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Investigation of Unusual Mortality Events in Florida Marine Turtles

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PROJECT TITLE: INVESTIGATION OF UNUSUAL MORTALITY EVENTS IN FLORIDA MARINE TURTLES

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BACKGROUND

From 5 October 2000 to 24 March 2001, 49 debilitated loggerheads associated with an epizootic were found in southern Florida from Manatee County on the west coast through Brevard County on the east coast (Fig. 1). Most (34) were found in the Florida Keys (Monroe County). By month, these loggerheads were found most often in November 2000 and January 2001 (Table 1). There was no apparent spatial trend in the discovery of the debilitated turtles through time. From the onset of the epizootic through its conclusion, effected turtles were found throughout south Florida. The size class distribution of loggerheads associated with the epizootic was not different than that for loggerhead strandings found in south Florida during the previous ten years (Fig.2). However, the number of dead or debilitated loggerheads found during the epizootic (N=189) was almost six times greater than the average number found in south Florida from October through March during the previous ten years ($\bar{x}$=33.4) (Fig. 3).

The number of dead and debilitated loggerheads found during the epizootic should be considered a minimum number affected as not all turtles would have been discovered, reported, and documented. Additionally, because carcasses (and presumably debilitated turtles) may be transported 100's of km by currents (Epperly et al., 1996), their locations at the time of discovery may have no longer represented the distribution of the turtles at the time of death or debilitation. However, given the observation that many of the turtles appeared to be acutely debilitated, we believe the distribution of strandings roughly identified the area of the epizootic.

There were 189 loggerhead strandings documented in south Florida (Palm Beach County - Manatee County) during the epizootic when the previous ten-year average for the same time and place had been a little over 33 (33.4). After determining that no other unusual mortality factors appeared to have been operating during the epizootic, we estimate that 156 (189 - 33) of the strandings were likely to be attributed to the epizootic. Furthermore, strandings may represent as little as 7% of the at-sea mortality of sea turtles (Epperly et al., 1996). Considering this, we estimate that the overall mortality associated with the epizootic was between 156 and 2229 loggerheads.

INITIAL PRESENTATION AND TREATMENT

Thirty-seven of the loggerheads associated with the epizootic were submitted to the Sea Turtle Hospital, Marathon, Florida. A thorough physical examination was performed on each turtle and a standard database was collected. Blood was collected from each animal for performing complete blood counts and for determining levels of plasma biochemicals. Radiographs were also taken of each turtle. To evaluate the respiratory system, each turtle was obtunded. The index case was treated for salt water drowning with furosemide, amikacin and penicillin. Because the turtles were too weak to hold their heads out of water, all turtles were housed indoors in individual pools without water. The temperature was maintained at 80F/26C. Turtles were force-fed and received fluids, antibiotics, antihistamines, steroids,

immunostimulants, and anthelmintics. The last six turtles arriving at the Sea Turtle Hospital were treated for organophosphate toxicity. Both 2-PAM and atropine were started using mammalian protocols.

The hallmark presentation was that of varying degrees of paresis. The more affected animals were unable to move voluntarily, had no corneal, palpebral or menace response, and no swallowing or gag reflex. Several of the turtles had corneal lesions (ulcers and plaques). Deep pain was absent in the periphery, but was present proximally. Major clinical pathological findings in most turtles included leukocytosis (primarily a heterophilia), hyperglycemia and hypermagnesemia. Most turtles had either unilateral or bilateral interstitial pneumonia, rales and mucoid respiratory discharge. Bronchoscopy on several patients demonstrated hyperemia, mucoid fluid accumulation and plaques in the trachea and bronchi. In severe cases brownish, caseous plugs were seen occluding both the upper and the lower airways.

NEUROLOGICAL EXAMINATION

Three loggerheads associated with the epizootic were given a neurological examination. The examination included an evaluation of the integrity of the cranial nerves and peripheral reflexes. Additionally, electromyography was also performed.

The two most affected turtles had depressed mentation with no menace response in either eye. There was little eye movement in general, a lack of palpebral reflexes, and reduced jaw tone. The animals could not chew or swallow. There was loss of neck retraction and neck muscle tone, slight to no response to superficial nociceptive stimuli, and generalized lack of limb movements, muscle tone, and flexor reflexes. Some tail and cloacal reflexes were present. There was no response to sensory stimuli along the dorsal or ventral shell, and no righting response could be elicited when the turtles were placed on their backs. A generalized lower motor neuron disorder was suspected including a polyneuropathy (generalized involvement of cranial and spinal nerves), neuromuscular junction blockade, or polymyopathy (thought to be less likely due to severity of paralysis).

Prolonged insertional activity was present without positive sharp waves or fibrillation potentials. There was little evidence of the motor unit action potentials usually found on electromyograms in unanesthetized animals. No evoked muscle response to stimulation of the sciatic nerve was found on either side. The limb did not move nor did the unanesthetized turtle act as if it experienced any discomfort. A normal loggerhead turtle was also evaluated. The normal turtle had an evoked muscle response and a motor nerve conduction velocity of 48.8 m/sec. This animal was anesthetized for the nerve stimulation due to the discomfort of the procedure. A polyneuropathy associated with axonal degeneration was thought unlikely due to the lack of fibrillation potentials and positive sharp waves. The lack of evoked muscle responses to nerve stimulation suggested a neuromuscular junction blockade such as found in tick paralysis,

coral snake envenomation, botulism or drugs such as curare. A last possibility would be a demyelinating polyneuropathy that has spared axons but resulted in a conduction block.

The third affected turtle was recovering and although he was very weak, he had bilateral palpebral reflexes, some neck retraction and some limb movements. His flexor reflexes were also still very depressed. When laid on his back he showed little righting reaction. His EMG was the same as the others.

NECROPSIES

Detailed necropsies were conducted on 21 of the loggerheads associated with the epizootic. During necropsy, organs were generally removed in bloc, and the skull was removed to extract the brain. The head was also sectioned longitudinally on the mid-line for examination of the nasal cavity. The spinal cord was removed using a band saw and a rotating necropsy saw. After it became apparent that adult trematodes were present in the brain and spinal cord, these organs were examined under a dissecting microscope for evidence of endoparasitism. Tissue sections (approximately 0.5 cm wide) from all major organ systems were fixed in 10% neutral buffered formalin for 24 to 48 hr, embedded in paraffin, sectioned at 5 to 6 μm, and stained with hematoxylin and eosin and as necessary, with a variety of stains for bacteria, fungi, myelin and collagen (Luna, 1968).

A section of liver and a swab of trachea from one turtle were collected for aerobic bacterial isolation. Specimens were inoculated onto a Columbia agar with 5% sheep blood, Columbia CNA agar with 5% sheep blood, and MacConkey agar (all from Remel, Lenexa, Kansas, USA) and incubated for 48 hr at 37 C with 5% CO2. Isolates were identified utilizing standard biochemical tests and the API 20E and NFT systems (BioMerieux Vitek, Inc., Hazelwood, Missouri).

