Short Communication

Occurrence of *Chiorchis fabaceus* (Trematoda: Cladorchiidae) in a wild Antillean manatee (*Trichechus manatus manatus*): New parasitological data for this imperilled sirenian

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ABSTRACT

Parasites impact wildlife populations and ecosystem health, thereby demanding regular monitoring. As part of the national conservation effort to preserve the free-ranging sirenians in Colombia, a parasitological survey was conducted between 2011 and 2023 on naturally deceased Antillean manatees (*Trichechus manatus manatus*). A total of 17 stranded carcasses were analysed for the occurrence of parasites. Herein, trematode adult stages were recovered from the stomach, small intestine, caecum and colon of an adult male manatee and microscopically analysed. Additionally, molecular analysis was conducted based on an almost complete coding sequence of the ITS1–5.8S-ITS2, partial sequence of 18S and 28S ribosomal DNA (rDNA) genes. Phylogenetic analysis clustered the trematode sequences within *Chiorchis fabaceus* species, thus constituting the first adult-based sequences isolated for the species. Presented data contribute to a better understanding of the sirenian parasite diversity.

1. Introduction

Manatees and dugongs, colloquially classified as sirenians, represent a large herbivorous aquatic mammal group inhabiting tropical and subtropical regions. Colombian free-ranging Antillean manatees (*Trichechus manatus manatus*) reside in the Orinoco River, the Caribbean coastline, and the Magdalena River Basin (MRB), and therefore of enormous importance for the conservation of this imperilled species (Debrot et al., 2023). Moreover, several reports evidence the devastating impact of anthropogenic pressure on wild manatees, mainly due to illegal hunting, watercraft collisions, sewage pollution, brevetoxicosis, accidental death in fishing nets, invasive alien species and habitat loss (Alvarez-Alemán et al., 2021; Bossart et al., 1998; Castelblanco-Martínez et al., 2009; Lazensky et al., 2021; Montoya-Ospina et al., 2001; O'Shea et al., 1985; Pyšek et al., 2020; Vélez et al., 2019; Wyrosdick et al., 2018). Regrettably, Antillean manatees in Colombia are currently in continuous decline, therefore seeming mandatory to strengthen national conservation programs (Castelblanco-Martínez et al., 2012). Manatees are robust long-living aquatic mammals considered as ideal sentinels for ecosystem health (Bonde et al., 2004). As other neotropical semiaquatic wildlife species, manatees should be considered as potential hosts for water-, food- and gastropod-borne parasites (Uribe et al., 2021; Vélez et al., 2018). Nevertheless, detailed knowledge on the life cycle, and epizootiology of manatee's parasites is still in its infancy and needing further investigations (Behringer et al., 2018; Vélez et al., 2019). There is also a lack of morphological and molecular information on manatee-specific parasites (Rivera-Pérez et al., 2024).

Trematodes are found all over the world, notwithstanding foodborne trematodiases are more prevalent in tropical and subtropical areas (Fürst et al., 2012; Robinson and Sotillo, 2022). The digenean trematodes are a diverse group of parasites with significant economic impact as a major cause of morbidity and mortality in animals and humans (Esch, 2002; Toledo and Fried, 2019). Moreover, the global trematode diversity is significantly underestimated, and thus trematode-

derived diseases have been neglected for years (Chai and Jung, 2022). At least one digenean trematode has been reported for most wildlife taxa, however the complex life cycles remain not fully described, and therefore the trematodes infecting wildlife species are neglected or still unknown (Bolek et al., 2019; Esch, 2002). Consequently, the sirenian trematodes life cycle of the species *Chiorchis fabaceus* and *Chiorchis groschafti*, *Moniligerum blairi*, *Nudacotyle undicola*, *Pulmonicola cochleotrema*, *Solenorchis travassosi* (syn. *Indosolenorchis hirudinaceus*), and *Zygocotyle lunata* are partially unsolved since larval stages and/or intermediate hosts remain undescribed (Bando et al., 2014; Blair, 1980; Vélez et al., 2019). Hence, the current study aims to present the first adult stage description of *C. fabaceus* from a free-ranging manatee in Colombia through necropsy-based approaches on morphometry and further molecular identification.

