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ANIMAL SCIENCE

Pathological changes by spirorchiid eggs in hawksbill sea turtle (*Eretmochelys imbricata*) stranded off brazilian coast

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Abstract: *Eretmochelys imbricata* (hawksbill turtle) is classified as a critically endangered species at world level; in Brazil, it is listed among the threatened species. Spirorchiids are parasites of the circulatory system of chelonians which may cause serious lesions in the various tissues of the host due to deposition of eggs in the bloodstream. In this context, the aim of the present study was to describe the pathology caused by spirorchiid eggs in *E. imbricata* from the Brazilian over a five year period. A total of 29 animals were analyzed, of which nine (31%) presented lesions associated to spirorchiids eggs. The lesions were: granulomatous enteritis in six (66.66%), granulomatous splenitis in five (55.55%), granulomatous hepatitis in three (33.33%), granulomatous pneumonia in three (33.33%), granulomatous pancreatitis in two (22.22%), and granulomatous adrenalitis in one specimen (11.11%). Concluded the main lesion in *Eretmochelys imbricata* was giant-cell granulomatous inflammatory reaction to the parasite's eggs. Animals exhibiting poor physical health were more susceptible to presenting such lesions.

Key words: giant-cell, granulomatous inflammatory reaction, hawksbill turtle, parasitology

INTRODUCTION

The hawksbill turtle *Eretmochelys imbricata* Linnaeus, 1766 is distributed worldwide and uses shallow tropical coastal waters as egg-laying and feeding grounds, preferring coral reefs and, to a lesser extent, subtropical waters (Spotila 2004, Marcovaldi et al. 2011). *E. imbricata* is one of the five sea turtle species found on the Brazilian coast. These turtles use the coast of the state of Espírito Santo as the feeding ground; however, egg laying occurs in Northeastern states (Marcovaldi et al. 2011). At present, this species of turtle is recognized worldwide as a critically endangered species (Mortimer & Donnelly 2015). It is also listed among the threatened species in Brazil according to the Ministry of the Environment and protected by federal law (Brasil 2018).

Spirorchiids (Digenea: Spirorchiidae) are parasites of the circulatory system of chelonians. Of the 19 known genera, 10 are exclusively found in sea turtles, and four of these are found in *E. imbricata* (Smith 1997, Werneck et al. 2014). The occurrence of these helminths of this family constitutes is a debilitating factor for sea turtles, which may even lead to death (Gordon et al. 1998, Santoro et al. 2007, Stacy et al. 2010). However, very little is known regarding the impact of this family of parasites on *E. imbricata* inhabiting the Brazilian coast.

In this context, the aim of the present study was to described the pathology of spirorchiid eggs in *E. imbricata* in Brazil over a 5-year period.

MATERIALS AND METHODS

Turtles inhabiting the shores of the states of Espírito Santo (18°42'58"S, 39°51'32"W) and north of Rio de Janeiro (22°57'58"S, 42°01'40"W), Brazil, observed in the respective monitoring programs, were included. The animals were either found dead after being stranded or died in rehabilitation centers over a 5-year period from 2010 to 2014.

During necropsy, samples of the esophagus, stomach, intestine, liver, pancreas, heart, lung, spleen, adrenal gland, kidney, and urinary bladder were collected; fixed in 10% buffered neutral formalin for at least 48 h; processed according to routine histological techniques; embedded in paraffin; cut in 5-µm-thick sections; and stained with hematoxylin and eosin (HE). All analyses of the viscera were authorized by federal licenses for activities with scientific purposes (SISBIO) 12431-4. Unfortunately, no techniques were used to collect parasites of the family Spirorchiidae during necropsy.

As described by Stacy et al. (2010), the body condition index of the sea turtles was evaluated as good, intermediate, or bad based on the musculoskeletal conditions. The body index was calculated by dividing the body weight (kg) by the cube of curved carapace length (CCL) (Stacy et al. 2010).

The tissue samples of *E. imbricata* were subjected to histopathological analysis to detect spirorchiid infection and assign a severity score. Only those samples positive for at least one parasite were included in the further analyses. The severity of infection was scored (1–3) as follows: 1, discreet (1-10 parasitic eggs and focal lesions); 2, moderate (10-30 parasitic eggs and multi-focal lesions); and 3, severe (more than 30 parasitic eggs and disseminated lesions).

