Bacterial and fungal pathogens in granulomatous lesions of *Chelonia mydas* in a significant foraging ground off southern Brazil

Bacterial and fungal pathogens in *Chelonia mydas* granulomatous lesions

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**Summary**

The green sea turtle *C. mydas* inhabit near-shore areas exposed to threatening anthropogenic activities. The granulomatous lesions in these animals may indicate infectious diseases that can be associated to environmental contamination and hazards to human health. This study aimed to characterise the granulomatous inflammation associated with bacterial and fungal infection, and also the cause of death in *C. mydas* off Paraná state, a critical foraging ground for juvenile green turtles in southern Brazil. From September 2015 to February 2019, a systematic monitoring was performed by the Santos Basin Beach Monitoring Project for sea turtles carcasses recover, necropsy, and cause of death diagnosis. The tissue samples were fixed in buffered formalin 10% for histochemical analysis and frozen for molecular analysis to fungi detection (Internal Transcribed Spacer region of the nuclear rDNA) and to bacteria detection (16S ribosomal gene). From a total of 270 *C. mydas*, granulomatous lesions were observed in different organs of 63 (23.3%) individuals. The histological analysis indicated lesions in 94 organs, affecting most respiratory and digestive systems. Bacteria were identified in 25 animals, including an acid-fast bacteria detected in one animal, and fungi in 24 green turtles. The presence of granulomatous disease was not associated with sex and body condition, nor the type of granulomatous lesion with the infectious agents evaluated. The fungi species included the genus Candida (*Candida zeylanoides*, n=3), Yarrowia (*Yarrowia lipolytica*, n=9; *Yarrowia deformans*, n*=*4; and *Yarrowia divulgata*, n=1), and *Cladosporium anthropophilum*, n=1). No species of bacteria was identified. All fungi species identified are saprobic, some are important to food and medical industries, but are also pathogens recorded from diseases in humans and other animals. Therefore, a long-term monitoring of those pathogens and the *C. mydas* health is necessary to indicate changes in environmental quality, and to assess possible zoonotic diseases and its effects.

Keywords: *Candida zeylanoides*, *Cladosporium* *anthropophilum*, infectious diseases, sea turtles, South America, *Yarrowia* spp.

Introduction

The sea turtles inhabit a diverse ecological niche of coastal and oceanic habitat, migrating among areas during their life cycle (Luschi et al., 2003). The juvenile’s green turtle (*Chelonia mydas*) may feed in near-shore areas, resulting in increased exposure to threatening anthropogenic activities. Some significant threats include bycatch in fishing gears, injuries from vessel an dredge collisions, chemical contamination, and potential infectious agents, as multidrug-resistant pathogens from runoffs discharges (Domiciano et al., 2017; Fuentes et al., 2020; Goldberg et al., 2019). Owing to threats, high mortality, and low population recovery, *C. mydas* is considered globally ‘endangered’ by the International Union for Conservation of Nature (IUCN, 2020).

The information about infectious diseases and their impacts on conservation status of *C. mydas* is scarce (Domiciano et al*.*, 2017). Aside from fibropapillomatosis associated with a Chelonid Alphaherpesvirus 5 (Hargrove et al., 2016), and parasitosis caused by trematodes of the family Spirorchiidae (Chapman et al., 2019), epidemiological aspects of other infectious and parasitic diseases are poorly known worldwide. Several species of bacteria and fungi have been identified in isolated cases of *C. mydas* lesions, but few studies had a systematic and continuous evaluation of sea turtles diseases. The limited studies including these pathogens identification were performed in free-ranging animals from USA (Innis et al., 2009, Work et al., 2015), Spain (Orós et al., 2003 and 2005) and Australia (Flint et al., 2010; Gordon et al., 1998; Raidal et al., 1998); in captive animals from USA (Brock et al., 1976), Australia (Glazebrook & Campbell, 1990), Thailand (Chuen-Im et al., 2010) and UK (Jacobson et al., 1979 and 1986; Homer et al., 1994).

Studies concerning the systematic evaluation of the presence of bacteria in lesions of *C. mydas* identified *Mycobacterium* spp., *Mycobacterium avium*, *Streptococcus* spp., *Micrococcus* spp., and *Shewanella* spp. in granulomatous pneumonia (Brock et al., 1976; Flint et al., 2010; Jacobson et al., 1986); *Citrobacter freundii, Aeromonas hydrophila*, *Vibrio alginolyticus*, *Vibrio* *parahaemolyticus*, *Micrococcus* spp., *Corynebacterium* spp., *Staphylococcus* spp., *Streptococcus* spp., *Aureobacterium* spp., and *Flavobacterium* spp. in caseous and ulcerative stomatitis (Chuen-Im et al*.*, 2010; Glazebrook & Campbell, 1990), rhinitis and secondary bronchopneumonia (Glazebrook & Campbell, 1990); *Vibrio alginolyticus*, *A. hydrophila, Shewanella* spp., *Flavobacterium* spp., *Staphylococcus* spp., and *Edwardsiella* spp. in ulcerative dermatitis (Chuen-Im et al., 2010; Glazebrook & Campbell, 1990); *Salmonella* spp. in systemic granulomatous infection (Raidal et al., 1998); *Salmonella enteritidis*, *Shewanella putrefaciens*, *Bacillus* spp., *C. freundii, Serratia marcescens*, *A. hydrophila*, *Citrobacter* spp., *Moraxella* spp*.*, *Escherichia coli,* *V. alginolyticus*, *Micrococcus* spp., *Chlamydophila* spp.,and *Aureobacterium* spp. in necrotising and granulomatous hepatitis (Chuen-Im et al., 2010; Glazebrook & Campbell, 1990; Gordon et al., 1998; Homer et al., 1994; Raidal et al., 1998); *M. avium* in granulomatous nephritis (Brock et al., 1976); *C. freundii* and *Chlamydophila* spp. in necrotising and granulomatous splenitis (Gordon et al., 1998; Homer et al., 1994); *S. putrefaciens*, *S. marcescens*, *A. hydrophila*, and *Chlamydophila* spp.in necrotising and fibrinous pericarditis(Glazebrook & Campbell, 1990; Gordon et al., 1998; Homer et al., 1994); and *Pseudomonas* *aeruginosa* in necrotizing adenitis (Glazebrook & Campbell, 1990).

The presence of fungi species identified in systematic evaluations in lesions of *C. mydas* were *Candida albicans* in caseous or ulcerative stomatitis (Chuen-Im et al., 2010), and *Sporotrichium* spp., *Cladosporium* spp., *Paecilomyces* spp., *Penicillium* spp., and *Fusarium* spp. in granulomatous pneumonia (Glazebrook & Campbell, 1990; Jacobson et al., 1979 and 1986); *Aspergillus* spp., *Penicillium* spp., *Fusarium* spp., and *Cephalosporium* spp. in granulomatous, necrotising or acute dermatitis (Jacobson et al., 1986); and *Aspergillus* spp. and *Cladosporium* spp. in granulomatous hepatitis (Jacobson et al., 1986).

