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Mycobacteriosis in captive wild animals and the public health

Micobacterioses em animais selvagens em cativeiro e a saúde pública

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ABSTRACT - Mycobacteria are bacterial infectious agents capable of causing chronic illness in humans and animals, and they are highly relevant due to their zoonotic nature. They have already been described as affecting several species of wild animals and can affect birds, mammals, and reptiles. However, the infection in wild animals is often underdiagnosed. With the strengthening of relationships between humans and wild animals, especially companion animals, it is essential to diagnose mycobacteriosis for greater control and surveillance over the agents. Thus, the present study aimed to identify positive cases of mycobacteriosis in captive wild animals necrosis at the Wild Animal Pathology Service (SEPAS), FCAV-UNESP, carry out anatomical-histopathological descriptions, and discuss the disease in these animals and the public health. The histochemical method (Ziehl-Neelsen and/or Fite-Faraco staining) identified sixteen positive animals, including twelve birds, three mammals, and one reptile. The main lesions were found in the liver, lungs, and intestines, which generally presented granulomas with acid-fast bacilli (AFB), which were revealed under special staining. This study highlighted the importance of diagnosing mycobacteriosis in wild animals and its relationship with public health, in addition to pointing out the main findings that could assist in future research on the subject.
Keywords: infectious disease, *Mycobacterium*, pathology, tuberculosis, zoonosis.

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INTRODUCTION

Mycobacteriosis is a chronic zoonosis with worldwide distribution, occurring mainly in emerging countries (TELL, WOODS & CROMIE, 2001; DHAMA, MAHENDRAN & TOMAR, 2008; DHAMA *et al.*, 2011; DA SILVA, MOURA & REIS, 2011). This disease is caused by a bacteria of the genus *Mycobacterium*, which are aerobic or microaerophilic, do not form spores, are immobile, have a rod shape, measuring 1 to 10 µm and are weakly gram-positive (DA SILVA, MOURA & REIS, 2011; DHAMA *et al.*, 2011; WILDNER *et al.*, 2011).

The clinical manifestations of this disease are divided into typical or tuberculous and atypical or non-tuberculous (HOOP, 1997; LOUREIRO *et al.*, 2013; MATOS, 2018) and have already been described affecting several animal species, including mammals, birds and reptiles, captive and free-living (HOOP *et al.*, 1993; TELL *et al.*, 2003; CONVERSE, 2007 *apud* LOUREIRO *et al.*, 2013). This disease can often be underdiagnosed in wild animals, mainly because of many asymptomatic cases that require greater attention to such individuals (HOOP *et al.*, 1993; VANDERHEYDEN, 1997; TELL *et al.*, 2003; CONVERSE, 2007 *apud* LOUREIRO *et al.*, 2013).

The mycobacteria with the most significant pathogenic relevance in humans and animals can be grouped into two complexes, namely the *Mycobacterium tuberculosis* complex (MTC) and the *Mycobacterium avium-intracellulare* complex (MAC) (GRANGE, YATES & BOUGHTON, 1990; MATOS, 2018). There are also other species of *Mycobacterium*, which do not belong to the complexes mentioned above but have health relevance as they affect several animal species, including humans (DA SILVA, MOURA & REIS, 2011; ALBERTTI *et al.*, 2015; MATOS, 2018).

The main mycobacteria that cause disease in wild animals do not commonly infect immunocompetent adult humans but can infect immunosuppressed humans and children, also acting as an occupational disease (TELL, WOODS & CROMIE, 2001; VALVASSOURA, 2011). This disease was responsible for the deaths of 1.4 million people worldwide, being the leading cause of death from infectious diseases in the world and one of the top ten due to general factors (WHO, 2020).

Given the relevance of this disease for production animals (cattle and buffaloes), in 2002, the National Program for the Control and Eradication of Brucellosis and Tuberculosis (PNCEBT) was created in Brazil, which in the scope of tuberculosis is based on periodic testing through tuberculariz ing of cattle and buffaloes only, and disposal of positive animals, not including wild animals (ALBERTTI, 2014; BRASIL, 2020). Furthermore, another control established was the mandatory notification of confirmed positive cases of paratuberculosis

(infection by *M. avium* subsp. *paratuberculosis*) in multiple species, tuberculosis in cattle and buffaloes, and confirmed positive cases of avian tuberculosis (BRASIL, 2013).

The shared environment between humans and wild animals is the main link for the interaction and consequent infection between them, with the most relevant sources of infection for humans being synanthropic animals and unconventional pets (TELL, WOODS & CROMIE, 2001). Diagnosing the infection is extremely important, both in humans and animals, so that appropriate measures can be taken and a complete overview of the epidemiology and actual severity of the situation can be obtained. The *ante mortem* or *postmortem* diagnoses are based on clinical signs, laboratory tests, anatomy and histopathological findings (TELL, WOODS & CROMIE, 2001; DHAMA *et al.*, 2011; VALVASSOURA, 2011; LOUREIRO *et al.*, 2013).

The *ante mortem* diagnosis, based on clinical signs, is too nonspecific. Furthermore, there is a shortage of diagnostic tests sensitive and specific enough for all animal species (VANDERHEYDEN, 1997; ARANAZ *et al.*, 1997 *apud* TELL *et al.*, 2003; DHAMA *et al.*, 2011; LOUREIRO *et al.*, 2013). With such difficulty, *postmortem* diagnosis is the most performed, based on macro and microscopic findings (TELL, WOODS & CROMIE, 2001; VALVASSOURA, 2011; MITCHELL, 2012). One of the definitive diagnoses of the disease is the visualization of acid-fast bacilli (AFB), evidenced under microscopy using Ziehl-Neelsen or Fite-Faraco (modified Ziehl-Neelsen) staining technique, as well as molecular methods, such as PCR (TELL, WOODS & CROMIE, 2001; DHAMA *et al.*, 2011; VALVASSOURA, 2011).

With the growing link between humans, unconventional pets, and synanthropic wild animals, surveillance, diagnosis, control, and prevention of agents become essential. Therefore, the objective of this study is to survey the occurrence of mycobacteriosis in captive wild animals belonging to the case series of the Wild Animal Pathology Service (SEPAS) of the São Paulo State University (UNESP), Faculty of Agricultural Sciences and Veterinary Sciences (FCAV), Campus of Jaboticabal, São Paulo, Brazil, describe the macro and microscopic aspects observed, when possible, identify the etiological and get involved, as well as point out risks of imminent human-animal transmission and vice versa and its importance for one health.

METHODOLOGY

Obtaining data

The present study was developed retrospectively, using the archive database of the Wild Animal Pathology Service (SEPAS) of FCAV-UNESP from its creation in 1994 until the end of 2021.

The database, meticulously compiled, comprises a binder, library, and laminate library, storing comprehensive information and materials on all animals received, necropsied, and microscopically evaluated by the service. The necropsy procedure, a careful and detailed process, includes macroscopic evaluation with photographic records. Organ fragments are collected during the procedure for freezing at -20ºC and for fixation in 10% buffered formalin.

The fixed fragments are cleaved into smaller fragments and undergo a dehydration process (under different alcohol concentrations) and diaphanization (under different concentrations of xylene). Afterward, the fragments are paraffinized and cut at a thickness of 3 µm to obtain histological slides, routinely stained with hematoxylin and eosin (H&E), and evaluated microscopically.

Upon the identification of histopathological lesions suggestive of mycobacteriosis, such as granulomas and changes in intestinal villi, mainly in birds, associated with inflammatory infiltrates (TELL, WOOD & CROMIE, 2001), a further microtomy of the paraffinized fragments is performed. This is followed using Ziehl-Neelsen and/or Fite-Faraco stains to confirm or rule out the presence of AFB in the evaluated tissues.

