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IMMUNOLOGICAL EVALUATION OF CAPTIVE GREEN SEA TURTLE (*CHELONIA MYDAS*) WITH ULCERATIVE DERMATITIS

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Abstract: Ulcerative dermatitis (UD) is common in captive sea turtles and manifests as skin erosions and ulcers associated with gram-negative bacteria. This study compared clinically healthy and UD-affected captive turtles by evaluating hematology, histopathology, immunoglobulin levels, and delayed-type hypersensitivity assay. Turtles with UD had significantly lower weight, reduced delayed-type hypersensitivity (DTH) responses, and higher heterophil:lymphocyte ratios. This study is the first to assay DTH in green turtles (*Chelonia mydas*) and suggests that UD is associated with immunosuppression.

Key words: *Chelonia mydas*, delayed-type hypersensitivity, heterophil:lymphocyte ratio, immunosuppression, ulcerative dermatitis.

INTRODUCTION

Captive green turtles (*Chelonia mydas*) are affected by a wide range of pathogens and diseases.¹¹ Ulcerative dermatitis (UD) is a condition that typically affects captive hatchlings in the first week of life. UD is characterized by pustules and ulcers that wax and wane on the back of the neck and on tips and edges of the flippers and tail. Prevalence can reach 100% with mortality ranging from 20% to 50%. Histopathology of skin lesions usually reveals diffuse infiltrates of heterophils and mononuclear cells in the dermis and epidermis associated with cell necrosis and microcolonies of gram-negative bacteria.^{10,11,13} The primary etiology of UD is unknown but various bacteria have been implicated on the basis of culture including *Citrobacter freundii*,¹⁷ *Enterobacteriace-*

ae,¹⁶ *Vibrio alginolyticus*, *Aeromonas hydrophila*, *Pseudomonas* spp. and *Flavobacterium* spp.¹³ However, in many cases these same agents were isolated from the skin of clinically healthy turtles and from tank water,¹⁴ leading some to think that UD might have had an immune-suppressive component.¹³ Understanding the pathophysiology of UD in captive sea turtles has the potential to enhance wild stocks because many captive turtles are often released into the wild, and it is in the best interest of such institutions to release animals in the peak of health.

An example of such an institution is Xcaret Park located at the Yucatan Peninsula (86°55'W, 21°20'N) facing the Caribbean 8 m above sea level. The park has a warm, subtropical climate with mean annual temperature of 27°C and average humidity of 74%. Xcaret Park has a 17-yr history of captive breeding and release of turtles into the Caribbean basin; however, the park has also had an intermittent history of UD in captive turtles. To gain a better understanding of the pathogenesis of UD in green turtles, this study set out to compare the humoral and cell-mediated immunological status of captive green turtles with and without UD.

MATERIAL AND METHODS

During the summer of 2008, 15 13-mo-old green turtles of unknown sex from the same clutch were randomly selected, marked with a uniquely numbered metal flipper tag on the right front flipper, weighed (to 0.1 kg), measured for curved carapace length (CCL) and width (CCW) (0.1 cm), and divided into 2 groups: Group 1 consisted of seven clinically healthy turtles,

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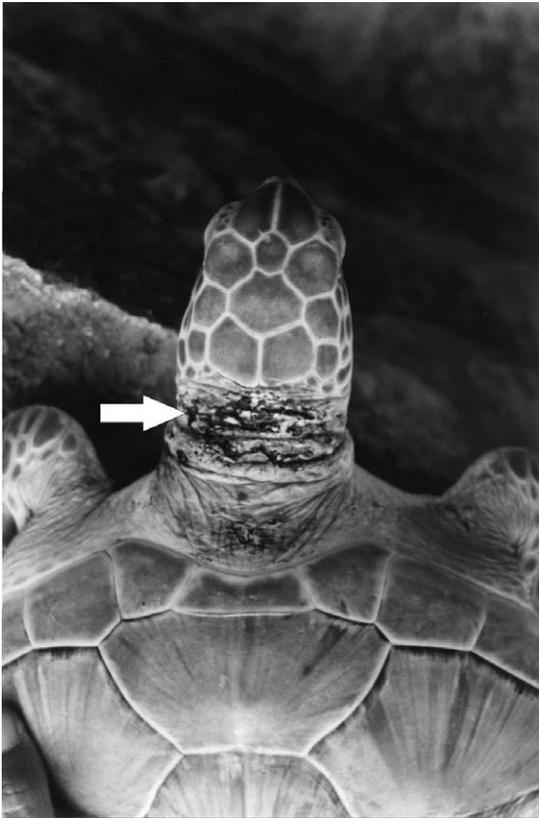