2. Materials and methods

2.1. Study area and sample collection

Between 2011 and 2023 a total of 32 deceased Antillean manatee carcasses were reported by local communities along the middle MRB and collected as part of the regional initiative for the conservation of manatees with the cooperation of non-governmental organizations, Colombian environmental authorities, and the Middle Magdalena Manatee Stranding Network (Table S1). The individuals were found dead floating in rivers, marshes, and wetlands in the departments of Antioquia, Bolívar, Cesar, and Santander. Based on the Köppen-Geiger climate classification manatee carcasses were retrieved from tropical monsoon climate in the Middle Magdalena Valley (Beck et al., 2018). Deceased manatees were morphologically identified and measured, thereafter assigned to 1 of 4 age classes based on body size (adults >226 cm, juveniles between >176 cm - < 225 cm, calves 141 - 175 cm and neonates <140 cm) in accordance with the Middle Magdalena Manatee Stranding Network guidelines and as described elsewhere (Arévalo-González et al., 2021; Mignucci-Giannoni et al., 2000; O'Shea et al., 1985The sex of animals was determined and the carcasses decomposition stage classified (Mignucci-Giannoni et al., 2000; Moore et al., 2020). The etiological death criteria were assigned by a member of the Species Survival Commission - Sirenia Specialist Group (IUCN SSC - SSG). A total of 17 Antillean manatee carcasses were necropsied and analysed during the study framework (Fig. 1). A complete evaluation of the manatees' skin was carried out searching for potential ectoparasites and external symbionts. The *in situ* macroscopic metazoan examination of the respiratory system (i. e., nasal cavity, trachea, bronchi, lungs, larynx), gastro-intestinal tract (i. e., throat, oesophagus, stomach, small and large intestine), pancreas, liver and bile ducts, heart, pulmonary arteries, spleen, and kidneys was performed by a parasitologist veterinary doctor. The collected metazoan parasites were immediately fixed in 90 % EtOH and RNAlater™ (Invitrogen™) preserved, incubated at 4 °C overnight, and stored at −20 °C until further analyses.

2.2. Parasite examination

2.2.1. Microscopical analyses

The 90 % EtOH preserved parasites morphometry was examined using the Olympus SZX7™ (Olympus Corporation, Tokyo, Japan) stereomicroscope system equipped with an Olympus DP27™ and SC30™ digital cameras. For digital pictures analyses the Olympus CellSens™ imaging software was used. The haematoxylin and eosin (H&E) staining procedure required the specimen dehydration with a graded ethanol series to 100 %. Thereafter, the specimens were embedded in paraffin and longitudinally cutting in serial-sections (6 μm-thick) with a Precision Rotary Microtome Lipshaw™ Type YSI-114. The sections were stained with haematoxylin and eosin (Sigma-Aldrich™) and observed under the light microscope above-mentioned. The adult parasite identification was made using general morphometry and original descriptions assisted by host species and location (Jones, 2005). Additionally, the whole specimens were carefully deposited on 10 mm diameter poly-L-lysine (Merck, Darmstadt, Germany) pre-coated glass coverslips (Nunc). Thereafter, the trematodes were fixed in 2.5 % glutaraldehyde (Merck) and thereafter post-fixed in 1 % osmium tetroxide (Merck), washed in double-distilled water (ddH₂O), critical point dried by CO₂-treatment and subsequently gold particles-covered as described for manatee parasite probes (Vélez et al., 2019). Afterwards, specimens subjected to SEM analysis were examined using XL30® scanning electron microscope (Philips, Hillsboro, USA) allocated at the Institute of Anatomy and Cell Biology, Justus Liebig University Giessen, Germany.
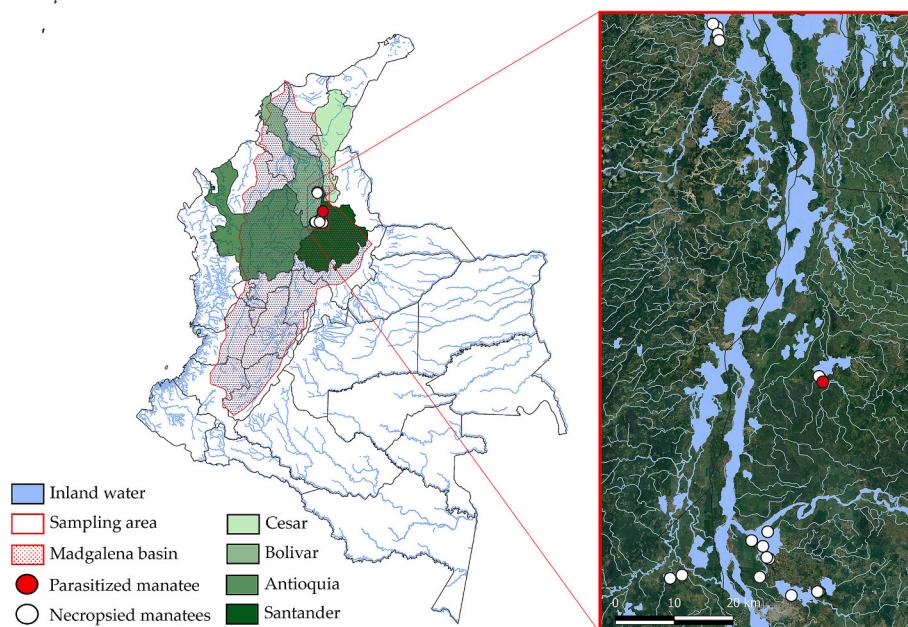

