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Leatherback Sea Turtle (*Dermochelys coriacea*) Embryo and Hatchling Pathology in Grenada, with Comparison to St. Kitts

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Abstract. – Globally, the hatch success of leatherback sea turtles (*Dermochelys coriacea*) is much lower than any other sea turtle species. Causes of embryonic mortality, a contributing factor to the low hatch success of the species, are poorly understood. Muscle necrosis, renal mineralization, and bacterial pneumonia are prevalent among embryos and hatchlings in St. Kitts, where hatch success is much lower (<∼ 20%) than the global average (∼ 50%), yet the significance of these conditions in the wider region is unknown. The objective of this study was to describe the pathology of hatchlings and embryos in Grenada as a comparison population for St. Kitts. In 2017, 20 hatchlings, 6 pipped hatchlings, 35 late stage embryos, and 5 early stage embryos were sampled for comprehensive postmortem examination from 12 leatherback nests on Levera Beach, Grenada and 2 nests on St. Kitts. Pathology affected 71% of nests and 44% of individuals from Grenada and 100% of nests and 56% of individuals from St. Kitts. Lesions observed in both populations included skeletal muscle degeneration and necrosis (19%), bronchopneumonia (8%), and renal tubular degeneration (5%) while chorioallantoitis (17%) and mycotic dermatitis (5%) were only observed in Grenada. Gonads were histologically classified as female in all instances (n = 60), suggestive of high incubation temperatures in nests at both locations. Further study is needed to find interventions that reduce the prevalence of perinatal pathology and to identify the basis for the large proportion of eggs that fail to develop as well as embryos that die without significant pathology.

Key Words. – leatherback sea turtle; *Dermochelys coriacea*; sea turtle pathology; embryo pathology; temperature; sex ratio

Leatherback sea turtles (*Dermochelys coriacea*) are the largest extant reptile with a global migratory range. They are a unique species of sea turtle that has a cartilaginous carapace, leathery skin, and feeds on gelatinous zooplankton, mainly jellyfish. An important role of nesting females is the transport of energy and nutrients from the sea to the nesting beaches (Bouchard and Bjorndal 2000). On a global level, leatherback sea turtles are currently listed as Vulnerable by the International Union for Conservation of Nature (IUCN) (Wallace et al. 2013). In the Wider Caribbean, leatherback nest abundance has significantly declined over recent decades (Northwest Atlantic Leatherback Working Group 2018), leading the IUCN to recently reclassify the Northwest Atlantic leatherback subpopulation as Endangered (The Northwest Atlantic Leatherback Working Group 2019). Fisheries bycatch (Eckert and Sarti 1997; Gilman et al. 2006; Soykan et al. 2008), habitat destruction (Fish et al. 2005; Chacón-Chaverri and Eckert 2007; Bourgeois et al. 2009), predation at various life stages (Nellis and Henke 2000; Pitman and Dutton 2004; Tapilatu and Tiwari 2007; Tomillo et al. 2010), pollution (Bugoni et al. 2001; Salmon 2003; Bourgeois et al. 2009; Mrosovsky et al. 2009), and low hatch success (Bell et al. 2004; Wallace et al. 2004; Tapilatu and Tiwari 2007) contribute to decreasing leatherback populations worldwide. Understanding factors which influence hatchling production is a priority research area for leatherback conservation.

Hatch success is the proportion of yolked eggs in a nest that hatch. The global average for hatch success of leatherbacks is around 50% (Rafferty et al. 2011), typically ranging from 45% to 65% (Eckert et al. 2012), and is the lowest of all the sea turtle species. Average hatch success of hawksbill (*Eretmochelys imbricata*, 78.6%; Ditmer and Stapleton 2012), green (*Chelonia mydas*, 80%–85%; Broderick and Godley 1996), loggerhead (*Caretta caretta*, 75%–83%; Broderick and Godley 1996), Kemp's ridley (*Lepidochelys kempii*, 78.7%; Johnson et al. 1999), olive

Table 1. Select reported hatch success for leatherback sea turtles at different nesting sites.

Location	Hatch success (%)	Reference
Australia, New South Wales	43, 78	Tavey 1993
Brazil	53.0, 78.0	Thomé et al. 2007
Costa Rica, Caribbean coast	53.2	Leslie et al. 1996
Costa Rica, Pacific coast	50.4	Rafferty et al. 2011
Costa Rica, Pacific coast	19.8	Bilinski et al. 2001
Costa Rica, Pacific coast	56.5	Bell et al. 2004
French Guiana	38.2	Caut et al. 2006
Grenada	34.0	Houghton et al. 2007
Indonesia	47.1	Tapilatu and Tiwari 2007
Malaysia	35.8–61.0	Chan and Liew 1996
Mexico, Pacific coast	35, 53	Martínez et al. 2007
St. Croix	46.7	Garrett et al. 2010
St. Croix	53.7–64.1	Eckert and Eckert 1990
Suriname	34.9, 63.7	Hilterman and Goverse 2003
Suriname	56.4 ± 9.5	Whitmore and Dutton 1985
Venezuela, Caribbean coast	47.2	Hernández et al. 2007

ridley (*Lepidochelys olivacea*, 73.7%; López-Castro et al. 2004), and flatback sea turtles (*Natator depressus*, 83%; Theissinger et al. 2009) are all generally much higher than those reported for leatherbacks (Table 1). In addition, in recent decades, hatch success has decreased at certain nesting sites (Tomillo et al. 2007; Rafferty et al. 2017; Northwest Atlantic Leatherback Working Group 2018).

In an attempt to identify a reason for low hatch success, Bell et al. (2004) performed a study on leatherbacks at Playa Grande, Costa Rica, which found embryo mortality rather than infertility was the basis for low hatch success. Causes for the mortalities were not investigated by Bell et al. (2004). However, several studies have investigated extrinsic factors which may cause embryo mortality, such as derangement in temperature, humidity, or gaseous content (Ackerman 1981; Wallace et al. 2004; Garrett et al. 2010). Nest erosion, inundation, and depredation also result in embryo mortality, but when nests fail to survive to term due to these factors, they are often excluded from reported estimates of hatch success (Eckert and Eckert 1990). Predators of leatherback eggs include army ants, dipteran fly larvae, locusts, mole crickets, and ghost crabs (Eckert et al. 2012). Despite the high prevalence of mortality in developing leatherbacks, there is a paucity of literature addressing their pathology.