Figure 1. Sea turtle with ulcerative dermatitis (UD) in the nape of the neck (arrow).

judged as such based on lack of visible skin lesions and good body condition as evidenced by weight, plastron convexity, and lack of morphologic or behavioral abnormalities after careful physical examination. Group 2 comprised eight turtles with severe (>3 lesions exceeding 1 cm diameter each) UD in various parts of the body surface (Fig. 1). Each group was placed in round cement tanks, 4.57 m long, 4.34 m wide, and 1.05 m deep containing 2,000 L of water with a continuous water flow of 2.8 l/min (total water turnover was 1 tank volume /12 hr). Temperature, salinity, and pH in the tanks during the 3-mo study averaged 26.3°C, 36 ppm, and 8.2, respectively. Both groups were fed with commercial turtle food (Sea Turtle Food, Silver Cup, Salt Lake City, Utah 84157, USA) with 35% protein, 5% fiber, and 3.5% fat, providing 3% of their live weight twice daily.

Three milliliters of blood were obtained from the cervical venous sinus²³ using a 21-gauge (39 mm) Vacutainer® needle and tubes with sodium heparin (BD Vacutainer, Becton Dickinson,

Franklin Lakes, New Jersey 07417, USA). Blood smears were prepared immediately, air-dried, fixed in absolute methanol, and stained with Wright's Giemsa for differential counts and determination of heterophil:lymphocyte (H:L) ratio.³⁹ Total white cell counts were done according to methods described by Natt and Herrick.²⁷

Biopsies were done on representative skin lesions using a 6-mm sterile biopsy punch (Acu-punch®, Acuderm Inc., Ft. Lauderdale, Florida 33309, USA) taking care to include the border between lesion and normal skin. The samples were fixed in 10% buffered formalin in labeled jars, embedded in paraffin, sectioned at 5 µm, stained with hematoxylin and eosin (H&E) or Gram stain, and reviewed under light microscopy.

Immunoglobulin precipitation was performed as described.⁶ One hundred milliliters of heparinized blood were obtained from the cervical venous sinus²³ of a clinically healthy adult male *Chelonia mydas*. The blood was centrifuged at 200 g for 15 min, plasma was separated and aliquoted into 14-ml polystyrene tubes (Falcon, Becton Dickinson, Franklin Lakes, New Jersey 07417, USA), and frozen at -20°C. Immunoglobulins were precipitated by adding an equal volume of 66% saturated ammonium sulphate solution at 4°C while stirring slowly. Precipitate was centrifuged at 4,000 g for 20 min at 4°C, resuspended in 25 ml phosphate-buffered saline (PBS), and precipitations repeated twice more. The third precipitate was resuspended in 10 ml PBS and dialyzed against 1,000 ml Tris buffer containing 0.1 M-NaCl 0.15 M, pH 8.0 (Tris-buffered saline, TBS), at 4°C overnight, aliquoted at volumes of 1 ml in Eppendorf® tubes (Axygen Scientific, Union City, California 94587, USA), and stored at -20°C until use.

