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Relationship of parasites (Trematoda: Spirorchidae) and their eggs to the occurrence of fibropapillomas in the green turtle (*Chelonia mydas*)

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Abstract: The prevalence of green turtle (*Chelonia mydas*) fibropapillomatosis is increasing in Hawaii, raising the possibility of long-term negative effects on the breeding population. As the etiology of this disease is unknown, our objective was to determine if any relationship exists between fibropapillomas and trematode eggs from the family Spirorchidae, whose presence in tissue sections has been noted for years. A process for tumor digestion and egg extraction was developed and a total of 61 tumors of all sizes was examined to determine the total numbers of eggs present and the species of spirorchid involved. Twelve samples of normal turtle tissue were also examined. Eggs of one to three spirorchid species (*Leardius learedi* Price, 1934; *Haplotrema dorsopora* Dailey, Fast and Balazs, 1993; and *Carettacola hawaiiensis* Dailey, Fast and Balazs, 1991) were recovered from all tumors and nontumored tissues digested. Five turtles were injected with spirorchid eggs and observed for 12 months. New tumors developed on two of the turtles at noninjected sites. Although attempts to induce fibropapillomas through injection were not successful, the presence of spirorchid eggs in every tumor examined raises additional questions regarding the relationship between these parasites and fibropapillomas in sea turtles.

Résumé : La prévalence de la fibropapillomatose augmente chez la tortue verte (*Chelonia mydas*) à Hawaii, ce qui fait craindre des effets négatifs à long terme sur la population reproductrice. L'étiologie de cette maladie étant inconnue, nous avons tenté de déterminer s'il existait une relation entre les fibropapillomes et les oeufs de trématodes de la famille Spirorchidae, dont la présence est observée dans les coupes de tissus de tortue depuis des années. Nous avons mis au point un procédé de digestion des tumeurs et d'extraction des oeufs et examiné au total 61 tumeurs de diverses tailles pour y déterminer le nombre total d'oeufs présents et les espèces de Spirorchidae en cause. Nous avons aussi examiné 12 échantillons de tissu normal de tortue. Nous avons retrouvé des oeufs de 1 à 3 espèces de Spirorchidae (*Leardius learedi* Price, 1934; *Haplotrema dorsoporal* Dailey, Fast et Balazs, 1993; *Carettacola hawaiiensis* Dailey, Fast et Balazs, 1991) dans toutes les tumeurs et tous les tissus sans tumeurs digérés. Nous avons injecté à cinq tortues des oeufs de Spirorchidae et nous les avons ensuite observées pendant 12 mois. De nouvelles tumeurs sont apparues chez deux des tortues ailleurs qu'au point d'injection. Si nous n'avons pas réussi à faire apparaître des fibropapillomes par l'injection, la présence d'oeufs de Spirorchidae dans chacune des tumeurs soulève de nouvelles questions quant à la relation entre ces parasites et les fibropapillomes chez les tortues de mer.
[Traduit par la Rédaction]

Introduction

Fibropapillomas (GTF) in the green turtle (*Chelonia mydas*) were first described more than 55 years ago from the Florida Keys (Lucke 1938; Smith and Coats 1938). Little work on this disease was done during the next 40 years. Beginning in 1982, cutaneous fibropapillomas were seen in green turtles in the Indian River Lagoon system in east central Florida, and between 1985 and 1986, 57% of green turtles collected from this locality were infected (Ehrhart 1991). During 1985, 35% of the 51 stranded green turtles examined throughout the Hawaiian Islands had fibropapillomas (Balazs 1986), the growths ranging from a few millimetres to more than 30 cm in diameter (Dailey and

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Table 1. Summary of turtles sampled.

Turtle No.	Collection locality	Date*	Weight (kg)	Carapace length (cm)	Sex
1	Oahu	3/2/92	18.6	58.8	F
2	Oahu	1/8/92	20.0	53.4	F
3	Maui	4/13/92	11.8	47.8	M
4	Oahu	8/11/92	21.4	56.7	F
5	Oahu	6/16/92	17.7	48.2	—
6	Oahu	6/16/92	41.8	67.3	—
7	Oahu	6/10/92	20.9	56.9	F
8	Oahu	9/22/92	18.2	56.0	F
9	Oahu	9/22/92	43.6	71.3	M

*Month/day/year.