This way, data from 3,487 captive animals, including birds, mammals, and reptiles, were evaluated.

Bacteriological methods and molecular analysis

Suggestive tissue samples were sent to the Department of Preventive Veterinary Medicine and Animal Health (VPS) of the School of Veterinary Medicine and Animal Science (FMVZ), University of São Paulo (USP), where the samples were procedures for bacteriological isolation and molecular analysis.

According to the recommendations and available during sample processing, two different decontamination techniques were used. Thus, a portion of the samples were decontaminated by the HPC (hexadecylpyridinium chloride) method according to the protocol adjusted by AMBROSIO *et al.* (2008) and another by the Petroff method. After decontamination, all samples were inoculated in duplicates in Stonebrink-Leslie and

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Lowenstein-Jensen media, incubated at 37ºC, and colonies were weekly observed for mycobacterial growth. Colonies from samples that were decontaminated by the HPC method were observed for 90 days (PAN-AMERICAN CENTER FOR ZOONOSIS, 1985), and those that underwent the Petroff method were observed for 60 days (HANCE *et al.*, 1989).

Colonies with morphology and characteristics compatible with mycobacteria were subjected to microscopic identification methods for acid-fast bacilli (AFB) using Ziehl-Neelsen (ZN) staining and molecular identification.

Statistical analysis

The number of positive captive birds, mammals, and reptiles compared to the number of negative individuals was meticulously subjected to Fisher's exact test. This rigorous analysis assessed whether or not a significant difference between the proportions of positives in these animal groups.

RESULTS

Between 1994 and 2021, 3,487 captive animals were necropsied, including 2,486 birds (71.3%), 818 mammals (23.5%), and 180 reptiles (5.2%). Based on the evaluations performed and described above, one Atlantic canary (*Serinus canaria*), three yellow-bellied siskin (*Carduelis xanthogastra*), two red siskin (*Carduelis cucullata*), four chestnut-bellied seed finch (*Oryzoborus angolensis*), one white-eared parakeet (*Pyrrhura leucotis*), one scarlet macaw (*Ara macao*), three tufted capuchin (*Sapajus apella*) and one red-eared slider (*Trachemys scripta elegans*) were identified as positive for mycobacteriosis, i.e., twelve birds, three mammals and one reptile (Table 1). The annual occurrence of these cases is shown in Figure 1.

Table 1. Number of animals received at SEPAS (1994-2021) and number of positive cases for mycobacteriosis in captive wild animals evaluated by the Service.

	Wild animals	Captive wild animals	Positive captive wild animals
Birds	890	2489	12 (0,5%)
Mammals	725	818	3 (0,4%)
Reptiles	136	180	1 (0,6%)
Total	1751	3487	16 (0,5%)

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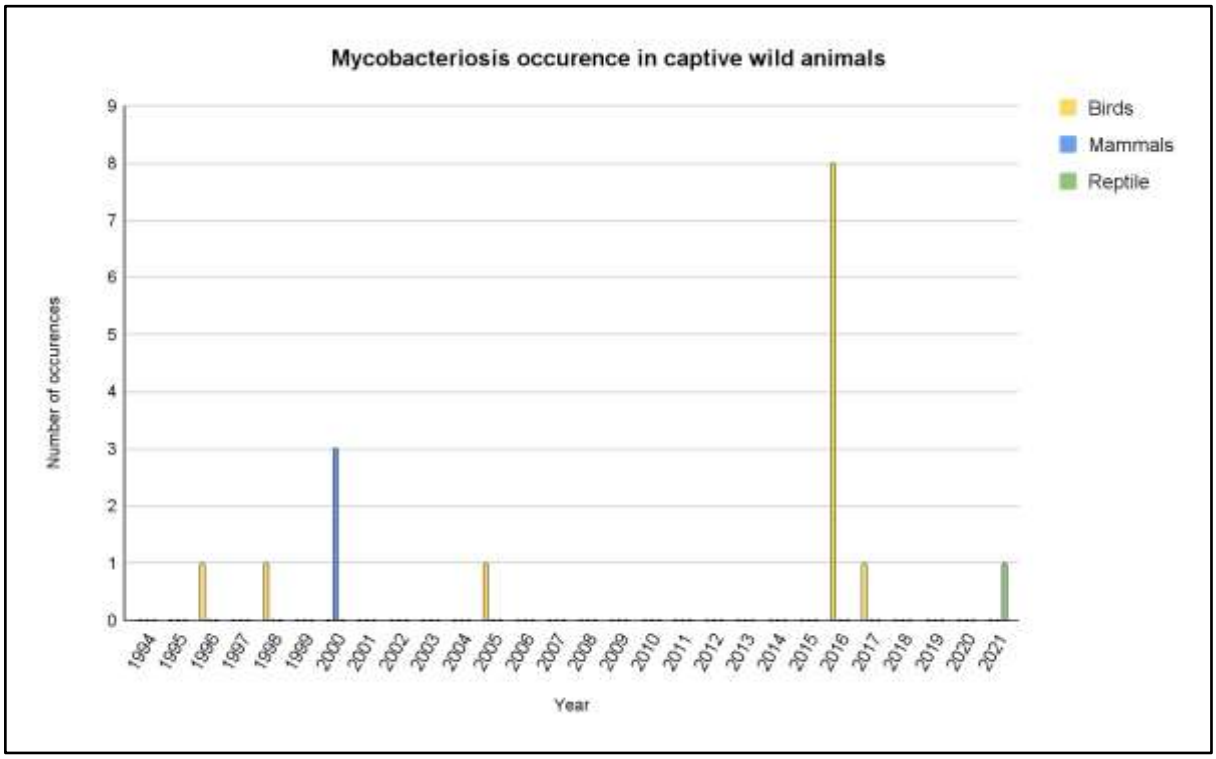

Figure 1. Graphical representation of the annual mycobacteriosis occurrence in captive birds, mammals, and reptiles necropsied at SEPAS from 1994 to 2021.

The occurrence of mycobacterial diseases varied from year to year without presenting a pattern. Furthermore, according to the data presented in Table 1, under Fisher's exact test, the value of P=0.8133 was obtained (Chart 1).

Chart 1. Fisher's exact test.

	Positive	Negative	Total
Birds	12 (0,48%)	2477	2489
Mammals	3 (0,37%)	815	818
Reptiles	1 (0,56%)	179	180
Total	16 (0,46%)	3471	3487

P= 0,8133

Birds

Ten of the twelve bird's positive cases were Passerines from commercial aviaries, and two were Psittaciformes, the white-eared parakeet (*Pyrrhura leucotis*) from a keeper, and the scarlet macaw (*Ara macao*) from a zoo.

All Passerines presented a similar history of progressive weight loss followed by death, while the Psittaciformes arrived with a history of a nodular mass in the facial region. Most positive birds were adults (92%) and males (50%).

During the necropsy, the most relevant findings were low body condition score (BCS) (thin to cachectic), hepatomegaly, splenomegaly, proventriculus dilatation, intestinal loops dilatation with hemorrhagic foci in the mucosa, and the presence of whitish to yellowish nodules in several organs (subcutaneous, heart, liver, spleen, lung, and air sacs). The occurrence rate of these findings in positive animals is shown in Figure 2.

The main histopathological alterations observed under hematoxylin-eosin (H&E) staining were in the liver, intestine, lung, kidney, brain, and heart. Of the 12 animals, 100% presented hepatic alterations, eight intestinal (67%), eight pulmonary (67%), seven renal (58%), seven cerebral (58%), and six cardiac (50%). The most significant hepatic alterations were represented in Figure 3, with inflammatory infiltrates and hepatocyte degeneration being the most prevalent. The other alterations in the organs above are represented in Figures 4 to 8.

