

The Potential Role of Natural Tumor Promoters in Marine Turtle Fibropapillomatosis

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Abstract.—Fibropapillomatosis (FP) in green turtles *Chelonia mydas* is a debilitating, neoplastic disease that has reached worldwide epizootic levels. The etiology of FP is unknown but has been linked to oncogenic viruses. Toxic benthic dinoflagellates (*Prorocentrum* spp.) are not typically considered tumorigenic agents, yet they have a worldwide distribution and produce a tumor promoter, okadaic acid (OA). *Prorocentrum* spp. are epiphytic on macroalgae and seagrasses that are normal

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components of green turtle diets. Here we show that green turtles in the Hawaiian Islands consume *Prorocentrum* and that high-risk FP areas are associated with areas where *P. lima* and *P. concavum* are both highly prevalent and abundant. The presence of presumptive OA in the tissues of Hawaiian green turtles further suggests exposure and a potential role for this tumor promoter in the etiology of FP.

Marine turtle fibropapillomatosis (FP) is a neoplastic disease that has been documented from the Atlantic Ocean (Florida, Brazil), Indo-Pacific region (Australia, Sri Lanka, Sarawak, Malaya, Bonin Islands [Japan]), Pacific Ocean (Hawaiian Islands), and Caribbean Sea (Cayman Islands, Puerto Rico, Dominican Republic, Virgin Islands, Barbados, Antigua and Barbuda, Central America) (Balazs 1991; Ehrhart 1991; Herbst 1994; Williams et al. 1994). Fibropapillomas are commonly found in green turtles *Chelonia mydas*, although loggerhead turtles *Caretta caretta* and olive ridley turtles *Lepidochelys olivacea* are also known to be affected (Jacobson et al. 1991; Herbst 1994). The tumors are benign, but growths of fibropapillomas can adversely affect locomotion, vision, swallowing, and breathing. Visceral fibromas can disrupt normal organ function to the extent that death ensues (Herbst 1994). Fibropapillomatosis was first reported from a captured turtle in 1938, when 1.5% (3/200) of free-ranging green turtles in Key West, Florida, were affected (Smith and Coates 1938). In the last 20 years, there has been a dramatic increase in the prevalence of FP in green turtles in the Hawaiian Islands, Florida, and in the Caribbean Sea. In the Hawaiian Islands, there is a clearly defined distributional pattern of FP with up to 92% prevalence in Keneohe Bay, Oahu (Balazs 1991). In 1995 in Florida Bay, prevalences of up to 60% were reported in green turtles and 11% in loggerheads (B. Schroeder, National Marine Fisheries Service, personal communication). In the Caribbean, increases in FP have also been noted since the mid-1980s, particularly in Puerto Rico and Colombia (Williams et al. 1994).

Possible causes of FP include infectious agents such as oncogenic viruses (Jacobson et al. 1991; Herbst et al. 1995; Casey et al. 1997; Quackenbush et al. 1998) and parasites (Dailey and Morris 1995; Aguirre et al. 1998), genetic predisposition (Herbst 1994), toxicants (Aguirre et al. 1994), ultraviolet radiation (Smith and Coates 1938), and other as yet undefined synergistic environmental factors (Herbst 1994). Herpesvirus was detected in fibropapillomas from Florida green turtles by the use of electron microscopy (Jacobson et al. 1991), and experimental transmission of fibropapillomas was achieved by inoculating disease-free green turtles with cell-free tumor homogenate (Herbst et al.

1995, 1996). Recent findings include the presence of retrovirus in both tumored and nontumored green turtles and the presence of three closely related herpesviruses in association with fibropapillomas in green turtles from Hawaii and Florida and in olive ridleys from Costa Rica (Casey et al. 1997; Quackenbush et al. 1998). Attempts to culture virus have not yet been successful, nor have Koch's postulates been fulfilled. The fact that tumors have been experimentally induced in animals using oncogenic viruses or cell-free filtrates in laboratory studies does not necessarily infer a single cause-effect relationship in what may be a multifactored or multistep neoplastic process occurring in the wild.

Harmful microalgae produce potent toxins that can cause acute mortality of humans and aquatic animals (Steidinger 1993; Baden et al. 1995). However, the long-term effects of microalgal biotoxins are less clear. Some biotoxins act as tumor promoters in mammals (Fujiki and Suganuma 1993; Falconer and Humpage 1996) and are suspected for chronic effects or as tumorigenic agents in aquatic animals (Landsberg 1995, 1996). The role of microalgal tumor promoters in marine systems is largely unknown, and their potential role in FP has not been considered by other investigators. Because toxic algal blooms tend to be acute in nature and lead to fast-acting shellfish poisoning events or mass mortalities of aquatic organisms, many of the potential chronic effects of biotoxins produced by either planktonic or benthic microalgae are unclear.

