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INCLUSION BODIES IN RED BLOOD CELLS OF HAWAIIAN GREEN TURTLES (*CHELONIA MYDAS*)

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EXECUTIVE SUMMARY

A total of 43 Romanowsky-stained, thin blood smears from juvenile green turtles (*Chelonia mydas*) in the Hawaiian Islands were examined for the presence of hemoparasites. In addition, 5 thin blood smears from hatchlings were also examined. Using light microscopy 40/43 juvenile and 4/5 hatchling specimens demonstrated the presence of erythrocytic inclusion bodies. No electro-dense bodies were identified by electron microscopy. These inclusion bodies were compared with similar structures identified in sea turtles nearly 60 years ago. Further research is required to determine the true nature of these inclusion bodies, the transmission cycles, the identification of an intermediate host, and their relationship to green turtle fibropapillomas.

PREFACE

Prepared under contract as part of the Southwest Fisheries Science Center Honolulu Laboratory's research program on marine turtles, this report provides the results of an attempt to identify inclusion bodies in red blood cells of the green turtle (*Chelonia mydas*). Although no electro-dense bodies were identified by electron microscopy, the inclusion bodies seen by light microscope were similar to *Rickettsia*-like disease causing organisms seen in other reptiles. It is possible that this suspected organism could play a causative role in the occurrence of tumors affecting the green turtle.

The incidence of life-threatening tumors on green turtles in the Hawaiian Islands has increased to epidemic proportions during recent years. A similar situation exists among green turtles at certain sites in Florida, the Caribbean, and other locations worldwide. The cause of this disease, known as fibropapillomatosis, remains unknown. Death appears to be the usual result of the disease and therefore the impact on the afflicted populations may have serious consequences. The disease represents one more threat to the survival of green turtles worldwide. The nature of this disease and its cause must be determined in order to develop a long-term disease management program. The present report by Dr. Alonso Aguirre constitutes progress in determining the cause of the disease, which must be followed up with additional studies to ascertain the true nature of the *Rickettsia*-like organism.

Because this report was prepared by an independent investigator, its statements, findings, conclusions and recommendations do not necessarily reflect the views of the National Marine Fisheries Service, NOAA.

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INTRODUCTION

Intraerythrocytic inclusion bodies may be caused by a wide variety of agents including viruses, bacteria, fungi, and parasites. Viral and rickettsial agents have been observed as intraerythrocytic structures in some reptile species (Telford and Jacobson, 1993). *Tunetella emydis*, a *Rickettsia*-like organism formerly classified as a piroplasmid, was reported in a Brazilian green turtle (Carini, 1937). Many hemoparasites have been described infecting the blood of terrestrial reptiles including *Haemogregarina* spp., *Hepatozoon* spp., *Haemoproteus* spp. (*Haemocystidium*), *Plasmodium* sp., *Karyolysus* sp., *Lainsonia* sp., *Leukocytozoon* sp., *Trypanosoma* spp., and others. Most of these organisms are transmitted by leeches, ticks, other bloodsucking invertebrates, and by mite ingestion (Lauckner, 1985; Barnard, 1986; Frye, 1991).

Hemoparasites of marine reptiles, however, have been largely undescribed and no recent information is available on the blood parasites of sea turtles. An isolated study reported the presence of unidentified intraerythrocytic *Hepatozoon*-like haemogregarines in 7/12 Galapagos marine iguanas (*Amblyrhynchus cristatus*) (Ayala and Hutchings, 1974).

During a series of epidemiologic studies designed to determine the etiology of green turtle fibropapillomas (GTFP), intracytoplasmic inclusion bodies were identified by light microscopy in the erythrocytes of green turtles (*Chelonia mydas*) collected during 1991-92 in Kaneohe Bay, Island of Oahu, Hawaii (Aguirre 1992, 1993). In addition, these structures were observed in blood films of hatchlings collected at French Frigate Shoals, Hawaii, in 1992. The objective of this study was the screening of blood films collected from Hawaiian green turtles by light and electron microscopy to determine the presence, ultrastructure, and nature (viral, bacterial, or parasitic) of these inclusion bodies.