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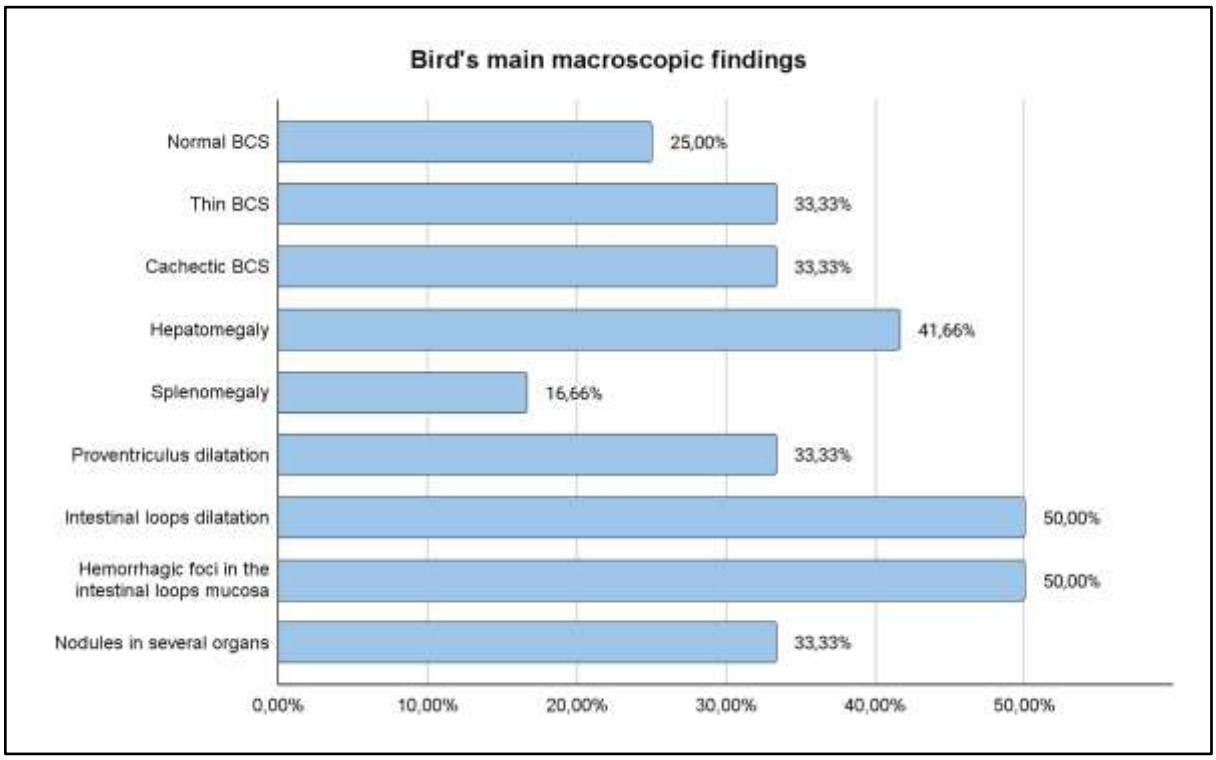

Figure 2. Graphical representation of the percentage occurrence of the most relevant macroscopic findings observed in positive birds. BCS = Body Condition Score.

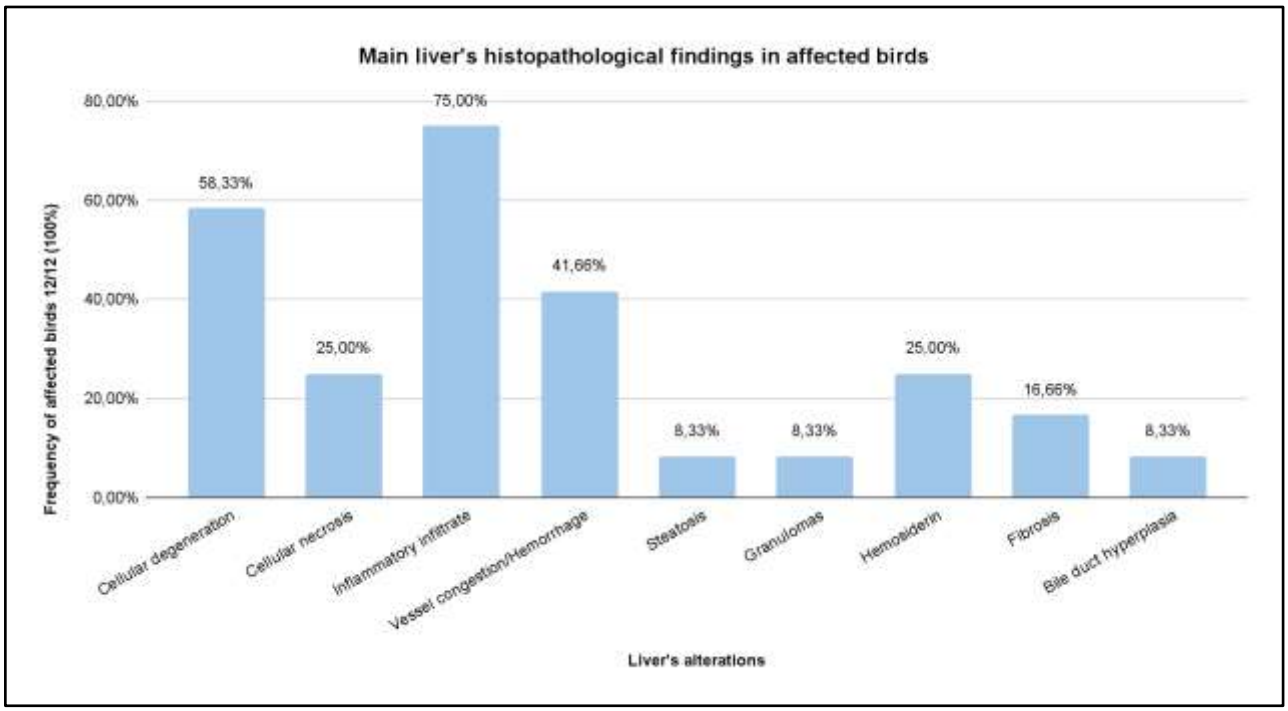

Figure 3. Graphical representation of the percentage occurrence of the main liver's histopathological findings in birds affected (hematoxylin-eosin (H&E) staining).

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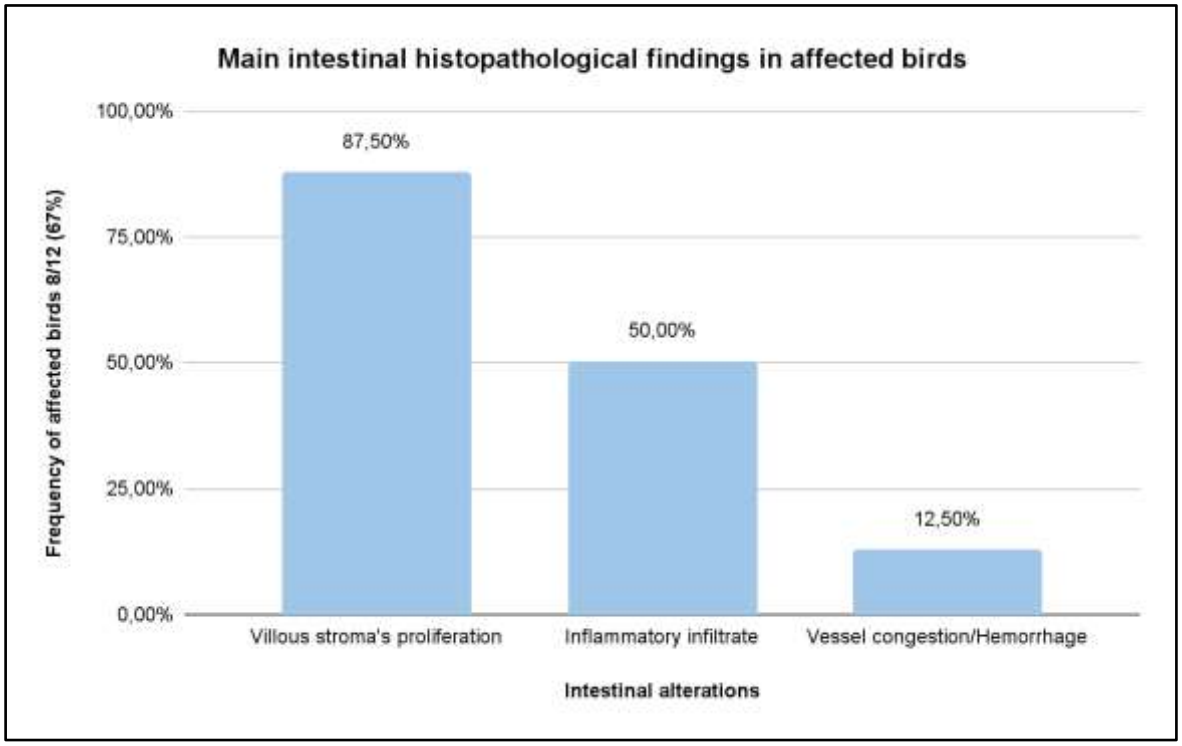