Turtles ranged in size from 5.1 to 157 kg with a straight carapace length of 231 to 984 mm. Fifteen turtles were females and three were males; the gender of three turtles was not recorded or determined.

Initially, turtles received for necropsy were in fair to good physical condition, with moderate to abundant fat stores, and a prominent paired thymus. Towards the latter part of the epizootic, especially after turtles had been treated for an extended period of time, the turtles were emaciated, there was loss of fat stores and muscle mass, the plastron was sunken and there was a greater epibiotic growth (barnacles)with moderate growth of green to brown algae. The eyes were markedly sunken within their orbits. Areas of skin within the axillary and inguinal regions were sometimes mottled pink to red. The carapace contained locally extensive areas of scaling and crusting that could be peeled off easily. The thymus was ≈25% of the expected size. The corneas of some turtles were cloudy, associated with a fibrin plaque on the epithelial surface and moderate bacterial growth along the mucosal surface.

The nasal turbinates varied from pink/tan to being lined by moderate amounts of yellow crusty material. The trachea varied from having a pink/tan smooth mucosal surface to a red granular surface with a small amount of thin red-tinged fluid and scant friable tan material. In three turtles, the trachea was lined with a yellow/green to grey/tan friable rubbery membrane, that in multiple areas almost entirely occluded the tracheal lumen. The lungs varied from pink and spongy to mottled red to red/brown and meaty. There were moderate amounts of white foam within the major bronchi. Grey friable granular to rubbery exudate was scattered within airways.

The coelomic cavities usually contained between 300 and 500 ml of moderately viscous red-tinged fluid. Excess pericardial fluid (25-50 ml) was present in a few turtles.

The stomach and intestines contained small to moderate amounts of clear yellow-tinged mucoid fluid admixed with flocculent tan mucoid material and dark green gelatinous material. In a few turtles, numerous 0.2 x 0.4cm red flat metazoan parasites were attached to the gastric mucosa. Helminths were also occasionally found in the intestinal tract. Gross lesions in the digestive tract included focal 1-3 cm diameter tan to dark brown raised fibrinous plaques on the mucosa surface of the oropharynx, esophagus, stomach and intestine, esophageal dilatation, and foci of intestinal mucosal hemorrhage.

Gross lesions of verminous encephalomyelitis included the presence of a few to many, slightly raised, tan 1-2mm diameter nodules and multiple pinpoint to 4mm diameter black foci on the meningeal surfaces. Under a dissecting microscope, numerous (>50), ≈20 micrometer diameter dark brown spherical helminth ova were visible through the meninges of the brain and spinal cord. One or more 2cm threadlike transparent helminths with black internal viscera and dark serpentine gastrointestinal tracts were present in meningeal blood vessels of the brain.

Seventeen of the twenty-one turtles had lesions consistent with neurologic to generalized spirorchidiasis. Multiple 8 µm, oval, globular to hollow trematode ova with a hyaline tan to yellow/brown wall were present within or adjacent to capillaries in white and grey matter of the brain. Multifocally, the ova were encompassed by granulomas composed of multinucleated giant cells. Small granulomas were also scattered throughout the meninges. Elsewhere, there were perivascular aggregates of macrophages with cytoplasmic refractile pale tan granules (ova fragments). In some areas of the white matter, there was marked gliosis with frequent satellitosis. Increased numbers of mononuclear cells were present in the Virchow-Robins' spaces of multiple capillaries. In areas of mixed leukocytic inflammation, glial nodules sometimes centered on necrotic neuronal cell bodies that contained indistinct nuclei and hypereosinophilic cytoplasm. Multiple capillaries within the meninges and neuropil of six turtles contained cross sections through metazoan parasites with no cuticle, multiple vitelline glands and multiple aggregates of dark brown granular pigment, and paired ceca (trematodes). There were mild to moderate multifocal infiltrates of lymphocytes in the meninges. There was multifocal meningeal fibrosis and mineralization. Some fibrotic foci were arranged in whorls. The meningeal space was multifocally widened and loosely arranged (edema). Special staining for myelin (Luxol Fast Blue) did not reveal significant demyelination of axons.

In the spinal cord, multiple small granulomas centered around parasite ova similar to those present within the brain were scattered throughout the white and gray matter, and the meninges. There was occasional neuronal necrosis. In longitudinal section, axons in locally extensive areas of the spinal cord were dilated and empty, or some contained digestion chambers with myelomacrophages. Multiple blood vessels within the meninges of three turtles, including one in which trematodes were not found in the brain contained adult trematodes similar to those present in the brain. Locally extensive to coalescing lymphoplasmacytic infiltrates and macrophages with intracytoplasmic refractile yellow/brown granules (ova fragments) were present within the meninges, especially in areas adjacent to vascular channels containing spirorchid adults. Clear spaces (edema) multifocally expanded the meninges.

The pituitary gland of two turtles contained trematode ova and adult trematodes in capillaries. Adult trematodes were not found in the brain of these turtles.

Sections of sciatic nerve, brachial plexus, optic chiasm, and cranial nerves were examined in 14 of the 17 turtles with neurospirorchidiasis. Multiple internodal myelin sheaths appeared to be swollen with clear spaces. Increased numbers of fibroblasts and collagen fibers (trichrome stain) were present within the endoneurium adjacent to multiple axons, and the epineurium was expanded by clear spaces (edema). Occasional mild to focally moderate infiltrates of lymphocytes were present in the epineurium, sometimes centering on capillaries. Occasional trematode ova, sometimes within a granuloma, were present in capillaries. Macrophages comprising these granulomas often contained intracytoplasmic yellow granular material. Endothelial cells lining some capillaries were plump with hypochromatic nuclei. Special staining for myelin (Luxol Fast Blue) did not reveal significant demyelination of axons.

There was multifocal skeletal muscle degeneration, edema and atrophy. Multiple skeletal muscle fibers were hypereosinophilic and there was loss of resolution of the striations. Other fibers were swollen and fragmented. There was mild to moderate variation in diameter of myofibers, several of which had internalized nuclei. Clear spaces within the endomysium (edema) separated individual skeletal muscle fibers. Perimyseal edema was present also. In a few turtles, there were 10x7x3cm to 7x5x5cm dark red foci with a dull granular center or tan caseous material within the skeletal muscle of the pectoral and cervical regions. These areas were comprised of degenerate, necrotic or atrophied muscle fibers encompassed by a mixed infiltrate of heterophils, macrophages and lymphocytes.