MATERIALS AND METHODS

Field Sampling

Blood specimens were collected from 43 wild green turtles caught in Kaneohe Bay, and 5 hatchlings collected at French Frigate Shoals from the same nest of a turtle known to have GTFP (Aguirre 1992, 1993).

Samples from the juveniles were taken from the bilateral dorsal cervical sinuses, as described by Owens and Ruiz (1980). Small samples from the hatchlings were taken by cardiac puncture following procedures similar to those described by Stephens and Creekmore (1983). The hatchlings were then held for two days to

confirm their well-being before being released back into the wild. Thin smears from fresh blood were air dried and immediately fixed in absolute methanol. Slides were numbered and stored for later staining and examination.

During 1992, blood specimens (0.5 ml) from 6 turtles were fixed in Karnovsky's solution (phosphate buffered 4.0% formaldehyde-1.0% glutaraldehyde). These specimens were held at 4°C until processed for both light and transmission electron microscopy (McDowell, 1978).

Laboratory Techniques

The blood smears were analyzed for the presence of parasites or blood cell abnormalities. Romanowsky-stained blood smears were examined for *Trypanosoma* spp., intraleukocytic *Leucocytozoon*-like hemoparasites and microfilariae under low power (100x) for 10 min each. *Haemoproteus* spp., *Hepatozoon* spp., *Plasmodium* spp., and hemogregarines were examined at high power, oil immersion (1000x and 5000x) light microscope during 10-15 min or 50 fields (10,000 erythrocytes). In addition, five randomly selected blood smears were stained using Machiavello's method for the identification of rickettsial organisms (Frye, 1991).

Blood specimens for electron microscopy were washed with 0.2 M Sorenson's phosphate buffer Ph 7.3 and postfixed in 1.0% osmium tetroxide for one hour. Then, blood cells were washed through two changes of ddH_2O, dehydrated through a graded acetone series, infiltrated with and embedded in Medcast-Araldite 502 Resin® (Ted Pella Inc., Redding, CA). Ultrathin sections from the specimens of two turtles were placed on copper grids, stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (Hayat, 1986).

RESULTS

Blood films from 29/32 turtles collected during 1991 contained purple intracytoplasmic inclusions of 0.1-0.4 μm in diameter in their erythrocytes (Fig. 1). Single, multiple, large, and small forms were observed. Tiny and discrete punctate bodies, large and more diffuse inclusions, as well as "signet-ring" intraerythrocytic inclusions were also identified.

Infected red blood cells with inclusion bodies in a smear averaged rates of 48%, ranging from 5% to >75% (Table 1). All 11 blood smears collected during the 1992 season presented *Tunetella*-like inclusions in large numbers (>50% of cells infected), and highly refractile inclusions similar to *Plasmodium* were observed in 4/11 blood films (Table 2). In addition, smears from the 5 hatchlings presented both *Tunetella*-like (3/5) and

Plasmodium-like (1/5) inclusions. In most cases, these inclusions varied in size and shape as reported by Brumpt and Lavier (1935) (Fig. 2).

Over 1,000 erythrocytes from blood specimens of turtles 039 and 040 (Table 2) were examined under the electron microscope and no electron-dense lesions or inclusion bodies were identified. Some smooth endoplasmic reticulum and an occasional Golgi apparatus were observed.

No microfilaria or hemogregarines were identified under low or high power light microscopy in any of the blood films examined. Selected Machiavello's-stained blood films were negative for Rickettsiae.

