. (1998) found tumors in the lungs, kidneys, heart, spleen, stomach, and liver but did not quantify relative frequency. Herbst (1994) found that the most commonly tumored internal organs in 52 green turtles from Florida were the lungs (77%), kidneys (69%), heart (38%), gastrointestinal tract (31%), and liver (23%). In Puerto Rico, lung and kidney were the most commonly affected organs in green turtles (Williams et al. 1994). Our findings were similar except that we rarely found hepatic tumors. Aguirre et al. (1998) did not see tumors in muscle and bone while Herbst (1994) did not see tumors in the spleen. In our study, tumors in the spleen were more common in turtles where three or more internal organs were affected suggesting that the spleen is one of the last organs to be colonized by tumors, perhaps because of its high population of immune cells. Tumors have not been reported in the central nervous system of turtles with FP, indicating this site is somehow protected. Investigating why this is so may shed more light on the pathogenesis of FP.

The lack of a significant relationship between numbers of external and internal tumors suggested that development of internal tumors did not depend on a minimum number of external tumors. We did not see any turtles with internal tumors that did not also have external tumors. However, elucidating the adverse health effects of internal tumors on turtles or determining if internal tumors can only develop in presence of external tumors is problematic, given that all our sample consisted of stranded turtles with FP. We had few opportunities to necropsy non-tumored turtles, and determining if live turtles with no external tumors have internal tumors requires techniques (endoscopy, magnetic resonance imaging, X-ray) that have their limitations in terms of sensitivity of detection and ease of use in the field. Developing blood markers that could reliably detect the presence of internal tumors, as done for some human cancers (Ye et al. 2003), could allow for further elucidation of the role of internal tumors in FP.

Like external tumors, cross-sectional area of internal tumors increased with size of turtles. Whether internal tumors arise as metastases from the skin or oral cavity, or as independent events within viscera merits investi-

gation. In mammals, some malignant skin tumors metastasize to the lymph nodes, lung, and liver (Pulley & Stannard 1990). Green turtles lack lymph nodes (Wyneken 2002), and metastases may favor the lung and kidney, these being the organs more commonly affected in green turtles with FP. Alternatively, internal tumors could arise secondary to multicentric viral-induced initiation. This idea is supported by the fact that herpesvirus genome has been found in internal tumors but not in nearby normal tissue (Quackenbush et al. 1998).

Gross morphology of internal tumors seen in this study was similar to those observed by others (Norton et al. 1990, Aguirre et al. 1998) and comprised a fibroblastic and connective tissue component regardless of organ affected. The propensity for lungs, kidneys, and intestines to have polycystic fluid-filled tumors may be a reflection of the vascular or lymphatic anatomy of these organs that allows for fluid accumulation. Necrosis in large tumors was probably secondary to loss of vascular supply (Cheville 1988). The right atrium was a favored site for tumor formation in the heart of turtles. Given the lack of data regarding other internal connective tissue tumors of reptiles, the only comparisons available are for mammals. In dogs, hemangiosarcomas form most often in the right atrium (Robinson & Maxie 1993) whereas in humans, myxomas are most common in the left atrium and cardiac sarcomas in the right (Hackel & Reimer 1990). Fibromas in the lungs are rare in humans (Spencer 1985). Fibromas in the kidneys occur in older dogs (Maxie 1993) but are rare in humans (Schreiner & Kissane 1990). Our diagnosis of fibrosarcomas of low-grade malignancy in the heart was based on morphology. Future studies to corroborate this could include attempts to culture heart tumors and observe behaviors of the tumor in culture or detection of protein (Mayall et al. 1997) or genomic (Cadile et al. 2001) markers of malignancy in tumor tissues. Aguirre et al. (in press) also noted fibrosarcomas in bones of green turtles with FP from Hawaii.

Stranded turtles with FP were in poor body condition and burdened by parasites as evidenced by emaciation, serous atrophy of fat and atrophy of liver, and inflammation with vascular flukes. These findings mirror those of Aguirre et al. (1998). Turtles stranded with FP also have additional complications including decreased organ weights (Eames et al. in press) and bacterial infections (Work et al. 2003). All turtles necropsied in this study had varying severity of infection with vascular flukes with pathology similar to that described by Aguirre et al. (1998). Necrosis of the salt gland could have been secondary to dehydration as evidenced by poor body condition, tacky musculature, and mostly empty gastrointestinal tracts except for small amounts of very firm feces.

Elucidating the role of vascular flukes, bacteria, viruses, and loss of body condition in turtles with FP will require development of reliable assays that can measure intensity of infection with the aforementioned agents. Vascular flukes can cause mild to severe pathology in turtles (Gordon et al. 1998). Serological assays exist to measure antibodies to vascular flukes in green turtles (Graczyk et al. 1995, Herbst et al. 1998); however, these tests do not reflect intensity of infection. Existing serological tests for FP-associated herpesvirus are laborious and of unknown specificity and sensitivity (Herbst et al. 1998). Improvements are also needed in quantifying body condition in green turtles and intensity of fluke infections, especially inflammatory response of host to eggs.

The geographic distribution of strandings favored Oahu and Maui. Length of coastline does not explain the differences in numbers of strandings because Oahu's coastline (where most turtles stranded) is shorter than that of Maui. Differential probability of sighting of stranded turtles is also not a likely explanation for the stranding patterns observed here. Although Oahu and Maui are the most populated islands, we found significantly more turtles stranding on the northeast and northwest shores of Oahu that have relatively lower populations than the southern shore. Geographic patterns of stranding could be due to higher densities of turtles, higher prevalence of FP, or prevailing currents that happen to wash turtles onto those particular coasts. Geographic disparities in prevalence of FP exist in non-stranded turtles as well. For example, FP is virtually absent in turtles on the west coast of the island of Hawaii (Balazs & Pooley 1991, Work et al. 2001). In addition to elucidating the pathophysiology of FP by developing more refined laboratory tools, it will be critical to devise creative ways to explain geographic disparities in distribution of FP. More complete understanding of the demographics and ecology of FP will lead to a better understanding of how to manage this disease.

*Acknowledgements.* We thank S. Eames, S. Murakawa, and D. Parker for valuable assistance in procuring animals and assisting with necropsies. The following were instrumental in recovery of strandings: M. Rice, J. Coney, L. Hallacher, D. Brown, S. Hau, J. Olivera, D. Heacock, B. Puleloa, Hawaii Preparatory Academy, University of Hawaii Marine Option Program, and State of Hawaii DLNR. We are grateful to G. Antonelis, A. Foley, F. Morado, J. Seminoff, L. Sileo, C. Whitow, and anonymous reviewers who provided constructive comments on the manuscript.

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*Editorial responsibility: Peernel Zwart,  
Utrecht, The Netherlands*

*Submitted: January 25, 2004; Accepted: June 14, 2004  
Proofs received from author(s): November 2, 2004*