Parasitic eggs in the tissue samples were morphologically identified as described by Wolke et al. (1982) and Chapman et al. (2019). Type 1 eggs are yellowish-brown, elongated (276 µm × 37 µm), and possess two processes, one of which is often curved. Type 2 eggs are ovoid (135 µm × 67 µm) and possess a sharp, short terminal process (±0.8 µm). Type 3 eggs are yellowish-brown, smaller and rounder (45 µm × 30 µm) than the other two types of egg, and lack the processes. The eggs observed by histopathological examination were evaluated using NIS-Elements BR and a Nikon Eclipse 80i microscope (Kurobane Nikon Co., Ltd, Otawara, Tochigi, Japan).

Descriptive statistics was used to analyze the histomorphological location of the lesions and to define the sites where the turtles were found. Chi-square test was applied to compare CCL, body mass, and body weight of the animals between the groups with and without lesions. Pearson's correlation coefficients were calculated for the association of lesion occurrence with CCL, body mass, body weight, and body condition. All statistical analyses were performed using Minitab® version 15.1.1.0, with a 5% significance level.

RESULTS

Of the 29 *E. imbricata* necropsied and microscopically evaluated, nine (31%) presented lesions associated with the eggs of parasites from the family Spirorchiidae; of these nine, eight (89%) were females (seven juveniles and one adult) and one (11%) was male (juvenile). All nine turtles with lesions were found stranded alive on the beach.

In turtles with spirorchiidiosis eggs, mean ± SD (range) CCL was 0.392 ± 0.47 (0.310–0.765)

m, body weight was 5.79 ± 2.79 (2.05–28.00) kg, and mean body index was 96 (75–133). There were differences ($p < 0.05$) in the way of being stranded between turtles with and without lesions. Parasitized turtles were more frequently found alive on the beaches. There were no differences ($p > 0.05$) in the sex, body condition, CCL, body mass, and body weight between turtles with and without spirorchiidiosis. Lesion occurrence was positively correlated with being stranded on the beach alive ($r = 0.37$, $p = 0.0251$), indicating that parasitized turtles are more likely to be stranded alive. Lesion occurrence was negatively correlated with body condition ($r = -0.335$, $p = 0.0381$), indicating that turtles with a poor body condition are more likely to have spirorchiidiosis.

Of the nine turtles with spirorchiidiosis, five (55.5%) were found in Espírito Santo [two in Guarapari (20°40'00"S, 40°29'51"W) and one each in Anchieta (20°48'21"S, 40°38'44"W), Aracruz (19°49'13"S, 40°1624"W), and São Mateus (18°42'58"S, 39°51'32"W)] and the remaining four (44.44%) were found in Rio de Janeiro [two in São Francisco de Itabapoana (21°18'07"S, 40°57'41"W) and one each in Cabo Frio (22°52'46"S, 42°01'07"W) and Arraial do Cabo (22°57'58"S and 42°01'40"W)].

Gross examination revealed whitish nodulation on the serosa of the intestine in 55.55% (5/9) and of the lungs in 22.22% (2/9) of the parasitized turtles; all animals presented with a poor body condition characterized by muscular atrophy.

Pathologies caused by the spirorchiid eggs were characterized by granulomatous reactions, with giant cells surrounding the parasitic eggs, and average severity score (Table I). These lesions were classified according to the affected organ and distributed among the infested animals as follows: granulomatous enteritis in six (66.66%), granulomatous splenitis in five (55.55%), granulomatous hepatitis in three (33.33%), granulomatous pneumonia in three (33.33%), granulomatous pancreatitis in two (22.22%), and granulomatous adrenalitis in one (11.11%). The most common average severity score was 1. Five turtles presented with inflammatory reactions in more than one organ. Tissue lesions caused by spirorchiid eggs in *E. imbricata* are presented in Figure 1.

Table I. Histopathology of parasitized *Eretmochelys imbricata* tissue samples and average severity score of spirorchiid infections. Tissues positive for at least one parasite were here included. Severity score is expressed as follows: 1, discreet; 2, moderate; and 3, severe.

Animal	Lungs	Spleen	Liver	Pancreas	Intestine	Adrenal gland
9175	1		1		1	1
9176	1				1	
9307			1	1	1	
9595	1	1	1	2	1	
9657		1			1	
10265		1				
10346		1				
10598					1	
10636		1				

The eggs were yellowish-brown and elongated, classified as type 1. In most cases, they were distributed focally, evoking an inflammatory response. Egg length was 165 µm, as measured for a single egg appearing whole in the histopathological section. Mean egg width was 39 (20–52) µm, as measured for several eggs distributed in the tissues.