Most of the bacteria and fungi cited above are ubiquitous and part of the microbiota of respiratory and digestive systems of sea turtles and other reptiles (Nardoni et al., 2008; Santoro et al., 2006). However, they can cause secondary diseases, including zoonotic diseases, and were also associated with traumas caused by fishing gear or debris ingestion (Cantas and Suer, 2014; Chuen-Im et al., 2010; Ebani et al., 2017; Orós et al., 2005; Sandoval-Denis et al., 2016; Work et al., 2003). Additionally, the systemic infection was the cause of death in most cases cited above (Gordon et al., 1998; Raidal et al., 1998; Chuen-Im et al., 2010).

In reptiles, granuloma is considered a hallmark of inflammatory response to a variety of bacterial, fungal, and parasitic infections (Stacy & Pessier, 2007). The histological classification includes three types of granuloma: ‘heterophilic’ associated with extracellular pathogens – most bacteria and fungi – or with injuries that lead to infiltration of heterophils; ‘histiocytic’ usually associated with obligate intracellular bacteria, but also observed in fungi infection and major macrophage infiltrate; and ‘chronic’ that may result from a persistence of heterophilic or histiocytic granulomas with fibrous connective tissue and scattered infiltrate (Stacy & Pessier, 2007).

Notwithstanding above, we estimated the prevalence and types of granulomatous diseases in *C. mydas* off Paraná state, southern Brazil. Further, we sought to detect and identify bacteria and fungi agents and the possible association with granuloma type and with the cause of death. Lastly, we identified potential hazards to human and environmental health.

Materials and Methods

Study area and sampling

The studied area encompasses the Paranaguá Estuarine Complex (PEC) and the adjacent coastline (25°44’S and 48°29’W) off Paraná state, southern Brazil. Since September 2015, a daily beach monitoring was carried out by the Santos Basin Beach Monitoring Project (Projeto de Monitoramento de Praias da Bacia de Santos - PMP-BS[[1]](#footnote-1)) for sea turtles carcasses recover.

The *C. mydas* found stranded dead were autopsied. The carcasses decomposition state was classified in ‘fresh’, ‘moderate’, and ‘advanced’. The curved carapace length (CCL) was measured to nearest 0.1 cm, the specimens in fresh or moderate decompositions were weighed (body mass to the nearest 0.1 Kg), and the body condition index (BCI) was derived (Bjorndal & Bolten, 1989; Work et al., 2015). Additionally, visual body condition was estimated and classified in ‘good’, ‘fair’, ‘poor’, and ‘emaciated’, according to volume or skeletal muscle and fat tissue (adapted from Work et al., 2015), and grouped in ‘better conditions’ (good and fair) and ‘worst conditions’ (poor and emaciated). All *C. mydas* were macroscopically evaluated, and granulomatous lesions were noted. Sample tissues from different organs were systematically collected, fixed in 10% buffered formalin solution, routinely processed, embedded in paraffin, and stained with hematoxylin and eosin (HE) for histological analysis or frozen at - 20°C for molecular analysis. Furthermore, gonad samples were evaluated for sex and stage of development classification.

The cases that had the autopsy, microscopic analysis and cause of death diagnosis performed from September 2015 to February 2019 were included in this study. All granulomatous lesions were classified as ‘heterophilic’, ‘histiocytic’ or ‘chronic’ in HE (Stacy & Perssier, 2007). Then, histochemical techniques including Grocott (for fungi), Gram – modified Brown and Brenn (for bacteria), and Ziehl-Neelsen (for acid-fast bacteria) were used to detect the etiological agents. The cases of parasitic granulomas were not considered in this study. The cause of death was classified as: ‘anthropogenic’, associated with harmful human activities (e.g. bycatch in fishing gears, debris ingestion, boat collision, and vandalism with related secondary sepsis); ‘euthanasia’; ‘natural’ associated with infectious diseases; and ‘unknown’ associated with no obvious diagnosis. The possible primary or secondary contribution of granulomatous lesions to cause of death was evaluated.

Molecular Analysis

The DNA of frozen tissue diagnosed with granulomatous lesions was extracted using Wizard Genomic DNA Extraction Kit (Promega, USA) in accordance with the manufacturer´s instructions. Two microliters of the extracted DNA was submitted to Polymerase Chain Reaction assay for fungi detection using ITS1 and ITS4 primers to amplify the internal transcribed spacers 1 and 2 and the 5.8S of the nuclear rDNA (White et al., 1990), and for bacterial detection using FD1 and RD1 primers to amplify ribosomal 16S gene (Weisburg et al., 1991). The PCR mixture (25 µl) for fungi detection comprised 1.5 µl (20 pmol.µl-1) from each of the primers cited above, 2.5 µl of deoxyribonucleotide triphosphate (dNTP; 200 mM.µl- of each dNTP – Invitrogen, Life Technologies, Carlsbad, USA), 0.2 µl (1 unit) of Platinum Taq DNA polymerase (Invitrogen, Life Technologies, BRA), 2.5 µl PCR buffer (1x, 20 mM Tris–HCl pH 8.4 and 50 mM KCl), 1.0 µl (1.5 mM.µl-1) of MgCl2, 2.5% DMSO, and ultrapure sterile water to final volume. The PCR mixture (20 µl) for bacteria detection comprised 1.5 µl (20 pmol.µl-1) from each of the primers cited above, 0.4 µl of dNTP (200 mM.µl-1 of each dNTP – Invitrogen, Life Technologies, Carlsbad, USA), 0.2 µl (1 unit) of Platinum Taq DNA polymerase (Invitrogen, Life Technologies, BRA), 2.0 µl PCR buffer (1x, 20 mM Tris–HCl pH 8.4 and 50 mM KCl), 1.0 µl (1.5 mM.µl-1) of MgCl2, 2.5% DMSO, and ultrapure sterile water to final volume. The positive controls were obtained from colonies and were extracted as described for tissue samples. Amplification was performed in a thermal cycler (Applied Biosystems 2720 Thermal Cycler, USA) with previous described cycling profiles (Weisburg et al., 1991; White et al., 1990). The PCR products were analysed by electrophoresis in a 2% agarose gel in Tris-Borate-EDTA buffer, pH 8.4 (89 mM Tris; 89 mM boric acid; 2 mM EDTA), stained with ethidium bromide (0.5 µg/ml), and visualised under ultraviolet light. The PCR products were purified using the PureLink Quick Gel Extraction kit (Invitrogen, Life Technologies, BRA), quantified using the spectrophotometer SLIPQ026 – L-Quant Quantifier (Loccus Biotecnologia, BRA), and sequenced using an ABI3500 Genetic Analyzer sequencer with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) with the primers used in PCR assay. Sequence quality analysis and contig assembly were performed with PHRED and CAP3 (<http://asparagin.cenargen.embrapa.br/phphs>) software, respectively. Similarity searches were performed against sequences deposited in GenBank using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Nucleotide (nt) sequence identity matrix was constructed using the BioEdit software version 7.2.6.1. Phylogenetic tree based on the nt sequences was obtained using the neighbor-joining method with the kimura two-parameter model using MEGA software version 7.0.26. The bootstrapping probabilities were calculated using 1,000 replicates.

