Vol. 159: 49–63, 2024
https://doi.org/10.3354/dao03800

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published August 1



# Verminous bronchitis and pneumonia by nasal trematodes in Greater Caribbean manatees from Puerto Rico

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ABSTRACT: Five adult Greater Caribbean manatees *Trichechus manatus manatus* were found stranded on various coasts of Puerto Rico; 2 stranded alive and 3 stranded dead. Clinical signs observed in live-stranded manatees included emaciation, weakness, bradypnea, arrhythmia, and nasal mucus discharge. Postmortem examinations revealed serosanguinous, mucohemorrhagic, or suppurative exudate in bronchi associated with luminal adult *Pulmonicola cochleotrema* (range: 18–182 trematodes), accompanied by pulmonary abscesses in 2 cases. Histologically, we observed eosinophilic bronchopneumonia of varying severity (n = 4) and chronic erosive to eosinophilic tracheobronchitis (n = 4) with squamous metaplasia (n = 3) and intralesional trematodes and eggs. The trematode identity was confirmed and compared through molecular analysis for the amplified 18S rDNA fragment. Comorbidities included enteric chiorchosis (n = 5), gastric heterocheilosis (n = 4), malnutrition (n = 4), trauma related to watercraft collision (n = 3), systemic toxoplasmosis (n = 1), acute bacterial peritonitis (n = 1), and interstitial nephritis (n = 1), suggesting that immunosuppression was a predisposing factor for lower respiratory tract pulmonicolosis. Based on lesion severity, clinical signs, and the presence and absence of other findings to explain death, this condition was considered the primary cause of death in 1 manatee, a contributory cause of death in 3 manatees, and an incidental finding in 1 individual. These clinicopathological descriptions will facilitate the diagnosis and clinical management of pulmonicolosis in *T. manatus*, a species endangered with extinction.

KEY WORDS: Disease · *Trichechus manatus manatus* · *Pulmonicola cochleotrema* · Pulmonicolosis · Endoparasite · Infection

## 1. INTRODUCTION

The American manatee *Trichechus manatus* is an herbivorous, fully aquatic mammal of the Order Sirenia that inhabits shallow coastal waters, rivers, and estuaries in tropical and subtropical regions of the Americas (Self-Sullivan & Mignucci-Giannoni 2012). This species is divided into 2 subspecies: the Florida manatee *T. m. latirostris* and the Greater Caribbean manatee *T. m. manatus* (Domning & Hayek 1986, Mignucci-Giannoni et al. 2024). Like other aquatic mammals, manatees are long-lived and considered

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sentinels of coastal health and indicators of emerging health problems affecting aquatic animals, the environment, and humans (Bossart 2011, Bonde et al. 2012). Florida and Greater Caribbean manatees are considered Vulnerable and Endangered with extinction, respectively (Deutsch & Valade 2024, Morales-Vela et al. 2024), and therefore protected by law. In Puerto Rico, the current manatee population is estimated to be between 312 and 535 individuals (Collazo et al. 2019).

Anthropogenic activities are a significant threat to the survival of the species, including habitat loss, watercraft injuries, entanglement with fishing gear, and, in some regions, hunting (Mignucci-Giannoni et al. 2000, Owen et al. 2018, Alvarez-Alemán et al. 2021). Disease also plays an essential role in causing stranding and mortality, but in Greater Caribbean manatees, these have been less described than in Florida manatees. Diseases causing mortality in manatees include cold stress syndrome (Bossart et al. 2002), pneumonia (Bossart et al. 2004, Bonde et al. 2012, Klećkowska-Nawrot et al. 2019), pleural empyema (pyothorax) (Attademo et al. 2020), gastroenteritis (Buergelt et al. 1984, Bonde et al. 2012), pyelonephritis (Keller et al. 2008), disseminated toxoplasmosis (Bossart et al. 2012, Smith et al. 2016), salmonellosis (Vergara-Parente et al. 2003, Vorbach et al. 2017), and brevetoxicosis (Bossart et al. 1998). Calf deaths due to malnutrition associated with abandonment, maternal separation, and unexpected parasitic infection have also been reported (Walsh & Bossart 1999, Mignucci-Giannoni et al. 2000, Bossart et al. 2004, De Meirelles 2008, Weisbrod et al. 2021).

Sirenians serve as hosts for various ecto- and endoparasites (Beck & Forrester 1988, Coy-Otero 1989, Mignucci-Giannoni et al. 1999a,b, Carvalho et al. 2009, Colón-Llavina et al. 2009, Hernández-Olascoaga et al. 2017, Owen et al. 2018, Vélez et al. 2018, Lucot et al. 2020, Gonzalez et al. 2021). The most common metazoan endoparasite is the ascarid nematode *Heterocheilus tunicatus* in the lumen and mucosa of the stomach and small intestine (Beck & Forrester 1988, Bonde et al. 2012). The trematodes *Chiorchis fabaceus* and *C. groschafti* are found in the lumen of the small and large intestines (Rivera-Pérez et al. in press), and *Nudacotyle undicola* and *Moniligerum blairi* are found in the lumen and mucosa of the small intestine. *Pulmonicola cochleotrema* (previously *Cochleotrema cochleotrema* Travassos & Vogelsang, 1931) is somewhat common in the nasal cavity (Blair 2005, Bonde et al. 2012, Owen et al. 2018) of subadult and adult manatees (Forrester 1992). The helminths of aquatic mammals are regarded as part of the typical fauna of the host (Measures 2018), and those of the manatee are mainly considered innocuous and do not usually cause significant disease (Geraci & Lounsbury 2005, Owen et al. 2018). Helminth infections do not usually cause clinical signs or pathology in healthy aquatic mammals (Dierauf 1990), and deaths due to endoparasites and their associated lesions are considered unusual in sirenians (Owen et al. 2018). However, endoparasites can opportunistically cause diseases linked to stranding in immunologically compromised animals (Measures 2018). As an example, some calf mortality in Florida manatees has been attributed to necrohemorrhagic enteritis caused by *N. undicola* or *M. blairi*, often with a concurrent diagnosis of immunosuppressive conditions such as brevetoxicosis, watercraft injury, or cold stress syndrome (Beck & Forrester 1988, Weisbrod et al. 2021).

