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Occurrence of endoparasites in wild Antillean manatees (*Trichechus manatus manatus*) in Colombia

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ABSTRACT

The recognized impact of parasites in wildlife populations demands surveillance of endangered species like the Antillean manatees (*Trichechus manatus manatus*) in Colombia. We conducted a parasitological survey in four rescued sea cows in order to document the parasite diversity of this sirenian in the Caribbean wetland of Colombia and contribute to the molecular characterization of its trematodes. The flukes *Chiorchis fabaceus*, *Nudacotyle undicola* and the protozoans *Eimeria manatus* and *E. nodulosa* were identified in analysed faecal samples. For *C. fabaceus* and *N. undicola*, partial regions of ribosomal RNA genes were amplified and sequenced in order to infer their phylogenetic relations. The current study constitutes a new sirenian host (*T. manatus manatus*) record for the genus *Eimeria* and the trematode *N. undicola*.

1. Introduction

Given that the Antillean manatee (*Trichechus manatus manatus*) populations in Colombia are continuously decimated (Trujillo et al., 2013) it seems imperative to strengthen the conservation programs. Therefore, the manatee health status has to be monitored especially on the level of pathogenic infections. Concerning parasitic infections, earlier studies reported on several metazoans (Beck and Forrester, 1988; Mignucci-Giannoni et al., 1999; Bossart et al., 2012) and protozoans (Lainson et al., 1983; Upton et al., 1989; Borges et al., 2011, 2017; Bossart et al., 2012; Bando et al., 2014) in manatees. Most of these studies were conducted on captive manatees or on carcasses thereby hardly reflecting the actual status of healthy free-ranging animals (Beck and Forrester, 1988; Mignucci-Giannoni et al., 1999; Borges et al., 2016). Due to their diving activities, apnea capacity and the typical nature of their habitats (turbid rivers laden with tannins and muddy wetlands), it is difficult to observe and sample free-ranging manatee continental populations in South America. Furthermore, to date, the pathological significance of parasites in manatees and respective life cycles are almost unknown (Beck and Forrester, 1988) and parasitological studies have been limited to the morphological description and identification of eggs and adult specimens in faecal or tissue samples. Consequently, no studies are available on the molecular identification of manatee parasites. Moreover, reports on natural occurring *Eimeria* infections in manatees are limited and included merely three studies reporting on *E. trichechi* infections in the Amazonian manatee (*T. inunguis*) (Lainson et al., 1983) and on *E. manatus* and *E. nodulosa* infections in Florida manatees (*T. manatus latirostris*) (Upton et al., 1989; Bando et al., 2014). In regard to our knowledge there are only two reports on *N. undicola* infections, both in the Florida manatee (Dailey et al., 1988; Bando et al., 2014). Therefore, the current report constitutes the first description of *Eimeria* species and *N. undicola* in Antillean manatees and thus extents their geographical distribution to manatee populations in the Caribbean wetland from Colombia. This study also adds useful molecular information for further research seeking to extent the knowledge on pathological, ecological and epidemiological aspects of manatee trematode parasites.

2. Materials and methods

Faecal samples of four rescued Antillean manatees were collected after spontaneous defecation during routine clinical examinations within the OMACHA Foundation and the 'Corporación Autónoma Regional de los Valles del Sinú y del San Jorge (CVS)' program for rehabilitation and conservation of manatees in Santa Cruz de Lorica, Department of Cordoba, Colombia (9°13′25.79″ N; 75° 50′ 33.92″ W) (Fig. 1). The sampling procedure was in concordance with the Guidelines for the Treatment of Marine Mammals in Field Research of The Society for Marine Mammalogy.

The samples were submitted for coprological analyses including the

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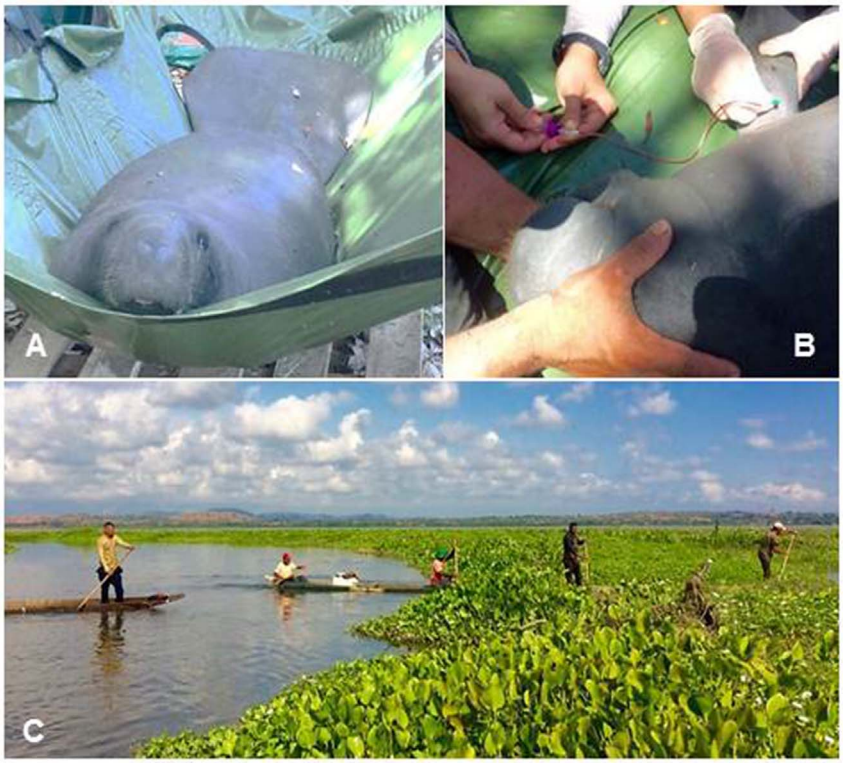