Prorocentrum lima, *P. concavum*, *P. hoffmannianum*, and *P. belizeanum* are benthic dinoflagellates that produce the tumor promoter okadaic acid (OA) (Murakami et al. 1982; Dickey et al. 1990; Aikman et al. 1993; Morton et al. 1998), which inhibits protein phosphatase types 1 and 2A. Inhibition of these enzymes increases protein phosphorylation that (1) affects intracellular processes including metabolism, contractility, gene transcription, maintenance of cytoskeletal structure, receptor-mediated signal transduction, and cellular division; (2) stimulates the expression of certain proto-oncogenes; (3) activates H1 kinase in vitro; and (4) induces various mitosis-specific events (Bialojan and Takai 1988; Fujiki et al. 1989; Haystead et al. 1989; Herschman et al. 1989; Ya-

mashita et al. 1990; Sakai and Fujiki 1991; Fujiki and Suganuma 1993; Honkanen et al. 1994). Okadaic acid has been experimentally shown to induce skin papillomas and carcinomas in mice and adenomatous hyperplasia and adenocarcinomas in the glandular stomach of rats in two-stage carcinogenesis experiments (Suganuma et al. 1990a; Fujiki and Suganuma 1993). Conversely, OA inhibits tumor promotion in vitro, reverts some oncogene-transformed cells, and inhibits H1 kinase expression and cell proliferation (Schönthal and Feramisco 1993). Until now, the potential of OA contributing to FP has not been investigated.

Green turtles show high fidelity toward their feeding sites, where they graze on specific seagrass and macroalgal substrates (Balazs et al. 1994a, 1994b, 1998a, 1998b; Russell and Balazs 1994). Benthic *Prorocentrum* have a worldwide distribution (Steidinger 1997), are common in tropical ecosystems, and are epiphytic on macroalgae and seagrasses (Fukuyo 1981; Anderson and Lobel 1987; Bomber et al. 1989) that are normal components of green turtle diets (Mortimer 1981; Garnett et al. 1985; Russell and Balazs 1994). Because *Prorocentrum* species are probably consumed by turtles grazing on macroalgae or seagrass, green turtles are potentially exposed to OA. We set out to document the distributions of *Prorocentrum* species known to produce OA and the presence of FP in turtles and to determine if there was any association between the two in the Hawaiian Islands.

Methods

Substrate collections and processing.—Macroalgae, seagrasses, or cyanobacteria were sampled from eight specific and well-known turtle foraging locations in the Hawaiian Islands from May 20 to August 25, 1997 (Table 1). The goal was only to detect the presence or absence of known OA-producing *Prorocentrum* spp. on those food items commonly eaten by green turtles in these foraging locations. The most abundant forage species were therefore specifically targeted and collected. Selected substrate species not eaten by turtles were also collected for reference. Single food species, which differed from location to location but were also potential substrates for *Prorocentrum*, were sought in stands as pure as possible. Approximately 100 g of each substrate species was carefully hand picked with minimal disturbance to avoid dislodging of microepiphytes. Substrates were hand collected by scuba diving, snorkeling, or wading and placed in plastic zip-lock bags. Sufficient ambient seawater was added to each bag to

keep the substrate moist. Labeled zip-lock bags were shipped in coolers at ambient temperature overnight and processed within 48 h of collection.

Zip-lock bags were shaken vigorously 20 times to dislodge attached microepiphytes (Ballantine et al. 1985, 1988) and the total volume was decanted and measured. From this volume, 50 mL was then fixed with 5 mL of formalin, and the sample was analyzed for dinoflagellate species and abundance. From each fixed sample, one 2-mL aliquot was placed into a settling chamber and left to settle for 12 h. The entire bottom of the chamber was then screened by transects to determine dinoflagellate composition and abundance. An inverted Zeiss microscope at 400 \times and 640 \times magnification was used. Duplicates were evaluated. Wet weight of macroalgae, seagrass, or cyanobacteria was determined gravimetrically. The number of cells per gram wet weight of macroalgal, seagrass, or cyanobacterial substrate was estimated by determining the volume : weight ratio of the individual samples.

Pathological examination.—Stranded turtles judged by two veterinarians to have no chance of survival were euthanatized and necropsied according to standard methods (Work and Balazs 1998). For each turtle, sex, straight carapace length, weight, and tumor status was recorded. Tumor severity was ranked according to standard criteria (Balazs 1991) with scores of 0–4 in which 0 means tumors are absent and 4 indicates extreme affliction. Tissues were collected immediately after euthanasia and stored separately in sealed plastic bags at -70°C . Kidney and liver from 12 tumored and 6 nontumored green turtles were processed for OA analysis.