The digenetic trematode *P. cochleotrema* is usually confined to the nasal cavity but can be found in the trachea, bronchi, and bronchioles when parasite loads are high (Bonde 1985, Beck & Forrester 1988). In the nasal cavity, it may be associated with chronic rhinitis. A single report of verminous pneumonia was attributed to *P. cochleotrema* in a Florida manatee, but lesions were not described (Buergelt et al. 1984). Pneumonia was clinically suspected in Brazil in Greater Caribbean manatees that had heavy nasal infections of *P. cochleotrema* and produced atypical respiratory noises (Borges et al. 2017a,b).

Here, we present the first comprehensive clinical and pathological descriptions of verminous tracheobronchitis and pneumonia due to *P. cochleotrema* infection in stranded Greater Caribbean manatees from Puerto Rico.

## 2. MATERIALS AND METHODS

### 2.1. Rescue and stranding events history

Five adult Greater Caribbean manatees were found stranded in 4 different geographic locations on the coasts of Puerto Rico between 2018 and 2022.

#### 2.1.1. Case 1

On 5 February 2018, an adult female manatee was found stranded on shore 2.5 km west of río Cañas Abajo in Juana Díaz (17.98917° N, 66.47400° W) (Fig. 1A, Table 1). The manatee was 267 cm in length, 272 kg in mass, lethargic, with a body condition of

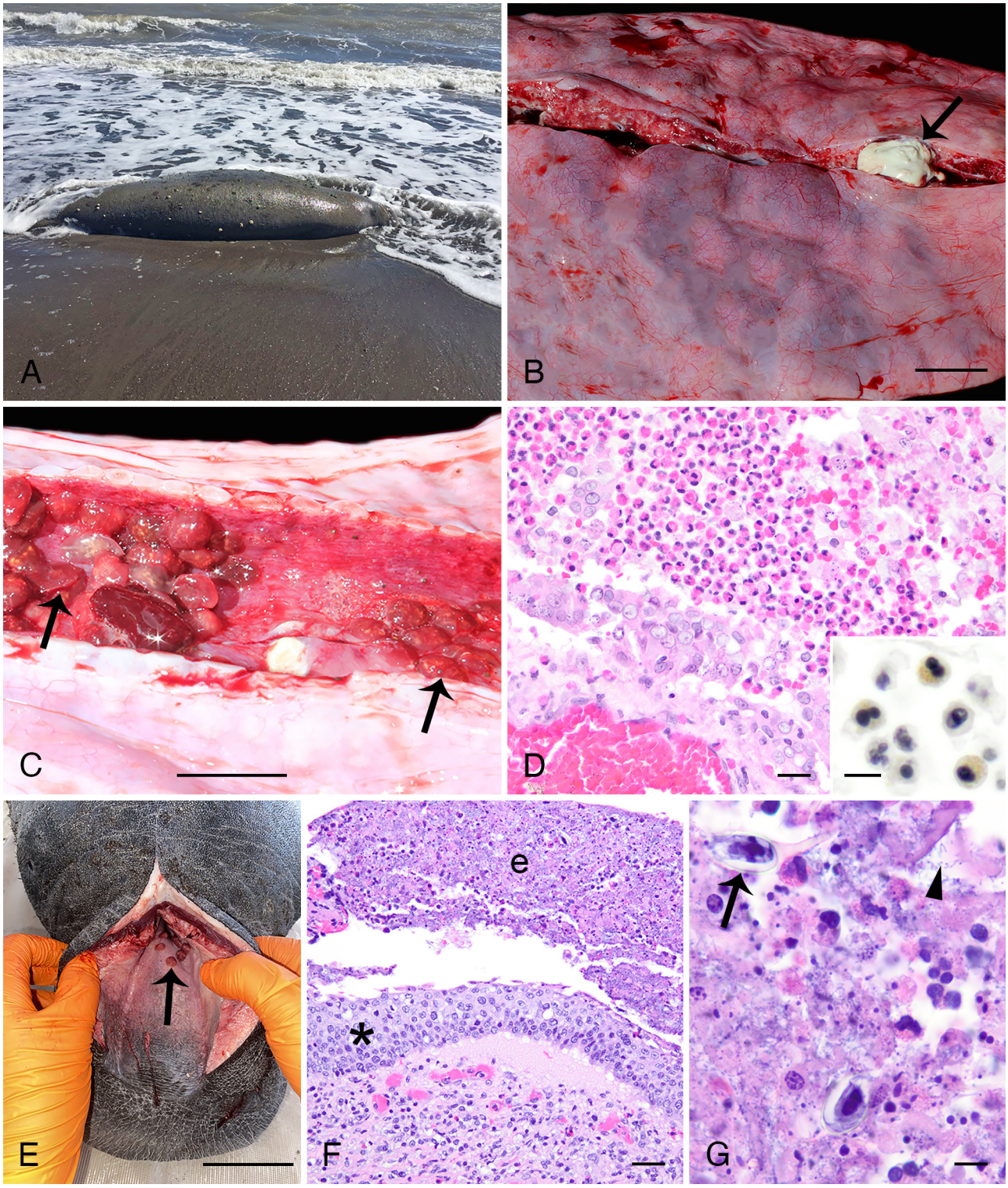


Fig. 1. Pulmonary pulmonicolosis in stranded Greater Caribbean manatees *Trichechus manatus manatus* in Puerto Rico. (A) Case 1: manatee stranded alive with respiratory distress. Photo by Grisel Rodríguez-Ferrer. (B) Postmortem examination showing a pulmonary abscess (arrow) associated with *Pulmonicola cochleotrema* infection; scale bar = 3 cm. (C) Large numbers of *P. cochleotrema* in the bronchus (arrows); scale bar = 1 cm. (D) Microscopically, bronchioles are filled with heterophils mixed with eosinophils (reddish granular cytoplasm with Luna stain; inset, scale bar = 15 µm). HE stain; scale bar = 20 µm. (E) Case 3: heavy *P. cochleotrema* infection in a manatee that was stranded. There are relatively low numbers of intranasal *P. cochleotrema* (arrow); scale bar = 5 cm. (F,G) Chronic eosinophilic bronchitis associated with heavy tracheobronchial *P. cochleotrema* infection observed grossly (HE stain). (F) Mucosa shows squamous metaplasia (asterisk, scale bar = 40 µm) and the exudate (e) contains (G) necrotic debris, eosinophils, bacilli (arrowhead), and trematode eggs with polar filaments (arrow); scale bar = 8 µm