Changes in the nasal cavity included focally extensive superficial erosion and individual necrosis of the keratinized squamous epithelium, and multifocal moderate hyperplasia of pseudostratified columnar mucosal epithelium. A small number of spirorchid eggs or breakdown products (yellow/tan pigment) of ova were scattered throughout the connective tissue stroma, and the stroma contained multifocal mild to moderate perivascular lymphocytic infiltrates. In three turtles with fibrinonecrotic tracheitis, the mucosal lining was extensively ulcerated, and the propria-submucosa was covered by a moderate layer of brightly eosinophilic homogeneous to fibrillar serum and fibrin with admixed necrotic cells, heterophils, lymphocytes, macrophages,

and mixed colonies of bacteria with scattered fungal yeasts. A mixed population of bacteria, with no predominant organisms were isolated from the tracheal lesion. In areas of intact epithelium, there was multifocal anisokaryosis, squamous metaplasia and hyperplasia. Several epithelial cell nuclei had marginated chromatin and vague glassy intranuclear inclusions, but virus was not detected by electron microscopy. Small aggregates of lymphocytes inflitrated the congested propria-submucosa. Pulmonary inflammation was mild to severe. Within air spaces there were various amounts of fibrin, erythrocytes, sloughed epithelial cells, macrophages, syncytial cells, heterophils and erythrocytes. There was multifocal colonization of Gram-positive bacterial cocci and Gram negative bacteria within affected air spaces. Epithelial lining was multifocally necrotic and ulcerated or hypertrophied and hyperplastic, and occasional enlarged cells with multiple, closely arranged nuclei (syncytial cells) were present. The interstitium was thickened, and there were scattered trematode ova. Multifocally, multinucleated giant cells or organized granulomas within the interstitium surrounded ova or fragments of refractile brown material. The collagenous stroma was expanded by clear spaces (edema) and various numbers of scattered to aggregated heterophils, lymphocytes and macrophages. In chronic lesions there was moderate to marked smooth muscle hyperplasia and mild interstitial fibrosis.

There was segmental mixed leukocytic to granulomatous inflammation in the gastrointestinal tract. Fibrinonecrotic esophagitis and enteritis were present in two turtles, characterized by locally extensive necrosis and ulceration of the mucosal epithelium. Necrotic regions were covered by a thick layer of abundant fibrin and deeply eosinophilic necrotic cellular debris that extended into the propria submucosa. A mixed population of bacteria was present in the surface exudate. Focally extensive gastric and intestinal mucosal ulceration were colonized by mixed bacteria and subtended by granulation tissue with multiple large foreign body granulomas. Multifocally, nodular aggregates of multinucleated giant cells within the gastrointestinal lamina propria surrounded parasite ova. There was multifocal erosion of the mucosa of the small and large intestine, and the lamina propria contained multifocal to coalescing infiltrates of moderate to large numbers of lymphocytes. The submucosa was segmentally edematous and multiple small to large granulomas surrounded trematode ova or fragments of ova. There was vacuolation of the myenteric plexuses of three turtles. Scattered granulomas with encysted cestodes were also found in the tunica muscularis of the stomach and intestine.

Other lesions included lymphocytic epicarditis and myocarditis, with multifocal fibrosis and parasitic granulomas surrounding spirorchid eggs. There was multifocal aortic lymphocytic vasculitis. In the kidney, there was multifocal chronic fibrosing, lymphoplasmacytic, tubular and interstitial nephritis, glomerular sclerosis and granulomas with intralesional spirorchid eggs. There was multifocal subacute to granulomatous splenitis, with intralesional degenerate parasite ova. In livers of chronically ill turtles there were reactive melanocytic centers, with hemosiderosis. Bacteria was not isolated from the liver submitted from one turtle. Serous surfaces of coelomic viscera were multifocally lined by plump markedly hypertrophied mesothelial cells, and there were multifocal subserosal mixed leukocytic infiltrates. In thyroids of six turtles, there was colloidal cyst formation with intrafollicular birefringent crystalline

material, scattered granulomas with intralesional degenerate parasite ova, lymphocytic infiltrates, or fibrosis and follicular atrophy. In four turtles, the cause of illness or death was due to gastroenteritis (2), pneumonia and laryngotracheitis (1), or septicemia secondary to severe bacterial dermatitis (1).

ELECTRON MICROSCOPY

Nerve and muscle specimens from 11 loggerheads associated with the epizootic collected either under anesthesia or post-mortem were sent to the Comparative Neuromuscular Laboratory at the University of California, San Diego. Fresh frozen muscle specimens were shipped by an overnight service on dry ice. Fixed muscle specimens were collected into 10% formalin, and nerve specimens were either placed in 10% formalin or in 2.5% glutaraldehyde in phosphate buffer. In addition to the material from the affected turtles, muscle and nerve specimens were submitted from five control animals that had died of unrelated causes.

Frozen muscle biopsy specimens (8-mm-thick sections) were evaluated using a standard panel of histochemical stains and enzyme reactions. Glutaraldehyde-fixed nerve biopsy specimens were postfixed in osmium tetroxide, and dehydrated in serial alcohol solutions and propylene oxide prior to embedding in araldite resin. Sections (1 mm) were stained with toluidine blue for light microscopy and ultrathin sections stained with uranyl acetate and lead citrate for electron microscopy.

Muscles from the right and left forelimbs and hindlimbs were evaluated. Several trematode ova were present within muscle fibers, in some cases surrounded by granulomatous cellular infiltration. Mild interstitial edema was also observed in several muscles. There was no evidence of overt lymphocytic inflammation or denervation. Thick sections from the sciatic and ulnar nerves showed scattered nerve fibers with varying degrees of demyelination, including nerve fibers with thinner than expected myelin sheaths and fully demyelinated fibers. Multifocal areas of endoneurial edema were also present. Similar but more marked abnormalities were identified within a single specimen from a nerve root. Ultrastructural examination confirmed the presence of demyelinated nerve fibers and edema. Axonal degeneration was not observed. Regeneration was not obvious. These findings, including the absence of axonal degeneration within the nerve biopsy specimens and neurogenic atrophy within the muscle biopsy specimens, are consistent with a demyelinating neuropathy.

PARASITOLOGY

The brains of 20 and the spinal cords of 15 loggerheads associated with the epizootic were examined for spirorchiid trematode eggs and adults. The cranium was opened with a striker saw and the brain was removed. The spinal cord was exposed by cutting through the bone surrounding then lifting the cord out of the body. The entire brain was examined grossly as was

the spinal cord. The brain was cut in half by making a sagittal incision through the brain. One portion was fixed in 10% buffered formalin and the remainder was examined under a stereoscope from 8 to 40 viewing magnification and using a double headed fibre optic. A portion of the spinal cord was examined in a similar manner. Egg presence and relative densities were noted. Samples of the eggs were placed on a glass slide wet mounts for viewing at higher magnification, and viewed. Adults in tiny vessels were best distinguished by seeing two very thin, dark streaks lying parallel to each other for a relatively short portion of a blood vessel. These were the digestive ceca of the adult fluke. Efforts were made to remove these from the blood vessels with fine insect pins made into probes. We also used jewelers forceps and iridectomy scalpels. Nothing was satisfactory as only segments of the worms were recovered.