Data analyses

The qui-square test was used to verify the relationship between granuloma and biological features (sex and visual body condition); and granuloma types and etiological agents (fungi and bacteria). Additionally, the t-test was used to verify differences between BCI and (i) groups of visual body condition, and (ii) cause of death (associated or not to granuloma inflammation). The IBM SPSS Statistics version 25 was used for statistical analysis; p-value <0.05 was considered significant.

Results

In total, 270 *C. mydas* were autopsied, gross and histological analysis were performed, and the cause of death was established. The granulomatous lesions were observed in different organs from 63 (23.3%) animals. Of the affected animals, 41 carcasses were ‘fresh’, 19 in ‘moderate’, and three in ‘advanced’ decomposition states. The average CCL was 37.7 ± 7.2 cm ( ± SD), the weight was 5.8 ± 3.3 Kg (*n* = 49/63; 77.8%), and the BCI derived was 1.19 ± 0.26 (*n* = 49/63; 77.8%). The visual body condition was ‘good’ in five animals, ‘fair’ in 23, ‘poor’ in 20, ‘emaciated’ in 12, and three animals were not evaluated owing to advanced decomposition. The frequency of ‘better’ (*n* = 28) and ‘worst’ body conditions (*n* = 32) was similar and it was not associated with granuloma presence (*p* = 0.204). Confirming the visual assessment, BCI of ‘better’ body conditions ( ± SD = 1.38 ± 0.2) was different of ‘worst’ body conditions ( ± SD = 1.03± 0.1; *p* = 0.001). Additionally, the BCI of animals that died from granulomatous lesions ( ± SD = 1.19 ± 0.2) or other causes ( ± SD = 1.20 ± 0.3) was similar (*p* = 0.107). Furthermore, 53 (84.1%) animals were females, nine (14.3%) were males, and in one animal the sex was not identified. No relationship between sex and granulomatous inflammation was observed (*p* = 0.424).

The gross findings observed in the organs with granulomatous lesions confirmed microscopically are described in Table 1 and lesions demonstrated in Figure 1. The distribution of lesions varied, including focal (*n* = 9), focally extensive (*n* = 1), multifocal (*n* = 32), multifocal to coalescing (*n* = 6), and disseminated (*n* = 28); and the intensity included mild (*n* = 19), mild to moderate (*n* = 14), moderate (*n* = 16), moderate to severe (*n* = 14), and severe (*n* = 13).

The occurrence of microscopic granulomatous lesions (*n* = 94) varied among organs, and similarly to gross findings it was higher in lung (*n* = 39), followed by liver (*n* = 18), stomach (*n* = 8), kidney (*n* = 6), small intestine (*n* = 5), spleen (*n* = 3), brain and meninge (*n* = 3), skin (*n* = 2), pancreas (*n* = 2), large intestine (*n* = 2), spinal cord (*n* = 1), thyroid (*n* = 1), thymus (*n* = 1), adrenal (*n* = 1), heart (*n* = 1), and salt gland (*n* = 1). The granulomatous lesions were observed exclusively in lung in 28 animals (28/39; 71.8%), liver in seven animals (7/18; 38.9%), kidney in five animals (5/6; 83.3%), small intestine in two animals (2/5; 40%); and stomach in two animals (2/8; 25%). However, granulomatous lesions were observed in multiple organs in 19 (30.2%) animals, mainly including respiratory and digestive systems (Table 2).

According to the type of granuloma, heterophilic granuloma was observed in 47 organs (50%; 25 lungs, 7 livers, 3 kidneys, 2 stomachs, 2 small intestines, and 2 large intestines, and 1 heart, spleen, salt gland, thyroid, brain, and spinal cord, each) (Figure 2A to 2C, and 2E), and histiocytic granuloma was observed in 38 organs (40.4%; 11 lungs, 7 livers, 6 stomachs, 2 kidneys, 2 skins, 2 spleens, 2 small intestines, 2 brains, 2 pancreas, and 1 thymus and adrenal, each) (Figure 2D). The chronic granuloma was observed in 1 small intestine (1.1%) and accompanied by a heterophilic granuloma in 1 lung (1.1%; Figure 2F). Additionally, heterophilic and histiocytic granulomas were observed in the same organ in 7 cases (7.4%; 4 livers, 2 lungs, and 1 kidney).

All three histochemical techniques were performed in 88 organs of 59 animals, but the number of samples varied according to technique owing to granuloma loss during slides preparation. The Grocott stain was performed in 91 organs of 60 animals. The fungi hyphae were observed in 25 organs (25/91; 23 lungs, 1 stomach and 1 small intestine) of 24 animals (24/60; 40%) (Figure 3A to 3C). Only 1 animal had fungi hyphae in different organs (lung and stomach) and 1 animal had pigmented and non-pigmented fungi coinfection in a lung. The morphology indicated septate and non-septate, large or thin hyphae, and the dichotomy varied between 45º and 90º. A small number of hyphae (2 to 3 hyphae) were observed in half of the cases.

The Gram stain was performed in 90 organs of 60 animals and 37 organs (37/90; 9 lungs, 8 livers, 4 kidneys, 3 spleens, 2 brains, and 1 heart, stomach, small intestine, large intestine, pancreas, skin, salt gland, thyroid, thymus, adrenal, and spinal cord, each; Figure 3D and 3E) of 25 animals were positive (25/60; 41.7%). From the total, Gram negative bacteria were observed in 24 organs, and Gram positive in 9 organs, but both Gram negative and positive were observed in 4 organs. In 5 animals, bacteria were observed in more than one organ, including 3 cases of Gram negative and positive coinfection – 1. Lung, liver, and stomach, Gram positive and negative; 2. Liver and salt gland, Gram positive and negative; 3. Lung, liver, spleen, skin, thyroid, and adrenal, Gram positive and negative; 4. Brain and spinal cord, Gram negative; and 5. Liver, brain, spleen, and heart, Gram negative. The Ziehl-Neelsen stain was performed in 89 organs of 59 animals and only 1 small intestine was positive (1/59; 1.7%; Figure 3F).

Coinfection of fungi and bacteria were observed in 3 lungs, and bacteria and acid-fast bacteria were observed in 1 small intestine. Additionally, coinfection of fungi and bacteria in different organs of the same animal were observed in 2 cases. In 33 organs (33/88; 37.5%) of 27 animals (27/59; 45.8%) the presence of etiologic agents was not identified by histochemical staining.

The relationship between heterophilic and histiocytic granuloma and the three etiological agents was significant (*p* = 0.001), but considering that acid-fast bacteria was positive in only one case, the analysis was remade using only bacteria and fungi infection. After that, no relationship was observed between the granuloma type and the etiological agent.