Gel filtration chromatography was used to separate Green turtle 17S IgM from immunoglobulins (7S and 5.7S IgY). Briefly, a 2.5 × 100-cm column (Pharmacia Fine Chemicals Inc., Piscataway, New Jersey 08854, USA) was packed with Sephadex® G-200 (Sigma Chemicals Company, St. Louis, Missouri 63103, USA) and equilibrated with TBS. Five milliliters of the immunoglobulin precipitation was placed into the column and eluted with TBS at a flow of 50/ml and 1-ml fractions were collected using a fraction collector (Retriever III Model 328, ISCO, Lincoln, Nebraska 68504, USA) coupled to an ultraviolet (UV) detector (DV-64 spectrophotometer, Beckman Coulter Inc., Brea, California 92822-8000, USA). Fractions containing pooled 17S IgM, 7S, and 5.7S IgY were reduced by heating (100°C) in a sample buffer with 2-

mercaptoethanol, resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) 12%,¹⁹ and identified based on molecular weight.^{2,32} Fifty-milliliter aliquots of purified pooled 7S and 5.7S IgY were concentrated with powdered sugar to a final volume of 5 ml and dialyzed against 1,000 ml PBS at 4°C overnight, filtered (0.22 µm), protein concentration determined by the method of Bradford,⁵ and 1-ml aliquots were stored at -20°C.

To generate anti-turtle IgY conjugates, an adult, nonpregnant female adult goat was inoculated subcutaneously with 0.5 ml (3 mg) of turtle pooled 7S and 5.7S IgY mixed with 1 ml of Freund's incomplete adjuvant (Sigma Chemicals Company) after banking 5 ml of preinoculation serum. The goat was bled 8 days after first injection. Fifteen days after first injection, 2 doses of 0.5 ml (3 mg) of turtle Ig in PBS were applied to the same animal intravenously at intervals of 8 days after taking a postinoculation blood sample and was subsequently bled every 15 days and serum assayed for antibodies against goat IgG by capillary agglutination.¹² Once goat anti-turtle IgG titers reached 1:1,000, 100 ml of blood were obtained by jugular venipuncture, blood was centrifuged at 200 g for 15 min, and serum decanted and aliquoted at volumes of 10 ml and frozen at -20°C. Goat IgG was purified from serum as previously described.⁶ Briefly, a sample of 5 ml of immunoglobulins was eluted through a Sephadex G-200 column coupled to a fraction collector. Protein-rich fractions were identified by UV absorbance (280 nm), identified by SDS-PAGE, concentrated, dialyzed against PBS, aliquoted, and stored at -20°C.

Indirect enzyme-linked immunosorbent assay (ELISA) was used to detect turtle pooled 5.7S and 7S IgY in plasma.⁸ Briefly, 96-well ELISA plates (MaxiSorp, Nunc Thermo Scientific, Pittsburgh, Pennsylvania 15275, USA) were incubated for 2 hr with 50 µl/well in triplicate of green turtle plasma diluted 1:20 in 100 mM carbonate buffer, pH 9.5. Fifty microliters/well of chicken plasma diluted 1:20 in carbonate buffer and 50 µl/well of carbonate buffer served as negative controls. Plates were then washed 4 times with PBS-Tween® 5%, blocked for 1 hr with 100 µl/well of skimmed milk 5% (DIFCO, Becton Dickinson, Franklin Lakes, New Jersey 07417, USA). After four washes with PBS-Tween 5%, plates were incubated 1 hr with 50 µl/well of goat anti-turtle Ig (dilution 1:500) in skimmed milk 1%, washed 4 times with PBS-Tween 5%, and incubated for 1 hr with 50 µl/well of 1:1,000 of horseradish perox-

idase conjugated donkey anti-goat IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, 19390, USA) in skimmed milk 1%. After eight washes with PBS-Tween 5%, color development was done by incubating 100 µl/well of 2,2'-azino-di-3-ethylbenziazoline sulfonate substrate (ABTS, Reagents National Veterinary Services Laboratories, Ames, Iowa 50010, USA) in the dark for 15 min and reading at 405 nm with an ELISA Reader (Micro Reader 4 plus, Hyperion Inc., Miami, Florida 33186, USA). All ELISA steps were done at 37°C except for color development, which was done at 27°C. Positive optical density (OD) values were those exceeding 2 standard deviations of pooled negative controls. The conversion of OD units to micrograms of turtle Ig was done using a standard curve composed of 50 µl/well of purified turtle pooled 5.7 and 7S IgY (6 mg/ml) diluted in a carbonate buffer in serial, twofold dilutions ranging from 1:50 to 1:3,200.