In St. Kitts, mean hatch success is typically $<\sim$ 20% (K.M.S, *pers. comm.*). Hatch success is much lower in St. Kitts than other islands in the region, such as Grenada at ~ 30% (2015) and ~ 32% (2016) (K.E.C., *pers. comm.*), Trinidad and Tobago at 58% ± 26% (2012) (Theodorakou 2013), and St. Croix at 46.7% (2007) (Garrett et al. 2010). The low hatch success values in St. Kitts do not include additional nests that fail due to tidal inundation, sand mining, depredation, and poaching (K.M.S., *pers. comm.*). Similar factors contribute to nest failure in Grenada. A beetle species has been observed in association with unhatched eggs of Grenadian leatherback nests (K.E.C., *pers. comm.*), but its role in hatch success is unknown.

In 2015–2016, postmortem examinations were performed on nonemergent hatchlings and unhatched embryos in St. Kitts in order to identify lesions which might explain embryo mortality and poor hatch success. Lesions were identified in 38% of study turtles and included renal mineralization (24%), bacterial pneumonia (12%), and skeletal muscle degeneration and necrosis (7%) (Hill et al. 2019). Dead-in-nest leatherback hatchlings of Florida's Atlantic coast have similar lesions at different prevalences, as well as other lesions not described in St. Kitts (Miller et al. 2009), suggesting that while pathology is not homogenous across nesting sites, some lesions may have a role in perinatal mortality throughout the region. Grenadian nests have lower hatch success than the global average, but higher hatch success than St. Kitts, and comparisons of perinatal pathology between these nesting sites may help clarify the regional significance of certain lesions.

The objectives of this study were to 1) describe the pathology of hatchling and embryo mortalities in Grenada, 2) compare pathology of embryo and hatchling mortalities in Grenada to St. Kitts, and 3) identify the beetles in Grenadian nests and clarify their role as predators vs. scavengers. Comprehensive descriptions of the pathology underlying unexplained embryo and hatchling deaths at multiple Caribbean nesting sites is a basic initial step to understanding fatal diseases or environmental factors which may impede hatchling production for the Northwest Atlantic subpopulation. Without this information, it is difficult to propose mitigations that may reduce mortality of endangered perinatal leatherbacks.

METHODS

Nesting Populations. — The study was undertaken at St. Kitts and Grenada during 2017. All nests laid between 31 March and 29 May 2017 in St. Kitts and 20 May and 4 June 2017 in Grenada were enrolled in the study if they could be found again at the time of excavation.

In St. Kitts, the nesting beaches were patrolled nightly during the leatherback sea turtle nesting season. The monitoring of the nesting beaches in St. Kitts occurs from February through July (Northwest Atlantic Leatherback Working Group 2018). During this time frame the beaches of Keys (17°20′N, 62°43′W) and North Friars (17°21′N, 62°43′W) were monitored for adult and hatchling leatherback emergence events. Any encountered nesting females were recorded and identified via tags. Data

Table 2. Classification of developmental stages of leatherback sea turtles (*Dermochelys coriacea*) examined from nests in St. Kitts and Grenada based on Miller et al. 2017.

Sample type	Description
No growth signs or development (NGSD)	Embryo not discernable macroscopically
Early stage embryo	Any developmental stage from visible pigmented iris to embryo size equal to or smaller than yolk sac
Late stage embryo	Embryo larger than yolk sac and front flippers able to wrap around yolk sac within egg
Pipped hatchling	Pipped hatchling dead inside shell
Dead-in-nest hatchling	Hatchling found dead inside of nest
Dead-outside-nest hatchling	Hatchlings found dead on beach with tracks leading to nest
Unknown	Unknown status due to predation or decomposition

collected, if available, included the date the nest was laid, Global Positioning System (GPS) coordinates of the nest, the flipper tag numbers for both right and left flippers, and the passive integrated transponder (PIT) tag number. Once the nest was located, it was marked with a stake labeled with the date of lay for ease of relocation. The GPS coordinates along with the observation of hatchling emergence were used to relocate the nests after hatching events.

In Grenada, monitoring of Levera Beach (12°13′N, –61°37′W) occurs from March through August. The Monel (National Brand & Tag, Newport, KY) flipper tag numbers for both right and left flippers, the PIT tag number, and the location of the nest were recorded during nesting when present. Levera Beach is divided into 25-m or 30-m zones with endpoints marked as letters of the alphabet in order from A–Z following the beach from the entrance point. There are two K points, K1 and K2, to accommodate for a bend in the beach. During patrols throughout the season, the distance from the nest to the two nearest posts that demarcated the zone endpoints were measured with transect tape in order to find the nest's location by triangulation at a later date. In both nesting locations, nests were manually relocated if they were laid in a location prone to inundation or erosion.

Nest Excavation. — Nests were excavated in St. Kitts between June and August of 2017 and in Grenada between 18 and 22 August 2017. At both sites, nests were excavated within 48 hrs of the observation of emergence or 70 d after date laid. Excavation date, emergence date, nest location, nest lay date, and GPS coordinates were recorded with each sample. Levera Beach nest information gathered included the excavation date, emergence date, nest location, female PIT tag number, female right and left Monel flipper tag numbers, and whether it was relocated or not.