Protein phosphatase inhibition assay.—Tissues were prepared for analysis and tested by a colorimetric protein phosphatase enzyme assay. This assay is nonspecific for a suite of protein phosphatase inhibitors, including OA and derivatives (Cembella et al. 1995). Samples were homogenized and notice of specific sublocalization was not undertaken. A simple method to detect all protein phosphatase inhibitors is to use a functional inhibition assay and a colorimetric detection system, for example, the *p*-nitrophenyl phosphate (*p*NPP) reaction using the enzyme protein phosphatase 1 (PP1). The assay is a microtiter plate assay based on a recombinant protein phosphatase expressed in *Escherichia coli* (rPP1/*p*NPP). Within the active binding site of the PP1 enzyme, an engineered PP1 that has a PP2 binding site is used (the PP2a enzyme has not been cloned as of yet).

TABLE 1.—Distribution of *Prorocentrum* (*P. lima* and *P. concavum* combined) on macroalgae, seagrass, or cyanobacteria substrates at various sites in the Hawaiian Islands, 1997. Data are mean \pm SE numbers of *Prorocentrum* per gram of substrate; $N = 4$. An absence of data means substrate not found at that site at the time of collection.

| Substrate species ^a | Collection site (date) | | | | | |
|-----------------------------------|--------------------------------|--------------------------------|----------------------------------|------------------------------|-----------------------------|--------------------------------|
| | Palaau, Molokai (Jul 22) | Honokowai, Maui (Aug 25) | Kaneohe Bay, Oahu (May 20) | Waikiki, Oahu (May 21) | Poipu, Kauai (Jul 15) | Puako, w. Hawaii (Jun 9) |
| <i>Acanthophora spicifera</i> * | 13.3 \pm 8.4 | 27.5 \pm 9.7 | 108.8 \pm 37.2 | | 1.0 \pm 1.0 | 0 |
| <i>Amansia glomerata</i> * | | | | 8.5 \pm 3.6 | | |
| <i>Dictyosphaeria cavernosa</i> * | | | 1.0 \pm 0.4 | | | |
| <i>Dictyota acuteloba</i> | 36.8 \pm 27.1 | | | | | |
| <i>Gelidiopsis variabile</i> * | | | | | | 0 |
| <i>Gracilaria salicornia</i> * | | | 7.8 \pm 0.6 | 1.3 \pm 0.5 | | |
| <i>Halimeda discoidea</i> * | | 27.0 \pm 15.6 | | | | |
| <i>Halophila hawaiiensis</i> * | 4.8 \pm 4.8 | | 121.5 \pm 7.8 | | | |
| <i>Hypnea musciformis</i> * | | 0 | | | | |
| <i>Pterocladia capillacea</i> * | | | | | 0 | |
| <i>Sargassum polyphyllum</i> * | | | | 8.5 \pm 4.9 | | |
| <i>Spyridia filamentosa</i> * | 225.0 \pm 106.0 | 123.8 \pm 30.9 | | | | |
| <i>Turbinaria ornata</i> * | | | | 34.5 \pm 20.1 | | 0 |
| <i>Ulva reticulata</i> * | | 0 | | | 2.3 \pm 2.3 | |
| <i>Ahnfeltia concinna</i> | | | | | | |
| <i>Alsidium</i> sp. | | | | | | 4.0 \pm 2.3 |
| <i>Caulerpa</i> sp. | 2.5 \pm 2.5 | | | | | |
| <i>Centroceros clavulatum</i> | | | | | | 8.3 \pm 7.0 |
| <i>Chondria tenuissima</i> | | | 157.3 \pm 60.0 | | | |
| <i>Cladophoropsis luxurians</i> | | | | 0 | | |
| <i>Enteromorpha</i> sp. | | | | | 0 | |
| <i>Euclima denticulatum</i> | | | 2.0 \pm 0.7 | | | |
| <i>Lynghya majuscula</i> | | | | 0 | | |
| <i>Microdictyon japonicum</i> | | | | 102.2 \pm 50.8 | | |
| <i>Padina japonica</i> | 160.8 \pm 61.4 | | | 62.5 \pm 24.4 | | |
| <i>Sargassum</i> sp. | | 0 | | | | |
| <i>Schizothrix calcicola</i> | | | 3.3 \pm 1.74 | | | |
| <i>Tolypocladia calodictyon</i> | | 139.0 \pm 27.7 | | | 0 | |

^a Asterisk indicates substrate consumed by Hawaiian green turtles.