Western blot was used to test the specificity of goat anti-turtle Ig. Briefly, pooled turtle 17S IgM, 7S, and 5.7S IgY was resolved in precast SDS-PAGE gel (NuPAGE® Novex® 4-12% Bis-Tris, Invitrogen, Carlsbad, California 92008, USA) under nonreducing conditions and electrotransferred to nitrocellulose membrane. Membranes were blocked 1 hr with 0.1% PBS-Tween 20 and 5% skim milk at 27°C with gentle agitation, washed three times with PBS-Tween 0.1% pH 7.2, incubated with goat anti-turtle Ig (1:500 dilution) overnight at 4°C, washed three times with PBS-Tween, incubated 1 hr with horseradish peroxidase (HRP)-donkey anti-goat IgG (Jackson ImmunoResearch Laboratories Inc.) at 37°C, washed three times with PBS-Tween 0.1%, and membranes visualized with DAB substrate.

Cell-mediated immunity was assessed as described by Binns³ and Kim.¹⁸ Briefly, 50 µg of phytohemagglutinin (PHA, Sigma Chemicals Company) in 100 µl of PBS were inoculated into a skin fold to the left side of the cloaca using a 13-mm needle while 100 µl of PBS were inoculated in the contralateral side as a control. Skin thickness was measured prior to inoculation using a digital micrometer (Mitutoyo de Mexico, Naucalpan 53370, Estado de Mexico, Mexico) and the inoculation site marked with indelible ink. The kinetic response to PHA (skin induration) was measured at 24, 48, and 72 hr. At 48 hr postinoculation, the injection sites were locally anesthetized with 0.1 ml of 2% Xylocaine, skin biopsies taken with a 6-mm biopsy punch, fixed in 10% neutral buffered formalin, and processed for

Table 1. Morphometrics, hematology, immunoglobulins, and delayed-type hypersensitivity results for turtles with (UD positive) and without (UD negative) ulcerative dermatitis (UD). Values with asterisk are significantly different.

	UD negative		UD positive	
	Mean \pm SD	Range	Mean \pm SD	Range
Weight (kg)	4.41* \pm 0.67	3.39–5.32	3.52 \pm 0.4	2.8–3.96
Curved carapace length	33.67* \pm 1.76	30.6–35.8	30.76 \pm 1.15	29.3–32.4
Curved carapace width	27.69* \pm 1.53	25–29.7	26.1 \pm 1.18	24.9–28
White blood cells	15,221 \pm 5,229	8,750–21,400	17,375 \pm 3,737	11,000–21,000
Heterophil–lymphocyte	0.731* \pm 0.095	0.593–0.846	1.659 \pm 0.43	0.959–2.129
Total Ig (μ g/ml)	22.74 \pm 14.82	11.18–53	14.7 \pm 4.01	11–24
Delayed-type hypersensitivity (mm)	1.713* \pm 0.67	0.43–2.61	0.53 \pm 0.12	0.21–1.12

histopathology as previously described. Giemsa stain was used to distinguish heterophils from mononuclear cells.²⁹

Morphometrics and immune status data were compared using a nonparametric *t*-test because data did not fit assumptions of normality.²⁶

RESULTS

Clinically healthy turtles had significantly greater CCL, straight carapace length, and weight ($P < 0.01$). No significant difference was seen for total white cell counts; however, the H:L ratio was significantly higher ($P < 0.0001$) in UD-positive turtles (Table 1). Biopsies of UD-positive turtles revealed severe, full-thickness, diffuse coagulation necrosis and spongiosis of the epidermis with occasional erosion accompanied by abundant heterophilic and sparse histiocytic infiltrates in the epidermis and dermis associated with Gram-negative bacteria (Fig. 2a–c). Gel filtration separated turtle 17S IgM from pooled 7S and 5.7S IgY (Fig. 3) and goat IgM from IgG as confirmed by SDS-PAGE. On western blot, goat anti-turtle Ig reacted with two proteins of 178 kDa and 127 kDa, corresponding to IgY 7S and IgY 5.7S, respectively (Fig. 4). No significant difference was seen in IgY levels between clinically healthy and UD-positive turtles (Table 1). Clinically healthy turtles had significantly ($P < 0.0008$) more-pronounced cutaneous reactivity to PHA versus those with UD (Table 1). Clinically healthy turtles injected with PHA had an optimum time to measure the maximum delayed hypersensitivity response at 48 hr, indicating kinetics similar to that reported for other species. Histologically, prominent perivascular mixed lymphocytic and histiocytic infiltrates were seen (Fig. 2e–f) which were largely absent in UD-affected animals (Fig. 2d).