DISCUSSION

Histopathological studies of *E. imbricata* are scarce. Glazebrook et al. (1989) and Glazebrook & Campbell (1990) have reported microscopic lesions caused by spirorchiid eggs in two hawksbill sea turtles from Australia. Through histological analysis, Dutra et al. (2012) reported lesions caused by parasitic eggs in the serosa of the intestine, lungs, heart, and liver in one hawksbill *E. imbricata* from Brazil. No lesions in the heart were detected in the present study. Lesions reported in the literature were acute, while those observed in the present study were chronic, characterized by granulomatous formation with giant cells. Santoro et al. (2015) analyzed one turtle found in Costa Rica and reported the presence of tissue lesions; histologically, they observed granulomas with

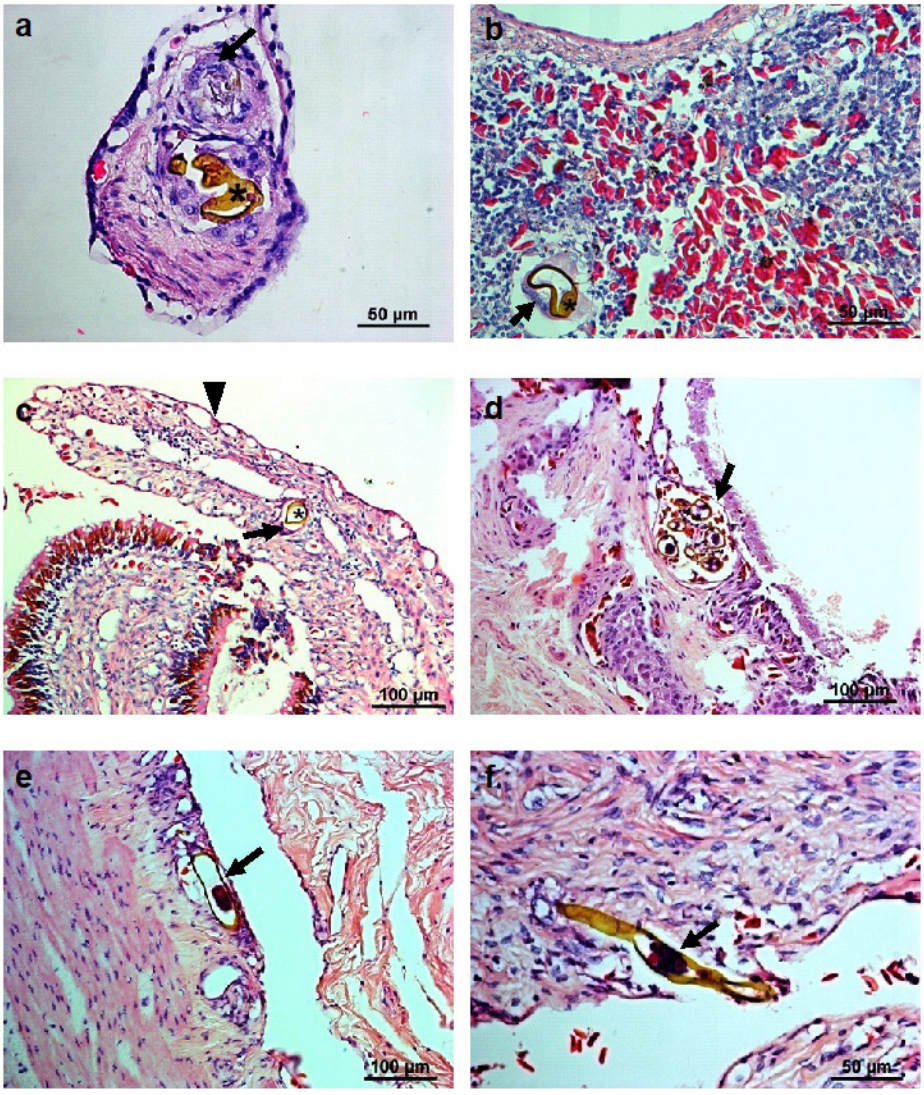

Figure 1. Tissue lesions cause by spirorchiids eggs in *Eretmochelys imbricata* stranded on the coasts of the states of Espírito Santo and Rio de Janeiro (Brazil). (a) Granulomatous pneumonia parasite eggs (*) with giant cells (arrow). (b) Granulomatous splenitis due spirorchiids egg (*) with giant cells (arrow). (c) Granulomatous enteritis due spirorchiids egg (*) with giant cells (arrow) in the lamina propria of eroded villus (arrowhead). (d) Granulomatous enteritis due spirorchiids egg, with an egg cluster in the serosa (arrow). (e and f) Yellowish-brown, elongated, thin-walled spirorchiid eggs (arrow), classified as type 1, in the intestine [hematoxylin–eosin staining (HE)].