Substitution of this binding site results in a 100-fold increase in sensitivity over the PP1 assays alone and is used exclusively for tissue assays. A sample that is inhibitory (i.e., contains a protein phosphatase inhibitor) will result in an absorbance reading that is less than that of the control, which is known to be free of any such inhibitor. Samples containing 10–100 ng OA spikes were used routinely and reproducibly detected. Sample activity was determined by verification against a standard curve for OA concentration.

Statistics.—All statistical analyses were performed with Sigma Stat (Jandel Scientific). Comparisons were made between pairs of groups by *t*-test.

Results

Distribution of Prorocentrum Species, Substrate Specificity, and FP Prevalence

Twenty-five species of macroalgae, two species of cyanobacteria, and one species of seagrass were examined (Table 1) and at least 12 species of ben-

thic dinoflagellates were found (K. A. Steidinger and J. H. Landsberg, unpublished data). Some substrate species that are preferred food items for green turtles were present in some locations but absent in others. For example, at the collection time and at the sites sampled, *Acanthophora spicifera*, which is usually a predominant species, was present in Molokai, Maui, Oahu, Kauai, and Puako, west Hawaii, but was absent from Kahaluu and Punalu'u in west and east Hawaii, respectively. *Pterocladia capillacea* was usually present in west Hawaii, whereas it was absent, for example, at study sites in Kaneohe Bay, Oahu, and Palaau, Molokai (Table 1). *Prorocentrum lima* and *P. concavum* were present on 21 substrate species, including *Acanthophora spicifera*, *Halophila hawaiiensis*, and *Spyridia filamentosa*, that are also major components of green turtle diets (Table 1). There was a species-specific difference in abundance of *Prorocentrum* spp. by geographical location (Table 1). In areas where FP is highly prevalent, *Prorocentrum* spp. (combined *P. lima* and

TABLE 1.—Extended.

| Substrate species ^a | Collection site (date) | |
|-----------------------------------|----------------------------|------------------------------|
| | Kahaluu, w. Hawaii (Jun 9) | Punalu'u, e. Hawaii (Jul 23) |
| <i>Acanthophora spicifera</i> * | | |
| <i>Amansia glomerata</i> * | | |
| <i>Dictyosphaeria cavernosa</i> * | | |
| <i>Dictyota acuteloba</i> | | |
| <i>Gelidiopsis variabile</i> * | | |
| <i>Gracilaria salicornia</i> * | | |
| <i>Halimeda discoidea</i> * | | |
| <i>Halophila hawaiiensis</i> * | | |
| <i>Hypnea musciformis</i> * | | |
| <i>Pterocladia capillacea</i> * | 2.3 ± 2.3 | 0 |
| <i>Sargassum polyphyllum</i> * | | 0 |
| <i>Spyridia filamentosa</i> * | | |
| <i>Turbinaria ornata</i> * | | |
| <i>Ulva reticulata</i> * | 0 | 0 |
| <i>Ahmfeltia concinna</i> | | 0 |
| <i>Alsidium</i> sp. | | |
| <i>Caulerpa</i> sp. | | |
| <i>Centroceros clavulatum</i> | | 0 |
| <i>Chondria tenuissima</i> | | |
| <i>Cladophoropsis luxurians</i> | | |
| <i>Enteromorpha</i> sp. | | 0 |
| <i>Euclima denticulatum</i> | | |
| <i>Lyngbya majuscula</i> | | |
| <i>Microdictyon japonicum</i> | | |
| <i>Padina japonica</i> | 0 | |
| <i>Sargassum</i> sp. | | |
| <i>Schizothrix calcicola</i> | | |
| <i>Tolypocladia calodictyon</i> | | |

P. concavum) were found on 57.1–100% of the substrate species examined with mean numbers from 178.3 to 279.9 cells/g of wet substrate at Kaneohe Bay, Oahu; Honokowai, Maui; and Palaau, Molokai (Table 2). In areas where FP is absent, *Prorocentrum* spp. were found on 0–40% of the substrate species examined with mean numbers from 0 to 2.3 cells/g of substrate at Puako, Punalu'u, and Kahaluu, Hawaii (Table 2).