Balazs 1987). These growths in turtles can result in reduced vision, disorientation, blindness, and physical impairment of normal swimming, feeding, and breeding.

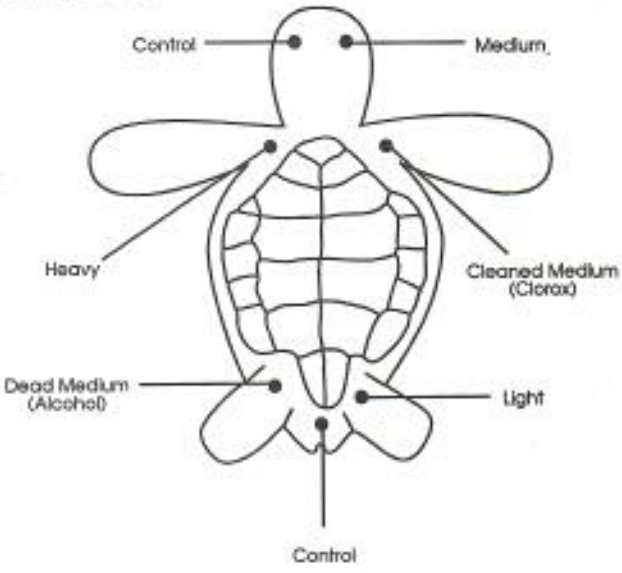
From 1986 to 1988, 10 green sea turtles from the Hawaiian islands of Lanai, Maui, and Oahu were surveyed for parasites and tumors. The results of this survey indicated that Hawaiian green turtles were heavily parasitized and that their tumors contained large numbers of trematode eggs from the family Spirorchidae (Dailey et al. 1991, 1992, 1993). The etiologic agent for these fibropapilloma tumors is still the object of speculation. One suggested theory is a parasite–tumor relationship. The present study was undertaken to look at this relationship by quantifying the size and location of all tumors examined and by noting egg type and number per tumor.

Materials and methods

Turtles were hand-captured from Kaneohe Bay, Oahu, Hawaii, by personnel of the National Marine Fisheries Service and the tumors measured and excised prior to the animal's release. Turtles found stranded on shore and which demonstrated no chance for survival were killed for necropsy using pentobarbital sodium (Table 1).

Tumors were surgically removed from all turtles. Live turtles received a lidocaine nerve block before tumor removal. Most of the live turtle samples consisted of small tumors and the animals did not require sutures after their removal. All tumors were measured and weighed prior to digestion processing. Large tumors from freshly killed turtles were cut into smaller pieces to facilitate digestion. Tumors were digested in 1 : 10 000 pepsin from porcine stomach mucosa. Approximately 9.5–10.0 g pepsin was mixed with 50 mL of water for the digestive process, giving a concentration that was deemed adequate for the digestion through trial and error. The tumor tissue was placed in plastic containers with perforated lids and incubated at 40°C for 48–72 h. After digestion, eggs were extracted by passing through a Flukfinder. The Flukfinder (trade mark name by Visual Difference, 5051-C Old Pullman Rd., Moscow, ID 83843) consists of a double filtration container with stacked top and bottom stainless steel screens of 100 and 40 μm, respectively. Eggs trapped by the bottom

Fig. 1. Injection sites on experimental green turtles (ventral view).



screen were backwashed into a beaker using a squeeze bottle. The collected material was then centrifuged for 5 min at 1550 rpm. The residue was then fixed with several drops of 10% formalin and stored until it could be examined using a compound microscope for total number of eggs and species represented. Eggs per gram weight of tissue was calculated. For control samples, normal tissue was collected by punch biopsy and processed in the same manner as tumor material.

Usually, most digestion was complete in 48 h except for some remaining fibrous tissue. Occasionally, some larger pieces had to be incubated for longer periods. Incubation times of up to 4 d did not affect the egg extraction procedure. Spirorchid eggs were incubated and observed for 5 d in this solution with no apparent effect.