Methods used for excavation were similar in both St. Kitts and Grenada. Once the location of the nest was identified via GPS coordinates and using stakes placed during nesting in St. Kitts, or the triangulation method using the nearest two posts in Grenada, a hole approximately 50–100 cm deep was dug by hand to expose the nest chamber. All of the contents of the nest chamber were removed with gloved hands and removed material was organized into groups of 10 along the surface. The nest contents were sorted and counted as eggshells, shelled albumin globs (SAGs), unhatched yolked eggs (depredated or decomposed eggs), and hatchlings. For the unhatched yolked eggs, each individual egg was opened to classify the stage of development it reached prior to death. The developmental stage was categorized as either no growth signs or development (NGSD), early stage embryo, late stage embryo, pipped hatchling, dead-in-nest hatchling, dead-outside-nest hatchling, or unknown (Table 2).

The presence of predators encountered in the nests was recorded. In addition, any coagulated eggs in the nest were counted. Coagulated eggs were those that had firm and opaque albumin or yolk (Fig. 1). Nests were categorized as "failed" if all yolked eggs were observed in the NGSD stage. Hatch success was calculated for each excavated nest by dividing the number of empty shells in a nest by the total number of eggs excluding SAGs (Eckert et al. 1999; Hilterman and Goverse 2003).

Sample Collection. — Following the developmental staging for all the individuals (i.e., classification as NGSD, early embryo, late embryo, or hatchling), up to 7 early embryos, late embryos, and hatchlings were collected from excavated nests containing these stages. If embryos and eggs were too decomposed to allow meaningful histopathologic evaluation, its presence was recorded but they were excluded from sampling.

Postmortem examinations were performed on the embryos and hatchlings, and tissues were carefully examined for gross pathology throughout the process. The necropsy was initiated by removing the turtle from their eggshell and yolk sac (if the yolk sac was external) followed by placing an incision into the plastron at the left axillary region. The incision was extended around the circumference of the plastron to detach it from the rest of the body. Once the plastron was detached from the rest of the body, the pectoral muscles were removed for better visualization of the underlying viscera. If the yolk sac was internal, it was removed by severing the omphalomesenteric duct. Gross abnormalities were recorded, and tissue samples were collected for histopathology from the heart, gastrointestinal tract, liver, gallbladder, lungs, kidneys, gonads, fetal membranes, muscles, integument, and cranium.

The gastrointestinal tract was removed from the body by cutting at the attachment to the esophagus and the most distal portion of the large intestine visible. Samples taken included the stomach and the small intestine. The heart

Figure 1. Coagulated leatherback sea turtle (*Dermochelys coriacea*) egg sampled from a nest in Grenada in 2017. The albumin and yolk are firm and opaque.

was transected at the base of the greater vessels and an edge of the liver incorporating the gallbladder was extracted. A microtome blade was used to cut 3-mm-thick cross-sections of the turtle just caudal to the axilla which included the lungs, spine, and paravertebral and body wall musculature. Samples of the kidneys and gonads were collected using the process outlined for the lungs. Sagittal slices of the cranium were collected by removing the mandible with the blunt sharp dissection scissors and then a parasagittal transection was made 2 mm from the midline, just medial to the eyes on each side of the remaining cranium to produce 4-mm-thick sections. All collected tissue samples were fixed in 10% neutral buffered formalin for 48 hrs in tissue cassettes.

Histopathology Processing. — Formalin-fixed tissue samples were routinely processed for histology, embedded in paraffin blocks, and sectioned at 4 μm thick and stained with hematoxylin and eosin (H&E). Additional histochemical stains were utilized when indicated by microscopic lesions. Brown-Brenn and Brown-Hopps Gram stains were used to identify bacteria, Periodic acid–Schiff stain was used to identify fungi, and Ziehl-Neelsen acid fast stain was used to identify mycobacteria. Von Kossa stain was used to determine the presence of minerals in the tissues.

Sex Determination. — The sex of the embryos or hatchlings was determined histologically by observing the cortical epithelial cells of the gonads (Ceriani and Wyneken 2008). When a simple cuboidal layer was seen at the outer layer of the cortex, the individual was classified as a female. When the outer layer of the cortex had a simple squamous layer, it was classified as a male. Sex was categorized as undetermined where there was insufficient quantity of adequately preserved gonad in a section to accurately assess.

Nest Predator Identification. — Any potential predators identified within the nest during excavation were recorded. Beetles within Grenadian nests were identified at the University of Georgia with the assistance of a key (Watrous and Triplehorn 1982). Dissection and examination of the male genitalia were used to confirm the identification. Voucher specimens were deposited in the University of Georgia Collection of Arthropods (UGCA).

RESULTS

Study Population. — There were 21 nests on the beaches monitored in St. Kitts for the nesting season of 2017, which was well below the historical average. Only 2 nests were observed being laid and these were accurately marked for excavation. Of the 21 nests, 14 (66.7%) could not be found and 4 (19.0%) were lost to tidal inundation, leaving only 3 (14.3%) that could be excavated. Of the 3 excavated nests, samples were collected from 2 nests because 1 nest, which was relocated, was a failed nest that yielded no embryos (i.e., all eggs were NGSD). One of the sampled nests was located on Keys Beach and the other was located on North Friars Beach; both nests were laid by unidentified females.

On Levera Beach in Grenada, there were a total of 412 confirmed nests for the 2017 nesting season. Eighty-three nests were laid during the nest enrollment period and 17/83 (20.5%) nests were excavated. These represented all

Table 3. Number of nests and each developmental stage of leatherback sea turtles (*Dermochelys coriacea*) sampled from the main nesting beaches in St. Kitts and Grenada in 2017.

		Developmental stage			
	Nests	Early embryo	Late embryo	Pipped hatchling	Hatchling
St. Kitts (n = 18)	2	0	5	0	13
Grenada (n = 48)	12	5	30	6	7
Total	14	5	35	6	20

nests that had not yet been excavated during the time period the pathology team was present in Grenada. Five of 17 excavated nests could not be sampled for the study because they contained no hatchlings or embryos: 2 failed nests (i.e., all unhatched eggs were NGSD stage) and 3 nests that were severely predated. Thus, 12/17 (70.6%) excavated nests were sampled for this study. Six of the 103 (5.8%) females that nested on Levera Beach in 2017 were identified and one nest from each was sampled. The remaining nests sampled by this study (6/12, 50%) were laid by unidentified females.