Fig. 1. Geographical location map of sampling area within the Magdalena River basin (MRB) in Colombia.

2.3. Molecular phylogeny

To characterize the metazoans collected during necropsy procedures, the specimens were rehydrated in descendant concentrations of EtOH. Thereafter, gently washed 5 times in 1× PBS solution, lysate in ALT buffer with 20 mg/ml proteinase K and incubated at 56 °C. Total DNA was obtained following the manufacturer instructions for the DNeasy Blood & Tissue Kit® (Qiagen, Dusseldorf, Germany). Almost complete ITS1–5.8S-ITS2 sequence, partial ribosomal regions of the small subunit (18S rDNA) and the large subunit (28S rDNA) were amplified using the specific primers as described elsewhere (Littlewood and Olson, 2014; Vélez et al., 2019). The DNA fragments were PCR-amplified following the thermocycle profiles as previously described (Vélez et al., 2018). The PCR amplicons were isolated from a preparative agarose gel using the HiYield Gel/PCR DNA Extraction Kit (Süd-Laborbedarf, Gauting, Germany). The PCR gene segments were bi-directionally sequenced by LGC Biosearch Technologies (Berlin, Germany). SeqManPro 7.1.0 (DNASTAR Inc., USA) was used to *in silico* edit and assembled the contig sequences. The alignments were conducted using the online version of MAFFTv. 7 (available at https://mafft.cbrc.jp/alignment/server) (Katoh and Standley, 2013). Sequences of representative trematode species within the different trematode superfamilies were included to reveal the phylogenetic position of analysed specimen. The phylogenetic relationships was inferred using the Neighbour-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) are shown next to the branches (Felsenstein, 1985). Nucleotide sequence divergences were calculated using best-fitting tested according to the corrected Akaike information criterion in IQ-TREE (Kalyaanamoorthy et al., 2017). For multiple substitution distance correction and the length branch units indicated the number of base substitutions per site (Kumar et al., 2018). Evolutionary analyses were conducted in MEGA11 to reveal the 18S rDNA, 28S rDNA and ITS1–5.8S-ITS2 phylogenetic position of the manatee isolated parasite (Tamura et al., 2021).