Table 1. Summary of clinicopathologic findings in cases of lower respiratory pulmonicolosis in 5 adult Greater Caribbean manatees from Puerto Rico. *Contributory cause of death; **primary cause of death

| Case no. | Presentation | Associated respiratory lesions | | Presumptive role in mortality | Comorbidities | Death category |
|---|---|---|---|---|---|---|
| | | Tracheo-bronchitis | Pneumonia | | | |
| 1 | Live stranding, Weakness, apnea, bradycardia, mucoid nasal discharge | Marked | Severe | Primary cause of death | Malnutrition*, gastric heterocheilosis, enteric chiorchosis | Natural: other (disease) |
| 2 | Dead stranding Pregnant | None | Severe | Contributory cause of death | Watercraft collision injuries (acute)**, gastric heterocheilosis, enteric chiorchosis | Human related: watercraft collision |
| 3 | Live stranding, Weakness, apnea dehydration, muffled heart sounds | Marked | Mild | Contributory cause of death | Systemic toxoplasmosis**, malnutrition*, watercraft collision injuries (chronic)*, interstitial nephritis, gastric heterocheilosis, enteric chiorchosis | Natural: other (disease) |
| 4 | Dead stranding | Moderate | None | Incidental finding | Bacterial peritonitis**, malnutrition*, gastric heterocheilosis, enteric chiorchosis, | Natural: other (disease) |
| 5 | Dead stranding | Marked | Severe | Contributory cause of death | Watercraft collision injuries (acute)**, malnutrition*, multipathogen superficial dermatitis, enteric chiorchosis | Human related: watercraft collision |

emaciation reflected in a noticeable delineation between the head and neck (a distinctive neck called a 'peanut head') (Castelblanco-Martínez et al. 2021), and a profoundly sunken abdomen with abdominal folds. There were no external lesions or injuries, but the body was covered with acorn barnacles *Chelonibia testudinaria* (Mignucci-Giannoni et al. 2022) and old scars. The animal was cold to the touch and dehydrated, oral mucous membranes were bright pink, and upon auscultation, the pulse was weak with bradycardia (36 bpm). Extreme bradypnea was evident, with 1 breath every 20 min (normal manatee respiration rate is 2–4 times every 5 min; Wong et al. 2012). Emergency triage was immediately implemented, including providing an intramuscular injection of doxapram hydrochloride (1 mg $kg^{-1}$, i.m., Dopram injection,® West-Ward Pharmaceuticals) to help stimulate breathing and body heat management. The animal was rapidly and humanely transported to the Caribbean Manatee Conservation Center (CMCC) rehabilitation facility while hydrating subcutaneously with 1000 ml of lactated ringer's injection USP (®Baxter Health Care) and 1000 ml of 0.9% sodium chloride injection USP (®Baxter Health Care) sterile solutions.

During the monitoring, the animal continued to be apneic and had a bilateral yellow mucoid nasal discharge. The manatee was diagnosed with shock, and additional intramuscular injections of doxapram and ceftazidime (22 mg $kg^{-1}$, i.m. twice, Fortaz, ®GlaxoSmithKline) were given. Upon arrival at the rehabilitation center, the animal was placed on a 24 h watch and supported at the surface with the aid of technicians and volunteers. Despite all efforts, the manatee died the following morning.

#### 2.1.2. Case 2

On 20 September 2020, a dead adult female manatee was found floating in Guánica Bay (17.95983° N, 66.90983° W). The 309 cm long carcass was in an advanced state of decomposition (condition codes as in Geraci & Lounsbury 2005, Raverty et al. 2018), and

was confirmed to be pregnant with a 137 cm long fetus. The manatee had a deep skin laceration attributed to a watercraft propeller injury on the tail but had no fractured or dislocated bones.

#### 2.1.3. Case 3

On 18 July 2021, a live adult female manatee was found stranded on shore, 1.2 km NNE of Punta Santiago in Humacao (18.17369° N, 65.73944° W). Upon arrival at the beach, we observed the manatee being aggressively harassed by a group of males trying to mate. The 305 cm, 389 kg animal was lethargic and extremely weak, with a body condition of emaciation (Castelblanco-Martínez et al. 2021), and had skin abrasions and wounds in the dorsal-posterior region of the body morphologically consistent with an approximately 1 wk old cutaneous watercraft collision injury. Ulcerated areas of these wounds were oblong (40 cm × 32 cm) with raised and pale borders and were bordered by subcutaneous hemorrhage. There were also foci of subcutaneous hemorrhage in the dorsal-posterior region before the peduncle, and noticeably worn nails were observed on both flippers. There were several large, encrusted ulcers with raised rounded edges along the caudal ventrum, from the umbilicus to the vulva, and ventral left flipper, suggestive of abrasions from prolonged contact with the benthos. The animal was dehydrated, with pale oral mucous membranes, apneic, and muffled heart sounds on thoracic auscultation. Emergency triage was immediately implemented, including providing the animal with an intramuscular injection of doxapram to help stimulate breathing and body heat management. The animal was rapidly and humanely transported to the CMCC rehabilitation facility. During transport, the animal went into respiratory arrest and died despite cardiopulmonary resuscitation attempts and the administration of intracardiac epinephrine (0.05 mg $kg^{-1}$, i.m., epinephrine injection, ®Sparhawk Laboratories).

Minutes after death, a blood sample was successfully collected from the venous plexus in the pectoral flipper. Serum biochemistry reflected an increase in liver enzyme lactic dehydrogenase (3215.0 mg $dl^{-1}$; reference interval ranges, 261–580 mg $dl^{-1}$, Mignucci-Giannoni & Alsina-Guerrero 2022), interpreted as either liver or muscle injury. Prerenal azotemia was characterized by elevated blood urea nitrogen (28 mg $dl^{-1}$; reference interval ranges, 2.2–6. mg $dl^{-1}$), creatinine (2.29 mg $dl^{-1}$; reference interval ranges, 1.3–2.0 mg $dl^{-1}$), and uric acid (5.40 mg $dl^{-1}$; reference interval ranges, 0.3–1.3 mg $dl^{-1}$), with hypersthenuria (urine specific gravity 1.050; reference interval ranges, 1.003–1.017, Cabrias-Contreras et al. 2021). There was marked hyperlipidemia (triglycerides and cholesterol [376 and 228 mg $dl^{-1}$; reference interval ranges, 88–137 mg $dl^{-1}$ and 90–143 mg $dl^{-1}$]), possibly representing redistribution of fat to support the metabolic process. Mild hypoalbuminemia (2.5 mg $dl^{-1}$; reference interval ranges, 3.7–4.5 mg $dl^{-1}$] was interpreted as malnutrition (as reflected by the poor body condition), response to inflammatory processes, and possible acute blood loss by trauma.