Figure 4. Graphical representation of the percentage occurrence of the main intestinal histopathological findings in birds affected (hematoxylin-eosin (H&E) staining).

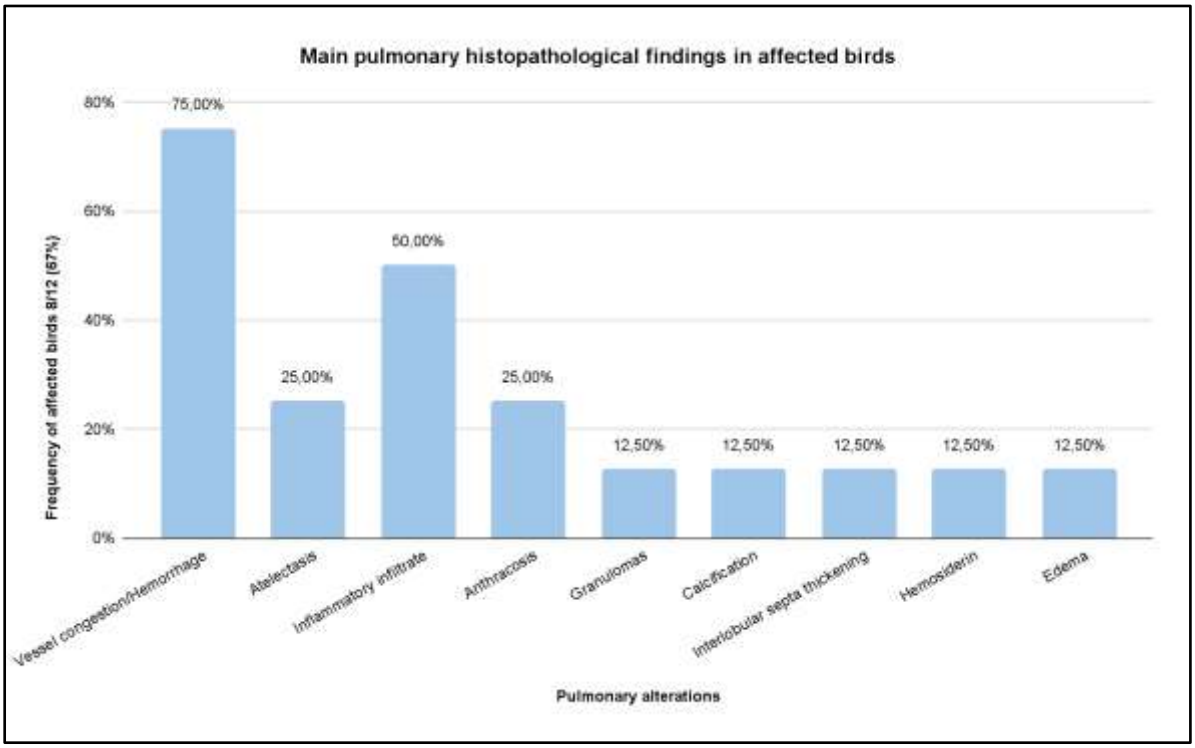

Figure 5. Graphical representation of the percentage occurrence of the main pulmonary histopathological findings in birds affected (hematoxylin-eosin (H&E) staining).

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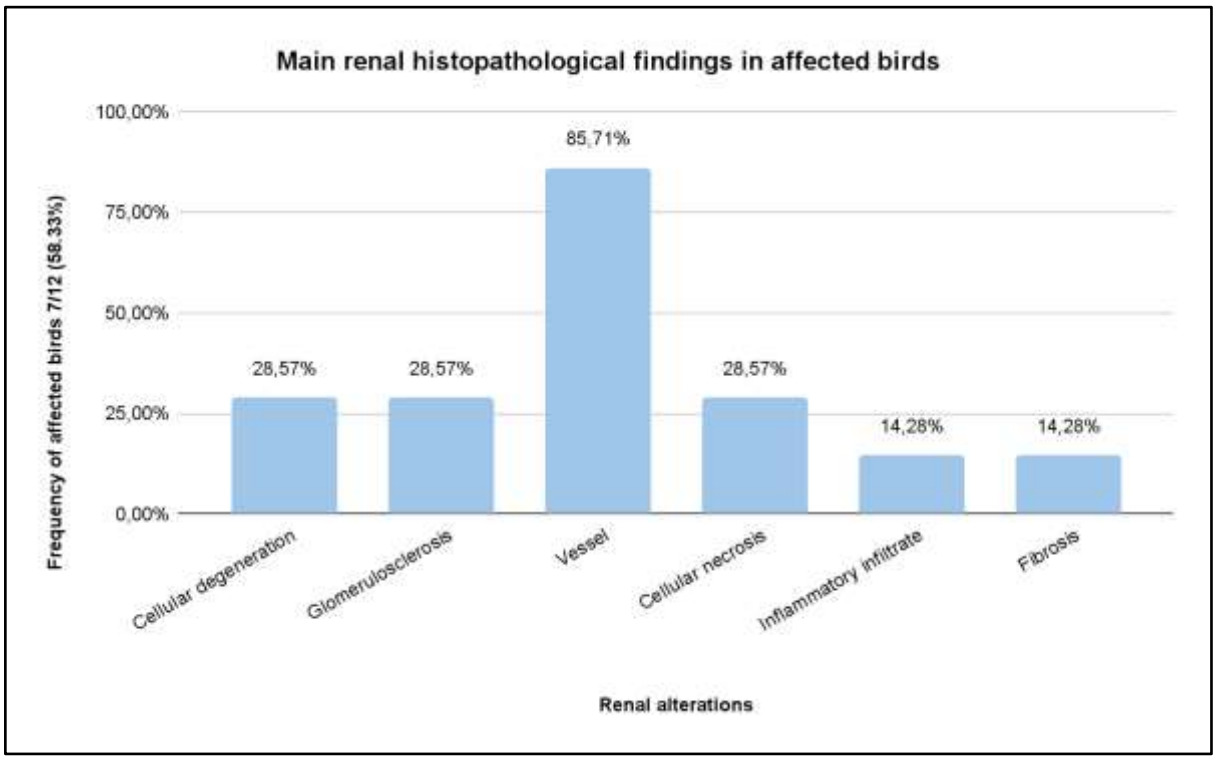

Figure 6. Graphical representation of the percentage occurrence of the main renal histopathological findings in birds affected (hematoxylin-eosin (H&E) staining).