DISCUSSION

Ulcerative dermatitis is a common problem in captive-raised sea turtles in the Caribbean and Australia.^{13,14} In this particular case, the prevalence of the disease in captive turtles at Xcaret Park (96.5%) approached that seen in captive-raised turtles in Australia (100%).¹³ Glazebrook and Campbell¹³ explained the high prevalence of skin disease as a result of captivity-induced immunosuppression along with biting, which likely opened cutaneous portals of infections with the subsequent colonization of skin lesions by bacteria, ultimately leading to dermatitis. In this study, the sites where the turtles bit each other (tip of the tail and flippers) did not completely coincide with the distribution pattern of the erosive skin lesions, suggesting that other causes in addition to trauma might contribute to UD in turtles. Histology revealed an invasion of Gram-negative bacteria; however, in spite of a chronic course that lasted several months, the inflammatory response was comprised mainly of heterophils causing extensive tissue damage due to degranulation but without apparently limiting the bacterial invasion. This contrasts with the description of the kinetics of experimental inflammation in snakes²⁹ where mononuclear cells predominate in chronic inflammatory processes. In this study, the histology suggested a chronic, active process whereby continuous infection by bacteria created a heterophilic response mixed with a mild histiocytic infiltrate.

Low mononuclear cells were confirmed by the elevated H:L ratio in UD-affected turtles compared to clinically healthy turtles. The H:L ratio is a good indicator of stress in birds^{15,27} and reptiles.^{1,30} An elevated H:L ratio in the presence of a normal white cell count in UD-affected turtles suggested a chronic depletion of peripheral blood