#### 2.1.4. Case 4

An adult female manatee was found dead on the same day, in the same location, and a few hours after the rescue of Case 3. The 267 cm, 254.5 kg manatee had a body condition of thin (Castelblanco-Martínez et al. 2021), and a noticeably dilated abdomen, with extremely watery feces exuding from the anus. The carcass was in a fresh state of decomposition, but scavenger damage in the dorsal-caudal region was consistent with shark bite marks. The animal was immediately transported to the CMCC for postmortem examination.

#### 2.1.5. Case 5

On 27 November 2022, an adult male manatee was found stranded dead on the shore, 0.2 km west of río Mameyes in Río Grande (18.38822° N, 65.75173° W). The 251 cm long carcass, in a thin body condition, was in a fresh state with a slight delineation between the head and neck (Castelblanco-Martínez et al. 2021), old scars, and nasal frothy discharge. During the salvage of the carcass, personal watercraft (jet ski) races were observed at high speed in the same area of the stranding event. Trauma to the forehead resulting in subdermal hemorrhage was consistent with a possible jet ski collision.

### 2.2. Postmortem examination and histopathology

Postmortem examinations were performed following the protocol of Bonde et al. (1983). Tissues from all cases were collected and preserved in 10% neutral-buffered formalin for subsequent histopathology examination following the protocol of Slaoui & Fiette (2011). Tissues sampled included skin, fat, cutaneous

trunci and rectus abdominis muscles, esophagus, stomach, cardiac gland, spleen, duodenal ampulla, pancreas, small intestine, cecum, and large intestine. Also, tissues from the heart, liver, gall bladder, kidneys, urinary bladder, uterus, ovaries, penis, testicles, mammary and adrenal glands, lymph nodes, trachea, bronchi, and lungs were collected. Formalin-fixed tissues were processed using standard methods, embedded in paraffin, sectioned 4 µm thick, and stained with hematoxylin and eosin for evaluation using light microscopy. Cause of death was ascribed to the injury or disease process believed to most closely precede death (also referred to as 'proximate cause of death'). Other pathological findings that were considered influential to the occurrence of death were classified as contributory causes of death. Those pathological findings that were deemed unlikely to have significant health impacts based on lesion severity or expected physiological significance were considered incidental findings. Malnutrition was diagnosed when there was a combination of poor body condition and empty proximal gastrointestinal tract, with fat depletion or serous atrophy of fat, and in some cases, transudative cavitary effusions. Death categories were classified according to criteria used by Mignucci-Giannoni et al. (2000), Bonde et al. (2012), and the Florida Fish and Wildlife Conservation Commission manatee rescue and mortality statistics (www.myfwc.com/research/manatee/rescue-mortality-response/statistics/mortality/categories/).

### 2.3. Nasal trematode DNA amplification and molecular analysis

DNA was extracted from *Pulmonicola cochleotrema* specimens collected from the different manatee cases using EURx GeneMATRIX Tissue DNA Purification Kit following the instructions of the manufacturer. Novel primers were designed by eye from alignments of the 18S rDNA reported by Rivera-Pérez et al. (2024) for *P. cochleotrema* and related Opisthotrematidae infecting sirenians. Three new primers were designed to amplify multiple variable regions from the 5′ end of the 18S rDNA and be specific for the known 18S sequences for the Opisthotrematidae from sirenians. The new primers were used in pairs or in combination with a universal primer (Table 2). The cycling conditions for the PCR were as follows: 94°C for 4 min; followed by 35 cycles at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s; and a final elongation at 72°C for 7 min. The DNA amplicon size was verified through 1% agarose gel stained with ethidium bromide. The PCR products were purified using EURx GeneMATRIX PCR Clean-Up Purification Kit and sequenced by Apical Scientific in Selangor, Malaysia. The sequence data were assembled using the alignment software Clustal X (www.clustal.org/clustal2) to confirm the identity of the sampled trematodes as *P. cochleotrema*.

## 3. RESULTS

### 3.1. Pathological findings

#### 3.1.1. Case 1

Postmortem examination revealed heavy infection by *Pulmonicola cochleotrema* and multifocal chronic suppurative bronchopneumonia with intrabronchial trematodes. Grossly, there were 2 poorly defined masses bilaterally in the caudo-ventral lobes of the lungs, which contained suppurative exudate (abscesses; Fig. 1B) and few trematodes. We found 103 *P. cochleotrema* in the bronchi, including the lower part, in the secondary and tertiary bronchi, which were distended with similar exudate (Fig. 1C), serous froth, and blood. Adjacent pulmonary parenchyma was red and firm. The trachea was filled with serous froth, and the nasal cavities contained some *P. cochleotrema* but were otherwise unremarkable. The

Table 2. List of the 18S rDNA primers used for molecular identification of the nasal trematode *Pulmonicola cochleotrema*. Tm: melting temperature

| Primer | Primer sequence (5′–3′) | Tm (°C) | GC content (%) | Paired primer(s) | Amplicon size (bp) |
|---|---|---|---|---|---|
| OP-18-F | ACA GAA CCA ACC GGA TGC AG | 57.8 | 55 | OP-18-r1<br>OP-18-r2 | 602<br>967 |
| OP-18-r1 | AGG CAC CTG GAA GCA TGC CGA C | 64.5 | 63.6 | OP-18-F | 602 |
| OP-18-r2 | ACT GCC CGT GAG GCC AAT AGT G | 61.7 | 59.1 | 390f (Freeman et al. 2013)<br>OP-18-F | 778<br>967 |

gastrointestinal tract was empty, except for the large intestine and rectum, which had hard and dry brownish digesta. A few *Heterocheilus tunicatus* were in the stomach lumen, and several *Chiorchis groschafti* were found in the large intestine.

Histologically, the pulmonary masses comprised areas of severe multifocal chronic eosinophilic and fibrinous bronchopneumonia (Fig. 1D). These abscessed regions microscopically consisted of cavitated areas of necrosis, filled with eosinophils and heterophils and partially lined by respiratory epithelium. In the bordering lung, the bronchi, bronchioles, and alveoli were filled with eosinophils and fewer heterophils, lymphocytes, plasma cells, macrophages, multinucleate giant cells, fibrin, and protein. Patchy eosinophil and lymphocyte infiltrate with mild fibroplasia expanded the alveolar septa, peri-bronchiolar, and bronchial stroma. Bronchioles and bronchi had segments of erosion, mucocyte hyperplasia, and squamous metaplasia. Other than mild eosinophilic gastritis, presumptively attributed to gastric heterocheilosis, no other lesions were identified. The primary cause of death was ascribed to pulmonary pulmonicolosis (Table 1), and malnutrition was considered a contributory cause of death.