spirorchiid eggs in the mucosal, submucosal, and muscular layers of the stomach, intestine, gallbladder, and liver. In the present study, granulomas were observed, but they were not found in the stomach. The gallbladder was not evaluated in the present study.

In the present study, nine of the 29 turtles (31%; eight juveniles and one adult based on CCL) exhibited lesions caused by spirorchiid eggs, and these turtles probably inhabited open-sea environments. Aznar et al. (1998) and Valente et al. (2009) have hypothesized that arrival at the continental shelf facilitates the contact between immunologically naive animals and parasites. Therefore, the first contact of the animals with such parasites constitutes a new challenge for their immune system.

The turtles examined in this study were from the states of Espírito Santo and Rio de Janeiro. Within the Brazilian territory, juvenile turtles are distributed throughout the North–Northeast coast and, to a lesser extent, in the South–Southeast (Marcovaldi et al. 2011). As the feeding grounds, these turtles use beaches in São Paulo, on the north coast, and in Ceará as well as the oceanic islands of Fernando de Noronha (Pernambuco), Atol das Rocas (Rio Grande do Norte), Abrolhos (Bahia), and Arvoredo (Santa Catarina) (Gallo et al. 2006). Moreover, as the Island of Trindade, located in Espírito Santo, serves as a feeding ground for *E. imbricata*, turtles are found on beaches in other municipalities of this state. However, although there are no reports of animals feeding in Rio de Janeiro, this state may be along the migratory rout of these turtles, and parasitized turtles are likely stranded on beaches in this state.

In the present study, nodules measuring 1-2 mm in diameter and containing parasite eggs in the tissues were observed on the serosa of the intestine in necropsy; such lesions have been described in *Chelonia mydas* (Gordon et al. 1998, Flint et al. 2009, Chen et al. 2012), *Caretta caretta* (Wolke et al. 1982), *E. imbricata* (Glazebrook & Campbell 1990, Dutra et al. 2012, Santoro et al. 2015), and *Lepidochelys olivacea* (Santoro et al. 2007, Jerdy et al. 2016). Moreover, such lesions have been observed in the pleura, the adventitious layer of the aortic arch and pericardium (Glazebrook et al. 1981), as well as in the lung parenchyma (Goldberg et al. 2013).

Microscopic tissue analysis revealed granulomatous reactions in various organs. According to Glazebrook et al. (1981), such reactions occur when the eggs laid by adult parasites reach the bloodstream, are disseminated in an embolical manner, and are retained in the arterioles and capillaries throughout the body. In this study, five turtles presented an inflammatory reaction in more than one organ, probably due to a high parasitic load.

In this study, the affected organs included the intestine, spleen, liver, lungs, pancreas, and adrenal glands. Because the spleen, liver, and lungs present an extensive network of blood vessels, they are more prone to egg deposition, particularly in the arterioles (Flint et al. 2009). In addition, the stroma of the intestine, pancreas, and adrenal glands also present blood vessels, allowing egg deposition. Similar to observations in the present study, parasites eggs have been observed in the lungs, spleen, and intestines of *L. olivacea* (Santoro et al. 2007, Jerdy et al. 2016, 2019) and *Caretta caretta* (Ribeiro et al. 2017). These observations may be related to the parasitic load of the animals.

The dissemination of parasitic eggs with granulomatous inflammatory responses in various organs corroborated the previous reports of lesions caused by spirorchiids in other sea turtles, including *C. mydas*, *C. caretta*, and *L. olivacea* (Glazebrook et al. 1981, Wolke et al. 1982, Dyer et al. 1991, Santoro et al. 2007,

Stacy et al. 2010, Chen et al. 2012, Dutra et al. 2012, Santoro et al. 2015, Jerdy et al. 2016, Ribeiro et al. 2017), in addition to *E. imbricata*. The predominant average severity score was 1, which is different from that reported by Santoro et al. (2020) in *C. caretta*. The spleen was the organ with the highest average severity score, followed by the intestine.