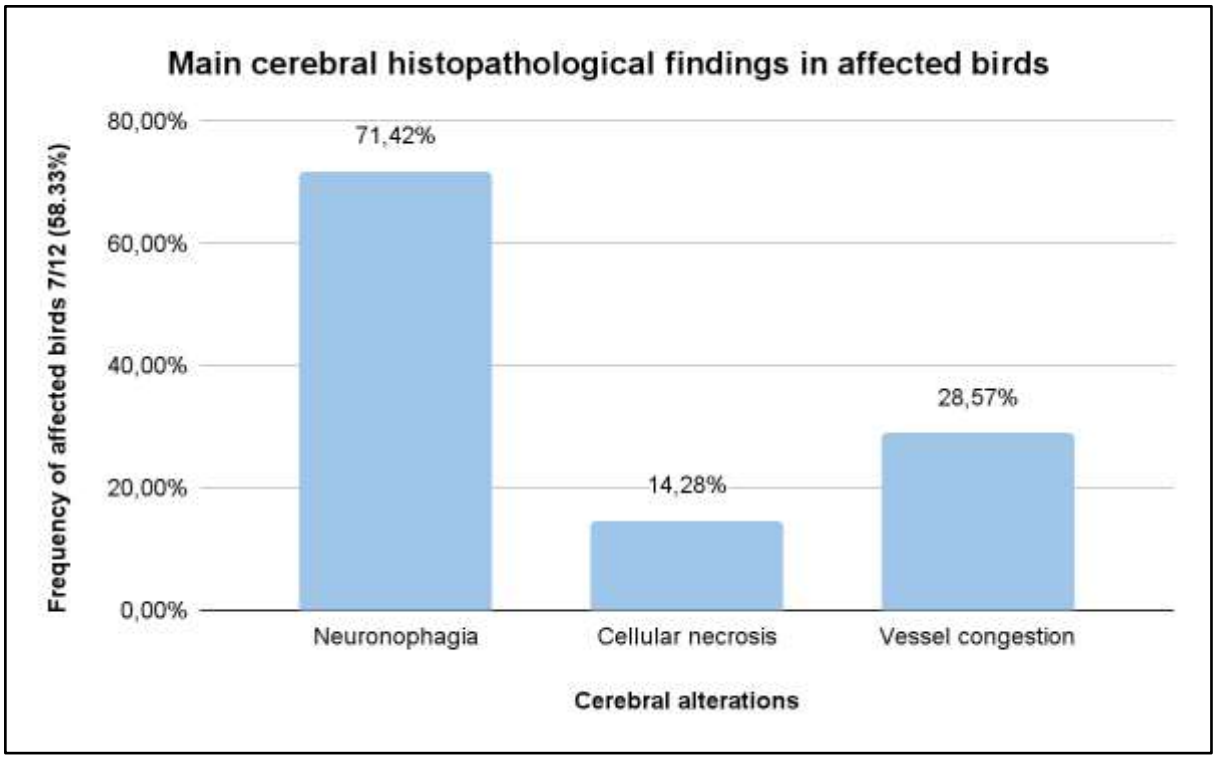

Figure 7. Graphical representation of the percentage occurrence of the main cerebral histopathological findings in birds affected (hematoxylin-eosin (H&E) staining).

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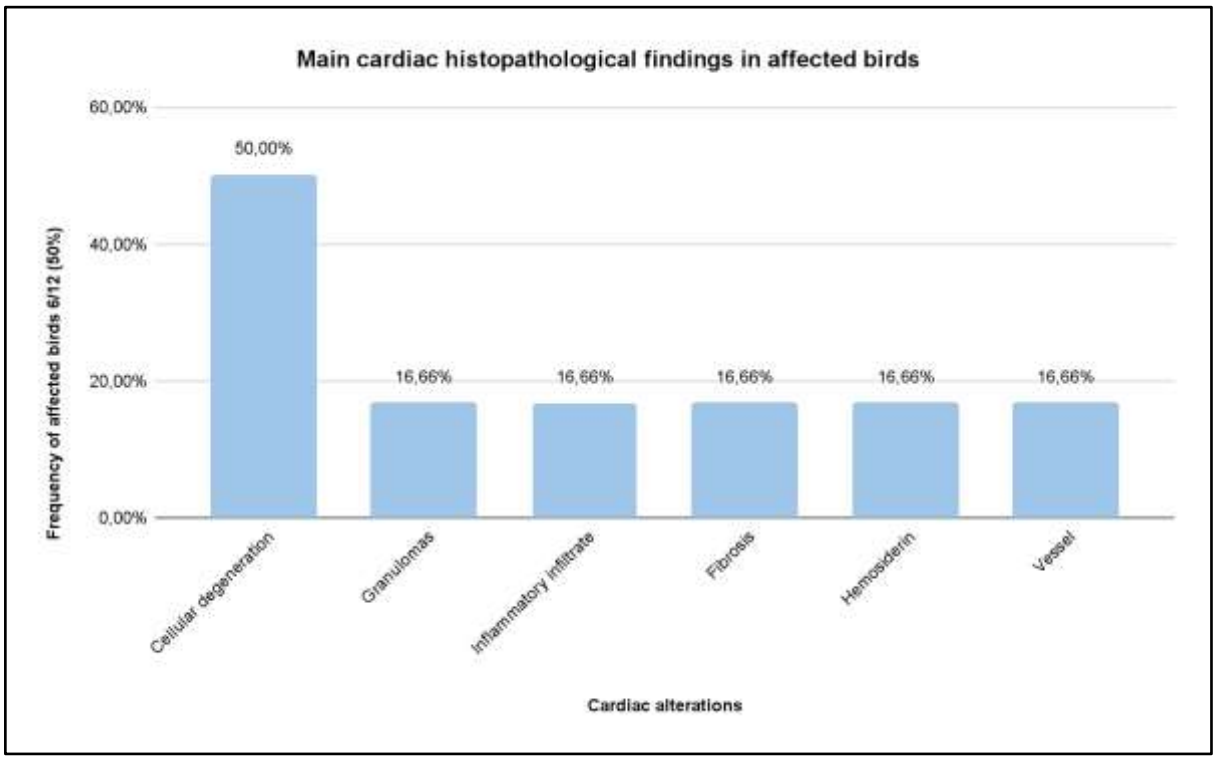

Figure 8. Graphical representation of the percentage occurrence of the main cardiac histopathological findings in birds affected (hematoxylin-eosin (H&E) staining).

The detection of acid-fast bacilli (AFB), using Ziehl-Neelsen staining, was possible in 11 animals (92%), mainly in the lungs (67%), liver (58%), intestine (58%), kidney (50%), heart (50%) and brain (33%) (Figure 9).