### 3.1.2. Case 2

Postmortem examination showed moderate infection of *P. cochleotrema* and associated severe eosinophilic and fibrinous bronchopneumonia. Grossly, bronchi contained serosanguinous fluid and 45 *P. cochleotrema* in the secondary or tertiary bronchi. The stomach contained 63 *H. tunicatus* nematodes, and the small and large intestines contained approximately 400 *C. groschafti.* Histologically, bronchi, bronchioles, and alveoli were filled with edema, fibrin, a mixture of heterophils and eosinophils, fewer macrophages, and cellular debris. The respiratory epithelium was obscured by postmortem decomposition, but similar leukocytes multifocally expanded the submucosa of bronchi. The primary cause of death was attributed to watercraft injury, and pulmonary pulmonicolosis was considered a contributory cause of death.

### 3.1.3. Case 3

Postmortem examination showed heavy infection by *P. cochleotrema* with associated eosinophilic and erosive tracheobronchitis and mild acute eosinophilic, histiocytic, and fibrinous bronchopneumonia. Grossly, 7 *P. cochleotrema* were in the nasal cavity (Fig. 1E), 2 in the trachea, and 118 in the bronchi. Those in the trachea and bronchi were accompanied by mucoid to hemorrhagic exudate, and the lungs were dark red, wet, heavy, and firm. *H. tunicatus* nematodes (n = 78) were found in the stomach and duodenal ampulla, associated with acute ulceration in the stomach, and approximately 1000 *C. groschafti* were in the small and large intestines. Other findings included a stomach devoid of ingesta, serous atrophy of retroperitoneal fat, focal abdominal nodular fat necrosis, hydropericardium, multifocal subacute cutaneous ulcers, focal perivulvar hyperkeratosis, and milk in the mammary glands.

Histologically, the submucosa of the trachea and bronchi was expanded with eosinophils, heterophils, histiocytes, and lymphocytes in a background of fibroplasia. The mucosa bordering luminal trematodes was segmentally eroded or thickened with squamous metaplasia, coated in fibrinonecrotic exudate containing mixed bacilli and adult trematodes and eggs (Fig. 1F,G). Eggs were oval, approximately 10–15 × 20–25 µm, with polar filaments up to 65 µm long and golden-brown to purple, slightly thin walls that were refractile and birefringent. Bronchus-associated lymphoid tissue was depleted. Areas of bronchopneumonia consisted of alveoli filled with macrophages, eosinophils, heterophils, fibrin, and cell debris. Other histological findings included severe multifocal necrotizing lymphadenitis, enteric leiomyositis, interstitial pneumonia, myocarditis, hepatitis, adrenalitis, and mastitis attributed to the protozoan *Toxoplasma gondii*. There was also pulmonary arterial thrombosis, serous atrophy of fat, subacute ulcerative dermatitis, mild chronic interstitial nephritis, marked eosinophilic enterocolitis associated with severe *C. groschafti* infection, and hyperplasia of submandibular lymph nodes. The primary cause of death was attributed to systemic toxoplasmosis, whereas contributory causes of death were pulmonary pulmonicolosis, previous watercraft injury, and malnutrition.

### 3.1.4. Case 4

Postmortem examination revealed a light infection of *P. cochleotrema* associated with moderate chronic eosinophilic bronchitis. A few *P. cochleotrema* trematodes were in the nasal cavity. The trachea and bronchi contained serosanguinous fluid with scant blood clots and 18 *P. cochleotrema.* The lungs were diffusely red, wet, and heavy. The stomach and duodenal

ampulla contained 57 *H. tunicatus*. The large intestine was highly distended by gas accumulation with watery and few pasty feces and approximately 500 *C. groschafti*. Other gross findings included large intestinal rupture with severe acute fibrinous peritonitis, mild serosanguinous pleural effusion, and serous atrophy of pericardial and perirenal fat.

Histologically, there was moderate chronic eosinophilic bronchitis with intraluminal *P. cochleotrema.* Clotted blood and mucus containing a single adult trematode were found in the bronchus. Affected bronchial segments had a marked increase in submucosal lymphocytes and eosinophils. Foci of epithelial attenuation and eosinophilic exocytosis were observed. Other microscopic lesions included severe acute fibrinous peritonitis with intralesional mixed bacteria and plant material and a chronic renal infarct. The cause of death was attributed to bacteremia and peritonitis secondary to gastrointestinal rupture of unknown cause. Intestinal rupture has been recently associated with aggressive copulatory action by male manatees (Dill 2023, Hylton 2023). Malnutrition was considered a contributory cause of death, and bronchial pulmonicolosis was considered an incidental finding.

#### 3.1.5. Case 5

This animal was heavily infected by *P. cochleotrema* associated with chronic eosinophilic bronchitis and fibrotic bronchopneumonia. We observed 125 *P. cochleotrema* trematodes in the nasal cavity, 81 in the trachea, and 101 in the bronchi. The latter were accompanied by serous froth mucus (Fig. 2A), and the lungs were reddened, wet, heavy, and firm. Approximately 30 yellowish miliary nodules between 2 and 4 mm in size with cloudy yellowish exudate were located in the caudal lung lobes, and the affected parenchyma was red and firm (Fig. 2B). The stomach and duodenal ampulla were empty and contained 4 *H. tunicatus*. The cecum and both small and large intestines contained approximately 400 *C. groschafti*. Other gross findings included hydropericardium, serous atrophy of pericardial fat, and hydrothorax.

Histologically, bronchi had marked chronic eosinophilic and erosive bronchitis with intralesional bacilli and *P. cochleotrema* adults and eggs. The submucosa was expanded with lymphoid follicles and markedly increased lymphocytes, heterophils, and eosinophils (Fig. 2C). Similar leukocytes and histiocytes were sparsely distributed within lumina, which were filled with mucus containing *P. cochleotrema* eggs and gram-positive bacilli (Fig. 2D). The lungs had severe multifocal eosinophilic and fibrosing bronchopneumonia with intralesional *P. cochleotrema* eggs. Multifocally septae and peribronchiolar stroma were expanded with fibroplasia, which occasionally impinged bronchiolar lumina (bronchiolitis obliterans) (Fig. 2E). Eosinophils, heterophils, fibrin, and proteinaceous edema containing a few *P. cochleotrema* eggs filled the bronchioles and alveoli (Fig. 2F). Severely affected areas had necrosis of alveolar septae. Other lesions included large intestinal trematodiasis by *C. groschafti* infection, mild focal granulomatous hepatitis of unknown etiology, and focal epidermal hyperplasia with superficial algae, hyphae, diatoms, and unidentified metazoan organisms (consistent with helminths and arthropods) (as in Suárez-Morales et al. 2010, Gonzalez et al. 2021). Given the extensive subcutaneous hemorrhage on the head and historical context, traumatic head injury was considered the primary cause of death (presumptively by a jet ski collision), whereas pulmonary pulmonicolosis and malnutrition were contributory causes of death.