3. Results

3.1. Morphometrical parasite identification

During the necropsies neither ectoparasites nor external symbionts were detected in the skin of evaluated manatees (Fig. 2A). The peritoneum- and thoracic cavity macroscopical examination also resulted in the absence of macroscopic parasites. One of the 17 examined carcasses was found to be infected with trematode which constitutes 6 % of the analysed individuals. Thereafter the gastrointestinal examination of an adult male Antillean manatee the presence of trematodes in the posterior portion of the stomach, the small intestine to the caecum and the first portion of the colon (i. e., ascendant colon) were evidenced. During the necropsy light pink to reddish parasites were retrieved throughout the gastrointestinal tract. Most trematode specimens were collected from the ascendant colon (80.5 %; *n* = 1167) and caecum (10.5 %; *n* = 153). The remainder parasites were distributed along the stomach and small intestine (9 %; *n* = 130) of examined manatee. Furthermore, a low body condition, absence of food content in the anterior portion of the gastrointestinal tract, multiple gastric petechial areas, and perforated ulcers were noticed in the individual. Moreover, hyperreactive and swollen mesenteric lymph nodes (i. e., mesenteric lymphadenitis) were macroscopically noticed during the necropsy procedure (Fig. 2B). In addition, the gastrointestinal examination of the individual unveiled no macroscopic evidence of cestodes, acanthocephalans, nor nematodes.

The digenean trematode specimens' body were flattened dorsoventrally, elongate, conical, and cylindrical (Fig. 3A). Also, a bluntly rounded posterior end and a slightly tapered anterior end without oral lobes was evidenced. Tegument folds and grooves were slightly visible around the mouth opening area in the dorsal surface view. Distinctly a circular-shape subterminal large ventral sucker was noticed and the genital pore was located ventrally at mid-level of body (Fig. 3A-C). A well-developed sucker was noticed in collected parasites (Fig. 3 and Fig. S1). Herein described specimens showed a lack of a genital sucker. Additionally, collected trematodes were characterized by the absence of oral sucker as other amphistomes within the superfamily Paramphistomoidea (Tandon et al., 2019). The analysed specimens (*n* = 1141) mean morphometric measurements were total body length of 5.64 mm (±0.87), body breadth of 3.97 mm (±0.45), and a sucker opening of 1.14 μm (±0.07) x 1.17 μm (±0.11). The scanning electron microscopy (SEM) analyses showed an elongate middle to large robust body with maximum width at middle-body level and aspinose smooth tegument with folds and grooves in the acetabular region. Adult parasite stages exhibit a double-walled deeply seated sucker opening with post acetabular region cobble shaped, but no papillae were observed (Fig. S1). The mean depth of the subterminal sucker was 1 mm (±0.08). Based on the trematode characteristic traits and the parasite distribution within the host, the specimens described herein correspond well with digenean trematodes of the family Cladorchiidae (Jones, 2005).

3.2. Molecular phylogenetics

Additionally, to morphologically identify the digenean trematode within the family Cladorchiidae, fragments of almost complete coding sequence of the ITS1–5.8S-ITS2, partial sequence of 18S and 28S ribosomal DNA (rDNA) genes were analysed to stablish the precise phylogenetic relationship of manatee collected trematodes. The last version of BLAST+ 2.15.0 (Basic Local Alignment Search Tool) was used to compare the novel characterized nucleotide sequences, thus inferring the evolutionary relationships within the NCBI GenBank database (Sayers et al., 2022). Nucleotide sequences isolated in this study are