Protein Phosphatase Inhibition Assay

Presumptive OA concentrations in the kidneys of tumored turtles ranged from 24.2 to 670.5 µg/g (Table 3). Results of the liver analyses using this assay were not available because of processing interference. Turtles rated as severe (*N* = 6) to extreme affliction (*N* = 2) for FP had a significantly higher concentration of presumptive OA in the kidney (mean 258.7 ± 76.7 µg/g, range 67.6–670.5 µg/g, *N* = 8) than moderately rated tumored turtles (mean 53.1 ± 16.2 µg/g, range 24.2–81.1 µg/g, *N* = 4, *P* < 0.05). However, nontumored turtles also had presumptive OA levels in the kidney (mean 134.9 ± 45.3 µg/g, range 33.7–287.9 µg/g, *N* = 6) that were not significantly different from severely tumored turtles (*P* > 0.05). Except for one turtle from west Hawaii (Table 3), nontumored turtles were all from presumptively high-risk OA exposure areas in Oahu and Maui.

Nontumored turtles were smaller (mean straight carapace length = 48.4 ± 3.9 cm, range 39.4–65.6 cm, *N* = 6) than tumored turtles (mean straight carapace length = 64.8 ± 3.6 cm, range 49.9–88.9 cm, *N* = 12, *P* < 0.05). This is a critical point in interpreting our data.

TABLE 2.—Site distribution, proportion of *Prorocentrum*-positive samples, and mean number of *Prorocentrum* cells in relation to the distribution and prevalence of green turtles with fibropapillomatosis (FP).

| Site | Prevalence of turtles with FP ^a | Number of substrate species with <i>Prorocentrum</i> | Number of substrate species sampled ^b | % of samples with <i>Prorocentrum</i> | Mean number of <i>Prorocentrum</i> (cells/g) on substrates consumed by turtles |
|-------------------|--|--|--|---------------------------------------|--|
| Palaau, Molokai | +++ | 6 | 6 | 100.0 | 279.9 |
| Kaneohe Bay, Oahu | +++ | 7 | 7 | 100.0 | 239.1 |
| Honokowai, Maui | +++ | 4 | 7 | 57.1 | 178.3 |
| Waikiki, Oahu | ++ | 6 | 8 | 75.0 | 52.8 |
| Poipu, Kauai | ++ | 2 | 5 | 40.0 | 3.3 |
| Puako, Hawaii | – | 2 | 5 | 40.0 | 0.0 |
| Kahaluu, Hawaii | – | 1 | 3 | 33.3 | 2.3 |
| Punalu'u, Hawaii | – | 0 | 6 | 0.0 | 0.0 |

^a From Balazs (1991, unpublished data), Balazs et al. (1994a, 1994b, 1998a, 1998b). Tumor prevalence: – is rare (<1%); + is low (1–10%); ++ is medium (11–50%); and +++ is high (51–100%).

^b Different substrate species were present at each location (Table 1) as determined by local conditions. If present, comparable species were sampled.

TABLE 3.—The distribution of presumptive okadaic acid (OA) in and characteristics of tumored and nontumored turtles from the Hawaiian Islands. Size is SCL = straight carapace length; sex is F = female, M = male, U = unknown; tumor score is 0 = none, 1 = slight, 2 = moderate, 3 = severe, 4 = extreme affliction.

| Sample number | SCL (cm) | Sex | Stranding location | Tumor score | Presumptive OA ($\mu\text{g/g}$) in kidney |
|---------------|----------|-----|----------------------------|-------------|--|
| 12463 | 71.5 | F | Mauna Lei, Lanai | 4 | 149.7 |
| 12451-2 | 85.1 | F | Kihei, Maui | 4 | 96.8 |
| 12468-1 | 53.2 | F | Haleiwa, Oahu ^a | 3 | 67.6 |
| 12469-1 | 56.0 | M | Magic Island, Oahu | 3 | 124.4 |
| 12454-1 | 56.9 | M | Kahala, Oahu | 3 | 670.5 |
| 12477-1 | 58.3 | F | Hickam, Oahu ^b | 3 | 227.2 |
| 12478-1 | 59.2 | M | Reef Runway, Oahu | 3 | 513.8 |
| 12451-1 | 73.3 | M | Maalaea, Maui | 3 | 219.5 |
| 12379-1 | 49.9 | M | Kanaha, Maui | 2 | 26.1 |
| 12455-1 | 58.2 | M | Paia, Maui | 2 | 81.1 |
| 08-13-97 | 67.0 | M | Kihei, Maui | 2 | 81.1 |
| 15029 | 88.9 | U | Iao, Maui | 2 | 24.2 |
| 07-04-97 | 39.4 | M | Au'au, Maui | 0 | 230.2 |
| 09-01-97A | 43.4 | F | Kaneohe Bay, Oahu | 0 | 45.6 |
| 08-30-97 | 43.7 | F | Maili, Oahu | 0 | 38.3 |
| 07-06-97 | 44.6 | M | Kawela Bay, Oahu | 0 | 287.9 |
| 09-01-97B | 53.4 | M | Kaneohe Bay, Oahu | 0 | 33.7 |
| 07-29-97 | 65.6 | F | Honaunau, Hawaii | 0 | 173.9 |

^a Haleiwa Boat Harbor.