Samples with bacteria and fungi detection in histochemical analysis were submitted to PCR assay to identify the etiological agent, but the negative cases (for the three stains) were also evaluated owing to possible identification. From 16 positive cases for fungi detection in lungs, 14 cases were positive and 2 cases were negative in PCR assay. Phylogenetic and nucleotide sequence analysis of 10 cases indicated presence of *Candida zeylanoides* (*n* = 2), *Yarrowia divulgata* (*n* = 1), *Yarrowia lipolytica* (*n* = 3), *Yarrowia deformans* (*n* = 3), and *Cladosporium* *anthropophilum* (*n* = 1) (Figure 4). From 24 positive cases for bacteria detection, 6 cases were positive and 18 were negative in PCR assay. Owing to quality of sequences, it was no possible to identify any species of bacteria by nt sequence analysis. Furthermore, 8 cases negative for all stains tested was positive for fungi in PCR assay (*Y. lipolytica*, *n* = 6; *Y.* *deformans*, *n* = 1; and *C. zeylanoides*, *n* = 1) and 3 cases negative for all stain tested was positive for bacteria in PCR assay. The sequences of *Yarrowia lipolytica*, *Yarrowia divulgata*, *Candida zeylanoides*, and *Cladosporium* *anthropophilum* described in this studyexhibited 100% of nt identity with representative strains from these fungal species, already the *Yarrowia deformans* sequences presented 99.4 to 100% of nt identity with representative strains.

The *causa mortis* of animals with granulomatous lesions was classified as ‘natural’ in 33 cases (52.4%), ‘anthropogenic’ in 20 cases (31.7%), ‘euthanasia’ in 4 cases (6.3%), and ‘unknown’ in 6 cases (9.5%). The granulomatous lesions were associated with the cause of death in 35 animals (55.5%), considering ‘primary’ lesions in 14 cases (14/35; 40%) and ‘secondary’ in 21 cases (60%). The comparison between primary or secondary cause of death associated with granulomatous lesions and other causes indicates a diversity of organs affected by bacteria and different pathogenicity of some fungi species (Table 3).

Discussion

The systematic health assessment of free-ranging *C. mydas* using macroscopic, histological, and molecular evaluation is scarce worldwide (Chapman et al., 2016 and 2019; Flint et al., 2010; Jones et al., 2016; Work et al., 2010 and 2015). Previous studies have investigated general diseases and the associated cause of death, but this is the first effort focused on granulomatous lesions caused by bacterial and fungal infection in *C. mydas*. Thus, comparisons about biological data, lesion prevalence, and affected organs are limited for the species.

The range of CCL observed is within previous studies in the area – from 30 to 58 cm –, comprising the juvenile post recruitment stage that uses the area for feeding and development (Andrade et al., 2016; Fuentes et al., 2020). The proportion of total female and male stranding was approximately 6 times higher for females than males (data not shown), and it was similar for animals with granulomatous inflammation (~4 times higher for females than males). In general, body condition was not related with the presence of granulomatous inflammation, nor death caused by these lesions. It reinforces that animals in ‘better’ body condition can also die of infectious diseases with no visual changes in volume of fat or skeletal muscle tissue, differing from previous studies with *C. mydas* (Labrada-Martagón et al., 2010; Work et al., 2015).

The granulomatous lesion is a hallmark for inflammatory reaction in fishes, reptiles and birds and the gross lesions observed and described as whitish, semi-solid and firm, well-delimitated is consistent with reptile’s granuloma and abscesses (Montali, 1988; Stacy & Pessier, 2007). Notwithstanding, it is important to note that 40.4% of the affected organs had unspecific or no gross lesions identified during autopsy, indicating that microscopic evaluation is necessary for a better understanding of granulomatous disease in *C. mydas,* particularly to identify the cause of death and its contributors. The most affected organs encompassed the respiratory and digestive systems, and it was similar to *C. mydas* from USA and Australia, and to Kemp´s Ridley sea turtles (*Lepidochelys kempii*) from USA, but granulomatous lesions were more often associated with systemic parasitic infection than bacterial and fungal infections (Flint et al., 2010; Innis et al., 2009; Work et al., 2015).

The prevalence between fungal or bacterial granulomas was similar in this study, but a greater diversity of organs affected was observed in bacterial infection. This characteristic may be related to the systemic infections observed or secondary to parasitosis, considering parasitosis coinfection. Additionally, acid-fast bacterial infection was rare and it reinforces rare or nonexistent cases reported in previous studies with sea turtles (Brock et al., 1976; Flint et al., 2010; Orós et al., 2005).

The granuloma type may differ according to pathogen extracellular or obligate intracellular habitat (Stacy & Pessier, 2007). The heterophilic granuloma was predominant and it was possible to observe both extracellular fungi and bacteria in heterophilic and histiocytic granuloma. No obligate intracellular bacteria were observed in histiocytic granuloma, but limited identified species precluded a better understanding of immunological reaction considering the pathogen habitat. Although bacterial infection was observed in 24 samples by histochemistry, only 6 samples were positive in PCR assay, and none presented sufficient quality for identification. The electropherogram analysis indicated overlapping peaks and possible mixed infection, making it impossible to identify species.

In this study, the molecular analysis was used as a complementary technique to histochemical fungi detection. Identified species of fungi are rare opportunistic, emerging pathogens (Khosravi et al., 2013; Sandoval-Denis et al., 2016; Zieniuk and Fabiszewska, 2019), circulating in marine environment and affecting *C. mydas* health population off Paraná state. It reinforces the importance of sea turtle health assessment, once the etiologic agents may spread throughout individuals during migrations in the south-western Atlantic Ocean (Coelho et al., 2018).

The Candida genus is considered commensals to humans, but some may cause lesions and invasive candidiasis that ranges from minimally symptomatic candidaemia to fulminant sepsis (Cruz, 2010; Khosravi et al., 2013; Pappas et al., 2018). The *C. zeylanoides* was isolated from rare cases of candidaemia, onicomycosis, dermatitis, arthritis, pneumonia, endocarditis, and pus from larynx carcinoma wound in humans and dermatitis in southern right whale (*Eubalaena australis*) (Arshad, Garcia and Khaja, 2017; Crozier 1993; [Dorko](https://link.springer.com/article/10.1007%2FBF02817657#auth-1) et al., 2002; Jautová et al., 2001; Mouton et al., 2009). This is the first study that has identified *C. zeylanoides* in free-ranging *C. mydas*, associated with primary or secondary cause of deaths. Added to *Candida albicans* isolated from caseous and ulcerative stomatitis in captive *C. mydas* (Chuen-Im et al., 2010), *C. zeylanoides* may affect sea turtle and human health.