Fig. 2. The general necropsy procedure of adult Antillean manatee (*Trichechus manatus manatus*) male. (**A**) Supine positioning of de-skinned carcass shows the absence of ectoparasites, mayor scars and/or ventral trauma. (**B**) Transverse incision of the ascendant colon shows the presence of multiple non-segmented metazoan parasites (white arrowhead) that were collected *in situ*. Notice the hyperreactive and swollen mesenteric lymph nodes (red arrowhead). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
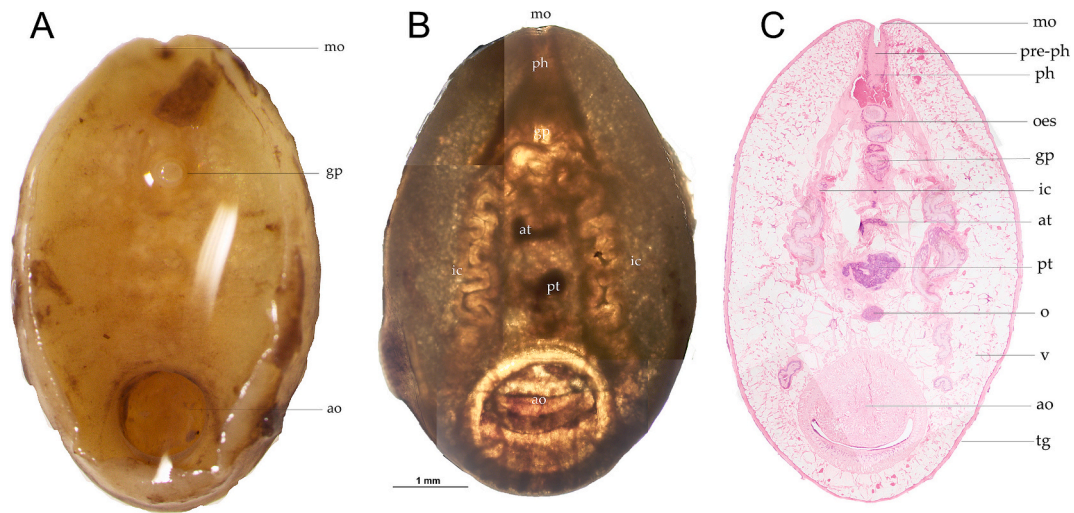
Fig. 3. Microphotograph images illustration of *Chiorchis fabaceus* adult specimen recovered from an adult male Antillean manatee (*Trichechus manatus manatus*) gastrointestinal tract in Cienega de Paredes (Santander), Colombia. (**A**) Ventral view of whole specimen. (**B**) Whole-mounted close-up merged photograph of adult trematode. (**C**) Longitudinal haematoxylin and eosin (H&E) stained section. Notice the small mouth opening (mo), pre-pharynx (pre-ph), pharynx (pH), oesophagus (oes), genital pore (gp) opening at the anterior one-third, intestinal cecum (ic), anterior (at) and posterior testes (pt), ovary (o), vitellaria (v), the large muscular caudal acetabular opening (ao) or ventral/caudal sucker and tegument (tg).

available in the GenBank, EMBL and DDBJ under the accession numbers: OR687246, OR687247, OR687248, and OR687299. Searches revealed that *Chiorchis fabaceus* (MF370224) was the closest match with high similarities of 99 %, 98 %, and 98 % respectively for the first three sequences above-mentioned. The phylogenetic analysis showed that trematode parasites collected from the Antillean manatee clustered within representatives of *Chiorchis fabaceus* (Fig. 4).