Five turtles captured from Kaneohe Bay were selected for experimental infection (Table 2). Four of the turtles were free of any visible external tumors, while the fifth had numerous tumors present. The visibly tumored turtle was selected as part of the study to determine whether turtles with growths are sensitized by the infection and perhaps more prone to develop new growths. The turtles were maintained on a diet of previously frozen squid with vitamin–mineral supplements at the National Marine Fisheries Service's Kewalo Research Facility in Honolulu.

A source of viable spirorchid eggs was found in the serosal vessels of the small intestine of necropsied tumored turtles. Each blocked vessel contained a mass of 100–150 eggs. Viability was confirmed by observing miracidial activity (contractions) within the egg. Eggs maintained in sea water at ambient temperature (25°C) remained viable to 30 d. Eggs were injected subcutaneously in the same sites on all turtles (Fig. 1). The injections were divided into three dosages of eggs suspended in saline (0.2 mL): light (100–150), medium (300–500), and heavy (1000–1500), which were injected into the base of the right rear flipper site, the left lower eyelid site, and the base of the right front flipper site. The base of left rear

Table 2. Length and weight gains of turtles following experimental exposure to infection.

	Initial		12 month period			
Turtle	Carapace length (cm)	Weight (kg)	Carapace length (cm)	Gain	Weight (kg)	Gain
H924	42.2	10.0	51.9	+9.7	20.5	+10.5
V471	42.2	8.6	50.0	+7.8	18.2	+9.6
H951	41.7	10.9	46.9	+5.2	16.4	+5.5
V393	40.7	10.0	45.6	+4.9	14.5	+4.5
H921*	42.9	11.8	46.0	+3.1	15.0	+3.2

*Indicates turtle having visible external tumors.

Table 3. Number of spirorchid eggs found in tumors of green turtles 1, 2, 7, 8, 9.

Site	N*	Average tumor wt. (g)	Average no. of eggs/g by species†			Average total no. of eggs recovered	Average total no. of eggs/g tumor tissue
			Ll	Hd	Ch		
Cornea	3	2.6 (0.5–4.0)	231 (36–631)	82 (36–209)	5 (1–15)	1 127	318
Eye	5	2.4 (0.5–5.5)	41 (29–106)	23 (9–58)	0.8 (0.4–3)	458	170
Jaw	2	1.4 (1.0–1.7)	45 (38–51)	41 (34–47)	4 (3–5)	232	90
Neck	5	9.5 (1.5–20.0)	27 (1.5–78)	35 (4–86)	0.6 (0–1)	3 064	63
Right front flipper	11	47.1 (0.1–180.0)	6 (0.5–32)	33 (1–141)	4.4 (0–14)	18 263	43
Left front flipper	4	79.5 (1.0–137.0)	21 (1.0–5.8)	39 (5–113)	2.3 (0–8)	4 683	83
Right rear flipper	7	1.4 (1.0–3.0)	22 (7.0–490)	42 (19–95)	5.4 (0–13)	767	69
Left rear flipper	5	85.0 (0.6–347.0)	11 (0.8–20)	46 (5–107)	3.6 (0–15)	21 945	61
Cloaca	1	0.5	0	26	0	26	52
Glottis	1	3.2	6	28	0	34	11
Lung	6	6.1 (0.2–13.4)	122 (3–215)	51 (1–204)	2.6 (0–6)	3 988	176
Kidney	3	26.0 (5.5–74.0)	35 (15–51)	68 (11–83)	11 (1–32)	20 653	114
Liver	5	60.0 (1.5–144.0)	7 (2–15)	27 (1–90)	1.8 (0–4)	15 426	36
Total eggs/g by species			568	541	62		
% total eggs/g by species		49	46	5			

*N, number of tumors examined.

†Values are means followed by ranges in parentheses. Ll, *Leardius learedi*; Hd, *Hapalotrema dorsopora*; Ch, *Carettacola hawaiiensis*.

flipper site was injected with alcohol-killed eggs. The base of left front flipper site was injected with eggs disinfected with 10% Clorox to eliminate any microorganisms. Both sites received a medium dose. Two control sites (right lower eyelid, cloaca) were injected with saline solution. One turtle (V471) received an additional (heavy dosage) injection of eggs in saline at the right front flipper tag site.