Supporting the possible hazards cited above, the Yarrowia genus has shown to be closely related to *Candida* spp. (e.g. the teleomorph *Yarrowia lipolytica* and anamorph *Candida lipolytica*; Nagy et al., 2013; the teleomorph *Yarrowia deformans* and anamorph *Candida deformans*; Groenewald & Smith, 2013; Péter et al., 2019). The *Yarrowia* spp. can be found in nature, including surface and oiled areas in marine and terrestrial environment, food, and human microbiota (Chang et al., 2016; Zieniuk & Fabiszewska, 2019). It has been used in the food and medical industry owing to extracellular lipolytic and proteolytic activity and safety considering human health (Rywińska et al. 2013). However, information regarding pathology in humans is known, although limited (Groenewald et al., 2014; Péter et al., 2019; Zieniuk & Fabiszewska, 2019).

*Yarrowia lipolytica* is considered to cause rare opportunist lesions in severely immunocompromised or otherwise seriously ill people with other underlying diseases (Groenewald et al., 2014). In humans, this pathogen was associated with ocular candidiasis, necrotising dermatitis, lung, duodenal, and mesenteric masses, and cause secondary diseases to bacterial infection, polytraumatism, surgery, chemotherapy, immunotherapy, and fungemia associated with contaminated catheters (Groenewald et al., 2014; Zieniuk & Fabiszewska, 2019). *Yarrowia lipolytica* was identified 8 animals described herein, and it was associated with the primary or secondary cause of death in 4 cases. *Yarrowia deformans* and *Y. divulgata* are also isolated from food products and marine environment (Chang et al., 2016; Nagy et al., 2013), but information about pathogenicity is scarce. *Yarrowia deformans* was identified in 4 cases, and *Y. divulgata* was identified in 1 case in this study. *Yarrowia deformans* was associated with secondary cause of death in 1 case.

Fungi of the Cladosporium genus are mostly saprophytes, and some species are plant, fungi, and animal pathogens (Bensch et al., 2012; Sandoval-Denis et al., 2016). Although *Cladosporium* spp. has been isolated from granulomatous lesions in captive *C. mydas* (Jacobson et al., 1979 and 1986), the knowledge about pathologies in humans and other animals is still incipient (Sandoval-Denis et al., 2016). The *C. anthropophilum* is a saprobic fungus but can represent a clinically relevant fungus isolated from human bronchoalveolar lavage and cerebrospinal fluid, human foot skin and animal abscess (Sandoval-Denis et al., 2016). *Cladosporium anthropophilum* was associated with secondary cause of death in 1 case here and may affect sea turtle’s health.

The death of the specimens was associated with granulomatous inflammation in half of the cases assessed. It suggests that these lesions can be an important indicator of health and infectious diseases in stranded *C. mydas* off Paraná state, but also in other areas throughout the south-western Atlantic Ocean. Most of the species of fungi identified are found in nature and considered safe by food and medical agencies, however they also have been shown to affect the health of humans and wildlife, as the juveniles *C. mydas* assessed. Therefore, a continuous monitoring of these pathogens, including possible contamination of sewage discard from food or medical industry, and sea turtle´s diseases is crucial to understand potential hazards to environmental health and possible zoonotic diseases.

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Ethical statement

The authors confirm that the ethical policies of the journal, as noted on the journal’s author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received.A field permit was granted by the IBAMA/Ministry of Environment (ABIO N° 640/215) that encompassed the legislated ethics approval for tissue sampling from stranded sea turtles.

Data availability statement

These sequence data have been submitted to the GenBank databases under accession numbers MT670430-32; MT670918; MT671179-82; MT671193; MT671351-59, openly available in the GenBank database at https://www.ncbi.nlm.nih.gov/nucleotide/.

Conflict of interest statement

The authors have no financial or personal interests that could influence the content of this article. The authors also declare no competing interest. All authors have seen and approve the manuscript.

References

Andrade, M. F.,Domit, C., Broadhurst, M. K., Tolhurst, D. J., Silva‑Souza, Â. T. (2016). Appropriate morphometrics for the first assessment of juvenile green turtle (*Chelonia mydas*) age and growth in the south-western Atlantic. Marine Biology, 163, 1–15. *doi*: 10.1007/s00227-016-3031-7.

Arshad, H., Garcia, S., Khaja, M. (2017). Case report of invasive, disseminated candidiasis with peripheral nodular cavitary lesions in the lung. Respiratory Medicine Case Reports, 20, 34–37. *doi*:[10.1016/j.rmcr.2016.11.003](https://dx.doi.org/10.1016%2Fj.rmcr.2016.11.003" \t "pmc_ext).

Bensch, K., Braun, U., Groenewald, J.Z., Crous, P. W. (2012). The genus *Cladosporium.* Studies in Mycology, 72, 1–401. *doi:*[10.3114/sim0003](https://doi.org/10.3114/sim0003" \t "_blank" \o "Persistent link using digital object identifier).

Bjorndal, K. A., Bolten, A. B. (1989). Comparison of straight-line and over-the-curve measurements for growth rates of green turtles, *Chelonia mydas*. Bulletin of Marine Science, 45, 189–192.

Brock, J.A., Nakamura, R.M., Miyahara, A.Y., Chang, E. M. L. (1976). Tuberculosis in Pacific green sea turtles, *Chelonia mydas*. Transactions of the American Fisheries Society,105, 564*–*566. *doi*: 10.1577/1548-8659(1976)105<564:TIPGST>2.0.CO;2.

Cantas, L., Suer, K. (2014). Review: the important bacterial zoonoses in “*One Health*” concept. Frontiers in Public Health, 2, 1–8. *doi*: 10.3389/fpubh.2014.00144.

Chang, C-F., Lee, C-F., Lin, K-Y., Liu, S-M. (2016). Diversity of yeasts associated with the sea surface microlayer and underlying water along the northern coast of Taiwan. Research in Microbiology, 167, 35–45. *doi*:10.1016/j.resmic.2015.08.005.

Chapman, P. A., Owen, H., Flint, M., Traub, R. J., Cribb, T. H., Mills, P. C. (2016). Molecular characterisation of coccidia associated with an epizootic in green sea turtles (*Chelonia mydas*) in South East Queensland, Australia. PLoS ONE, 11, 2, e0149962. *doi*:10.1371/journal.pone.0149962.

Chapman, P.A., Cribb, T.H., Flint, M., Traub, R. J., Blair, D., Kyaw-Tanner, M. T., Mills, P. C. (2019). Spirorchiidiasis in marine turtles: the current state of knowledge. Diseases of Aquatic Organisms, 133, 217–245. *doi*:[10.3354/dao03348](https://www.researchgate.net/deref/http%3A%2F%2Fdx.doi.org%2F10.3354%2Fdao03348?_sg%5B0%5D=OEyyTZBeY8ukhdo1kfJL75D3pznjL4BcRYC873229DOzgTaS0vqjJmIuz93si71L6C3JBS5xAwzBsyqIWRDdOBL91Q.wwSZTXPNL3nayKT_rG2q-vvBMYyTu6VNcUfnrbtVzxr-8qX9IgjJ1DCwXq4o444gZRxJkEv47kKxhAb5Tyopyg).

