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Mycobiota from the eggs, nests and stillbirths of *Eretmochelys imbricata* Linneus 1766 (Testudines: Cheloniidae) in Pernambuco State, Brazil

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***Eretmochelys imbricata* Linneus 1766 was the subject of trade due to egg collection and consumption of the flesh of the females, being the fishery one of the main impacts towards the coastal area. The pathogens are also worrying factors of mortality of sea turtles especially those caused by fungi; these can cause the death of embryos and cutaneous mycoses. This study aimed to investigate the mycoflora isolated from soil, eggshells and stillbirths from *E. imbricata* in three beaches of Ipojuca (Brazil). We recorded data on the reproductive biology of the species after incubation of nests. Soil samples and fragments of eggshells were collected at the end of the nesting season for fungi identification. A total of eight species of fungi were identified by their morphological characteristics: *Aspergillus terreus*, *Aspergillus niger*, *Aspergillus flavus*, *Cladosporium cladosporioides*, *Nigrospora grisea*, *Fusarium solani*, *Fusarium lateritium* and *F. oxysporum* in the soil samples, eggshells and stillbirths. *Fusarium* was recorded in other studies interfering with the development of turtles embryos. The data from this study will provide information to support the management and conservation of sea turtles.**

Key words: Eggshell, fungi, *Fusarium*, Hawksbill, testudines.

INTRODUCTION

Eretmochelys imbricata Linnaeus 1766, commonly known as the hawksbill turtle, uses the beaches in the Brazilian states of Bahia, Rio Grande do Norte and Sergipe as its main nesting site (Marcovaldi et al., 2011); the breeding also occurring in the states of Pernambuco and Paraíba (Mascarenhas et al., 2003; Moura et al., 2012). In spite of its wide distribution, this chelonian is classified as critically endangered in the red list of the International

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Union for Conservation of Nature (Marcovaldi et al., 2011).

According to Gallo et al. (2006), one of the main anthropogenic impacts on chelonians is coastal fishing, which is considered the main cause of mortality of sea turtle. Other negative factors include the disorderly growth of coastal regions (artificial lighting, erosion, beach occupation by houses and hotels, vehicular traffic, the construction of ports and other marine structures) that degrade the marine environment and impact chelonian populations (Tuxbury and Salmon, 2005; Deem et al.,2007). Several studies also have reported finding plastic residues in the digestive tracts of sea turtles, resulting in their mortality (Thomas et al., 2002; Wabnitz and Nichols, 2010; Marcovaldi et al., 2011).

In addition to anthropogenic impacts, pathogenic agents have been factors causative of the mortality of sea turtles, including ectoparasites (leeches) and endoparasites (nematodes and trematodes) (Greiner, 2013), viral diseases (such as fibropapilloma, a debilitating or deadly disease for these animals, and now the focus of research investigations) (Baptistote et al 2005; Higgins, 2003; Rodenbush et al., 2014), respiratory diseases, and fungal infections (Cabañes et al., 1997; Phillott and Parmenter, 2001; Higgins, 2003; Coelho, 2009; Sarmiento-Ramírez et al., 2010).

Studies carried out to evaluate the influence of fungi on turtle eggs and hatchlings of *Caretta caretta* (Linneaus, 1758) have reported neonate mortality through mycoses caused by the fungal species *Fusarium solani* (Mart.) Sacc. (Phillott et al., 2004; Sarmiento-Ramírez et al., 2010) on Boa Vista Island (Cape Verde). These infections were considered responsible for the mortality of *C. caretta* embryos inside the nest (Sarmiento-Ramírez et al., 2010). Phillott et al. (2006) reported that these fungi degrade the calcium in sea turtle eggshells, facilitating the penetration of hyphae and consequently affecting the embryo. Cabañes et al. (1997) noted that *F. solani* isolated from chelonian rehabilitation tanks has caused cutaneous mycoses and weakened those turtles.

Fusarium species are actually a monophyletic “species complex”, that is *F. solani* species complex, including up to 60 species (O’Donnell, 2000; Short et al., 2013). This complex has a huge range of distribution and includes borne saprobes from soil and plant debris (O’Donnell et al., 2008; Short et al., 2011). Although, studies focusing on *F. solani* isolated from nests of sea turtles are still scarce (Sarmiento-Ramirez et al., 2014). *Fusarium* infections were identified in eggs of sea turtles in different stage of development, since the second week of incubation presenting initial infection, until advanced stage of incubation with evident mycelia inside the egg (Sarmiento-Ramirez et al., 2014).

According to Marcovaldi and Marcovaldi (1999), the turtle population on the Brazilian coast is considered one of the largest in the world, but additional studies investigating biotic and abiotic aspects that can influence the reproductive success of these animals are still needed, including the potential role of the mycobiota in the development of *E. imbricata*. In view of the impact caused in the reproductive success of this endangered species by fungi infections, we sought to isolate and identify fungi from nests of *E. imbricata* on the coast of Ipojuca (Pernambuco State, Brazil), due to provide data to the nesting conservation of this taxon.