Sixty-six turtles and 14 nests in total were examined by this study, including 40 embryos and 26 hatchlings (Table 3).

Hatch Success and Nest Observations. — Average (range, median, standard deviation [SD]) hatch success for the nests sampled (i.e., excluding failed nests that did not have embryos or hatchlings) on St. Kitts was 42.2% (39–44, 42.2, 3.6; n = 2), and on Grenada at 29.0% (0–65, 27.7, 21.1; n = 12) (Fig. 2).

NGSD eggs predominated in the nests sampled by this study (Fig. 3). For Grenada and St. Kitts nests combined, there was an average (range, median, SD) of 96.2 (63–128, 100.5, 21.0) total number of eggs, 18.3 (0–49, 19, 12.5) eggshells, 32.2 (17–55, 31, 11.8) SAGs, 21.5 (3–74, 13, 21.5) NGSDs, 2.4 (0–8, 1.5, 2.7) early stage embryos, 8.3 (0–43, 3, 11) late stage embryos, 0.9 (0–10, 0, 2.6) pipped hatchlings, 2.4 (0–10, 0, 4.1) hatchlings, and 11.9 (0–47, 6.5, 13.6) unknowns per nest. The presence of coagulated and opaque albumen and yolks (4/17, 23.5%) and infiltration by vines (1/17, 5.9%) were seen in the nests of Grenada. These have previously been observed in St. Kitts but were absent from the nests examined in 2017.

Nest Predator Identification. — Beetle (*Phaleria fulva* Fleutiaux & Sallé) infestation (5/17, 29.4%) (Fig. 4) and predation by ghost crabs (*Ocypode occidentalis*) (2/17, 11.8%) were observed in the nests in Grenada. Ghost crabs have been previously seen in Kittitian nests, but were absent in 2017, whereas beetles have never been documented there. Beetles within Grenadian nests were identified as the darkling beetle (family Tenebrionidae) *Phaleria fulva*.

Gross Pathology. — Gross lesions were evident in 6/66 (9.1%) embryos and hatchlings and were only observed

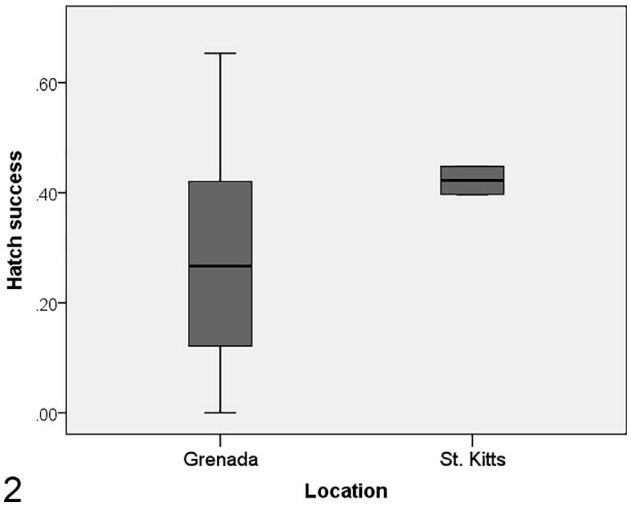

Figure 2. Box and whisker plot of hatch success for leatherback sea turtle nests excavated and sampled on St. Kitts (n = 2) and Grenada (n = 12) in 2017.

on Levera Beach. Gross lesions corresponded to microscopic lesions in many cases. One late stage embryo was found with leucism, holoprosencephaly with arhinia, a proboscis, and maxillary micrognathia (Fig. 5A) as well as chorioallantoitis with intralesional Gram-negative rods seen microscopically. White films and plaques that corresponded microscopically to fungal dermatitis were on the skin of 2 early stage embryos from the same nest (Fig. 5B). In one of these embryos, the white film also grossly involved the chorioallantoic membrane, which corresponded microscopically to heavy fungal and bacterial colonization without an inflammatory response and was interpreted as postmortem microbial growth. One hatchling had a skeletal malformation consisting of a midbody depression of the carapace on the left side, where its egg had been constricted by roots, and also had renal urate stasis. Two hatchlings that had red and collapsed lungs were both identified with bronchopneumonia with intralesional Gram-negative rods. One also had skeletal muscle degeneration and the other had microvascular thrombosis and multifocal necrosis of the liver.

Histopathology. — Histological assessment was performed on 743 tissues from the 66 sampled turtles including lungs (n = 65), liver (n = 65), kidneys (n = 65), heart (n = 64), skeletal muscle (n = 64), gastrointestinal tract (n = 64), gonads (n = 62), skin (n = 62), bone marrow (n = 56), brain (n = 52), gallbladder (n = 31), chorioallantoic membrane (n = 29), yolk (n = 20), salt glands (n = 13), thyroid (n = 9), thymus (n = 7), pancreas (n = 4), and spleen (n = 1). In 2 individuals, both from Grenada, sex was histologically undetermined, whereas 100% of the remaining 60 from St. Kitts and Grenada were female. Microscopic lesions affected 31/66 (47.0%) individuals, including 13/40 (32.5%) of the embryos and 18/26 (69.2%) of the hatchlings examined in this study. Both sampled nests in St. Kitts had pathology, and pathology was identified in 8 of the 12 nests sampled in