^b Hickam Air Force Base.

Discussion

In this pilot study, we did not specifically test for OA production by isolates of *Prorocentrum lima* and *P. concavum* (or other benthic *Prorocentrum* species) from the Hawaiian Islands. We assumed that these species produce OA since past studies have shown that OA production is a consistent feature of *Prorocentrum* (i.e., *P. lima*, *P. concavum*, *P. hoffmannianum*, *P. belizeanum*, and *P. maculosum*; Murakami et al. 1982; Yasumoto et al. 1987; Dickey et al. 1990; Morlaix and Lassus 1992; Aikman et al. 1993; Hu et al. 1993; Tomas and Baden 1993; Bourdeau et al. 1995; Sohet et al. 1995; Quilliam et al. 1996; Morton et al. 1998). There are no data on the comparative OA-producing potencies and potential tumorigenicities of OA produced by *P. lima* and *P. concavum* in the Hawaiian Islands. The dominance of *P. lima* in Poipu, Kauai, along with a medium FP prevalence in turtles versus the low level presence of *P. concavum* (data not shown) in Puako and Kahaluu, Hawaii, along with rare FP may indicate differential toxin production, potency, or tumorigenicity of the OA produced by the two *Prorocentrum* species. Environmental factors such as nutrient loading may influence toxin production by *Prorocentrum*; for example, production of OA can vary quite considerably depending upon the ambient concentrations of phosphorus (Morlaix and Lassus 1992;

Tomas and Baden 1993; Sohet et al. 1995). This may have significant implications for eutrophic coastal systems such as Oahu and Maui in the Hawaiian Islands and other known FP sites in Florida, the Great Barrier Reef, Australia, and in the Caribbean. In general, most cases of FP are found in nearshore grass and algae flats or lagoons near urban or agricultural activities that are associated with eutrophication (Balazs 1991; Ehrhart 1991; Herbst 1994).

In the Hawaiian Islands, green turtles are site-specific and consistently feed in the same areas (Balazs et al. 1994a, 1994b, 1998a, 1998b) on preferred substrates such as *Acanthophora spicifera*, *Halophila hawaiiensis*, *Spyridia filamentosa*, and *Pterocladia capillacea* (Russell and Balazs 1994), which vary by location and between islands. For example, *Acanthophora* is ubiquitous throughout most of the Hawaiian Islands but was absent from two of three sites sampled in Hawaii. In west Hawaii, *Pterocladia* is the most common forage item for green turtles (Balazs et al. 1994a) whereas *Acanthophora* or *Spyridia* are more commonly found and selected by turtles in Oahu, Molokai, and Maui (Balazs et al. 1994b). At each of the locations sampled, we specifically targeted preferential food items of turtles to determine their suitability as substrates for *Prorocentrum*. The absence of these macroalgal substrates from a par-

ticular area was not due to their being overlooked by our sampling but rather because they were rare or did not occur at these sites. Because green turtles are site-specific they will consume different species at the various locations (Balazs et al. 1994b).

The distribution and abundance of *Prorocentrum lima* and *P. concavum* also appear to vary by island in relation to these substrate assemblages. Because benthic dinoflagellates, including *Prorocentrum* species, have specific substrate preferences (Yasumoto et al. 1979; Bomber et al. 1989) their distribution and abundance will be influenced by the presence of preferred macroalgae or seagrass species. Based on our sampling in the Hawaiian Islands, it appears that *Prorocentrum* spp. prefer green turtle foods like *Acanthophora spicifera*, *Halophila hawaiiensis*, and *Spyridia filamentosa*. In addition, the growth of benthic dinoflagellates is influenced by biotic and physical factors such as turbidity, light, temperature, salinity, and wave action (Carlson et al. 1984; Bomber et al. 1985; Bomber et al. 1989; Grzebyk et al. 1994; Morton et al. 1994); their distribution and community composition will therefore vary according to those macroalgal assemblages present (Grzebyk et al. 1994). The absence of *Prorocentrum* spp. at particular locations is therefore a reflection of both the substrate species present and prevailing ecological conditions. For example, although *Prorocentrum* spp. colonize *Acanthophora*, there appear to be wide variations in abundance of these dinoflagellates by island with apparently more preferable conditions for *Prorocentrum* spp. on *Acanthophora* in Oahu than in west Hawaii. The general absence or rarity of *Prorocentrum* spp. in those areas sampled in Hawaii appears to be a reflection of both a lack of suitable substrates and, apparently, less favored optimal environmental conditions. Clearly, we need to conduct more intensive surveys to determine variability between sites in relation to ecological factors and substrate preferences.