Chuen-Im, T., Areekijseree, M., Chongthammakun, S., Graham, S. V. (2010). Aerobic bacterial infections in captive juvenile green turtles (*Chelonia mydas*) and hawksbill turtles (*Eretmochelys imbricata*) from Thailand*.* Chelonian Conservation and Biology, 9, 135–142. *doi*:[10.2744/CCB-0808.1](https://doi.org/10.2744/CCB-0808.1" \t "_blank).

Coelho, V. F., Domit, C., Broadhurst, M. K., Prosdocimi, L., Nishizawa, H., Almeida, F. S. (2018) Intra-specific variation in skull morphology of juvenile *Chelonia mydas* in the southwestern Atlantic Ocean.[Marine Biology](https://link.springer.com/journal/227), 165, 1–12. *doi*:10.1007/s00227-018-3429-5

Crozier, W. J. (1993). Two cases of onychomycoses due to *Candida zeylanoides*. Australasian Journal of Dermatology, 34, 23–25. *doi*:[10.1111/j.1440-0960.1993.tb00842.x](https://doi.org/10.1111/j.1440-0960.1993.tb00842.x" \t "_blank).

Cruz, L.C.H. (2010). Candida. CRUZ, L. C. H. *Micologia Veterinária* (2nd ed). Rio de Janeiro: Revinter. 163–178.

Domiciano, I. G., Domit, C., Bracarense, A. P. F. R. L. (2017). The green turtle *Chelonia mydas* as a marine and coastal environmental sentinels: anthropogenic activities and diseases. Semina, 38, 3417–3434. *doi*:[10.5433/1679-0359.2017v38n5p3417](http://dx.doi.org/10.5433/1679-0359.2017v38n5p3417).

Dorko, E., [Pilipčinec](https://link.springer.com/article/10.1007/BF02817657#auth-2), E.[, Tkáčiková](https://link.springer.com/article/10.1007/BF02817657#auth-3), L. (2002). Fungal diseases of the respiratory tract. Folia Microbiologia, 47, 302–304. *doi*:10.1007/BF02817657.

Ebani, V. V. (2017). Domestic reptiles as source of zoonotic bacteria: a mini review. Asian Pacific Journal of Tropical Medicine, 10, 723–728. *doi*: 10.1016/j.apjtm.2017.07.020.

Flint, M., Petterson-Kane, J. C., Limpus, C. J., Mills, P. C. (2010). Health surveillance of stranded green turtles in southern Queensland, Australia (2006–2009): an epidemiological analysis of causes of disease and mortality. EcoHealth, 7, 135–145. *doi*:[10.1007/s10393-010-0300-7](https://doi.org/10.1007/s10393-010-0300-7" \t "_blank).

Fuentes, M.M.P.B., Wildermann, N., Gandra, T.B.R., Domit, C. (2020). Cumulative threats to juvenile green turtles in the coastal waters of southern and southeastern Brazil. Biodiversity and Conservation. 29,1783–1803.*doi:*[10.1007/s10531-020-01964-0](https://doi.org/10.1007/s10531-020-01964-0).

Glazebrook, J.S., Campbell, R.S.F. (1990). A survey of the diseases of marine turtles in northern Australia. I. Farmed turtles. Diseases of Aquatic Organisms, 9, 83*–*95.

Goldberg, D.W., Fernandes, M.R., Sellera, F.P., Costa, D.G.C., Bracarense, A.P.F.R.L, Lincopan, N. (2019). Genetic background of CTX-M-15 producing *Enterobacter hormaechei* ST114 and *Citrobacter freundii* ST265 co-infecting a free-living green turtle (*Chelonia mydas*). Zoonoses and Public Health,66, 540–545. *doi*:10.1111/zph.12572.

Gordon, A. N., Kelly, W. R., Cribb, T. H. (1998). Lesions caused by cardiovascular flukes (Digenea: Spirorchidae) in stranded green turtles (*Chelonia mydas*). Veterinary Pathology, 35, 21*–*30. *doi*:[10.1177/030098589803500102](https://doi.org/10.1177/030098589803500102" \t "_blank).

Groenewald, M., Smith, M. T. (2013). The teleomorph state of *Candida deformans* Langeron & Guerra and description of *Yarrowia yakushimensis* comb. nov. Antonie van Leeuwenhoek,103, 1023-1028. *doi*:[10.1007/s10482-013-9882-8](https://doi.org/10.1007/s10482-013-9882-8" \t "_blank).

Hargrove, S., Work, T., Brunson, S., Foley, A.M., Balazs, G. (2016). Proceedings of the 2015 international summit on fibropapillomatosis: global status, trends, and population impacts. U.S. Dep. Commer., NOAA Tech. Memo., NOAA-TM-NMFS-PIFSC-54. 1-87. *doi*:10.7289/V5/TM-PIFSC-54.

Homer, B. L., Jacobson, E. R., Schumacher, J., Scherba, G. (1994). Chlamydiosis in mariculture-reared green sea turtles. Veterinary Pathology, 31, 1–7. *doi*:[10.1177/030098589403100101](https://doi.org/10.1177/030098589403100101" \t "_blank).

Innis, C., Nyaoke, A. C., Williams III, C. R., Dunnigan, B., Merigo, C., Woodward, D. L., Weber, E. S., Frasca Jr, S. (2009). Pathologic and parasitologic findings of cold-stunned Kemp´s Ridley sea turtles (*Lepidochelys kempii*) stranded on Cape Cod, Massachusetts, 2001­–2006. Journal of Wildlife Diseases, 45, 594–610. *doi*:[10.7589/0090-3558-45.3.594](https://doi.org/10.7589/0090-3558-45.3.594).

Jacobson, E. R., Gaskin, J. M., Shields, R. P., White, F. H. (1979). Mycotic pneumonia in mariculture-reared green sea turtle. Journal of the American Veterinary Medical Association, 175, 929-933.

Jacobson, E.R., Gaskin, J.M., Roelke, M., Greiner, E.C., Allen, J. (1986). Conjunctivitis, tracheitis, and pneumonia associated with herpesvirus infection in green sea turtles. Journal of the American Veterinary Medical Association, 189, 1020–1023.

Jautová, J., Virágová, S., Ondrasovic, M., Holoda, E. (2001). Incidence of Candida species isolated from human skin and nails: a survey. Folia Microbiologia, 46, 333–337. *doi*:[10.1007/bf02815623](https://doi.org/10.1007/bf02815623" \t "_blank).

Jones, K., Ariel, E., Burgess, G., Read, M. (2016). A review of fibropapillomatosis in Green turtles (*Chelonia mydas*). The Veterinary Journal, 212, 48–57. *doi*:[10.1016/j.tvjl.2015.10.041](http://dx.doi.org/10.1016/j.tvjl.2015.10.041).

Khosravi, A.R., Shokri, H., Nikaein, D., Erfanmanesh, A., Fatahinia, M., Helan, J. A. (2013). Evaluation of the pathogenicity of *Candida zeylanoides* in BALB/c mice. TurkishJournal of Veterinary and Animal Sciences,37, 408–413. *doi*:10.3906/vet-1105-40.