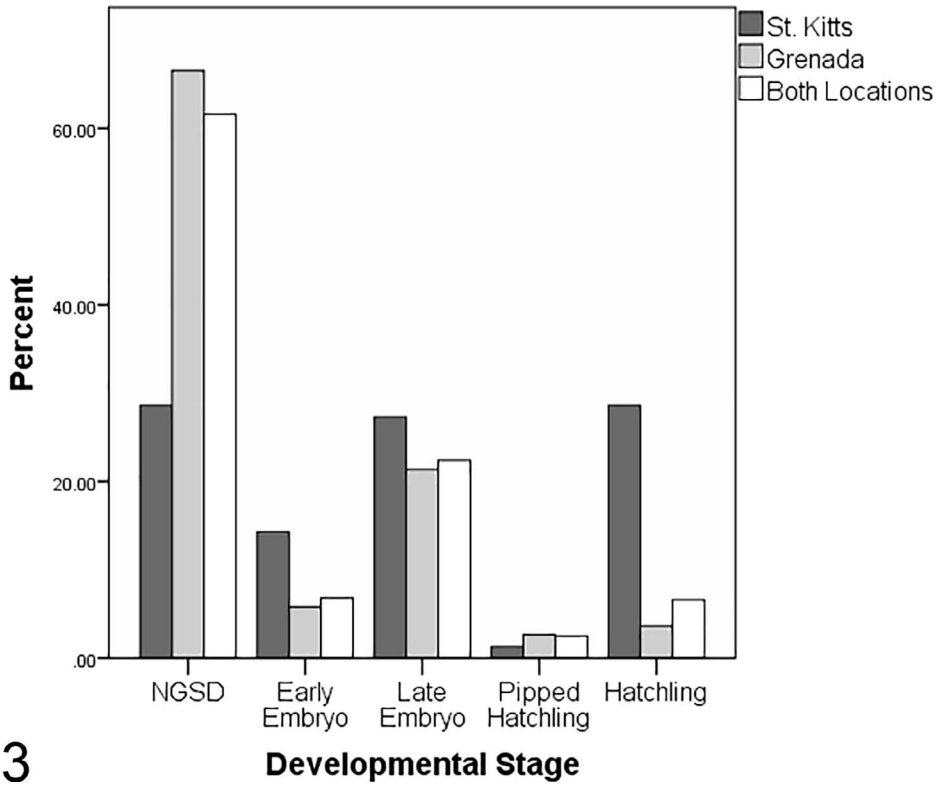

Figure 3. Percent of leatherback sea turtle developmental stages (as per Table 2) in nests excavated and sampled on Grenada (n = 12) and St. Kitts (n = 2) in 2017. NGSD = no growth signs or development.

Grenada. Across all nests sampled, no pathology was identified in 35/66 (53.0%) of individuals evaluated.

The most common microscopic lesions included bronchopneumonia, muscle degeneration, and chorioallantoitis (Table 4). Renal tubular degeneration, renal tubular amorphous urate accumulation without tophi formation (i.e. renal urate stasis), mycotic dermatitis, microvascular thrombosis, enteritis, and yolk sac atrophy were also observed. Skeletal muscle degeneration and necrosis were the most prevalent lesions in both St. Kitts and Grenada, affecting 50% (7/14) of nests and 18.8% (12/64) of embryos and hatchlings, and up to 3 individuals per nest. This lesion was discerned histologically as myofibers that were swollen with fragmented, vacuolated, and hypereosinophilic cytoplasm lacking cross-striation, nuclear pyknosis and karyorrhexis, and occasionally mineralization of cytoplasm (Fig. 5C). Degeneration and necrosis ranged from mild to moderate in severity and affected late stage embryos, pipped individuals, and hatchlings. Two individuals with skeletal muscle degeneration and necrosis had concurrent bronchopneumonia.

Figure 4. Darkling beetle (*Phaleria fulva* Fleutiaux & Sallé) (arrow), found infesting leatherback sea turtle (*Dermochelys coriacea)* nests in Grenada in 2017. Bar = 6 mm.

Chorioallantoitis was the next most prevalent with 17.2% (5/29) of individuals affected and 28.6% (4/14) of nests sampled, involving 1–2 individuals per nest. This lesion was only observed in Grenada. Histologically, chorioallantoitis consisted of heterophilic infiltrate within regions of necrosis and fibrin exudation, usually colonized by microorganisms. Gram-negative rods, both present extracellularly and within the cytoplasm of heterophils, were the only organisms found in the lesions of the early stage and late stage embryos (Fig. 5D) whereas fungus was also present along with the Gram-negative rods in the sample from the pipped hatchling. Fungal hyphae in this case were slender (2–4 μm in diameter), sparsely septate, and dichotomously branched at acute angles. Embryos and hatchlings with chorioallantoitis did not have any other associated microscopic lesions.