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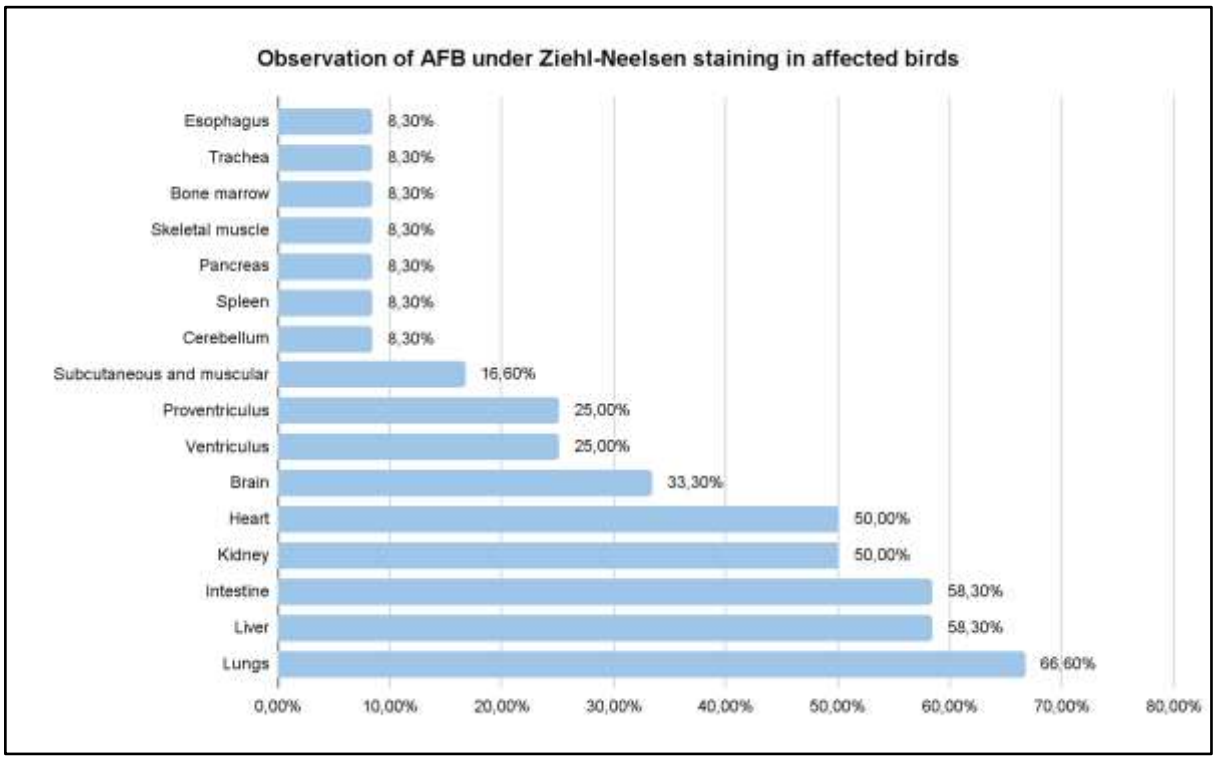

Figure 9. Graphical representation of the observation rate of acid-fast bacilli (AFB) under Ziehl-Neelsen staining in the organs of the birds analyzed in this study.

One of the microscopic changes that attracted the most attention due to its intensity was the proliferation of the villi stroma accompanied by a marked presence of acid-fast bacilli visualized under Ziehl-Neelsen staining (Figure 10).

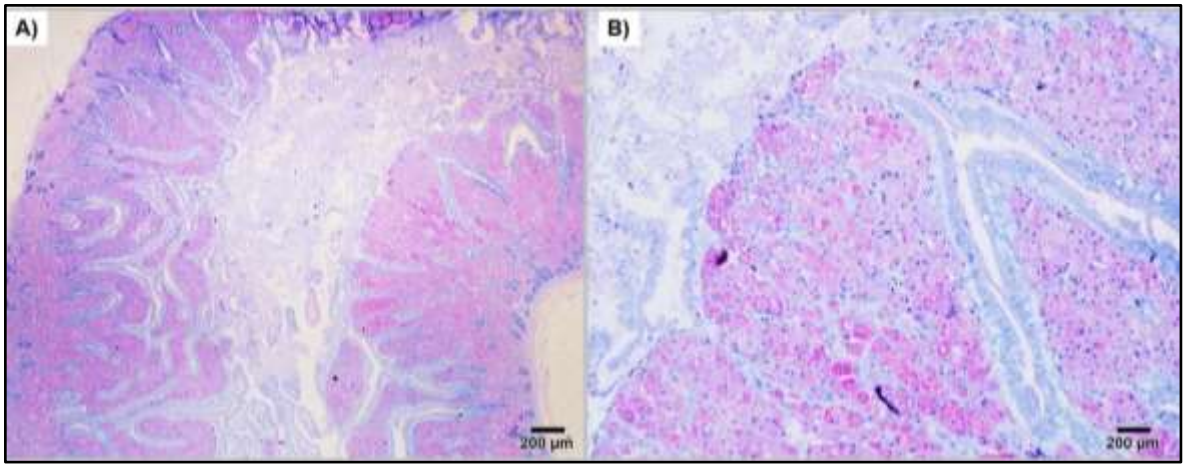

Figure 10. Photomicroscopy of the intestine of *C. xanthogastra* showing marked and diffuse proliferation of villous stroma and abundant presence of AFB (pink staining). A- obj.10x; B- obj.20x; Ziehl-Neelsen.

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Furthermore, on complementary examinations of scrapings of the proventriculus mucosa of the birds, five animals (41.66%) were infected by *Macrohabdus ornithogaster* (Figure 11). In the examinations of scrapings of the large intestine mucosa and the direct feces examinations, one bird (8.33%) was identified with coccidian infection.

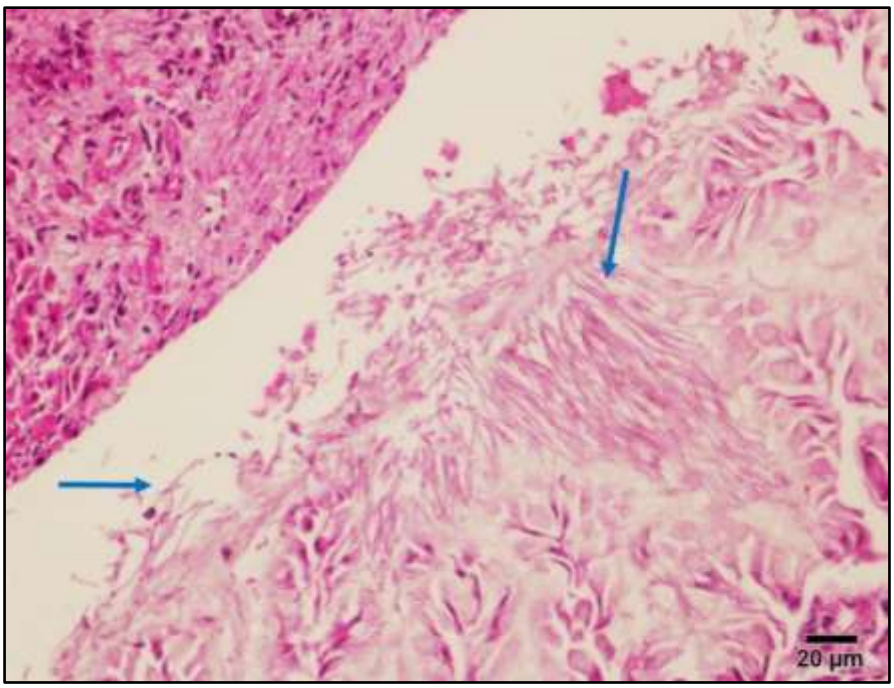

Figure 11. Proventriculus' photomicroscopy of *C. xanthogastra* with marked infection by *Macrorhabdus ornithogaster* (arrows) in the mucosa. (Obj. 40x, H&E).

Some animals also had their samples submitted to culture and isolation of the agent. Thus, was identified a yellow-bellied siskin (*C. xanthogastra*) infected by *M. genavense* type 1, the scarlet macaw (*A. macao*) by *M. avium*, and a chestnut-bellied seed finch (*O. angolensis*), that did not present positivity under Ziehl-Neelsen staining, was identified infected by the genus *Mycobacterium*.

Mammals

The three affected mammals were tufted capuchin (*S. apella*) adult males maintained like pets on a farm, in which raw cow's milk was offered to the animals.