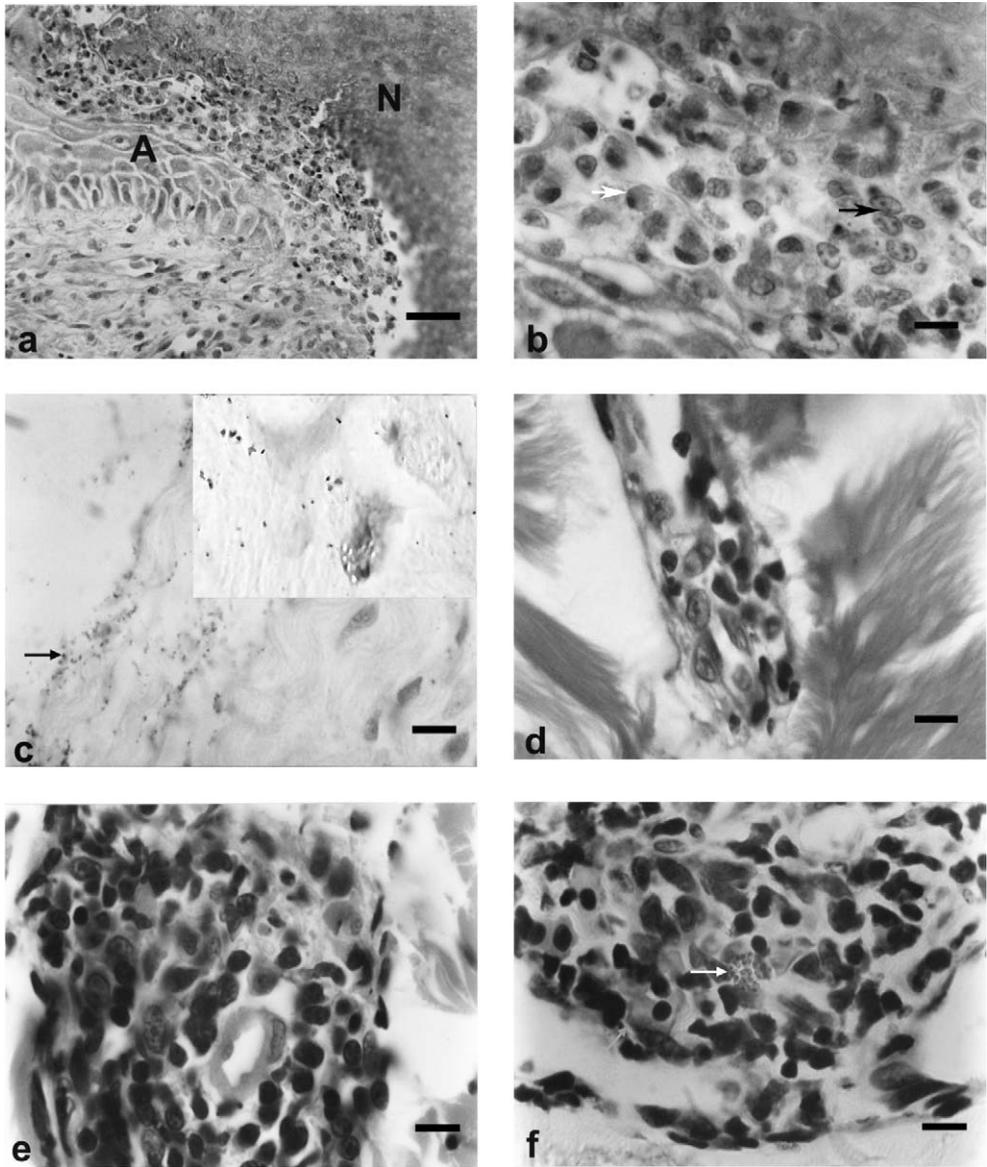


Figure 2. a. Histology of skin lesions. Note abundant granulocytic infiltrate separating necrotic debris (N) from the epidermis, manifest marked spongiosis, ulceration of epidermis (right), and dermal granulocytic and mononuclear infiltrates (A). Bar = 30 μ m. b. Close-up of dermis from A. Note abundant heterophils (white arrow) and smaller numbers of histiocytic infiltrates (black arrow). Bar = 6 μ m. c. Gram stain of skin lesion. Note microcolonies of gram-negative rods (arrow and inset) in dermis ($\times 100$). Bar = 6 μ m. d. Scant mixed lymphocytic-histiocytic perivascular infiltrate in skin biopsy of ulcerative dermatitis (UD)-positive turtle stimulated locally with PHA. Bar = 6 μ m. e. Marked mixed lymphocytic-histiocytic perivascular infiltrate of mononuclear cells in the skin biopsy of UD-negative turtles stimulated with phytohemagglutinin (PHA). Bar = 6 μ m. f. Giemsa staining of a skin biopsy of UD-negative turtle stimulated with PHA ($\times 100$). Note a granulocyte (white arrow, orange granules) among the abundant presence of mononuclear cells and some erythrocytes (red arrow).

lymphocytes. Elevated H:L ratios are also apparent in green turtles affected with other chronic diseases such as the neoplastic disease, fibropapillomatosis,^{1,7,31,33} seeming to validate this metric as an indicator of chronic stress in this species.

Delayed hypersensitivity tests using mixtures of antigens or mitogens have been used to assess cell-mediated immunity in mammals and birds.^{9,18,25} This work presents the first use of this test to compare immunologic status between

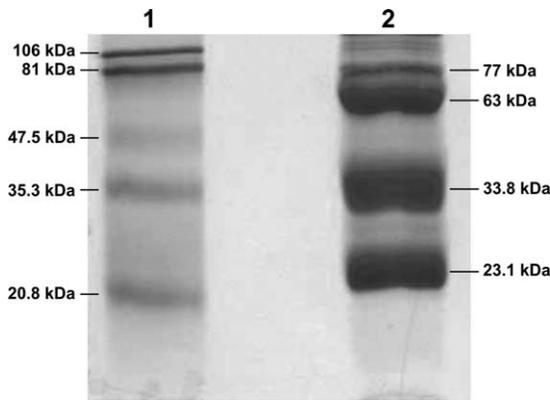


Figure 3. Denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showing (1) molecular weight marker, and (2) the bands corresponding to the light chains (23.1 kDa), the heavy chains of 17S IgM (77 kDa), 7S IgY (63 kDa), and 5.7S IgY (33.8 kDa).