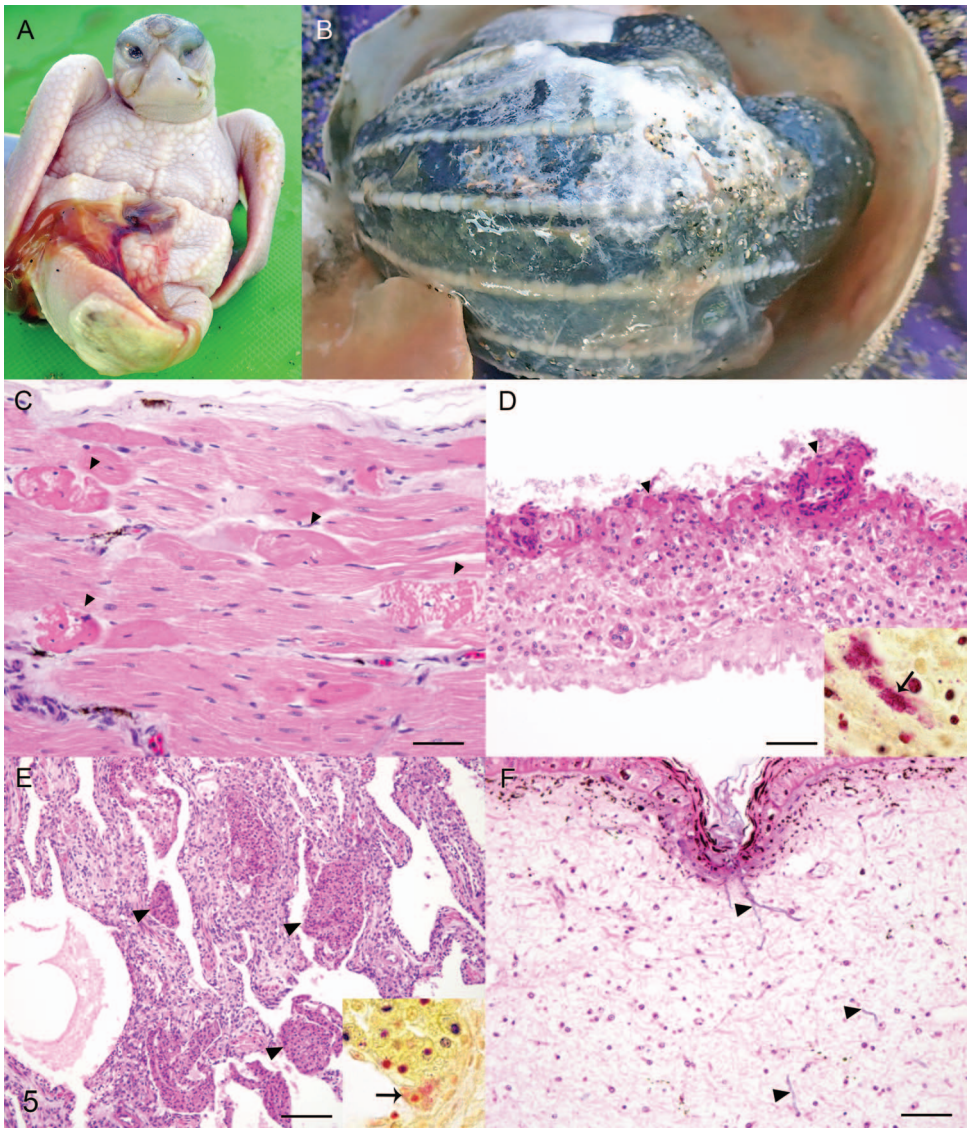

Figure 5. Gross and microscopic pathology of leatherback sea turtle (*Dermochelys coriacea*) embryos and hatchlings sampled from nests on Grenada and St. Kitts in 2017. (A) Leucism and holoprosencephaly with arhinia and maxillary micrognathia in a late stage embryo from Grenada. (B) Early stage embryo from Grenada with a white film covering the chorioallantoic membrane. Microscopically, the film consisted of mats of fungal hyphae and bacteria. (C) Skeletal muscle degeneration and necrosis in a hatchling from St. Kitts. Necrotic myocytes have fragmented and vacuolated sarcoplasm (arrowheads). Hematoxylin and eosin (H&E). Bar = 50 µm. (D) Chorioallantoitis in a late stage embryo from Grenada. Heterophils have infiltrated the chorioallantosis (arrowheads). H&E. Bar = 50 µm. Inset: Gram-negative rod-shaped bacteria (arrow) are within inflamed areas. Brown-Hopps gram stain. (E) Heterophilic bronchopneumonia in hatchling from Grenada. Clusters of heterophils and macrophages fill airways (arrowheads). H&E. Bar = 100 µm. Inset: Gram-negative rod-shaped bacteria (arrow) are within the cytoplasm of leukocytes. (F) Mycotic dermatitis in a late stage embryo from Grenada. Fungal hyphae (arrowheads) extend through the dermis accompanied by dermal edema and mononuclear leukocyte infiltrate. H&E. Bar = 50 µm. (Color version is available online.)

Bronchopneumonia was the next most prevalent pathology affecting 7.7% (5/65) of individuals and 21.4% (3/14) of nests, including up to 2 individuals per nest, involving nests at both study sites. Histologically, bronchopneumonia was heterophilic and granulomatous. Heterophils and macrophages, within a background of protein and necrotic cell debris, filled airways. Affected foci were congested. Severity of bronchopneumonia ranged from mild to moderate, involving ~ 20%–50% of the parenchyma. In 3 of the cases, lesions were bilateral. In 4 of the cases of bronchopneumonia, Gram-negative rods (Fig. 5E) were seen with Brown-Hopps stained slides. For the other case, intralesional microorganisms were not identified with special stains.

Mycotic dermatitis prevalence was 4.8% (3/62) of individuals and 7.1% (1/14) of nests. Only 2 early stage embryos and 1 late stage embryo showed mycotic dermatitis. Histologically, this was visualized as mats of fungal hyphae coating the epidermal surface and multifocally extending into the dermis. The affected dermis was expanded with regional edema and lightly populated by mononuclear leukocytes and reactive fibroblasts (Fig. 5F).

Table 4. Percent prevalence of lesions observed in leatherback sea turtle (*Dermochelys coriacea*) embryos and hatchlings collected from the main nesting beaches in St. Kitts and Grenada in 2017.

	Location		Developmental stage				
	St. Kitts	Grenada	Early embryo	Late embryo	Pipped	Hatchling	Total
Nests with pathology	100 (2/2)	67 (8/12)					71 (10/14)
Individuals with pathology	56 (10/18)	44 (21/48)	60 (3/5)	19 (10/35)	33 (2/6)	80 (16/20)	47 (31/66)
Muscle degeneration and necrosis	24 (4/17)	27 (8/47)	20 (1/5)	23 (4/34)	17 (1/6)	32 (6/19)	19 (12/64)
Chorioallantoitis	0 (0/1)	18 (5/28)	33 (1/3)	14 (3/22)	25 (1/4)	NA	17 (5/29)
Bronchopneumonia	11 (2/18)	6 (3/47)	0 (0/5)	0 (0/34)	17 (1/6)	20 (4/20)	8 (5/65)
Other[a]	6 (1/18)	8 (4/48)	0 (0/5)	6 (2/35)	0 (0/6)	15 (3/20)	8 (5/66)
Mycotic dermatitis	0 (0/17)	7 (3/45)	40 (2/5)	3 (1/34)	0 (0/6)	0 (0/17)	5 (3/62)
Renal tubular degeneration	6 (1/18)	4 (2/47)	0 (0/5)	0 (0/34)	0 (0/6)	15 (3/20)	5 (3/65)

[a] Other lesions include renal urate stasis ($n = 2$), microvascular thrombosis of the liver ($n = 1$), yolk sac atrophy ($n = 1$), enteritis ($n = 1$), and leucism with craniofacial malformation ($n = 1$).