We detected adult *Neospirorchis* sp. in 4 of 20 brains and 1 of 16 spinal cords. *Neospirorchis* eggs were seen in 19 of 20 brains and in 15 of 19 spinal cords. The relative numbers of eggs ranged from seeing scattered single eggs in vessels to masses of eggs probably numbering in the thousands. At times they distended segments of the vessel to a diameter of about 2 - 3mm. The eggs were consistent with those of *Neospirorchis* sp. *Neospirorchis* adults were also found in other sites in these loggerheads. These were found in the pancreas, major vessels arising from the heart and the heart. While adults of *Hapalotrema* and *Carettacola* sp. were isolated from some of these turtles, they was never found associated with the adults and their eggs were not found in the central nervous tissues either.

SEROLOGY

For antigen preparation, plaque purified LETV clones 292 and 221 were propagated in terrapene heart cell monolayer cultures (TH-1; ATCC No. CCL 50) and previously described D-1 green turtle cells (Herbst et al., 1998). Cell lines were grown in DMEM/F12 supplemented with 5% FBS and antibiotics in vented flasks in a 5% CO_2 humidified incubator at 28 C. Infected monolayers were scraped, freeze/thawed and then sonicated. After low speed centrifugation for clarification, virus was pellet by ultracentrifugation at 40,000 g for 1 hr at 4 C. Virus pellets were washed in phosphate buffered saline (PBS), repelleted, resuspended in PBS, and then sonicated for 60 sec. Virus titers were determined by plaque assay and were approximately 1.0 X 10^6 pfu/ml. Uninfected cells were scraped into medium, pelleted, resuspended in PBS, and sonicated to serve as uninfected cell lysate controls. Protein concentrations of virus and uninfected cell controls antigens were measured by Bradford assay protocol and were approximately 1.0 mg protein/ml and 3.25 mg/ml, respectively.

For ELISA assays, each well of a 96 flat bottom-well microtiter plate (Nunc-Immuno MaxiSorp, Nunc, Kamstrup, Denmark) was filled with 50 l of LETV or uninfected cell lysate control at a concentration of 5 g protein/ml, and then covered with sealing tape and incubated overnight at 4 C. The wells were washed in an automatic plate washer (ELX50, Bio-Tekko Instruments, Inc., Winooski, VT, USA) 4 times with 250 ul phosphate buffered saline 0.5% Tween 20 and 0.02% sodium azide (PBS/T) and then blocked overnight with 5% w/v non-fat dry

milk in PBS/T (PBS/TM) at 4 C. After washing, 50 µl of green turtle plasma samples diluted 1:25 in PBS/TM or LETV negative or positive control turtle plasma diluted 1:100 in PBS plus 2% v/v FBS (PBS/F) was added to the appropriate wells. Each plasma sample was incubated with LETV and uninfected cell lysate control for 1 hour at room temperature on a Nutator (Becton Dickinson, Sparks, MD, USA). The microplate was washed and 50 l of biotinylated monoclonal HL858 (anti-green turtle 7s IgG) (Herbst and Klein, 1995) diluted to a concentration of 1 g/ ml was incubated 1 hour at room temperature on a Nutator. After washing, 50 µl of alkaline phosphatase-conjugated streptavidin (Zymed Laboratories Inc., San Francisco, CA, USA) diluted 1:5000 in PBS/A was added to each well. The microplate was incubated and washed as described above, and then 100 µl of p-nitrophenyl phosphate disodium (1 mg/ml in 0.01 M sodium bicarbonate pH 9.6, 2 mM $MgCl_2$) was added to each well. The microplate was incubated in the dark without agitation at room temperature. The A_{405} of each well was measured after a 2 hour incubation in an ELISA plate reader (Spectra II, SLT-Labinstruments, Salzburg, Austria with DeltaSoft version 3.3 software, Biometallics, Princeton, NJ, USA). Optical density values were adjusted as follows. For each plasma sample, the optical density of the well coated with uninfected cell lysate was subtracted from the optical density of the well coated with virus to account for non-specific absorbance. Optical densities equal to or less than zero were adjusted to 0.001 for statistical analysis. An arbitrary cutoff value was based on optical density values obtained from 42 healthy captive-reared green turtles previously evaluated). The cutoff value was calculated as the highest optical density value obtained from healthy captive-reared green turtles plus three times the standard deviation (cutoff value=0.310).

Detection of herpesvirus by consensus primer PCR. Herpesvirus was detected by using sequential nested consensus-primer PCR (VanDevanter et al., 1996) to amplify an internal region of the herpesvirus DNA polymerase gene. The PCR was performed by using a Perkin Elmer Gene Amp 2400 thermal cycler. The first round of amplification included 100 ng sample template nucleic acid or 10 ng positive control template varicella-zoster virus DNA (VZV; Varivax, Merck). In some experiments, DNA from another green turtle herpesvirus, lung-eye-trachea disease-associated virus, (LETV) (Coberley et al., 2001) was used as an additional control template. Nucleic acid-free water was the negative control. A 2 ul aliquot of that amplification mixture was subsequently used as template for the second round of amplification. The products were electrophoresed through a 1.5% agarose gel in 1X TBE buffer, stained with ethidium bromide, and photographed under shortwave UV illumination. Samples containing herpesvirus yielded a 224 bp amplified fragment, which matched the size of the product obtained by using the positive control template. Serial dilution of VZV positive control template showed that under those conditions the assay had a limit of detection of about 1.1 herpesvirus genomic equivalents per 100 ng of sample nucleic acid.

No antibody to LET herpesvirus was detected in plasma of any of the samples evaluated and no herpevirus gene sequences were amplified from tissues evaluated.

HEAVY METALS, ORGANOPHOSPHATES, ORGANOCHLORIDES, AND CHOLINESTERASE

Fifteen metals (aluminum, arsenic, cadmium, chromium, copper, iron, lead, manganese, mercury, molybdenum, nickel, selenium, silver, thallium, and zinc) were assayed in the matched liver and kidney tissues of ten loggerheads that were associated with the epizootic.

Tissue samples were homogenized, portioned and pre-digested in nitric acid and hydrogen peroxide for all metals except total mercury which was digested in a warmed solution of concentrated sulfuric and nitric acids with potassium permanganate followed by an addition of potassium persulfate to scavenge excess permanganate. Analysis for aluminum, cadmium, chromium, copper, iron, lead, manganese, molybdenum, nickel, silver, thallium, and zinc was by inductively coupled plasma-atomic emission spectroscopy; for arsenic and selenium by electrothermal atomic absorption spectrometry with Zeeman background correction; and for mercury by cold-vapor atomic absorption. Due to the limitations of having tissues from only one animal outside of the incident under investigation, statistics for data from affected animals were limited to means, standard deviations and ranges. All results are reported as mg/kg dry weight. Dry weight values were obtained by freeze-drying tissue samples.