DISCUSSION

The morphology of the intraerythrocytic inclusion bodies observed in this study was similar or identical to the agent *Tunetella emydis*, a *Rickettsia*-like organism reported 58 years ago from *Clemmys caspica leprosa* in North Africa (Brumpt and Lavier, 1935). Carini (1937) described a second species of this genus in a Brazilian green turtle. The genus *Tunetella* has been synonymized with *Aegyptianella pullorum*, a rickettsia from the family Anaplasmataceae which infects red blood cells of several bird species (Carpano, 1939; Weiss and Moulder, 1983; Gothe, 1992). In this case, the specific name of this organism should be changed to *Aegyptianella emydis* (see Reichenbach-Klinke and Elkan, 1990).

The majority of the green turtle specimens obtained for this study are infected with an agent that displays a remarkable degree of pleomorphism. Many inclusions were round or pyriform with a sharply defined end; also, several small bodies appeared to have coalesced to form a solitary inclusion. These structures are similar to *Aegyptianella* inclusions and contain up to 26 initial bodies, constituting the actual parasitic bacteria (Weiss and Moulder, 1983). In several instances, these inclusions were highly refractile and are morphologically indistinguishable from *Plasmodium* sp. This parasite has been observed in the erythrocytes of many semiaquatic freshwater turtles and terrapins. The "signet-ring" inclusions observed in some specimens were similar to the intracytoplasmic bodies reported in freshwater emydids from southeastern United States (F. L. Frye, pers. comm., 1993).

Considerable controversy exists regarding the nature of these entities. This entire class of intraerythrocytic inclusions have been considered to be a variant of *Aegyptianella* (S. R. Telford Jr., pers. comm., 1992; F. L. Frye, pers. comm., 1993). This group of pleomorphic organisms are transmitted by argasid ticks and in most cases act as pathogenic, obligate

parasites of vertebrates (Weiss and Moulder, 1983). Sea bird ticks, *Ornithodoros* spp. and *Ixodes* spp., commonly occur in the soil at French Frigate Shoals where nearly all green turtles hatch in the Hawaiian Islands (Balazs and Pooley, 1991).

The negative results of the electron microscopic examination warrant further collection of ultrastructural data to determine the viral, rickettsial, or parasitic nature of these erythrocytic intracytoplasmic inclusion bodies. Research identifying possible arthropod vectors, the transmissibility of these agents, and their relationship to GTFP is recommended.

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TABLE 1.--Size, weight, sex, tumor score, and description of intracytoplasmic inclusion bodies for 32 green turtles (*Chelonia mydas*) sampled from Kaneohe Bay, Island of Oahu, Hawaii, 1991.