In all 3 cases of mycotic dermatitis, morphology of the fungal hyphae was similar to those described above in the inflamed fetal membranes of a pipped hatchling.

DISCUSSION

This study demonstrates that pathology is common in perinatal leatherbacks and adds to our knowledge of diseases which may contribute to poor hatch success among the species. Pathology was prevalent among embryos and dead-in and out-of-nest hatchlings of both Grenada (8/12, 67% of nests and 21/48, 44% of individuals) and St. Kitts (2/2, 100% of nests and 10/18, 56% of individuals). Unfortunately, the 2017 nesting season in St. Kitts was the slowest on record to that date, since monitoring began in 2003, in line with nesting declines observed throughout the region (Northwest Atlantic Leatherback Working Group 2018). This limited the sample size in St. Kitts and therefore the extent to which pathology could be compared among the two study sites for 2017. However, Hill et al. (2019) found that pathology was similarly prevalent, identified in 38% of the embryos and dead-in-nest hatchlings studied in St. Kitts in 2015 and 2016. Lesions observed in embryos and hatchlings from both islands include bronchopneumonia, skeletal muscle degeneration and necrosis, and renal tubular degeneration. Bronchopneumonia and skeletal muscle degeneration and necrosis were previously observed in St. Kitts by Hill et al. (2019). Lesions that were only seen in Grenada included chorioallantoitis (affecting 5/28, 17.9%, of embryos and pipped hatchlings) and mycotic dermatitis (affecting 3/45, 6.7% of embryos). Hill et al. (2019) described renal mineralization in 24% of leatherback embryos and hatchlings, but this lesion was not identified in the present study.

Skeletal muscle degeneration and necrosis (Fig. 5C) was the most prevalent lesion in both St. Kitts and Grenada (19%), and it affected all developmental stages examined. Skeletal muscle degeneration was seen at a prevalence of 32% in a pathology study on dead-in-nest hatchlings in Florida (Miller et al. 2009). Muscle degeneration is featured in exertional myopathy accompanying net-entanglement, as suspected in a juvenile green turtle (Phillips et al. 2015), and juvenile or sub-adult loggerhead sea turtles (Orós et al. 2005). It is possible that hatchlings are prone to exertion stress during hatching and emergence. Vitamin E deficiency and selenium deficiency can also cause muscle degeneration in many animals (Dierenfeld 1989; Miller et al. 2009), and in leatherback hatchlings is potentially linked to maternal nutrient levels or mercury exposure (Perrault et al. 2011). Alternatively, pathogenesis may involve hyperthermia.

Chorioallantoitis (Fig. 5D) affected 5/29 (17%) individuals evaluated and appears to be mainly caused by Gram-negative bacilli. This lesion affected early stage embryos (1/3, 33%), late stage embryos (3/22, 14%), and pipped hatchlings (1/4, 25%). Chorioallantoitis has not previously been described in leatherback or other sea turtle embryos and was only identified in Grenadian turtles. However, previous studies based in St. Kitts did not evaluate the chorioallantois (Hill et al. 2019), and only one fetal membrane sample from St. Kitts was evaluated by the present study. In the present study, 5 of the 6 cases appeared to be caused by Gram-negative rod-shaped bacteria. The route of infection presumptively involves bacterial penetration of the eggshell, as has been proposed for bacterial pneumonia in leatherback embryos (Hill et al. 2019).

Bronchopneumonia (Fig. 5E) was observed in both St. Kitts and Grenada (3/45, 6.7% of individuals from both countries) and appears to be consistently caused by Gram-negative bacilli. Bacterial pneumonia is problematic in reptiles and tends to be caused by opportunistic environmental Gram-negative bacteria (Schumacher 1997). Pneumonia affected 80% of the dead-in-nest hatchlings sampled in Florida (Miller et al. 2009) and has now been documented in St. Kitts for several years with prevalence as high as 17% (Hill et al. 2019), suggesting that pneumonia is an important disease of leatherback hatchlings throughout the Northwest Atlantic population. The histological pattern of pneumonia, where leukocytes are centered on airways, suggests an aerogenous route of

infection. Hill et al. (2019) diagnosed bronchopneumonia mostly in hatchlings and, similarly in the present study, bronchopneumonia was only present in hatchlings and pipped hatchlings (20% and 17% prevalence, respectively). These developmental stages are exposed to the air and environment outside of the eggshell, contrary to the early and late stage embryos that are confined inside the eggshell. Gram-negative rods which have been isolated from swabs of lung surfaces of leatherback hatchling and embryos affected with pneumonia include *Pseudomonas* spp., *Acinetobacter* spp., *Citrobacter* spp., *Enterobacter* spp., and *Salmonella* spp. in St. Kitts (Hill et al. 2019), and those that have been isolated from lung samples of dead-in-nest hatchlings in Florida include *Alcaligenes faecalis*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Pseudomonas mendocina*, and *Shewanella putrefaciens* (Miller et al. 2009). Further investigation into the identity of the Gram-negative rods causing pneumonia and chorioallantoitis is warranted to determine whether the bacteria are from the environment or from maternal reproductive tracts. Collection of samples for tissue culture from lungs showing gross changes of bronchopneumonia, such as red and collapsed lungs or those bearing nodules (Hill et al. 2019), could provide a more accurate approach for bacterial identification but may be complicated in a decomposing nest environment.