Of the fifteen metals assayed, eight were present in both the liver and kidney at detectable levels for all turtles (respective means of 44.83 and 69.19 mg/kg As, 35.74 and 139.46 mg/kg Cd, 54.88 and 6.64 mg/kg Cu, 8464.03 and 352.42 mg/kg Fe, 19.13 and 6.27 mg/kg Mn, 83.41 and 26.54 mg/kg Se, 140.14 and 129.59 mg/kg Zn, and 10.90 and 4.80 mg/kg Hg). Chromium was found in ten liver and three kidney samples (8.78 and 3.49 mg/kg). Silver was not detected in any kidney samples, but was determined in the livers (3.24 mg/kg) of two affected turtles. Thallium, on the other hand was present in seven kidney samples (4.78 mg/kg), but in no livers. Molybdenum was quantified in a single sample of liver (11.99 mg/kg) and kidney (4.05 mg/kg). Lead was determined in ten kidney (4.91 mg/kg), but in only three liver samples (11.38 mg/kg). Of the eleven turtles studied, Al and Ni were detected in fewer than four tissues for liver (20.15 and 4.65 mg/kg, respectively) and kidney (20.78 and 3.09 mg/kg, respectively). Mean concentrations of metals in the tissues of affected loggerheads are given in Table 2, along with the measured metal concentrations from the animal not involved in this particular episode. The tissues gave an average percentage moisture of 72.00 ± 7.14 for liver and 78.78 ± 4.55 for kidney.

While averaged concentrations of all but Al, As, Cd, and Th were higher in kidney than in liver, these trends did not necessarily hold when the tissues of the individual animals were compared. Typically, higher levels of As, Cd, and Pb were quantified in the kidneys than in livers, but in two animals for As and one each for Cd and Pb, this trend was reversed. One other animal had no detectable amounts of Pb in any tested tissue. Some metals gave expected trends. Copper, Fe, and Se were determined in all tissue samples and, in each case, were present in greatest concentration in the liver. Others came close. Chromium, Mn, Zn, and Hg each were generally found at higher levels in the liver than in the kidney, except for a few specific

individuals, in which the trend reversed. And others missed completely. Although Ag, Al, Mo, and Ni were not often detected in this set of samples, all were present in the tissues of two individuals. Aside from these two animals, Al and Ni were each quantified only once in different subjects. Thallium, also was not frequently determined, but when detected appeared only in the kidney. While little can be concluded regarding the actual levels of metals found in any turtle, it is reasonable to suggest that two of the turtles did not fit the normal profile for this sample set.

Samples of adipose and brain were collected to screen for organochlorine and organophosphorus pesticides. Liver and adipose samples collected from 39 turtles involved in the epizootic were screened for 30 organochlorine pesticides by GC-MS. Liver, fat, and brain samples collected from 6 loggerheads were analyzed for the presence of organophosphorus pesticides.

ALGAL TOXINS

Tests were conducted for the following neurotoxins: brevetoxins, ciguatoxin, (FMRI), domoic acid, and palytoxin (NOAA) as well as for protein phosphatase inhibition (FMRI).

Various tissues (kidney, liver, lung, and some urine and stomach contents) from twenty loggerheads were extracted twice in 100% acetone (1:2 w/v). The pooled acetone extracts were evaporated to dryness, redissolved in 80% methanol, and partitioned twice with hexane. The methanol fraction was then evaporated to dryness and redissolved in 100% methanol. Urine samples were centrifuged to clarify and extracted with C18 solid phase extraction columns (1 g, Supelco Discovery).

Competitive ELISA assays to detect brevetoxins (PbTx) were performed according to Naar et al. (in press). Briefly, 96-well microplates were pre-coated with brevetoxin, and any remaining binding sites in the wells were blocked. Samples and controls were then added to the wells and allowed to compete with the plate-bound toxin for anti-brevetoxin antibodies. After one hour, the wells were rinsed out; removing the samples and all antibodies except those attached to the plate-bound toxin. The antibodies remaining in the plate were then visualized using a series of steps: a biotinylated secondary antibody, a streptavidin-horseradish peroxidase (HRP) conjugate, and the HRP substrate o-phenylenediamine. This assay recognizes all congeners and metabolites of brevetoxin that have a PbTx-2-type backbone. The lower limit of quantification was 5 ng/g (5 ng/ml for urine).

Receptor binding assays were performed according to Van Dolah et al. (1994) to detect brevetoxins and/or ciguatoxins. Assays were performed in 96-well polystyrene plates. PbTx-3 competition curves were generated using 35 ?l [3H]PbTx-3 (10 nM), 35 ?l unlabeled PbTx-3 (10-6 - 10-11 M), and 140 ?l synaptosome preparation. For unknowns, 35 ?l of extracts in binding buffer were added in place of the unlabeled PbTx-3. The plates were then incubated for 1 h at 4ºC. After incubation, the mixtures were filtered onto Unifilter-96 GF/B filterplates

(Packard). Liquid scintillation cocktail was added to each well (30 ?l Microscint 20, Packard), and the plates were counted using a TopCount Microplate Scintillation Counter (Packard). This assay detects anything that binds to site 5 on the voltage-dependent sodium channel, and so would detect ciguatoxins as well as brevetoxins. The lower limit of quantification was approximately 30 ng/g (30 ng/ml for urine).

Extracts were also analyzed for okadaic acid (and other protein phosphatase inhibitors) using a colorimetric protein phosphatase inhibition assay modified from Tubaro et al (1996). In 96-well plates, sample extracts and okadaic acid controls were incubated both with and without added protein phosphatase 2A (PP2A, Upstate Biotechnology, Lake Placid, NY) in the presence of p-nitrophenol phosphate (PP2A substrate). After 2 h at room temperature the plates were read at 405 nm.

Portions of stomach contents and urine samples were sent to NOAA /NOS Center for Coastal Environmental Health and Biomolecular Research in Charleston S.C. to be screened for domoic acid and palytoxin. Receptor binding assays were performed according to Van Dolah et al. (1994) to detect domoic acid, and a modified hemolysis assay was used to screen for palytoxin.

Various tissue samples were also sent to North Carolina State University where they were analyzed for brevetoxins using micellar electrokinetic capillary chromatography with laser-induced fluorescence detection (MEKC-LIF; Shea, 1997).

Of the twenty loggerheads analyzed, eight tested positive for brevetoxins by ELISA. In four of these, more than one tissue type was positive. Values ranged from 3.6 to 26.5 ng/g (Table 3). The majority of positive samples were from the liver and stomach contents (Fig.2). These values seem low compared to turtle tissues from the 1996 red tide mortality event which contained brevetoxin levels from 10 to 570 ng/g.