Labrada-Martagón, V., Méndez-Rodríguez, L.C., Gardner, S.C., Cruz-Escalona, V.H., Zenteno-Savín, T. (2010). Health indices of the green turtle (*Chelonia mydas*) along the Pacific coast of Baja California Sur, Mexico. II. Body condition index. Chelonian Conservation and Biology, 9, 173–183. *doi*:[10.2744/CCB-0807.1](https://doi.org/10.2744/CCB-0807.1).

Luschi, P., Hays, G.C. and Papi, F. (2003). A review of long-distance movements by marine turtles, and the possible role of ocean currents. Oikos, 103, 293–302. *doi*:[10.1034/j.1600-0706.2003.12123.x](https://doi.org/10.1034/j.1600-0706.2003.12123.x).

Montali, R.J. (1988). Comparative pathology of inflammation in the higher vertebrates (reptiles, birds amd mammals). Journal of Comparative Pathology. 99, 1–27. *doi*:[10.1016/0021-9975(88)90101-6](https://doi.org/10.1016/0021-9975(88)90101-6" \t "_blank).

Mouton, M., Reeb, D., Botha, A., Best, P. (2009). Yeast infection in a beached southern right whale (*Eubalaena australis*) neonate. Journal of Wildlife Diseases, 45, 692–699. *doi*:[10.7589/0090-3558-45.3.692](https://doi.org/10.7589/0090-3558-45.3.692" \t "_blank).

Nagy, E., Niss, M., Dlauchy, D., Arneborg, N., Nielsen, D. S., Péter, G. (2013). *Yarrowia divulgata* f.a., sp. nov., a yeast species from animal-related and marine sources. International Journal of Systematic and Evolutionary Microbiology, 63, 4818–4823. *doi:*[10.1099/ijs.0.057208-0](https://doi.org/10.1099/ijs.0.057208-0" \t "_blank).

Nardoni, S., Papini, R., Marcucci, G.M., Mancianti, F. (2008). Survey on the fungal flora of the cloaca of healthy pet reptiles. Revue de Médecine Vétérinaire,159, 159–165.

Orós, J., Calabuig, P., Déniz, S. (2004). Digestive pathology of sea turtles stranded in the Canary Islands between 1993 and 2001. The Veterinary Record, 155, 169–174. *doi*:[10.1136/vr.155.6.169](https://doi.org/10.1136/vr.155.6.169" \t "_blank).

Orós, J., Torrent, A., Calabuig, P., Déniz, S. (2005). Diseases and causes of mortality among sea turtles stranded in the Canary Islands, Spain (1998–2001). Diseases of Aquatic Organisms, 63, 13–24. *doi*:[10.3354/dao063013](https://doi.org/10.3354/dao063013" \t "_blank).

Pappas, P.G., Lionakis, M.S., Arendrup, M.C., Ostrosky‑Zeichner, L., Kullberg, B.J. (2018). Invasive candidiasis. Nature Reviews Disease Primers, 4, 1–20. *doi*:10.1038/nrdp.2018.26.

Péter, G., Nagy, E.S., Dlauchy, D. (2019). Systematics, diversity and ecology of the genus *Yarrowia* and the methanol-assimilating yeasts. A. Sibirny (ed.), *Non-conventional Yeasts: from Basic Research to Application*. *doi*:10.1007/978-3-030-21110-3\_9.

Portaria MMA. (2020, April 20). PORTARIA MMA Nº 444, DE 17 DE DEZEMBRO DE 2014. Retrieved from <https://www.icmbio.gov.br/portal/images/stories/docs-plano-de-acao/00-saiba-mais/04_-_PORTARIA_MMA_N%C2%BA_444_DE_17_DE_DEZ_DE_2014.pdf>.

Raidal, S.R., Ohara, M., Hobbs, R.P., Prince, R. (1998). Gram-negative bacterial infections and cardiovascular parasitism in green sea turtles (*Chelonia mydas*). Australian Veterinary Journal. 76, 415–417. *doi*:[10.1111/j.1751-0813.1998.tb12392.x](https://doi.org/10.1111/j.1751-0813.1998.tb12392.x" \t "_blank).

Rywinska, A., Juszczyk, P., Wojtatowicz, M., Robak, M., Lazar, Z., Tomaszewska, L., Rymowicz, W. (2013) Glycerol as a promising substrate for *Yarrowia lipolytica* biotechnological applications. Biomass and Bioenergy, 48, 148–166. *doi*:10.1016/j.biombioe.2012.11.021.

Sandoval-Denis, M., Gené, J., Sutton, D.A., Wiederhold, N.P., Cano-Lira, J.F., Guarro, J. (2016). New species of *Cladosporium* associated with human and animal infections. Persoonia, 36, 281–298. *doi*:10.3767/003158516X691951.

Santoro, M., Hernández, G., Caballero, M., García, F. (2006) Aerobic bacterial flora of nesting green turtles (*Chelonia mydas*) from Tortuguero National Park, Costa Rica. Journal of Zoo and Wildlife Medicine, 37, 549–552. *doi*:[10.1638/05-118.1](https://doi.org/10.1638/05-118.1).

IUCN. The IUCN Red List of Threatened Species. Version 2020-1. (2020, August 3). Retrieved from <https://www.iucnredlist.org/search?query=Chelonia%20mydas&searchType=species>

Stacy, B.A., Pessier, A.P. (2007). Host response to infectious agents and identification of pathogens in tissue section. Jacobson, E.R. (Ed). *Infectious diseases and Pathology of Reptiles: color atlas and text*. Boca Raton: CRC Press, 257–298.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J. (1991). 16S ribossomal DNA amplification for phylogenetic study. Journal of Bacteriology,173, 697–703. *doi*:10.1128/jb.173.2.697-703.1991.

White, T.J., Bruns, T., Lee, S., Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Ed). *PCR protocols: a guide to methods and applications*. San Diego: Academic Press, 315–22.

Work, T.M., Balazs, G.H., Wolcott, M., Morris, R. (2003). Bacteraemia in free-ranging Hawaiian green turtles *Chelonia mydas* with fibropapillomatosis. Diseases of Aquatic Organisms, 53, 41–46. *doi*:10.3354/dao053041.

Work, T.M., Balazs, G.H. (2010). Pathology and distribution of sea turtles landed as bycatch in the Hawaii-based North Pacific pelagic longline fishery. Journal of Wildlife Diseases, 46, 422–432. *doi*:[10.7589/0090-3558-46.2.422](https://doi.org/10.7589/0090-3558-46.2.422" \t "_blank).

Work, T.M., Balazs, G.H., Summers, T.M., Hapdei, J.R., Tagarino, A.P. (2015). Causes of mortality in green turtles from Hawaii and the insular Pacific exclusive of fibropapillomatosis. Diseases of Aquatic Organisms. 115, 103–110.*doi*:10.3354/dao02890.

Zieniuk, B., Fabiszewska, A. (2019). *Yarrowia lipolytica*: a beneficious yeast in biotechnology as a rare opportunistic fungal pathogen: a minireview. World Journal of Microbiology and Biotechnology, 35, 1–10. *doi*:10.1007/s11274-018-2583-8.