MATERIALS AND METHODS

Study area

The study area was located on the beaches of Muro Alto, Cupe and Merepe (08°24'06"S and 35°03'45"W) in the municipality of Ipojuca, Pernambuco State, Brazil. The coastline there features areas of mangrove vegetation, Atlantic Forest remnants, flooded areas, vegetation shins, and restinga vegetation (sandy substrate, near-shore vegetation) (Santos, 2005). The Muro Alto Beach is characterized by sandstone reefs, with native vegetation behind the tidal zone. Cupe Beach has rock formations parallel to the coast that is breached in a number of locations, facilitating the entry of the waves that shape the coastline through sand deposition. Merepe Beach is 3.47 km long, without sandstone or coral reef barriers, and only sparse vegetation behind the tidal zone (Moura et al., 2012).

Sampling

Soil samples, eggshells, and stillbirths were collected from seven monitored nests of *E. imbricata* on the beaches at Merepe (3 nests), Cupe (3), and Muro Alto (1). Data of the seven nests were collected regarding the reproductive biology of the species determined after opening the nests, including: the numbers of eggs per nest, the numbers of live offspring, numbers of stillbirths (estimated from the number of offspring that had not completed their development), the numbers total of eggs originally laid (including those that had not completed their development), and the hatching success of each nest (Table 1); the numbers of nests per beach were also determined. These evaluations were carried out *in situ* during low tides during the rainy season in June/2011 on three beaches (Muro Alto, Cupe, and Merepe). The samples were collect from seven nests, which were opened to depths of approximately 50 cm, and soil samples, the shells of eggs and stillbirths were collected. The soil samples and egg fragments were placed in sterile bags, while stillbirths were sampled using sterile swabs rubbed on the skins of the animals (the swabs were subsequently held in sterile distilled water). All of the samples were transferred to the laboratory for proper processing.

Fungi isolation and identification

The soil samples were processed using the successive serial dilution technique proposed by Clark (1965), with modifications. Dilutions of the soil samples were made up to 1:1000 in Petri dishes containing Potato Dextrose Agar (PDA) with added chloramphenicol (100 mg/l). After dilution, the samples were sown into Petri dishes (as triplicates) containing PDA culture medium. The eggshell fragments were disinfected with sodium hypochlorite (3 min), rinsed 3 times with distilled water, and subsequently sown into Petri dishes containing PDA medium with 100 mg/l chloramphenicol (as triplicates). The swab samples from the stillbirths (1 ml aliquots) were sown into Petri dishes containing PDA with chloramphenicol (100 mg/l) (as triplicates). All the plates were

Table 1. Total of nests, number of living, stillborn and unhatching, and percentage of living.

Beaches	Nests	Living	Stillborn	Unhatching	% living
Merepe	1	2	0	111	1.76
Merepe	2	90	6	45	63.82
Merepe	3	26	0	85	23.42
Cupe	4	56	11	63	43.75
Cupe	5	0	0	97	0
Cupe	6	46	9	72	36.22
Muro Alto	7	100	4	11	86.96

Table 2. Fungi isolated from soil, stillbirths and unhatched eggs on the beaches Merepe, Cupe and Muro Alto (08°24'06'' S e 35°03'45'' W), in 2011.

Species/Beaches	Merepe			Cupe			Muro alto		
	Soil	Eggs	Stillbirths	Soil	Eggs	Stillbirths	Soil	Eggs	Stillbirths
Aspergillus flavus Link	X								
A. niger (Tiegh.)	X								
*A. terreus (*Thom)	X								
Cladosporium cladosporioides (Fresen.) G.A. de Vries						X			
Fusarium lateritium Nees					X				
F. oxysporum Schlecht.		X	X						
F. solani (Mart.) Sacc.	X	X	X		X	X			
Nigrospora grisea (Herbert) Barr							X		X

incubated in BOD incubator (26°C) during 72 h. To identify the fungal species, samples were transferred to specific media and their macroscopic and microscopic characteristics were subsequently evaluated using the specific literature.

RESULTS AND DISCUSSION

Eight fungal species were isolated and subsequently identified based on macrostructure and microstructure characteristics: *Aspergillus flavus, A. niger, A. terreus*, *Cladosporium cladosporioides, F. solani*, *F. lateritium*, *F. oxysporum*, and *N. grisea* (Table 2). A total of 58 x 10^3 g/ml of colony forming units were observed at Merepe Beach, followed by Cupe Beach (26 x 10^3 g/ml) and Muro Alto Beach (23 x 10^3 g/ml). Colonies of *F. solani* were more abundant on eggs, in the soil, and associated with stillbirths. The species identified from Merepe Beach were *A. flavus, A. niger, A. terreus* and *F. solani* isolated from soil; *F. oxysporum* and *F. solani* were also identified on eggs and Stillbirths. Cupe Beach had colonies from *F. solani*, *F. lateritium* and *Cladosporium cladosporioides* obtained from eggs and stillbirths. In samples from Muro Alto Beach only the species *N. grisea* was isolated from soil samples and on stillbirths. The genus Aspergillus occur commonly in soil in warmer climates, in compost, decaying plant matter, stored grain, and could survive in many environments, which are abundant in tropical and subtropical regions (Domsch et al., 2007; Rosa et al., 2002).