### 3.2. Nasal trematode molecular identification

The newly designed PCR primers reliably amplified the 18S rDNA target from all samples of *P. cochleotrema* collected in the present case studies and tested, with DNA from other trematodes (Heterophyidae and Renicolidae) not being amplified. The primers worked in all combinations (Table 2) and allowed a final consensus sequence of 967 bp for each trematode sampled.

The molecular identity of all the nasal trematodes collected in the present cases was confirmed using the Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (GenBank) (Altschul et al. 1990), which resulted in 100% identical sites, being the same genetic sequence of digenean reported by Rivera-Pérez et al. (2024) for the same region of ribosomal DNA.

## 4. DISCUSSION

This study presents the first confirmed reports of mortality-associated lower respiratory tract *Pulmonicola cochleotrema* infection in Greater Caribbean manatees from Puerto Rico and the first comprehensive pathological descriptions of associated tracheobronchitis and bronchopneumonia in the American manatee. Based on the severity of associated pathol-

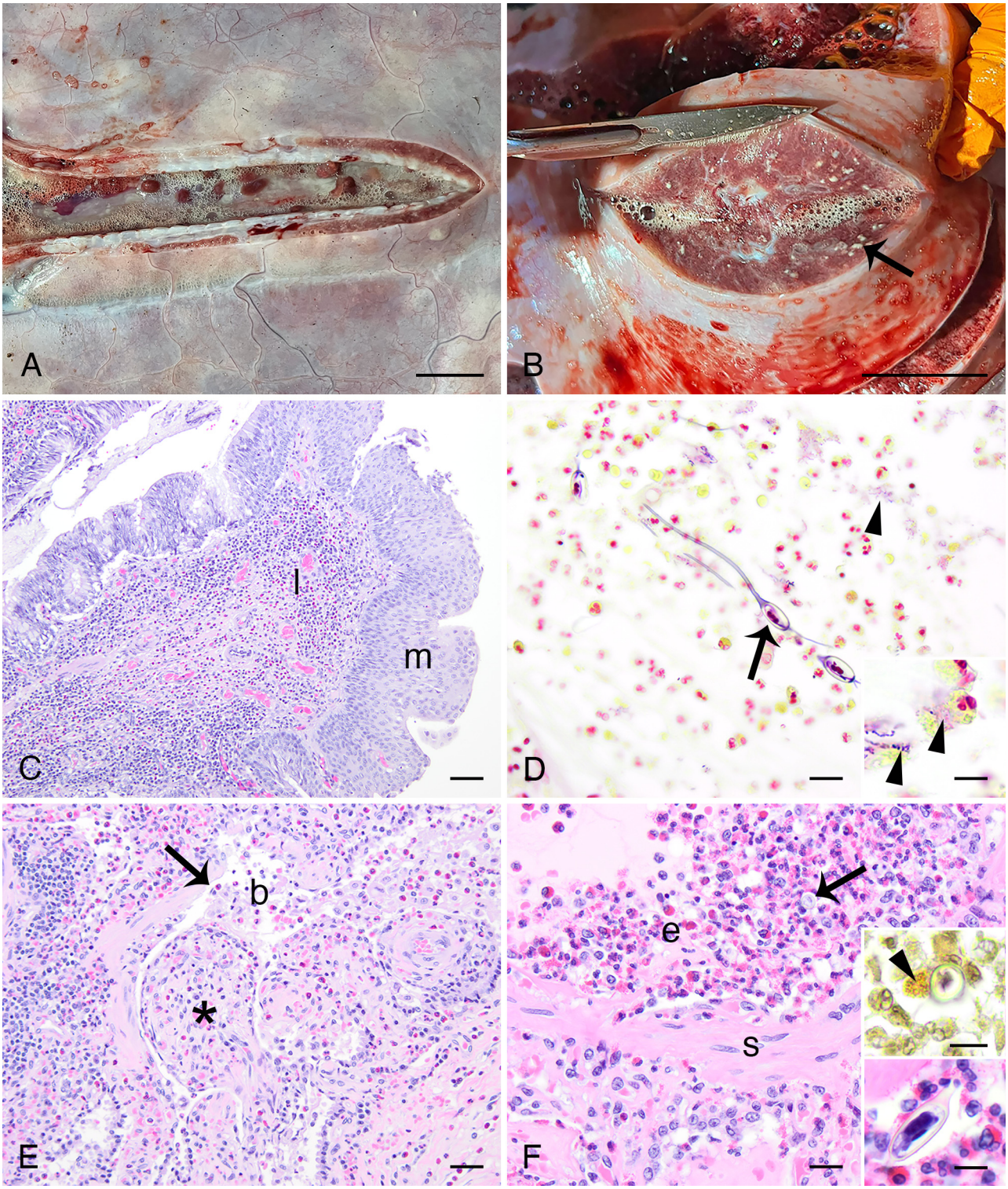


Fig. 2. Pulmonary pulmonicolosis in a stranded Greater Caribbean manatee *Trichechus manatus manatus* in Puerto Rico (Case 5). (A) Bronchi filled with frothy edema and several *Pulmonicola cochleotrema* associated with mucosal surface; scale bar = 1 cm. (B) Firm, reddened lungs have miliary yellow nodules that exuded thick yellow fluid on cut surface (arrow); scale bar = 3 cm. (C) Microscopically, there is marked peribronchiolar leukocytic infiltrate (l) and segments of mucosal squamous metaplasia (m); HE stain, scale bar = 100 µm. (D) The bronchial lumen contains leukocytes, necrotic cell debris, variably gram-positive bacilli (arrowhead, inset, scale bar = 10 µm), and trematode eggs (arrow); Hucker-Conn Gram stain, scale bar = 20 µm. (E) Bronchiole (b) with segmental loss of epithelium (arrow) and lumen containing similar leukocytes and focally impinged by fibrous polypoid projections (asterisk; bronchiolitis obliterans); HE stain, scale bar = 50 µm. (F) Eosinophilic and heterophilic pneumonia; exudate (e) consisting of eosinophils (inset upper arrowhead, Luna stain, scale bar = 10 µm), proteinaceous edema, and rare *P. cochleotrema* eggs (arrow, insets, scale bar = 10 µm) fill alveoli. S = alveolar septum; HE stain, scale bar = 20 µm

ogy and the presence or absence of alternative findings to explain death, lower respiratory pulmonicolosis was considered a primary cause of death in 1, a contributory cause of death in 3, and an incidental finding in 1 of the manatees.