Samples were negative for microalgal toxins by all other methods. The levels of brevetoxin found by ELISA were below the detection limit for the receptor-binding assay. Because ciguatoxin also binds to the same site 5 channel in the receptor-binding assay we can infer that the tests were equally negative for ciguatoxins. No brevetoxins were detected by MEKC-LIF, however that method is specific for PbTx-2, PbTx-3, cysteinyl PbTx-3, and oxidized cysteinyl PbTx-3. The ELISA may be detecting other brevetoxin metabolites.

GUT CONTENTS

The gut contents of twenty loggerheads associated with the epizootic were examined. The guts of the turtles were mostly empty, but almost all (19/20) had been feeding on cnidarians. To further explore the possibility that jellyfish toxicosis played a role in the loggerhead epizootic, John Higgins, a graduate student of Dr. Monty Graham (Dauphin Island Sea Lab) examined the

gut contents of four of the loggerheads associated with the epizootic to see if they contained an exotic, potentially toxic pelagic cnidarian (*Drymonema dalmatinum*) that was found around Florida at the time of the epizootic. The nematocysts from *D. dalmatinum* were found in three of the turtles. Jellyfish toxicosis remains a possible cause of the epizootic.

CONTROLS

Twelve loggerheads were submitted for necropsy during 2002 as potential control cases to compare with cases of loggerheads associated with the epizootic. Complete histopathological evaluations were conducted on all turtles. The gastrointestinal tracts and brains were removed and saved for studies of associated parasites. Portions of ulnar and sciatic nerves, and their nerve roots from most of the loggerheads were collected, fixed in Trump's solution for electron microscopy, and shipped to the University of California at San Diego for ultrastructural evaluation. Based on the results of the necropsies (to rule out the possibility that these turtles had a neurologic disease similar to that seen during the epizootic) five of the cases were selected to use as controls.

In addition to tissues from 39 loggerheads associated with the epizootic, the archived samples now available for study include the liver, kidney, adipose, and brain tissues from twenty additional animals (collected during detailed and gross necropsies in 2002) that were not involved in the epizootic.

Cholinesterase normals in loggerheads (to compare to those values found in epizootic loggerheads) were established from the plasma samples of 77 apparently healthy loggerheads captured in Florida Bay. Cholinesterase levels were determined in duplicate for each sample by immunoassay. In the method, m-nitrophenol acted as the colorimetric indicator (measured at 420 nm) for the presence of acetic acid, a product of the enzyme hydrolysis of acetylcholine. The mean level of activity was 27.79 Rapp Units/mL with a median of 25.48 Rapp Units/mL and standard deviation of 15.72. The values ranged from below the quantitation limit (1 Rapp Unit/mL) for one sample to a maximum of 62.04 Rapp Units/mL.

CURRENT ASSESSMENT

At this time it is impossible to attribute the illness and epidemic to any one specific pathogen or toxin. However, the neurospirorchidiasis has many similarities to neuroshistosomiasis reported in humans. Spirorchidae and Schistosomatidae represent sister groups of intravascular trematodes. Neuroschistosomiasis is well described and a range of neurological defects have been reported in humans. However in the literature, only one case of polyneuropathy could be found in a patient with neuroschistosomiasis. Areflexic flaccid paraplegia with spincter dysfunction and disturbance of sensation has also been described in CNS Shistosoma infection. It is possible that an immune response to either the trematodes or to the

release of previously hidden self-antigens (ie: myelin) may result in either the observed demyelination or to impairment of neuromuscular junction function. It is also possible that these parasites may act as vectors for some other pathogen. Algal toxins are known to cause neurologic disorders in a variety of marine animals but the results of the assays conducted in this study did not reveal any unusual conditions. Additionally, no fish or other species of marine turtles were known to be affected within the range of the ill loggerheads. If it was a toxin it would have to be localized in the prey base of these turtles, or these turtles have to be uniquely sensitive to this toxin. A final consideration is the possibility of jellyfish toxicosis. The loggerheads that were associated with the epizootic were known to have been feeding on jellyfish, particularly *Drymonema dalmatinun*.

LITERATURE CITED

COBERLEY, S.S., L. H. HERBST, L. M. EHRHART, D. A. BAGLEY, S. HIRAMA, E. R. JACOBSON, AND P.A. KLEIN. 2001. Survey of Florida green turtles for exposure to a disease associated herpesvirus. Diseases of Aquatic Organisms 47:159-167.

EPPERLY, S. P., J. BRAUN, A. J. CHESTER, F. A. CROSS, J. V. MERRINER, P. A. TESTER, AND J. H. CHURCHILL. 1996. Beach strandings as an indicator of at-sea mortality of sea turtles. Bulletin of Marine Science 59(2):289-297.

HERBST, L.H., E. C. GREINER, L. M. EHRHART, D. A. BAGLEY, AND P. A. KLEIN. 1998. Serological association between spirorchidiasis, herpesvirus infection, and fibropapillomatosis in green turtles from Florida. Journal of Wildlife Diseases 34(3): 496-507.

HERBST, L. H., AND P. A. KLEIN. 1995. Monoclonal antibodies for the measurement of class-specific antibody responses in the green turtle, *Chelonia mydas*. Veterinary Immunology and Immunopathology 46 (3-4):317-335.

LUNA, L.G. 1968. Manual of histologic staining methods of the Armed Forces Institute of Pathology. McGraw-Hill Book Company, New York, New York, 251 pp.

NAAR, J., A. BOURDELAIS, C. TOMAS, J. KUBANEK, P. L. WHITNEY, L. FLEWELLING, K. STEIDINGER, J. LANCASTER, AND D. G. BADEN. In press. A competitive ELISA to detect brevetoxins from *Karenia brevis* (ex *Gymnodinium breve*) in seawater, shellfish, and mammalian body fluid. Environmental Health Perspectives.

SHEA, D. 1997. Analysis of brevetoxins by micellar electrokinetic capillary chromatography and laser induced fluorescence detection. Electrophoresis 18: 277-283.

TUBARO, A., C. FLORIO, E. LUXICH, S. SOSA, R. DELLA LOGGIA, AND T. YASUMOTO. 1996. A protein phosphatase 2A inhibition assay for a fast and sensitive assessment of okadaic acid contamination in mussels. Toxicon 34:743-52.

VANDEVANTER D. R., P. WARRENER, L. BENNETT, E. R. SCHULTZ, S. COULTER, R. L. GARBER, T. M. ROSE. 1996. Detection and analysis of diverse herpesvirus species by consensus primer PCR. Journal of Clinical Microbiology 34:1666-1671.