Mycotic dermatitis (Fig. 5F) was uncommon (3/62, 5% of individuals), identified only in one nest from Grenada, and has not been observed in St. Kitts to date. Dermatitis was only seen in early and late stage embryos, which implies that the pathogen was able to penetrate the eggshell. Fungal cultures were not performed in this study and morphological features of fungal hyphae in histological sections are nonspecific, thus the identity of the organism(s) is unknown. Mycotic dermatitis was also observed in one hatchling in the Miller et al. (2009) study and was attributed to *Fusarium* spp. Infection of sea turtle eggs with *Fusarium* spp. has been a growing area of study in the search for factors threatening the survival of sea turtle nests (Sarmiento-Ramírez et al. 2010, 2014; Rosado-Rodríquez et al. 2016). Although *Fusarium* spp. have been isolated from nests with poor hatch success (Sarmiento-Ramírez et al. 2014), and from the interior of eggs that failed to hatch (Rosado-Rodríguez et al. 2016), its role remains debatable as the organism may thrive in a decomposing nest environment and may not necessarily be a cause of embryo mortality. While the morphology of the fungus in myocotic dermatitis and chorioallantoitis was nonspecific, it is consistent with *Fusarium* spp. (Guarner and Brandt 2011). The identification of pathology caused by fungal infection in the present study helps build a case for the role of mycoses in leatherback perinatal mortality, although fungal etiology appears to account for a minority of pathologies observed in perinatal leatherbacks of the West Indies. Furthermore, because the fungi and bacteria causing dermatitis and other lesions may represent saprophytes, it is possible that they are opportunists terminally contributing to morbidity while not primarily responsible for mortality.

Congenital anatomic abnormalities are infrequently reported in leatherbacks (Eckert et al. 2012) and have not yet been observed in St. Kitts (Hill et al. 2019). Two were documented on Levera beach in 2017, one with leucism and severe craniofacial malformation and another with a carapace deformation. Leucism is often associated with craniofacial malformations in sea turtles as seen in the present case (Bárcenas-Ibarra et al. 2015). Deformations (i.e., alteration in shape/position of a previous normally formed organ) are typically caused by external forces, in this case roots invading the nest and compressing the egg.

Darkling beetles (*Phaleria fulva* Fleutiaux & Sallé) infested 29.4% of the nests excavated in Grenada. While they have been observed in Grenadian nests previously (K.E.C., *pers. comm.*), they have not yet been observed in St. Kitts. Beetles of the *Tenebrionidae* family are scavengers known to feed on fungi and decaying animal matter; therefore, it seems likely that the beetles are feeding on decomposing material within the nest rather than predating on viable eggs.

Many nests (4/14, 29%) and individuals (35/66, 53%) in this study showed no gross or microscopic lesions. Extreme temperatures of the nest environment could inhibit proper development of the embryos and may cause death in the absence of gross or microscopic lesions. Suffocated or drowned nests due to flooding or increased precipitation could also halt the development of the eggs without morphological tissue changes (Magnusson 1982; Sepúlveda et al. 2006). Another possibility includes embryonic dehydration due to increased permeability in irregularly formed eggshells, as observed in *Caiman latirostris* (Fernández et al. 2013). While gross abnormalities of the eggshells were not evident in St. Kitts or Grenada, microscopic and mineral analysis of leatherback eggshells may also help elucidate whether there are abnormalities permissive to trans-shell bacterial transmission.

The development of females compared with males is at a ratio of 1:1 at the pivotal incubation temperature of around 29°C–30°C in leatherbacks (Mrosovsky 1994; Godfrey et al. 1996; Binckley et al. 1998). Skewed sex ratios could influence the survivability and genetic biodiversity of leatherback sea turtles. Although there is no "normal" primary sex ratio yet established for leatherback hatchlings, estimates for Atlantic nesting beaches have ranged from 50% to 76% female (Turtle Expert Working Group 2007; Laloë et al. 2016). Of the gonads histologically classified in this study ($n = 60$), 100% were identified as female, similar to findings of others (Binckley et al. 1998; Hill et al. 2019). Sex was classified using the histological methods described by Ceriani and Wyneken (2008). Direct examination of gonads using histology is considered the most accurate method for determining sex of neonatal sea turtles (Wibbels 2003). An immunohistochemical approach to

determine the sex of the embryos and hatchlings was considered, but that process was made unnecessary based on a study by Tezak et al. (2017) showing that there was 100% agreement between histology and immunohistochemistry. Regardless, these findings support the potential need to identify and implement effective mitigations to alleviate high nest temperatures such as shading, relocation, and hatchery translocation (Sieg et al. 2011; Esteban et al. 2018).

Another finding that raises concern for high nest temperatures was the presence of coagulated eggs. Coagulation and possible damage of the egg contents can be seen with desiccation (Boas 1927) and high nest temperatures (Romanoff and Romanoff 1949). The temperature at which yolk coagulates in chickens is 65°C (Romanoff and Romanoff 1949), but there is no established temperature for yolk and albumen coagulation in sea turtles. Interestingly, nests with coagulated eggs had a higher proportion of NGSD eggs than did those without, suggesting that the factors causing egg coagulation may also have inhibited embryo development.

Although embryos and hatchlings were the focus of this study, undeveloped yolked (NGSD) eggs constituted the majority of unhatched eggs observed, similar to a previous study in St. Kitts (Hill et al. 2019). These accounted for 20% of eggs in excavated nests sampled by this study and, in addition, 3/20 (15%) nests excavated for this study were failed nests constituted entirely of such eggs. Eggs with no signs of growth or development similarly predominated in Suriname (Hilterman and Goverse 2003) as well as Florida (Bell et al. 2004). Future studies that investigate whether NGSD eggs represent nonfertilized eggs rather than very early embryo mortality are needed, as this has been examined by only a single study in the Eastern Pacific (Bell et al. 2004).

In conclusion, this study demonstrates several lesions that are likely contributing to perinatal morbidity and mortality and reduced hatch success in Caribbean leatherback turtles. Comparisons to regions with above-average hatch success may help clarify the extent to which these lesions influence perinatal mortality. Research is needed to identify strategies that reduce the prevalence of embryonic and hatchling pathology in leatherbacks. Those rehabilitating or head-starting leatherback hatchlings should be mindful of the risk of diseases including skeletal muscle degeneration and necrosis, bacterial pneumonia, bacterial chorioallantoitis, and mycotic dermatitis, such that proper care can be provided for affected turtles.

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