Fig. 1. A) Clinical evaluation of a calf manatee as part of the conservation programme of the OMACHA Foundation in Colombia. B) Blood collection for health status evaluation. C) 'Ciénaga Grande de Lorica', Cordoba, flowers of water spinach (*Ipomea aquatica*), are a favorite manatee food.

standard sodium acetate acetic acid formalin (SAF) technique with ethyl acetate (Yang and Scholten, 1977) and a modified sedimentation technique. Samples were examined microscopically and illustrated via a digital camera. Additionally, carbol fuchsin-stained faecal smears were conducted (Heine, 1982) for the detection of *Cryptosporidium* spp. oocysts. Commercial coproantigen-ELISAs were also performed for the detection of *Giardia* and *Cryptosporidium* infections (ProSpecT®, Oxoid).

For further molecular analysis, eggs of the trematodes were isolated and subjected to an eggshell destruction process through five freezing and thawing cycles. Samples were treated with liquid nitrogen for 1 min and following submitted to a rapid temperature increase to 99 °C. Thereafter, DNA was extracted using the DNeasy Blood & Tissue Kit® (Qiagen, Germany). Partial ribosomal regions of the small subunit (SSU), the large subunit (LSU) and 5.8S were amplified using the following specific primers: WormA, NF1, 18S, WormB, (for the SSU), ZX-1, NC2, Plagi 28S-r1, D3A, D3B (for the LSU) and NC1 (for the 5.8 S) (Littlewood and Olson, 2001). The final PCR reaction volume consisted of 50 µL containing 5 µL DNA template, 0.5 µL of bovine serum albumin (BSA, 10 mg/mL Sigma-Aldrich), 1 µL of forward- and reverse-primer (10 pmol/µl) and 10 µl of 5x HOT FIREPol Blend Master Mix 7.5 mM $MgCl_2$ (Solis BioDyne, Tartu, Estonia). Reactions were performed in a Veriti 96 thermocycler (Life Technologies, Darmstadt, Germany) using the following cycling conditions: 95 °C for 15 min (initial denaturation), followed by 40 cycles of 95 °C for 20 s (denaturation), 54 °C for 30 s (annealing) and 72 °C for 2 min 30 s. PCR amplicons were isolated from a preparative agarose gel using the HiYield Gel/PCR DNA Extraction Kit (Süd-Laborbedarf, Gauting, Germany) and thereafter cloned into pDrive vector (Qiagen, Hilden, Germany). Isolated recombinant plasmid DNA and PCR amplicons were bi-directionally sequenced by LGC Genomics (Berlin, Germany). The obtained DNA sequences of *C. fabaceus* and *N. undicola* have been submitted to GenBank under the accession numbers MF370224 and MF538578, respectively. For phylogenetic analysis, the D2-D3 28S rDNA region was used. A redundant dataset of sequences was chosen from the highest scoring BLAST results of GenBank which were posteriorly aligned by MUSCLE and used for a phylogenetic analysis using maximum parsimony and maximum likelihood methods with 1000 bootstraps by means of Mega 7 (Kumar et al., 1994).

3. Results

Overall, the coprological analyses revealed two metazoan and two protozoan parasite species. Among the latter, neither *Cryptosporidium* nor *Giardia* infections were detected. The metazoan species consisted of monoxenous manatee trematodes, *C. fabaceus* (Diesing, 1838) and *N. undicola* (Dailey et al., 1988). Illustrations of the respective trematode eggs are shown in Fig. 2. Morphological characterization of *C. fabaceus* eggs (n = 21) revealed a mean length of 162 µm (137–175 µm) and a width of 115 µm (103–131 µm), with an ovoid shape and a well-defined operculum at one pole. The eggs contained a light brown granular content surrounded by a thin eggshell, as typical for trematode species (Fig. 2 A). *Nudacotyle undicola* eggs (n = 18) had a mean size of 16.9 µm (15.01–20.13 µm) x 9.0 µm (8.26–10.33 µm), with an ovoid shape and characteristic elongated filaments on each pole whose bases were of equal widths; the content was granular and slightly diaphanous (see Fig. 2D).

The protozoan stages found in this study corresponded to oocysts of two *Eimeria* species. The unsporulated oocysts from *E. manatus* (Upton et al., 1989) had a mean size of 9.51 µm (8.58–11.95) x 9.09 µm (7.11–11.31), were spheroidal, with a thin, translucent oocyst wall lacking any micropyle and enclosing a spherical sporoblast within circumplasm (Fig. 2 B). *Eimeria nodulosa* oocysts (Upton et al., 1989) had a mean size of 12.05 µm (6.81–15.92) x 10.93 µm (10.60–13.87 10.37 µm), were spheroidal or sub-spheroidal with characteristic knob-like protrusions on their oocyst wall surface (Fig. 2C).

The phylogenetic analysis was performed on a sequence dataset of the D2-D3 region of the 28S rRNA gene (*C. fabaceus*: 859 bp; *N. undicola*: 853 bp). The overall topology of the inferred phylogenetic tree (Fig. 3.) agreed well with the analysis of Olson et al. (2003). As expected, the assignment of the two trematode species to superfamilies in phylogenetic analysis corresponded to their morphological taxonomy classification. Thus, the genus *Chiorchis* grouped to the superfamily Paramphistomoidea and *Nudacotyle* to the Pronocephaloidea.