Lower respiratory pulmonicolosis presented a spectrum of lesions of varying severity and clinical significance. One case (Case 4) was limited to moderate chronic tracheobronchitis in association with luminal *P. cochleotrema* adults. This case reflected the typical expectation for host response to *P. cochleotrema* displacement to the lower respiratory tract and was considered an incidental finding. In contrast, the other 4 cases (Cases 1–3, 5) had more serious lesions, including multifocal mild to severe eosinophilic and fibrinous to fibrosing bronchopneumonia and generally more severe tracheobronchitis. These lesions were considered of sufficient severity to impact respiratory function and were associated with respiratory signs in 2 live-stranded manatees, supporting the view that they may be clinically significant. While the clinical significance of lower respiratory pulmonicolosis and other types of endoparasitism in sirenians is debated (Forrester 1992, Geraci & Lounsbury 2005, Measures 2018, Owen et al. 2018), we present unequivocal evidence with histological descriptions of how lower respiratory pulmonicolosis is of clinical importance in this species.

Bronchopneumonia was grossly evident in the 4 cases with this lesion, including bilateral abscesses in the caudal lung lobes with intrabronchial *P. cochleotrema* and hemorrhage (Case 1), and firm, reddened lungs with intrabronchial mucohemorrhagic exudate and trematodes (Cases 3 and 5). In the other case, only pulmonary edema was identified grossly with the presence of the intrabronchial trematodes (Case 2). This instance emphasizes the importance of conducting a comprehensive postmortem examination, including histological assessment, to accurately diagnose stranded animals and determine the extent of pathology associated with infectious organisms, including *P. cochleotrema*. While watercraft injury was considered the cause of death for Cases 2 and 5, we hypothesize that *P. cochleotrema* pneumonia may have predisposed them to watercraft collision by causing the manatees to spend increased time at the surface to breathe or by reducing mobility to escape the collision. This observation further emphasizes the importance of comprehensive postmortem examination for precise mortality data.

Histologically, the predominance of eosinophilic infiltrate in *P. cochleotrema* tracheobronchitis and pneumonia, as confirmed by Luna stain (Figs. 1D & 2F), is consistent with an inflammatory response to parasite infection and in contrast to typical response to bacterial infection. Verminous bronchopneumonia was also reported in a dugong *Dugong dugon* from Australia (Marsh et al. 2000) but was caused by *P. pulmonalis* infection, a nasal trematode taxonomically close to *P. cochleotrema* belonging to the same family, Opisthotrematidae (Blair 2005). The dugong described by Blair (2005) was in poor body condition, similar to the manatees in our study. However, the histopathological features of bronchopneumonia were not defined. While pathological descriptions are lacking for *P. cochleotrema*-associated lesions in American manatees, verminous tracheobronchitis caused by *P. pulmonalis* infection was reported in dugongs from Indonesia and Australia, and for the latter, considered to have an inconclusive role in mortality (Budiarso et al. 1979, Owen et al. 2012). Histologically, the affected dugongs had predominantly lymphoplasmacytic submucosal infiltrate of varying severity and mucosal papillary hyperplasia, squamous metaplasia, or ulceration (Budiarso et al. 1979, Owen et al. 2012). The features were generally consistent with changes observed in manatees, although 1 manatee (Case 3) had a higher severity of bronchial lesions associated with presumptive secondary bacterial infection. The lower respiratory tract lesions associated with *P. cochleotrema* in American manatees are also similar to those documented in association with the pterygoid sinus trematode *Nasitrema attenuata* in common bottlenose dolphins *Tursiops truncatus* (Kumar et al. 1975) and the nasal trematode *Prouterina wescotti* in a black bear *Ursus americanus* (Foreyt et al. 1996). Kumar et al. (1975) hypothesized that aspiration of trematode eggs was the main cause of pneumonia in the common bottlenose dolphin, and eggs were found in areas of fibrosing bronchiolitis in the black bear (Foreyt et al. 1996). Similarly, in Case 1, adult trematodes were grossly within lung abscesses, and trematode eggs were in alveolar exudate in the pneumonia of Case 5. Together, these findings support pathogenesis involving aspiration of *P. cochleotrema* life-stages. Bronchopneumonia may also represent lesion severity with an overflow of exudate from bronchi into lower airways or possibly a host-dependent hypersensitivity response to inhaled parasite-derived antigen. While cultures were not performed, the extent of abscessation seen grossly (Cases 1 and 5), the fibrin and heterophils seen microscopically in most lesions, and bacteria microscopically observed in association with eggs and trematodes (Cases 3 and 5) also suggest that associated bacterial infection may contribute to pathogenesis in

manatees. Therefore, the presence of specific bacterial endosymbionts harbored by the *P. cochleotrema* trematode may influence and contribute to the severity of the pathology, such as the inflammatory response in the tissues, as observed with other species of parasites and in other mammalian hosts (Kramer et al. 2008).

Previous reports documented *P. cochleotrema* in the lower respiratory tract of American manatees (Beck & Forrester 1988, Mignucci-Giannoni et al. 1999a,b, Carvalho et al. 2009) with severe trematode loads, mostly confined to the nasal cavity, but did not specify associations with clinical signs or lesions. In contrast, the present cases did not all necessarily have severe trematode loads yet still presented clinical signs or significant pathology in 4 of 5 manatees. In our study, the number of *P. cochleotrema* trematodes in the lower respiratory tract ranged from 18 to 182, versus over 490 in another study (Beck & Forrester 1988), without considering those in the nasal cavity. This disparity suggests that factors other than high parasite load may predispose to infection of the lower respiratory tract. Conversely, parasite load may be artefactually reduced when they are lost from the nasal cavity while the carcass is in the water or during transport (Beck & Forrester 1988). This factor is consistent with the number of trematodes documented in the present cases, where rescued manatees had a higher parasite load in the respiratory tract than some of the dead strandings. In addition, there is the possibility that parasites incidentally migrate into the lower respiratory tract postmortem, as demonstrated in Sprague-Dawley laboratory rats with the helminth *Hymenolepis diminuta* (Platt & Villanueva 1995). Therefore, those documenting the presence of *P. cochleotrema* in the lower respiratory tract of manatees in future studies should take the necessary steps to document associated lesions such as those described in this study to confirm their clinical significance.