One of them had a history of apathy, chronic cough, and progressive weight loss despite eating normally. The second one had a history of chronic suppurative abscess in the lateral cervical region, which, when scraped and stained with Ziehl-Neelsen, revealed abundant AFBs. Both underwent two intrapalpebral tuberculin tests (Figure 12) but did not

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react after 24, 48, and 72 hours. The hematological examinations showed anemia, and in the lungs' radiographs, diffuse changes were noted, with an increase in the density of the entire parenchyma (Figure 13).

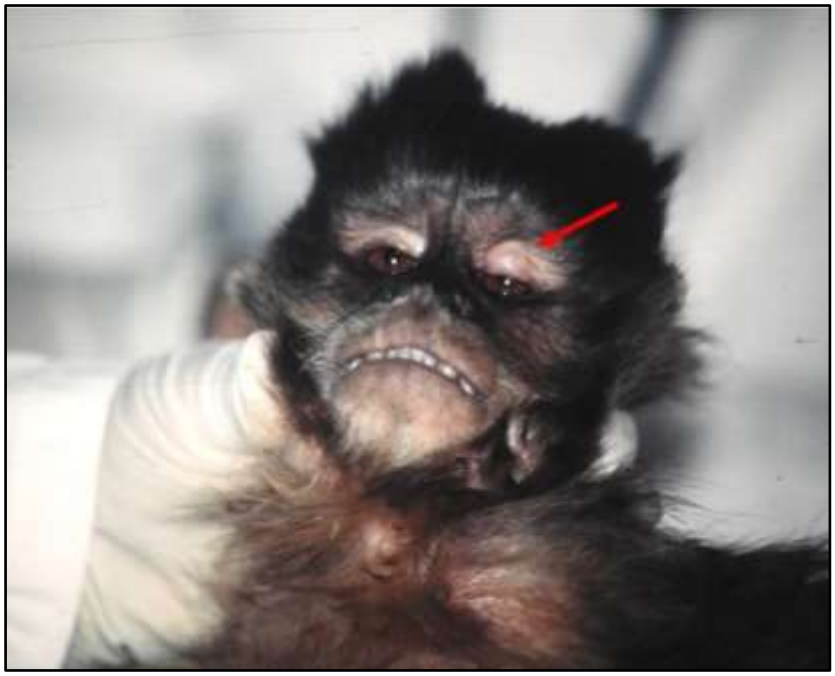

Figure 12. Photograph of sedated *Sapajus apella* immediately after intrapalpebral tubercularizing of the left eye (arrow).

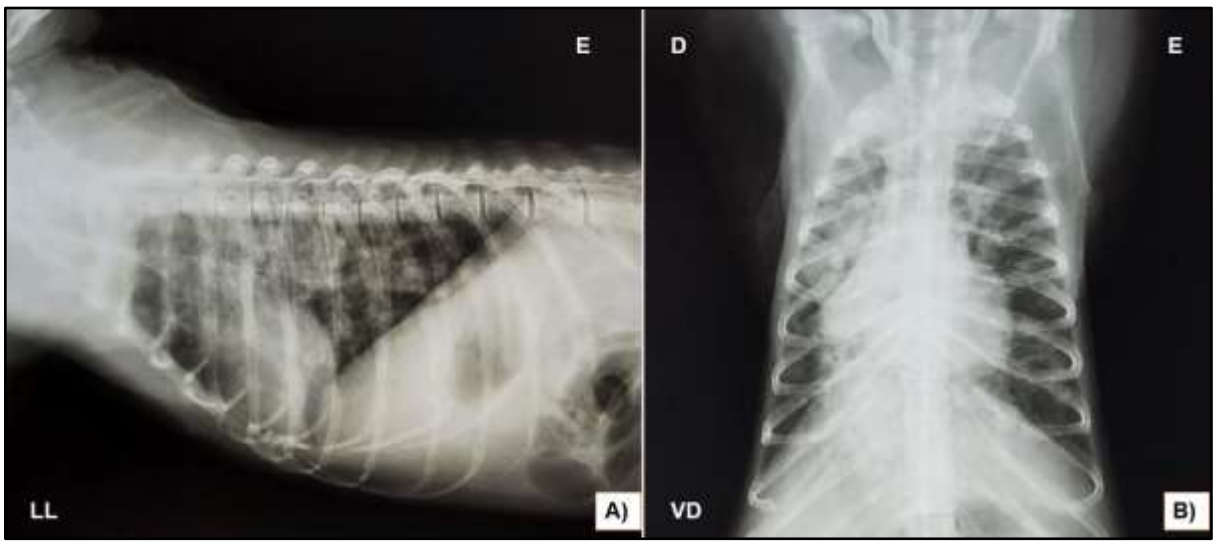

Figure 13. Radiographic image of the thoracic region of *Sapajus apella* with increased radiopacity in the bilateral lung parenchyma. Lateral-lateral projection (A) and ventro-dorsal projection (B).

Considering the zoonotic potential of the disease and the results of the complementary tests performed, the two animals and their contacts (three more individuals) were euthanized and submitted for necropsy. Of the contact animals, only one also tested positive.

The most relevant macroscopic findings in the positive animals were cachexia, enlarged lymph nodes (mandibular, submandibular, prescapular, and mesenteric) associated with a military aspect, lungs with whitish areas of firm consistency and nodules that presented a caseous appearance when cut, kidneys and intestines with whitish spots in the cortex and mucosa, respectively, and subcutaneous abscesses.

The most important microscopic finding was the presence of granulomatous processes in multiple organs, accompanied by intense mononuclear inflammatory infiltrate, cellular debris, and Langerhans giant cells. Under Ziehl-Neelsen staining, acid-fast bacilli (AFB) were observed in multiple organs.

Post-mortem tests, such as culture and molecular examination, confirmed infection by *M. bovis* in the three primates. In addition, two animals were also positive for toxoplasmosis in the indirect immunofluorescence test (IIF).

Reptiles

In the period study (27 years), only one captive reptile tested positive for mycobacteriosis in the SEPAS case series. The animal was an adult male red-eared slider (*T. scripta elegans*) from a zoo.

It referred to with a history of neck and limb edema, injuries from fights, and progressive weight loss. During the necropsy, the main changes observed were cachexia, perforated lesions in the plastron (suggestive of necrotic dermatitis), and increased volume in the limbs and neck. Abundant caseous matter, encapsulated or not, was also seen in the subcutaneous tissue of the limbs and neck, in addition to numerous yellow-white spherical formations in various organs, such as in the skeletal and cardiac muscles, in the right cerebral hemisphere, in the lungs, and in the liver (in capsule and parenchyma) (Figure 14).

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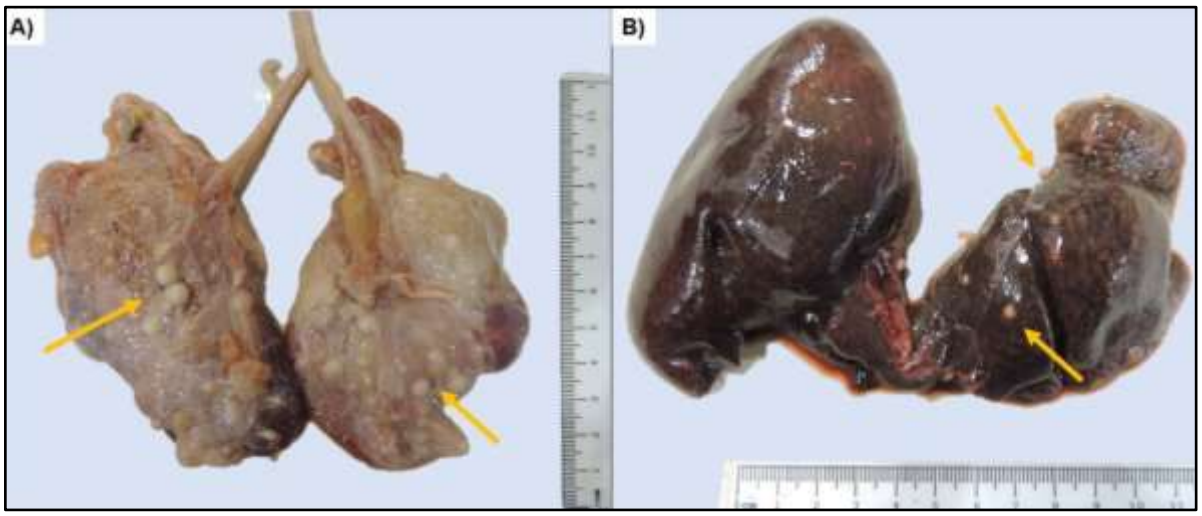

Figure 14. Necroscopic photographs of the lung (A) and liver (B) of *T. scripta elegans*, showing yellow to whitish spherical formations (arrows).