4. Discussion

Current study identified an adult Antillean manatee male infected with *Chiorchis fabaceus*. This trematode was the first described parasite in manatees almost two hundred years ago (Diesing, 1838). During the past century, the parasite was sporadically identified in Belize, Colombia, Dominican Republic, Florida, Mexico, and Puerto Rico based on the eggs morphology, but there are scarce descriptions and general
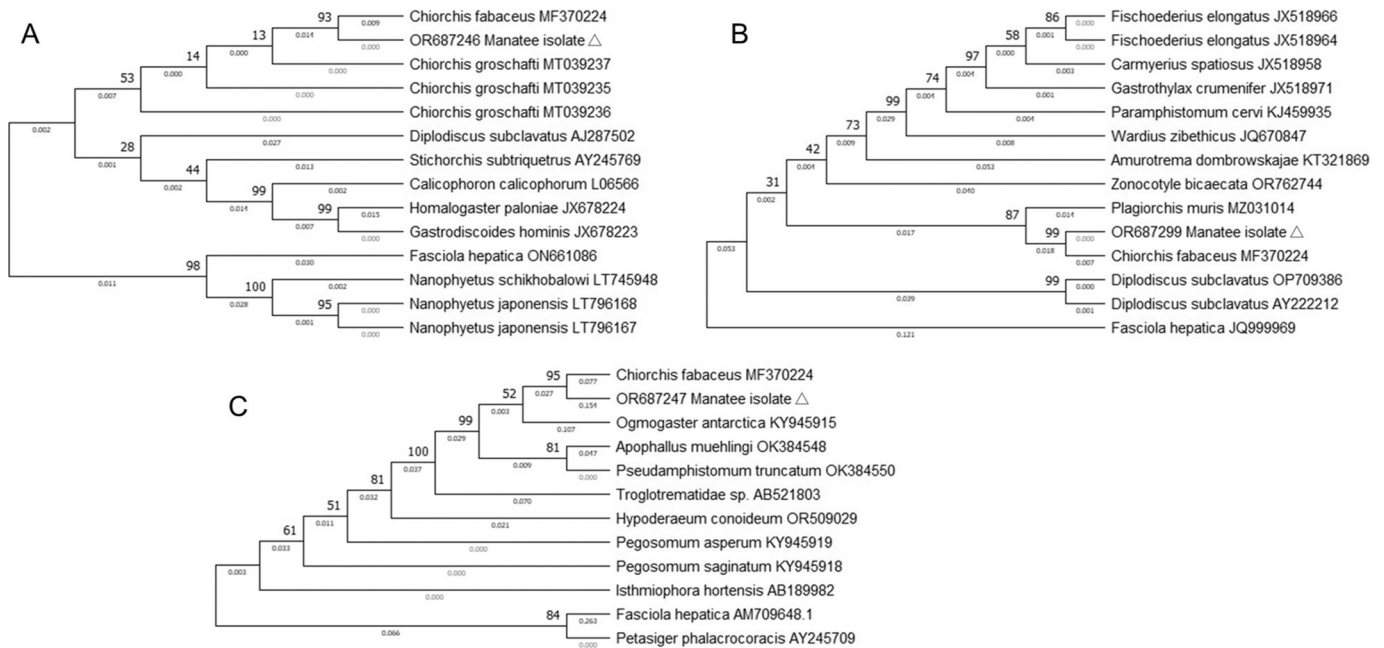
Fig. 4. Phylogenetic relationship of sequences-isolated from necropsied manatee parasite (green triangle). Optimal trees are inferred from 10,000 replicates represent the evolutionary history of analysed taxa. The percentage of trees in which the associated taxa clustered together is shown above the branches. The evolutionary distances are in the units of the number of base substitutions per site. (**A**) 18S rDNA phylogenetic analyses were computed using the Jukes-Cantor method. For (**B**) 28S rDNA and (**C**) ITS1 the Kimura 2-parameter method was used, the rate variation among sites was modelled with a gamma distribution (shape parameter = 1). All ambiguous positions were removed for each sequence pair. Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates were collapsed. The species *Fasciola hepatica* (ON661086, JQ999969, and AM709648), *Nanophyetus schikhobalowi* (LT745948) / *N. japonensis* (LT796168, and LT96167) and *Petasiger phalacrocoracis* (AY245709) respectively belonging to the Fasciolodae, Nanophyetidae, and Echinostomatidae were used as outgroups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

data on adult stages (Bando et al., 2014; Beck and Forrester, 1988; Bossart et al., 2012; Colón-Llavina et al., 2009; Lucot et al., 2020; Mignucci-Giannoni et al., 1999b; Mignucci-Giannoni et al., 1999a; Olivera Gómez, 2017; Vélez et al., 2019; Vélez et al., 2018). Herein, we describe the morphology of tegument, genital pore, and large ventral sucker of *C. fabaceus* adult stages. The parasites were recovered from the stomach and small intestine ($n = 130$), but the greatest number of specimens were concentrated in the caecum ($n = 153$) and colon ($n = 1167$) as described elsewhere (Beck and Forrester, 1988). It is also notable that manatee carcass evaluation shows a lower parasite diversity compared to coproparasitological samples analysed in Colombia (Vélez et al., 2019). However, necropsy procedure allows to collect *C. fabaceus* adult stages for the first time in Colombia. Morphological descriptions are still important although often not efficient for all trematode stages (Pitaksakulrat et al., 2022). During the past few decades molecular approaches for parasite identification have become an efficient, quick and reliable commonplace (Duflot et al., 2021). Therefore, the sister group relationships of *C. fabaceus* adult stages were inferred using ITS1–5.8S-ITS2, 18S and 28S rDNA genes.