The 2 live-stranded manatees were not clinically diagnosed with pulmonicolosis while alive, but pneumonia was suspected due to respiratory signs. Pulmonicolosis can be clinically diagnosed in manatees by visible expulsion of trematodes (Borges et al. 2017a), through observation of the nasal vestibule when the animal breathes, and auscultation of the respiratory tract to detect pneumonia by abnormal respiratory sounds such as crackles. In this study, the 2 rescued manatees had bradypnea (Cases 1 and 3) and nasal mucous discharge (Case 1), similar to a Florida manatee treated for pneumonia (Ball et al. 2016) and other aquatic mammals with respiratory helminths (Field 2022), providing support for the clinical diagnosis of pneumonia. The presence of concurrent nasal discharge containing trematodes is specifically suspicious for verminous pneumonia (Borges et al. 2017a,b) but was not observed in the present manatee cases. Radiographic, sonographic, and endoscopic studies can facilitate the diagnosis of pneumonia, tracheitis, bronchitis, and airway obstructions (Davis & Walsh 2018, Borque-Espinosa et al. 2020, Hall et al. 2022). The presence of eosinophilia on hematological examination can also raise suspicion of helminth parasitic infection (Cheepsattayakorn & Cheepsattayakorn 2014). These studies could not be performed in time for a definitive diagnosis due to the critical condition of both animals and death during transport, reducing the chances of complete clinical diagnostics within 24 h of rescue. However, eosinophilia must be assessed by a trained medical technologist, as manatee neutrophils are very similar morphologically to manatee eosinophils, leading to an erroneous diagnosis of eosinophilia (Mignucci-Giannoni & Alsina-Guerrero 2022).

Immunosuppression appears to be an important predisposing factor for lower respiratory pulmonicolosis in Greater Caribbean manatees, as is often the case when aquatic mammals succumb to infectious diseases (St. Leger et al. 2018), which can lead to weakness and malnutrition in the individual. Furthermore, the limited number of individuals in a population, such as the case in Puerto Rico, may contribute to decreased immune-system genetic diversity due to the population bottleneck, making them more vulnerable to acquiring infectious diseases (Rector et al. 2004). Conditions that are often implicated in causing immunosuppression of aquatic mammals include starvation (Bando et al. 2014), emaciation (Bossart et al. 2012, St. Leger et al. 2018), debilitating disease, brevetoxicosis (Duignan et al. 1995, Halvorsen & Keith 2008, Sulzner et al. 2012), cold stress syndrome (Bossart et al. 2002, Weisbrod et al. 2021) including calves, trauma related to watercraft collision (Bassett et al. 2020), or exposure to pollutants (Bonde et al. 2004). The Greater Caribbean manatees in this study had findings consistent with at least one of these conditions providing the basis for immunosuppression of the individuals, supported by their high frequency of concurrent infections, including systemic toxoplasmosis, mixed skin infections, and enterocolitis due to *Chiorchis groschafti* (Halvorsen & Keith 2008).

Climate change can influence the dynamics of diseases and impact the marine environment and aquatic mammals through various mechanisms, including changes in water temperature, quality and

chemistry, loss of food supply and their quality, increased atmospheric events, toxicity caused by harmful algal blooms, and the altered flow of terrestrial pathogens to the sea (Burge et al. 2014, Marsh et al. 2017, 2022, Deutsch et al. 2022). These factors can lead manatees to immunosuppression through environmental stress, making them vulnerable to contracting infectious diseases (Bonde et al. 2004, Marsh et al. 2017), resulting in behavioral, reproductive, and habitat changes (Marsh et al. 2022), and potentially culminating in epizootics or mass mortality events as observed in other aquatic mammals (Burge et al. 2014). While we cannot conclude from this study how climate change might specifically affect the incidence of lower respiratory pulmonicolosis in manatees, this condition was not diagnosed in Puerto Rican stranding surveillance in previous decades (Mignucci-Giannoni et al. 2000). Addressing the impact and understanding of climate change on disease dynamics in aquatic mammals and their environment is crucial to help conserve biodiversity and aquatic resources and promote animal and human health from a One Health perspective.

## 5. CONCLUSION

The pathological descriptions presented in this study will facilitate antemortem and postmortem diagnosis and management of pulmonicolosis in wild manatee populations, emphasizing the importance of completing comprehensive and thorough postmortem examinations on stranded aquatic mammals to detect unexpected causes and contributing factors to their mortality, and thereby improving the understanding of their diseases and current threats to survival. In addition, the valuable clinical and anatomical pathology information presented here will help encourage faster veterinary care and treatments to those rescued individuals, and lead to new strategies for better management of the species and its conservation and protection (Bonde et al. 2012, Weisbrod et al. 2021). The latter is instrumental in the conservation of the small and endangered population of manatees in Puerto Rico, which is significantly reduced in numbers in comparison to that of Florida and other populations in their natural range.

*Acknowledgements.* We thank G. Rodriguez, N. Jimenez, F. Guzmán, and their team from the Puerto Rico Department of Natural and Environmental Resources (PRDNER) for alerting us to these events and assisting in the rescue and salvage of the animals. We also thank the technicians, veterinarians, and especially the rescue volunteers of the Caribbean Manatee Conservation Center (CMCC) at the Inter American University of Puerto Rico for their participation and prompt attention to the strandings and for assisting in the collection of samples during the postmortem examinations. Support for this research was provided by a Ross University School of Veterinary Medicine Intramural grant (RUSVM grant ID #43011-2022) and a Morris Animal Foundation grant (MAF grant ID #D22ZO-822). The rescue, handling, and postmortem examinations of the manatees by the CMCC were permitted under US Fish and Wildlife Service (USFWS) permit #LOAFC 231088-A and PRDNER-Red Caribeña de Varamientos' Letter of Agreement 2020–2025. The present work was part of C.I.R.P.'s PhD thesis.

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*Editorial responsibility: Dave S. Rotstein, Olney Maryland, USA*
*Reviewed by: N. Castelblanco Martínez and 1 anonymous referee*

*Submitted: March 22, 2024*
*Accepted: June 10, 2024*
*Proofs received from author(s): July 28, 2024*