TURTLE NO.	SCL[1] (cm)	WGT[2] (kg)	SEX	TUMOR SCORE[3]	FINDINGS
001	40.3	8.6	UNK	0	a few erythrocytes with Tunetella-like inclusions; "Type 13" (Brumpt and Lavier, 1935)[4]
002	45.9	15.5	UNK	0	a few erythrocytes with Tunetella-like inclusions; "Type 20/21"
003	44.5	11.8	UNK	3	all erythrocytes contained stain precipitate-like surface objects (artifacts?); no inclusions observed
004	47.7	15.9	UNK	0	~17% of erythrocytes contained Tunetella-like inclusions, some refractile
005	40.7	10.0	UNK	0	~20% of erythrocytes contained Tunetella-like inclusions
006	42.0	10.0	UNK	0	a few erythrocytes contained inclusions, a rare one was refractile
007	44.5	11.8	UNK	3	no inclusions observed
008	53.6	22.3	UNK	3	<5% of erythrocytes contained inclusions
009	52.9	21.4	UNK	2	most erythrocytes contained well-stained Tunetella-like inclusions
010	51.4	17.3	UNK	0	~12% of erythrocytes contained well-stained Tunetella-like inclusions
011	55.2	25.4	UNK	0	~70% of erythrocytes contained well-stained Tunetella-like inclusions
012	62.3	UNK	UNK	2	intraerythrocytic inclusions present, but different from slides 009/010
013	40.1	9.5	UNK	0	thick blood film; no inclusions observed
014	41.6	10.4	UNK	0	Tunetella-like inclusions present "Type 27/29"
015	37.4	7.7	UNK	0	Tunetella-like inclusions present; "Type 30"
016	54.0	21.8	UNK	2	50% of erythrocytes contained inclusions of various shapes and sizes
017	52.8	20.4	UNK	0	thick blood film; Tunetella-like inclusions present in ~25% of erythrocytes; various shapes
018	40.7	10.0	UNK	0	Tunetella-like inclusions present; various sizes and shapes
019	38.8	7.7	UNK	0	Tunetella-like inclusions present; "Type 23"
020	44.8	13.6	UNK	0	>90% of erythrocytes contained Tunetella-like inclusions; varied in size and shape
021	39.8	8.6	UNK	0	~35% of erythrocytes contained Tunetella-like inclusions; varied in size and shape
022	43.9	11.4	UNK	0	most cells (90%) contained Tunetella-like inclusions
023	43.9	12.7	UNK	0	~40% of RBC contained Tunetella-like inclusions
024	50.5	17.7	UNK	1	thick blood film; ~45% of erythrocytes contained Tunetella-like inclusions; "Type 10"
025	42.2	8.6	UNK	0	Tunetella-like inclusions present in large numbers
026	40.8	9.5	UNK	0	small number of erythrocytes contained tiny punctate Tunetella-like inclusions; "Type 29"
027	43.5	11.0	UNK	0	small number of erythrocytes contained small, multiple Tunetella-like inclusions
028	68.3	UNK	UNK	3	small numbers of cells contained inclusions
029	53.9	UNK	UNK	3	Tunetella-like inclusions present in large numbers
030	55.3	UNK	UNK	4	Tunetella-like inclusions; quite variable in size
031	54.1	UNK	UNK	3	Tunetella-like inclusions present
032	66.3	UNK	UNK	2	many large and tiny Tunetella-like inclusions present; "Type 12/26"

[1]SCL= Straight Carapace Length
[2]WGT= Weight
[3]Tumor Scale from 1 to 4, 4 being the most severe
[4]*Tunetella emydis* inclusion types are described on Fig. 2

TABLE 2.--Size, weight, sex, tumor score, and description of intracytoplasmic inclusion bodies for 11 green turtles (*Chelonia mydas*) sampled from Kaneohe Bay, Island of Oahu, and 5 hatchlings sampled from French Frigate Shoals, Hawaii, 1992.

TURTLE NO.	SCL[1] (cm)	WGT[2] (kg)	SEX	TUMOR SCORE[3]	FINDINGS
038	42.7	10.5	UNK	0	inclusions seen, but many look more like Plasmodium than *Tunetella*
039	42.1	11.4	UNK	0	*Tunetella*-like inclusions present in large numbers
040	43.8	12.3	UNK	0	*Tunetella*-like inclusions present in large numbers
041	42.8	10.5	UNK	0	*Tunetella*-like inclusions present in large numbers
042	42.9	11.8	UNK	2	many tiny punctate *Tunetella*-like inclusions present; "Type 25/29" (Brumpt and Lavier, 1935)[4]
043	52.3	16.4	F	0	inclusions seen, but many look more like *Plasmodium* than *Tunetella*
046	56.1	18.2	F	3	relatively few erythrocytes contained *Tunetella*-like inclusions
047	52.3	16.8	F	3	highly refractile inclusions present; look like *Plasmodium*
048	71.3	43.6	M	3	highly refractile inclusions present; look like *Plasmodium*
049	52.9	20.0	F	3	*Tunetella*-like inclusions present in large numbers
050	66.9	37.5	M	3	*Tunetella*-like inclusions present in large numbers
081	HATCHLING		UNK	Mother+	*Tunetella*-like inclusions present in large numbers
082	HATCHLING		UNK	Mother+	very few cells on slide; no inclusions present
083	HATCHLING		UNK	Mother+	a few refractile *Plasmodium*-like inclusions seen
084	HATCHLING		UNK	Mother+	*Tunetella*-like inclusions present in large numbers
085	HATCHLING		UNK	Mother+	*Tunetella*-like inclusions present