Similar to the report in *C. mydas* by Glazebrook et al. (1981), only type 1 eggs were identified in the present study. The average egg length and width were 165.4 and 39.4 µm, respectively, and the eggs occurred in different organs. The length was lower than that (276 µm) reported by Wolke et al. (1982) and (324 µm) Jerdy et al. (2019), but the width was comparable (37 and 42 µm, respectively). This may because egg length was evaluated in the tissue rather than measuring directly from fecal samples. Jerdy et al. (2019) found type 1 and 3 eggs in their study.

Type 1 eggs are typical of the genera *Amphiorchis*, *Hapalotrema* (*Learedius*), and *Monticellius* (Wolke et al. 1982, Chapman et al. 2019), all of which have been reported to parasitize *E. imbricata* worldwide (Werneck et al. 2014). From the Brazilian coast, *Monticellius indicum* and *Hapalotrema postorchis* (Werneck et al. 2015a) have been reported in the same host. In addition, type 2 eggs of *Carettacola stunkardi* (Werneck et al. 2008, 2015a) as well as *Amphiorchis caborojoensis* parasites (Werneck et al. 2008, 2015a, Dutra et al. 2012) have been reported in *E. imbricata* on the Brazilian coast. In the present study, no adult parasites were found during necropsy; therefore, there were no sufficient data to precisely identify the parasitic species.

The correlation between poor physical condition and lesion occurrence is consistent with the trends reported by Glazebrook et al. (1981), Wolke et al. (1982), Dutra et al. (2012), and Chapman et al. (2019). Although these authors did not perform statistical analyses, their studies *C. mydas*, *C. caretta*, and *E. imbricata* revealed that the parasitized turtles presented with cachexia. However, there is no explanation for the correlation between the poor physical condition and lesion occurrence in the literature. Propose two reasons. First, the lesions caused by parasitic eggs occur because the turtles are vulnerable due to a potentially suppressed immune defense because of the stress of being stranded. Second, a high parasitic load compromises the physical health of the turtles, and they are stranded as a result. Notwithstanding, further studies are warranted to test these hypotheses. In this light, Chapman et al. (2019) have proposed the development of diagnostic and therapeutic strategies for live turtles.

Studies reporting lesions caused by the eggs of members of the family Spirorchiidae in Brazil are scarce. Some studies have described tissue alterations in *C. mydas* (Goldberg et al. 2013, Werneck et al. 2006, 2015a, 2015b, 2015c, Jerdy et al. 2019), *L. olivacea* (Jerdy et al. 2016, 2019), and *C. caretta* (Ribeiro et al. 2017, Jerdy et al. 2019). Adult parasites of *A. caborojoensis* and *C. stunkardi* have often been reported in young turtles (Werneck et al. 2008, Dutra et al. 2012).

According to Santoro et al. (2017, 2020), the impact of a parasite on its host may depend on geographical factors and vary over time. Systematic monitoring of spirorchiid infections using PCR for molecular characterization and phylogenetic analysis is warranted.

The infections in this study did not lead to death or a strong impact on the general health status of the turtles. Similar results have been reported by Marchiori et al. (2017) in *C. caretta*. To the best of our knowledge, the present study represents the largest evaluation of *E. imbricata* individuals in Brazil, and our data will allow for further comprehensive assessments of the

parasitological state of turtles in the states of Espírito Santo and Rio de Janeiro.

CONCLUSIONS

In conclusion, the key pathology related to spirorchiid eggs in *E. imbricata* was characterized by a granulomatous inflammatory response with giant cells surrounding the parasite eggs. Animals with poor physical health are more susceptible to developing such lesions.

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Author contributions

Maria Aparecida da Silva and Eulógio Carlos Queiróz de Carvalho designed the study. Maria Aparecida da Silva, Raphael Mansur Medina, Hassan Jerdy Leandro, Rachel Bittencourt Ribeiro Rodrigues and Mariah Bianchi Reis Gusmão Petronilha contributed to the acquisition of data. Eduardo Shimoda performed the data analysis. Maria Aparecida da Silva drafting of the manuscript. Renato Luiz Silveira, Max Rondon Werneck and Eulógio Carlos Queiróz de Carvalho made a critical revision of the manuscript. All authors gave final approval for publication.