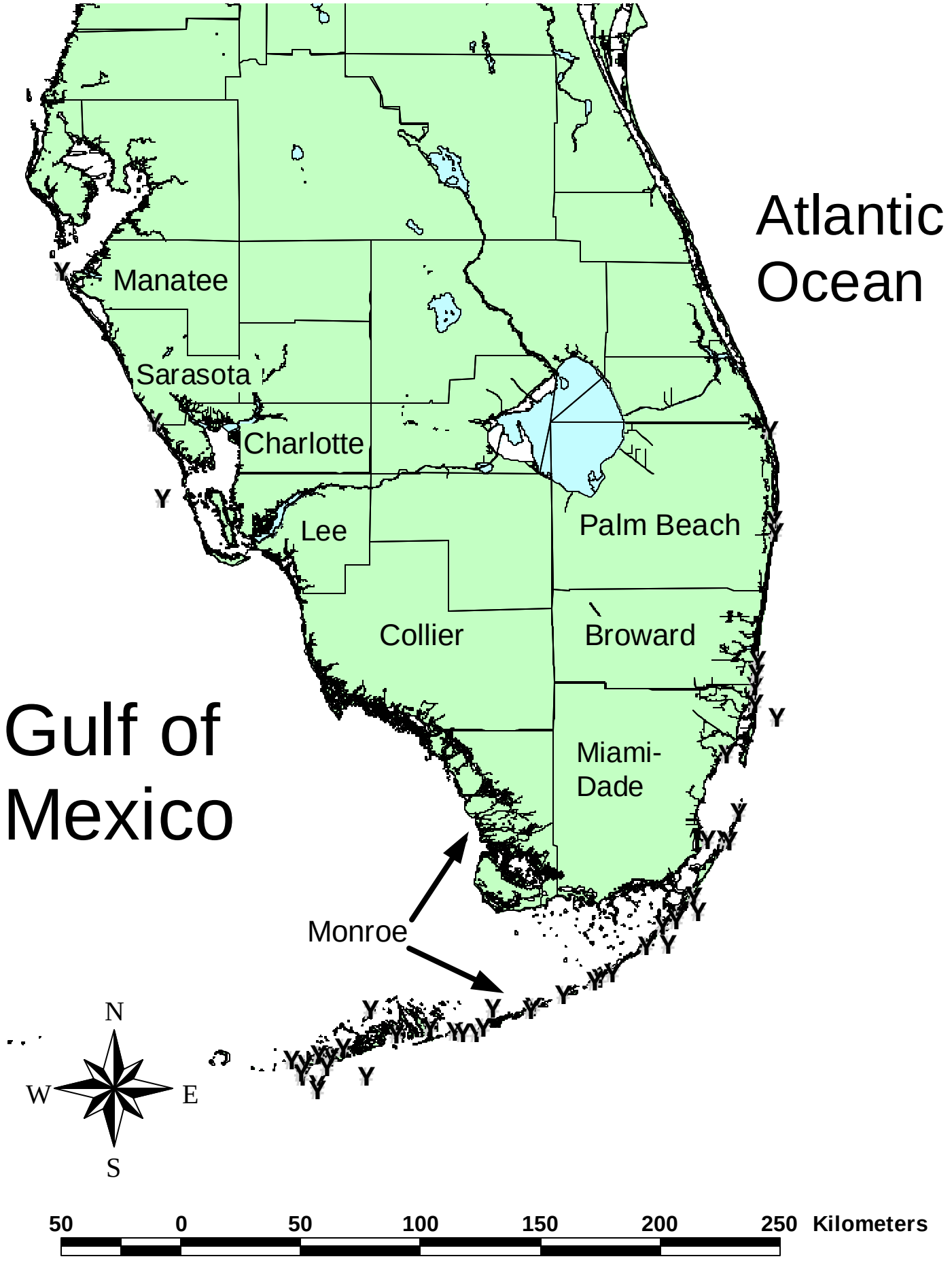
VAN DOLAH, F. M., E. L. FINLEY, B. L. HAYNES, G. J. DOUCETTE, P. D. MOELLER, AND J. S. RAMSDELL. 1994. Development of rapid and sensitive high throughput pharmacological assays for marine phycotoxins. Natural Toxins 2: 189-196.

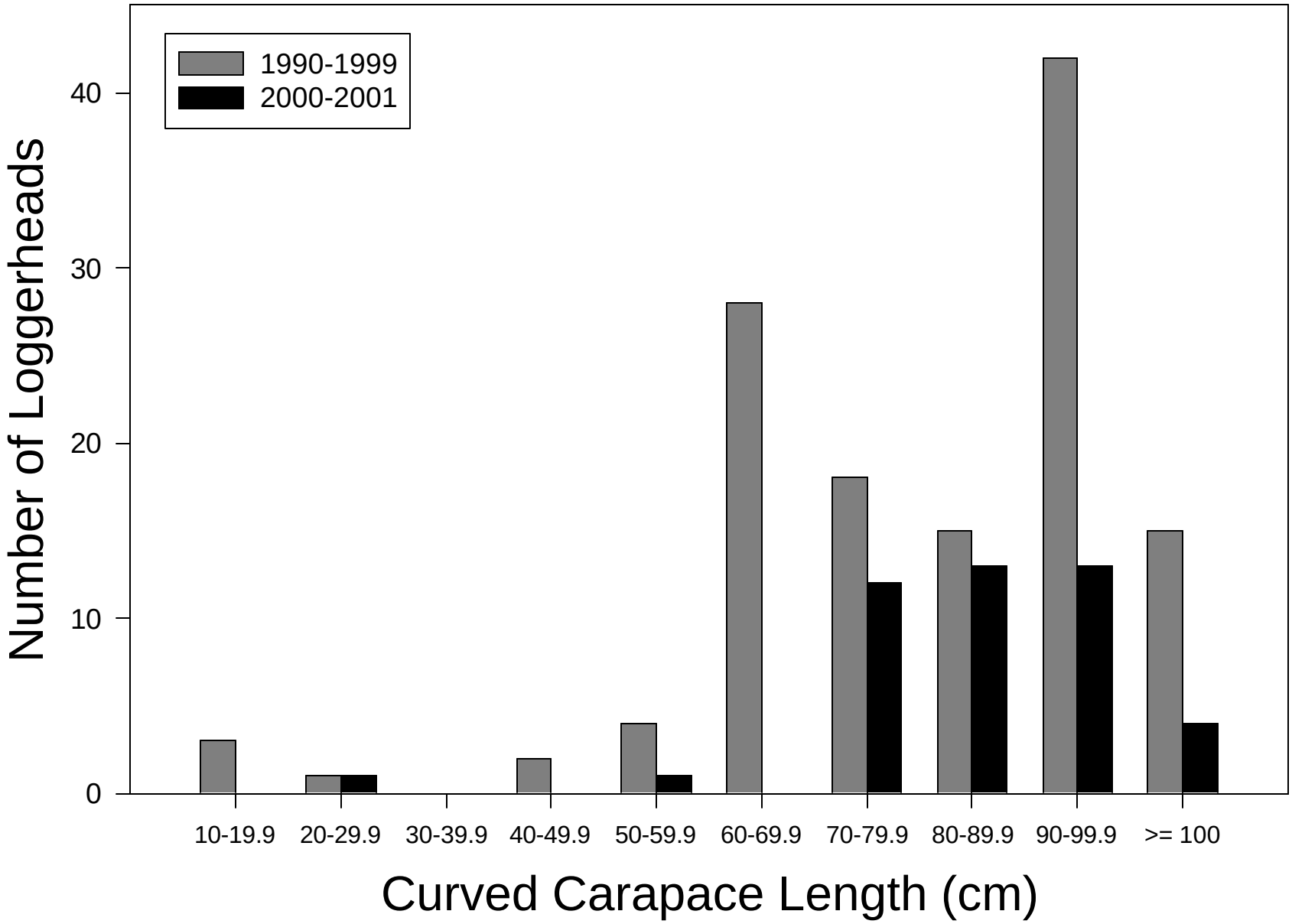
Fig. 1. Locations where debilitated loggerheads associated with the epizootic were found in Florida between 5 October 2000 and 24 March 2001. Monroe County includes the triangular piece of land between Collier and Miami-Dade and almost all of the chain of islands (the Florida Keys) to the south and southeast.