**Table 1**. Gross evaluation of *Chelonia mydas* organs with granulomatous lesions confirmed microscopically.

|  |  |
| --- | --- |
| **Gross evaluation** | **Organs (granulomas confirmed microscopically)** |
| No obvious lesion (n = 15 animals) | Liver (n = 5), lung (n = 4), stomach (n = 3), pancreas (n = 2), kidney (n = 1), small intestine (n = 1), brain (n = 1), skin (n = 1), and thymus (n = 1) |
| Non-specific lesion (edema, congestion/hyperemia, haemorrhage, degeneration, atrophy, and paled colour; n = 15 animals) | Lungs (n = 7), liver (n = 4), stomach (n = 3), kidney (n = 2), spleen (n = 1), brain (n = 1), and salt gland (n = 1) |
| Well-delimited lesions, whitish to yellow or black, most firms, between 0.1 and 5 cm diameter, round nodules or caseous plates of different sizes (n = 39 animals) | Lungs (n = 28), liver (n = 9), small intestine (n = 4), kidney (n = 3), stomach (n = 2), large intestine (n = 2), spleen (n = 2), adrenal (n = 1), brain (n = 1), spinal cord (n = 1), heart (n = 1), skin (n = 1), and thyroid (n = 1) |

**Table 2**. Granulomatous inflammation in multiple organs of *Chelonia mydas* stranded dead along the Paraná state, southern Brazil.

|  |  |
| --- | --- |
| **Individual identification** | **Organs** |
| #1066, #1615, #32871 | Lung and liver |
| #1525 | Lung, large intestine, and pancreas |
| #114847 | Lung and pancreas |
| #79591 | Lung and stomach |
| #40921 | Lung, liver, and stomach |
| #115031 | Lung, liver, spleen, and stomach |
| #84198 | Lung, liver, spleen, skin, thyroid, adrenal, and brain |
| #1839 | Lung and kidney |
| #112220 | Lung and skin |
| #42636 | Liver and salt gland |
| #52906 | Liver, stomach, and small intestine |
| #59708 | Liver and large intestine |
| #105893 | Liver and thymus |
| #47993 | Stomach and small intestine |
| #50039 | Stomach and large intestine |
| #56546 | Liver, spleen, heart, and brain |
| #49035 | Brain and spinal cord |

**Table 3**. Biological features and organs affected by granulomatous lesions in *Chelonia mydas* according to cause of death.

|  |  |  |
| --- | --- | --- |
| **Variable** | **Primary and secondary causes of death associated with granulomatous lesions (n = 35)** | **Other diagnoses (n =28)** |
| CCL (x ± SD) | 38.4 ± 7.3 | 36.9 ± 7.1 |
| BCI (x ± SD) 'good' condition | 1.39 ± 0.2 | 1.38 ± 0.2 |
| BCI (x ± SD) 'worst' condition | 1.08 ± 0.1 | 0.91± 0.1 |
| Organs affected by bacteria | lung (n = 8), kidney ( n = 4), liver (n = 4), spleen (n = 3), brain (n = 2), and salt gland, stomach, small intestine, large intestine, skin, spinal cord, thyroid, thymus, pancreas, adrenal and heart (n = 1) | liver (n = 4) and lung (n = 1) |
| % of organs affected by bacteria | 51.6% (32/62) | 1.6% (5/32) |
| Organs affected by fungi | lung (n = 12), stomach (n = 1) | lung (n = 11), small intestine (n = 1) |
| % of organs affected by fungi | 20.9% (13/62) | 37.5% (12/32) |
| Species identified | *Yarrowia lipolytica* (n = 4), *Candida zeylanoides* (n = 3), *Yarrowia deformans* (n = 1), and *Cladosporium anthropophilum* (n = 1). | *Yarrowia lipolytica* (n = 5), *Yarrowia deformans* (n = 3), and *Yarrowia divulgata* (n = 1) |

**Figure legends**

**Figure 1**. Macroscopic granulomatous lesions in different organs of *Chelonia mydas* off Paraná state, southern Brazil. **A**. Lung. Whitish, firm, multifocal, moderate to severe round nodule in parenchyma. **B**. Kidney. Yellowish, multifocal, severe, round caseous in parenchyma. **C**. Liver. Whitish, round, disseminated nodules (arrows) in parenchyma. **D**. Brain. Yellowish, multifocal to coalescent, moderate, caseous plaque in meninge and parenchyma (arrow). **E**. Stomach. Yellowishand blackened, multifocal to coalescent, severe, plaque in hyperemic mucosa. **F**. Large intestine. Yellowishcaseous mass, focally extensive, severe, adhered to mucosa, and associated to lumen obstruction.

**Figure 2**. Histology of granulomatous lesions in different organs of *Chelonia mydas* off Paraná state, southern Brazil (HE). **A**. Brain. Heterophilic granulomatous meningitis, multifocal, moderate to severe (4x). **B**. Kidney. Heterophilic granulomatous nephritis, multifocal, severe, with intralesional bacteria (20x). **C**. Skin. Histiocytic granulomatous dermatitis, focal, mild, with intralesional bacteria (20x). **D**. Lung. Heterophilic ( ) and chronic (\*) granulomatous pneumonia, multifocal, mild to moderate (4x).

**Figure 3.** Fungal or bacterial infection associated with granulomatous lesions in *Chelonia mydas* off Paraná state, southern Brazil (histochemistry and species identification). **A.** Lung. Random distribution of septate, branched, thin to large hyphae (Grocott, 20x; *Yarrowia deformans*). **B.** Lung. Random distribution of branched, large, true and pseudohyphae (Grocott, 100x; *Yarrowia divulgata*). **C.** Lung. Radious distribution of septate, branched, and large hyphae (Grocott, 20x; not identified). **D.** Kidney. Gram negative bacteria (Gram - modified Brown and Brenn, 40x; not identified). **E.** Kidney. Gram positive bacteria (Gram - modified Brown and Brenn, 20x; not identified). **F.** Large intestine. Acid-fast bacteria (Ziehl Neelsen, 100x; not identified)

**Figure 4**. Phylogenetic analysis based on fungal partial nucleotide sequences (nt 244 -453) of the internal transcribed spacer region of the nuclear rDNA gene from fungal sequences from this study (filled circle) and fungal representative strains available in GenBank. The tree was constructed using the neighbor-joining method and the Kimura two-parameter model as nucleotide substitution model. The bootstrap values are shown at the branch nodes (values < 60% are not shown). The scale bars at the bottom of the tree represent the number of nucleotide substitutions per site. The GenBank accession numbers of the strains are provided in parentheses.

1. PMP-BS - Projeto Monitoramento de Praias da Bacia de Santos (PMP/BS), a requirement set by the Brazilian Institute of the Environment (IBAMA) for the environmental licensing of the oil and natural gas production and transport by Petrobras. [↑](#footnote-ref-1)