The abundance of *P. lima* and *P. concavum* on substrates that turtles most commonly consume, such as *S. filamentosa*, *H. hawaiiensis*, and *A. spicifera*, closely parallels the prevalence of FP at individual sites. In highly abundant *Prorocentrum* areas, the potential for turtles to be exposed to OA is quite high. The potential for continuous consumption of *Prorocentrum* spp. and presumptively continuous exposure to OA through the diet is therefore a risk for green turtles in these susceptible areas. In other areas, such as west Hawaii,

where the dominant macroalgae in the turtle diets are mostly *Pterocladia*, *Ulva*, and red turfs, the incidence of *Prorocentrum* is low, thus reducing the risk of turtle exposure to OA. Even if *Prorocentrum* is present at low levels in some areas, turtles may not be at risk from OA exposure if they predominantly feed on those macroalgal species that are not a preferred substrate for *Prorocentrum*. The risk to turtles will therefore depend on the dominance and proportions of the macroalgal species consumed, the presence and abundance of *Prorocentrum* on those macroalgal species, and the concentration of OA to which the turtles are exposed. That the same macroalgal species can have very different benthic dinoflagellate assemblages and abundances in different geographical locations and that dominant green turtle food items, each with varying dinoflagellate assemblages, are on each island may be extremely critical with respect to the potential exposure of turtles to OA.

Nontumored turtles tend to be smaller (Balazs et al. 1994b) than tumored turtles. Hence, elevated presumptive OA in tumored turtles may simply be a function of time of exposure. It is currently unclear what the concentrations of OA in tissues represent in relation to potential tumor induction in turtles, and more detailed information on the toxicokinetics of OA is needed. Ten μg OA twice weekly (applied to the skin), after initiation by 7,12-dimethylbenz[*a*]anthracene (DMBA), produced skin papillomas in 93.0% of exposed mice after 16 weeks (Fujiki and Suganuma 1993). To our knowledge, the potential tumorigenic effect of OA through oral exposure has not been tested.

Benthic *Prorocentrum* are found in the Caribbean Sea (Ballantine et al. 1985; Carlson and Tindall 1985; Faust 1990, 1995), the southern North Atlantic Ocean (Bomber et al. 1985, 1989), the Indo-Pacific region (Bagnis et al. 1985; Grzebyk et al. 1994), and the Pacific Ocean (Fukuyo 1981). For the most part, these are the same areas where turtles with FP are currently found: the Caribbean Sea (Cayman Islands, Puerto Rico, Dominican Republic, Virgin Islands, Barbados, Antigua and Barbuda, Central America), the Atlantic Ocean (Florida, Brazil), the Indo-Pacific region (Australia, Sri Lanka, Sarawak, Malaya, Japan), and the Pacific Ocean (Hawaiian Islands) (Balazs 1991; Ehrhart 1991; Herbst 1994; Williams et al. 1994). Green turtles also occur farther north and south (Gulf of Mexico, Mediterranean Sea, Red Sea, and Bermuda), outside the range of the more tropical benthic *Prorocentrum* species (Faust 1995), where re-

ported incidences of FP in turtles are rare or non-existent (Teas 1991; Herbst 1994; A. Meylan, Florida Fish and Wildlife Conservation Commission, personal communication). However, *P. lima* is reported to be cosmopolitan (Steidinger 1997).

We hypothesize that the etiology of FP involves a tumor promoter such as okadaic acid that operates in conjunction with a tumor initiator such as a herpesvirus or retrovirus. The potential for dietary exposure to biotoxins cannot be underestimated and may be a dominant contributory factor to virus expression and FP in marine turtles. There are many examples of a multifactorial etiology in the development of neoplasia whereby potentially oncogenic viruses require interacting cofactors to express carcinogenicity (Jackson et al. 1993). Experimental models have demonstrated the interaction between oncogenic viruses and tumor promoters. In rodents, an *in vivo* relationship was demonstrated between the expression of endogenous papilloma virus, a tumor-promoting cocarcinogen such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and the development of skin papillomas (Amtmann et al. 1994). Increased virus yield of oncogenic herpesviruses has been demonstrated for *in vitro* cell lines using TPA (Zur Hausen et al. 1978). Okadaic acid significantly increased the frequency of transformed foci of mouse C3H/10T1/2 cells that were transfected with a plasmid containing full-length bovine papilloma virus (BPV-1) DNA (Tsang et al. 1991).