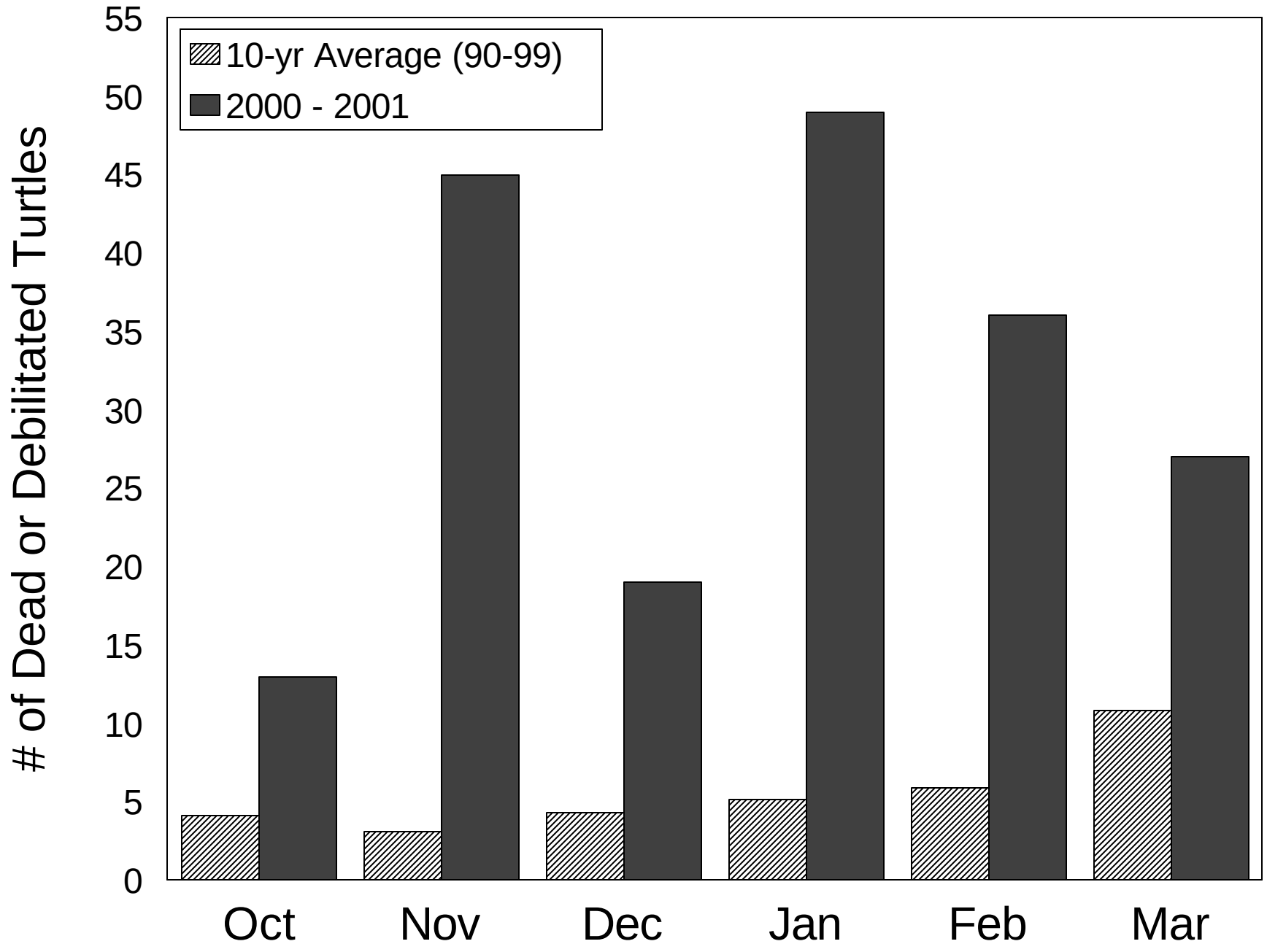
Fig. 2. The size class distribution of debilitated loggerheads associated with the epizootic (2000 - 2001) and the size class distribution of dead or debilitated loggerheads found during the previous ten years (1990-1999) in south Florida (Manatee County - Palm Beach County) from October through March. There was no difference in carapace lengths between the two groups (Kruskal-Wallis One-Way ANOVA, $P > 0.05$).

Fig. 3. The number of dead or debilitated loggerheads found each month from October to March in south Florida (Manatee County - Palm Beach County) during the epizootic (2000 - 2001) and the average number of dead or debilitated loggerheads found in south Florida during the previous ten years (1990 - 1999) from October through March.

Fig. 4. Brevetoxins detected in loggerhead tissues.


Atlantic Ocean
Gulf of Mexico
Manatee
Sarasota
Charlotte
Lee
Collier
Monroe
Palm Beach
Broward
Miami-Dade
N
W
E
S
50 0 50 100 150 200 250 Kilometers


1990-1999
2000-2001
Number of Loggerheads
0
10
20
30
40
10-19.9
20-29.9
30-39.9
40-49.9
50-59.9
60-69.9
70-79.9
80-89.9
90-99.9
>= 100
Curved Carapace Length (cm)


10-yr Average (90-99)
2000 - 2001
# of Dead or Debilitated Turtles
55
50
45
40
35
30
25
20
15
10
5
0
Oct
Nov
Dec
Jan
Feb
Mar