clinically healthy and UD-positive green turtles. In this study, the optimum time to measure the maximum delayed hypersensitivity response to intradermal PHA was 48 hr (Muñoz, unpubl. data), indicating kinetics similar to that reported for other species.^{18,24} All but one clinically healthy turtle showed a vigorous response to PHA whereas all UD-affected turtles responded weakly or not at all. PHA specifically stimulates T lymphocytes,²⁵ and the histology response seen in this study was similar to that of mammals.^{3,22,28} The PHA test may prove valuable to help assess T-cell response in sea turtles but probably needs further validation using other metrics of immunity as a complement.²⁰

There was no significant difference in pooled 7S and 5.7S levels between UD-positive and clinically healthy turtles; however, the assay used here measured total IgY levels and may have been insufficiently sensitive to detect more-subtle changes such as changes in particular classes of immunoglobulins. On the other hand, results suggest that cell-mediated immunity in captive turtles with UD might be depressed versus clinically healthy animals. This may explain why clinically healthy animals are larger, as the animals were raised under the same conditions and from the same clutch; chronic inflammation in a UD-affected animal probably siphons away resources that would normally be slated for growth. Determining whether smaller body size is caused by UD, or whether it is one of the factors characterizing individuals that may be predisposed to develop the disease, needs further

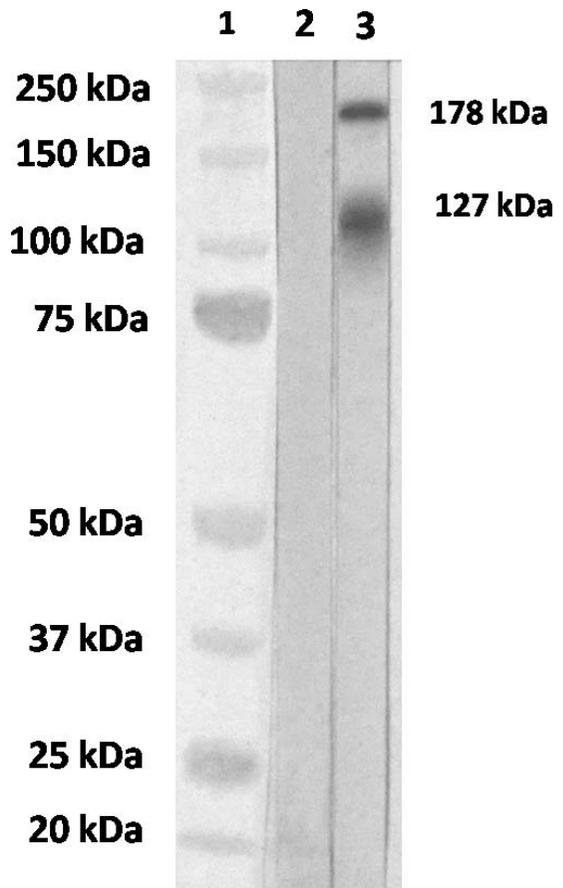


Figure 4. Western blot of sea turtle immunoglobulins. The immunoglobulins were separated in non-denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), electrotransferred to nitrocellulose membrane, and incubated with goat anti-turtle Ig or goat preimmune serum as a negative control. The goat anti-turtle Ig detected two proteins of 178 kDa and 127 kDa, corresponding to intact IgY 7S and IgY 5.7S, respectively. Molecular weight markers (lane 1), negative control (lane 2), and anti-turtle Ig (lane 3).

investigations. Similarly, future studies to understand whether there is a causal relationship between UD and immunosuppression in sea turtles may help enhance captive breeding and release efforts for this endangered species in the Caribbean.

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