All turtles were examined for tumor growth and their lengths and weights were measured every 2 weeks for 6 months and monthly thereafter for 1 year.

Results

A total of 61 tumors from both wild-caught and beached, necropsied turtles were digested and examined. These tumors were collected from both internal and external sources and ranged in weight from 0.2 to 347 g. Eggs were recovered from 100% of tumors examined, with egg numbers ranging from less than 1 to more than 845 per gram of tumor tissue. Turtles 1, 2, 7, 8, and 9 were heavily tumored, providing 93% of all material examined (Table 3). Turtle 3 had no external tumors and was included with the nontumored tissue sample (Table 4). Turtles 4, 5, and 6 were capture/release animals, which were used primarily for obtaining very small tumors (Table 5).

Experimental infections were induced in five turtles (Table 2). All experimentally infected turtles demonstrated rapid length and weight gains during the year in captivity. The tumored turtle (H921), however, showed less growth in both areas than the four nontumored turtles. Two turtles (V471, H951) developed new tumor growths that were histologically diagnosed as fibropapillomas. After 3 months, a tumor appeared at the control injection site (right lower eyelid) in V471, which grew to a maximum size of 9 × 4 mm over the 12-month period. During

Table 4. Number of spirorchid eggs found in nontumored tissue of green turtles.

Turtle No.	Site	Wt. (g)	Eggs by species*			Total eggs
			Ll	Hd	Ch	
1	Left front flipper	1.3	0	11	1	12
1	Neck	5.0	1	1	0	2
1	Left rear body	4.0	18	18	0	36
2	Neck	4.5	2	1	0	3
2	Right front flipper	2.5	7	21	0	28
2	Right rear flipper	18	2	2	0	4
3	Neck	113	1	0	0	1
3	Right front flipper	113	0	2	0	2
3	Right rear flipper	79.2	2	6	0	8
7	Kidney	30	496	544	2 224	3 264
7	Heart	77.5	2 650 +	43 999 +	159 +	46 808 +
7	Brain (entire)	4	367	906	0	1 273

*Ll, *Leardius learedi*; Hd, *Hapalotrema dorsopora*; Ch, *Carettacola hawaiiensis*.

Table 5. Number of spirorchid eggs found in tumors of green turtles captured and released.

Turtle No.	Site	Tissue wt. (g)	Eggs by species*			Total eggs	Total eggs/g
			Ll	Hd	Ch		
4	Eye	0.5	8	8	0	16	32
4	Shell	0.6	23	30	0	53	88
5	Right front flipper	0.4	2	0	0	2	5
6	Neck	2.1	8	59	0	67	32
Total eggs by species			41	97	0		
% total eggs by species			30	70	0		

*Ll, *Leardius learedi*; Hd, *Hapalotrema dorsopora*; Ch, *Carettacola hawaiiensis*.

month 11, the same turtle developed another growth in the left eye (medial canthus), which grew to 3 × 2 mm in the final month of observation. The second turtle (H951) also developed a tumor in the same area as V471 (medial canthus of left eye) at the same time (11 mo). This growth measured 4 × 3 mm by end of month 12.

Discussion

The factors (pathogen plus environmental insult) responsible for the development of fibropapillomas in green turtles have yet to be confirmed. Recently, Jacobson et al. (1991) reported the presence of a herpesvirus in two cases of GTF. They were unable, however, to fulfill Koch's postulate and demonstrate that a causal relationship existed between the herpesvirus and the tumors. They found no evidence of herpesvirus infection, as indicated by the presence of intranuclear inclusions, in any of the sectioned material originating from the 73 tumors from Hawaiian green turtles. Jacobson (1992) stated that trematode eggs from the family Spirorchidae were seen more often within the dermal capillaries of cutaneous GTF of Hawaii-caught turtles as compared to those from Florida. He indicated that trematode eggs were few in sections of tumor examined, and that multiple sections of a given tumor may need to be examined in order to clearly demonstrate the presence or absence of eggs.