The amount of fungi found was proportional to the birth index of the nests. The reproductive success of Muro Alto (86. 96%) was the higher between the beaches, presenting lower colony forming units and the least fungi diversity. Besides that, only the species *N. grisea* was found in the samples collected in this beach. However, Moura et al. (2012) recorded Merepe beach with significant occurrence of *E. imbricata* nests and higher reproductive success in comparison with the other beaches (Cupe and Merepe), therefore we suggest increasing the number of samples for future analysis due to correlate the incidence of fungi contamination and the birth index.

According to Mader (2006), some species of opportunistic fungi cause infections in marine turtles. These microorganisms are usually saprobes but can invade living tissue under favorable conditions, and studies have reported that some of these fungi species infects sea turtle eggs and cause embryo mortality. Patino-Martinez et al. (2012) reported similar results in the eggs of the sea turtle *Dermochelys coreacea* in Colombia, where *F. solani* and *F. oxysporum* were

identified by phylogenetic analyzes; these fungi were possibly affecting the phenotype of the hatchling (body size). The first way of contamination of the eggs by *Fusarium* species probably occur through the secretion present in the oviduct, as suggested by Phillot et al. (2002), likewise it might be the reason for fungi contamination in nests of other species that belong to Cheloniidae family, since several species of fungi have been isolated from the cloaca of females during breeding. The most possible via of infection of the microorganisms come from spores present in the nesting substrate into the coacla during the copula with males (Phillott and Parmenter, 2001; Phillott et al., 2002).

Several biological and abiotical parameters influence the reproductive success of sea turtles, simlarly it might create conditions more farovables to the development of fungi infections in the nests (Phillott et al., 2002). In the present study, *F. solani* and *F. oxysporum* were observed in infected eggs and probably interfering in the development of embryo and increasing the mortality of sea turtles, as reported by Phillott and Parmenter (2004). Phillot and Parmenter (2004) identified and analyzed the mycobiota in nests of *E. imbricata*, *Chelonia mydas, Natator depressus* and *Caretta caretta* in Australia and noted that *F. solani* and *F. oxysporum* were present on the eggshells. Sarmiento-Ramírez et al. (2010) also observed *F. solani* on the eggshells and embryos of *C. caretta* in Cape Verde, and determined that this specie was responsible for mass nest mortality. Phillott et al. (2006) likewise reported that calcium losses in eggs could be attributed to the presence of fungi and would interfere with embryonic development. Cabañes et al. (1997) reported skin fungal infections in weakened marine turtles in recovery tanks mainly caused by *F. solani*. According to Wiles and Rand (1987), skin mycoses are more frequent among sea turtles in captivity than in their natural environment.

According to Marcovaldi et al. (2011) these monitoring and nesting biology studies are of significant importance, because the data will help in improving the conservation plans for *E. imbricata* - classified as critically endangered in Brazil based on data available until the year 2009 and being considered for similar conservation status in other countries (IUCN, 2008). The hawksbill is among the most endangered species of sea turtles among the seven existing species in the world (Wallace et al., 2011).

Species of the genus *Fusarium* are not part of the normal microbiota of marine animals, as they are saprobic fungi that normally live alone or as plant pathogen (Frasca et al., 1996), but studies have shown that opportunistic infections caused by these fungi occur relatively frequently in humans and animals (such as sea turtles) (Rebell, 1981). *Colletotrichum acutatum* was identified as the causative agent in the death of a young specimen of *Lepidochelys kempii* in Florida in 2000 (Manire et al., 2002), emphasizing that the immune statuses of animals will determine their pathogen resistance. Elshafie et al. (2007) reported 14 species of fungi isolated from soil and eggs in *C. mydas* nests, with high incidence of *Aspergillus* species, especially: *A. flavus*, *A. niger*, *A. terreus*, *A. nidula*s, *A. fumigatus*, and *A. ochraceus*. Among these, *A. flavus*, *A. niger* and *A. terreus* were likewise isolated from the soil on the beaches at Merepe and Cupe (Ipojuca- PE), and it appears that fungal growth on eggs and on the sea turtles themselves, and the production of mycotoxins, will affect embryonic development and contribute to chelonian mortality.

Our research has shown the presence of *F. solani*; it could be considered another threat to sea turtle, especially on beaches with anthropic impacts, as the occupation of reproductive and consequently the decrease of spawning areas. Beaches that are more exposed to environmental pressures such as erosion, sediment movement, and anthropic pressure as pollution might been more susceptible to the colonization of these pathogenic fungi. The nests sampled in this study were located in beaches with high exploitation of tourism and under anthropic pressure.

We characterized the mycobiota present in the nest envionment of *E. imbricate*. Finding the pathogenic fungi infections, and understanding the environmental conditions that favor colonization by *Fusarium* and other pathogenic species of fungi within the nests of sea turtles still need to be clarified as well as their influence in the development of sea turtles.

Conflict of interests

The authors did not declare any conflict of interest.

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