[1]SCL= Straight Carapace Length
[2]WGT= Weight
[3]Tumor Scale from 1 to 4, 4 being the most severe
[4]*Tunetella emydis* inclusion types are described on Fig. 2

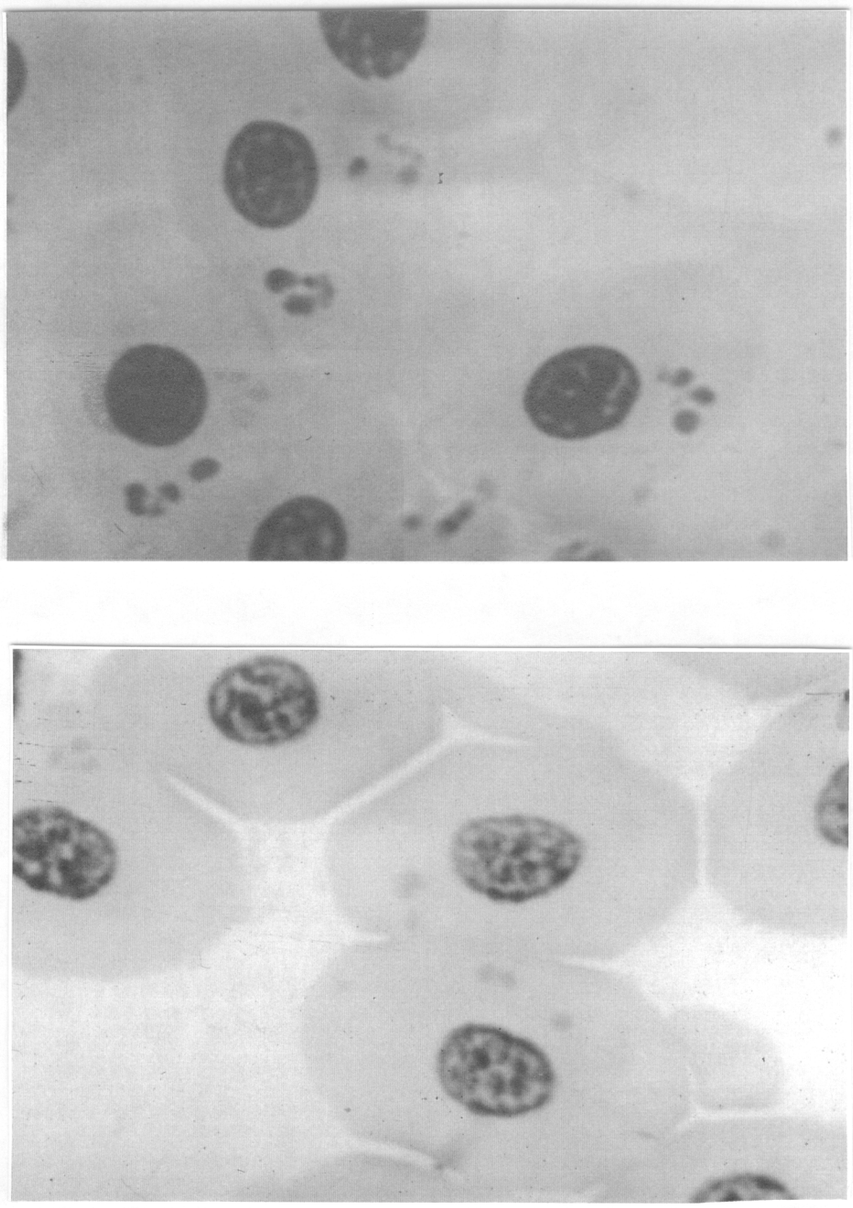

Figure 1.--*Tunetella*-like intraerythrocytic inclusion bodies identified in green turtles (*Chelonia mydas*) collected in Kaneohe Bay, Island of Oahu, Hawaii, 1991-92. Romanowsky stain; x 1000 oil immersion.