Closely related amphistomes cause mucosal lesions and acute gastroenteritis, decrease feed intake, productivity and economic losses (Anuracpreeda et al., 2015; Tandon et al., 2019). The clinical chiorchiosis in manatees manifests with loss of appetite and consequent low body condition, foetid diarrhoea, dehydration, emaciation, extreme weakness with consequent exhaustion, oedema and subnormal temperature (Conga et al., 2022; Sreedevi et al., 2017). Smaller manatee trematodes such as *M. blairi* and *N. undicola* are associated with grossly visible haemorrhage, nodular, necrotic, and rugose intestinal mucosa, histologically by lymphoplasmacytic ulcerative enteritis with the consequent submucosal oedema and crypt atrophy (Arnett-Chinn et al., 2013; Dailey et al., 1988; Panike et al., 2017; Weisbrod et al., 2021). The *C. fabaceus* muscular ventral acetabulum is not only larger but also deeper than other manatee trematodes. Consequently, chiorchiosis could lead to extensive intestine ulcerative areas due to strong sucking activities of ventral acetabulum, thus resulting in manatee multifocal necrotizing gastroenteritis as described in other monogastric semi-aquatic mammals infected with parasites of the family Cladorchiidae (Uribe et al., 2021). Consequently, this underestimated trematode should be considered as a concomitant cause of disease in manatees with high parasitic burden.

Since trematodes require obligate intermediate hosts, it would be appropriate to analyse in depth gastropods, bivalve molluscs, and polychaete annelids in the manatee habitats to better understand the life cycle of parasitic trematodes in sirenians. Moreover, trematode larval stages should be investigated in manatee dietary sources (Allen et al., 2018; Takoukam Kamla et al., 2021). The wildlife parasite biodiversity is of great importance in terms of host conservation, and also to understand wild animals as reservoirs of non-zoonotic and zoonotic parasites as reported elsewhere (Gómez and Nichols, 2013; Sparagano et al., 2021; Thompson et al., 2010; Uribe et al., 2023).

5. Conclusion

Megaherbivore aquatic animals like sirenians have a pivotal role as sentinels of ecosystem health, harbouring a plethora of non-zoonotic and zoonotic parasites, thus evidencing feasible pathogen interchange in the human-animal interphase (Bonde et al., 2004; Fricke et al., 2022; Wright et al., 2002). Nonetheless, the lack of knowledge on the life cycle, occurrence, and pathogenesis of manatee-specific parasites requires interdisciplinary efforts to comprehend the transmission routes and manatee's population health impact. In conclusion the present study adds new insights on the neglected *C. fabaceus* infection in Antillean manatees as a baseline for further parasitological research on this imperilled aquatic mammal species. Also, expanding the parasite distribution range to MRB within Colombia. Further research is necessary to understand the complex trematode- intermediate hosts (gastropod)

interactions in order to develop effective diagnostic field tests, and determine the parasite burden (Pfukenyi and Mukaratirwa, 2018).

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CRediT authorship contribution statement

Manuel Uribe: Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis. **Katherine Arévalo-González:** Writing – review & editing, Resources, Funding acquisition, Data curation, Conceptualization. **Anja Taubert:** Resources, Funding acquisition. **Carlos Hermosilla:** Writing – review & editing, Validation, Supervision, Data curation. **Jenny J. Chaparro-Gutiérrez:** Writing – review & editing, Validation, Supervision, Funding acquisition, Data curation.

Declaration of competing interest

None.

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