Pollutant-mediated stress causing morphophysiological changes such as cellular proliferation (i.e., skin tumor) is well documented in aquatic organisms (Giam and Ray 1987). Chemical pollutants impairing the immune system and causing stress have been listed as possible contributors to GTF in Hawaiian green turtles (Aguirre 1991, 1992; Balazs and Pooley 1991). Aguirre (1993), to determine the involvement of selected environmental pollutants on the etiology of GTF, examined 11 heavily tumored turtles from Hawaiian waters. He tested brain, fat, liver, and kidney tissues for any of the listed organochlorines, polychlorinated biphenyls, organophosphates, or carbamate insecticides in concentrations above stated method of detection limits. He also looked at concentrations of selenium and heavy metals. In all cases he found that concentration levels were below levels reported to be normal in other animal species.

The present study deteremined the total number of eggs that could be recovered from the entire tumor through digestion and identified the species of spirorchid trematodes involved and their degree of involvement. The results

indicate clearly that 100% of all tumors found during this study, regardless of size or location, contained spirorchid eggs. Not only did all tumors contain eggs, but the eggs were sometimes present in massive numbers, particularly in tumors located in internal organs (Table 3). A tumor on the cornea of turtle 2 contained 631 eggs/g tissue. Even the smallest tumors contained a significant number of eggs. A 0.6-g shell tumor had 88 eggs/g and a 0.5 g eye tumor had 32 eggs/g. As previously stated, internal tumors also contained large concentrations of ova. For example, a lung tumor weighing 2.5 g contained 243 eggs/g, while the average lung granuloma (internal growth) contained 176 eggs/g. The liver and kidneys were also tumored in some turtles, the liver tumors averaging 36 eggs/g, while kidney tumors were somewhat higher at 114 eggs/g. Massive numbers of eggs were also found in nontumored internal organs and the brain (Table 4). Although the significance of this information has yet to be determined, we feel it strongly suggests a direct link between GTF and spirorchid trematode infections.

Three species of blood fluke, *Leardius learedi* Price, 1934; *Hapalotrema dorsopora* Dailey, Fast and Balazs, 1993; and *Carettacola hawaiiensis* Dailey, Fast and Balazs, 1991, which are found in large numbers in Hawaiian green turtles, occupy niches similar to those occupied by members of the genus *Schistosoma* (i.e., the circulatory system). The finding in this study of a high egg count in brain tissue (318/g in turtle 7) demonstrates the extent to which these eggs are spread in the circulatory system. The effect on the turtle of such large numbers of eggs was examined by Wolke et al. (1982). During their study of loggerhead sea turtles (*Caretta caretta*) along the eastern Atlantic seaboard, they found eggs representing three genera of spirorchid trematodes: *Carettacola*, *Hapalotrema*, and *Neospirorchis*. Gross signs of pathology in heavily infected animals included cachexia, anemia, and enteritis. They also noted a similarity in the histopathological lesions to those present in homeotherms with schistosomiasis. These authors concluded that spirorchidiasis is prevalent in subadult loggerhead sea turtles and that it may be responsible for significant debilitation and mortality.

Although the mode of transmission of marine species of the family Spirorchidae has not been elucidated, the life cycle of *Spirorchis elegans* Stunkard, 1923, a species that lives in the blood vessels of freshwater turtles, is known (Goodchild and Kirk 1960). In the life cycle of *S. elegans*, the cercariae are shed from a snail and penetrate the mucous membranes of the eye, nose, mouth, and cloaca of turtles. Recent studies on snails inhabiting the feeding grounds of Hawaiian green turtles have, to date, not revealed the presence of spirorchid trematode larvae (M.D. Dailey, unpublished data). This observation indicates that the life cycle of marine spirorchids infecting sea turtles may not involve a snail intermediate host. Køie (1982) has found that *Aporocotyle simplex* Odhner, 1900, a sanguinicolid blood fluke of marine fishes, uses a polychaete as the sole intermediate host.

Preliminary work has begun on isolating the protein that causes the host to react to the ova. Findings to date indicate that a protein is present in the sterile saline solution in which eggs recovered from the serosa of tumored turtles have been cultured (Dr. J. Sakanari, School of Medicine, Department of Pathology, University of California, San Francisco, CA 94121, personal communication). Additional work is needed to find the specific protein involved and to confirm that it is parasite rather than host induced. Once isolated, this protein could be collected and tested to determine its role in GTF production.

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