In some cases, the action of cofactors may be to transform tumors from a benign to a malignant state. For example, the etiological agent of epithelial papillomas in cattle is a papillomavirus, but when animals consume bracken fern containing mutagenic and immunosuppressive cofactors, papillomas transform into squamous cell carcinomas (Campo and Jarrett 1986; Campo et al. 1994). Herbst and Klein (1995) discussed the strong likelihood that the etiology of FP would probably involve environmental cofactors. They discussed the fact that high FP prevalence is in affected nearshore habitats associated with agricultural, industrial, or urban development. Although they indicated that there did not appear to be a correlation with chemical contaminants, they suggested that nearshore habitats may contain other stressors that rendered turtles more susceptible to FP. Tumor-promoting biotoxins were not discussed.

Whereas available data implicate a link between the presence of *Prorocentrum* spp., presumptive OA, and FP, other possibilities must be considered. Cyanobacteria produce tumor-promoting com-

pounds such as microcystins (from *Microcystis*) and nodularins (*Nodularia*) (Carmichael and Falconer 1993; Falconer and Humpage 1996); however, they are principally found in freshwater or brackish habitats (Skulberg et al. 1993) or in temperate marine regions (Chen et al. 1993). Although the microcystins and nodularins, like okadaic acid, are protein phosphatase inhibitors (Honkanen et al. 1990; Yoshizawa et al. 1990), their mode of action appears to be different (Falconer 1993). Microcystins and nodularins do not easily penetrate epithelial cells nor are they tumor promoters on mouse skin (Matushima et al. 1990; Fujiki and Suganuma 1993). As yet no evidence exists for the involvement of microcystins or nodularins in the development of papillomas in aquatic animals.

In the marine environment, the cyanobacterium *Lyngbya majuscula* causes contact skin dermatitis in humans (Moikeha et al. 1971) and produces the tumor promoters aplysiatoxin, debromoaplysiatoxin, bromoaplysiatoxin, and lyngbyatoxin-a (Fujiki et al. 1984a, 1984b, 1985; Moore 1984). Like okadaic acid, lyngbyatoxin-a has been experimentally demonstrated to induce papillomas in two-stage mouse carcinogenesis experiments (Fujiki et al. 1984b). Lyngbyatoxins promote tumor growth through the activation of protein kinase C, not through protein phosphatase inhibition (Fujiki et al. 1990). Because the assay used in this study was specifically for the detection of protein phosphatase inhibitors, lyngbyatoxins are not currently implicated; also *Lyngbya* was only detected in a few of the Hawaiian field samples (K. A. Steidinger, unpublished data). However, the potential role of marine cyanobacteria and their chronic effects on animal health should certainly be considered.

In marine tropical systems, there are several classes of protein phosphatase inhibitors (Honkanen et al. 1994; Bagu et al. 1997) that are potential tumor promoters: the polyether toxin okadaic acid and derivatives (e.g., OA and dinophysistoxins DTX-1, DTX-3; Fujiki and Suganuma 1993), motuporin (a peptide inhibitor; de Silva et al. 1992), calyculin A (Kato et al. 1986), and dy-sidiolide (Gunasekera et al. 1996). Thus far OA and some derivatives and Calyculin A have been shown to act as tumor promoters in two-stage carcinogenesis experiments using mice (Suganuma et al. 1990b; Fujiki and Suganuma 1993). Whereas most marine protein phosphatase inhibitors are produced by sponges (Kato et al. 1990; de Silva et al. 1992; Gunasekera et al. 1996), among the microalgae, the production of OA and its derivatives has only been documented from the marine

dinoflagellates *Prorocentrum* spp. and *Dinophysis* spp. (Murakami et al. 1982; Cembella 1989; Dickey et al. 1990; Hu et al. 1993). In the specific enzyme assay used in this study, these would be the only known marine tropical protein phosphatase inhibitors in which activity should be demonstrated. Our assumption that the assay has detected presumptive OA is also based on corroborative field data on the distribution of benthic dinoflagellates *Prorocentrum* known to produce OA. Motuporin, calyculin A, and dysidiolide are not currently suspected because the sponges producing these compounds would not be a normal component of the Hawaiian green turtle diet (Russell and Balazs 1994).

We have presented preliminary evidence that there is an association between the distribution of FP in the Hawaiian Islands and the distributions of benthic *Prorocentrum* species known to produce OA. We have demonstrated that in high-risk FP areas, preferred macroalgae and seagrass in the turtle diet have a high incidence of toxic *Prorocentrum* species and that turtles are presumptively exposed to OA. The potential role of protein phosphatase inhibitors such as OA in tumor development in marine turtles should be further explored, either for direct tumorigenic effects, as cofactors, or as sublethal immunosuppressive factors that render animals susceptible to oncogenic viruses or other pathogens.

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