The main histopathological findings were granulomas in various stages of development in multiple organs, most of which were not accompanied by an adjacent inflammatory reaction and the presence of bacterial colonies distributed in various organs.

Gram-negative bacterial colonies were observed in the liver and heart under Gram staining. Ziehl-Neelsen staining was negative for AFB, but Fite-Faraco staining revealed foci of acid-fast bacilli (AFB) in the liver parenchyma (Figure 15).

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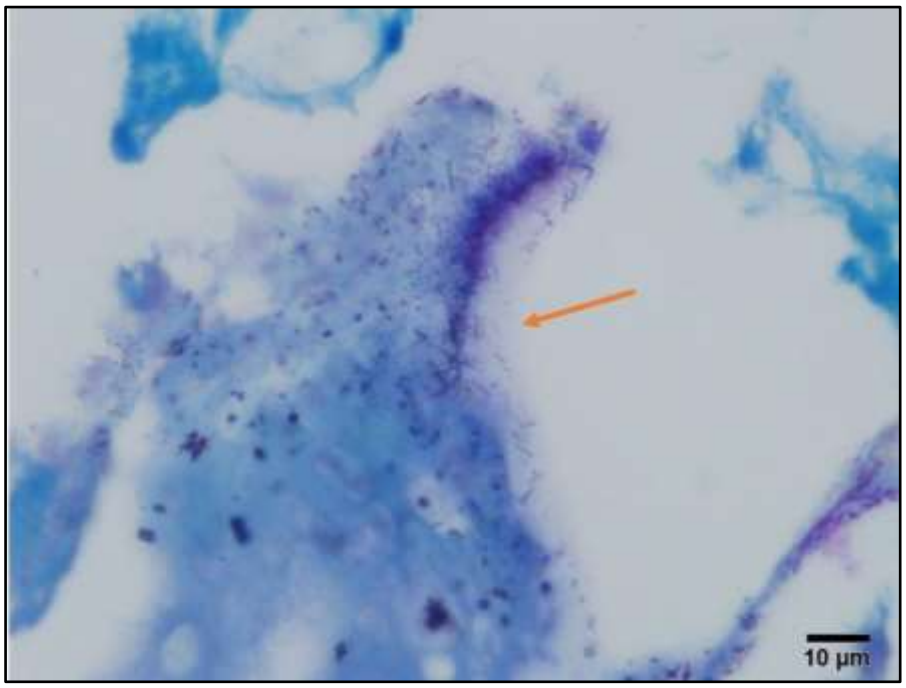

Figure 15. Photomicroscopy of *T. scripta elegans*' liver under Fite-Faraco staining (obj. 100x) showing the discrete presence of acid-fast bacilli (AFB) (arrow).

DISCUSSION AND CONCLUSION

The occurrence of mycobacteriosis in wild animals is often underdiagnosed, mainly due to the lack of specificity or absence of clinical signs and classic anatomical-histopathological findings presented by these individuals (HOOP *et al.*, 1993; VANDERHEYDEN, 1997; TELL *et al.*, 2003).

Fisher's exact test (P=0.8133) showed no significant difference between the proportions of positives in the animal groups. Thus, the difference in the percentage of mycobacteriosis occurrence in these groups is due to the composition of the total sample, in which the proportion of birds is more significant, followed by mammals and reptiles.

The most commonly affected organs in birds are the liver, spleen, and intestines. It is common to macroscopically observe animals with a low body condition score, subcutaneous nodules in the face region, mainly periocular, parenchymal organs with increased volume and granulomatous nodules, distended intestines, and thickened mucosa (TELL, WOODS & CROMIE, 2001). Regarding histopathological findings, the main ones are multifocal to coalescent granulomas in multiple organs and fusion or distension of intestinal villi associated with granulomas with inflammatory infiltrates containing macrophages, epithelioid cells, and Langerhans giant cells (TELL, WOODS & CROMIE, 2001).

The macro and microscopic findings of the birds in this study were compatible with those described in literature, mainly in the liver and intestines. However, no splenic changes were observed in most cases, while lesions in organs such as the lungs, kidneys, heart, and brain were more relevant.

Regarding the mammals' *Mycobacterium* sp. infection, the positive primates in this study presented several clinical alterations described as classic, although nonspecific, such as apathy, progressive weight loss, chronic cough, and skin abscesses (DA SILVA, MOURA & REIS, 2011; VALVASSOURA, 2011). The macroscopic and microscopic findings were also compatible with those described in the literature, mainly concerning cachexia, pulmonary, renal, and intestinal involvement, increased lymph node volume, and multifocal granulomas accompanied by inflammatory infiltrate and Langerhans giant cells. However, the central calcifications in the granulomas usually described in mammals were absent (VALVASSOURA, 2011).

Fite-Faraco staining confirmed the red-eared slider (*T. scripta elegans*) infection by *Mycobacterium* sp. only in the liver, which is also one of the organs that can be affected in reptiles (MITCHELL, 2012). However, in other organs such as the heart, lungs, and skin tissue, which presented granulomas, a change normally described in the literature, AFB was not observed; instead, Gram-negative bacterial colonies were present.

The susceptibility to mycobacteriosis is highly variable, related to the intensity of the infection and the number of stress factors to which the host is subjected (TELL, WOODS & CROMIE, 2001). In all animal groups, it was possible to observe the presence of diseases concomitant with mycobacteriosis, such as infection by *Macrorhabidus ornithogaster* and coccidiosis in birds, toxoplasmosis in primates and infection by gram-negative bacteria in the red-eared slider, a factor that can lead to immune failures and increase the susceptibility of hosts.

The mycobacteria species identified by molecular methods (*M. avium*, *M. genavense*, and *M. bovis*) have significant zoonotic relevance and can mainly infect immunosuppressed humans and children, in addition to acting as an occupational disease (TELL, WOODS & CROMIE, 2001; VALVASSOURA, 2011). It is important to highlight that one of the positive birds, despite being negative under Ziehl-Neelsen staining, presented positivity in molecular tests, raising an alert for identifying and diagnosing infected animals.

All the animals in this study were kept close to humans, which raises awareness of the importance of diagnosis, control, and prevention of disease in wild animals, especially those kept in captivity.

One of the main objectives of the National Program for the Control and Eradication of Brucellosis and Tuberculosis (PNCEBT) is based on an economic bias. The program includes cattle and buffalo, undermining its effectiveness by excluding other animal species (ALBERTTI, 2014; BRASIL, 2020). Thus, when carrying out a more meticulous evaluation of the current measures for the prevention and control of mycobacterial diseases implemented in the Brazilian territory, it is possible to identify some flaws, which could be mitigated by monitoring the disease in wild species, thus bringing numerous benefits, mainly for One Health, since such animals can act as sentinels. These measures would contribute to more significant epidemiological information on the disease and enable the creation of other strategies for eradicating, preventing, and controlling the disease.

The pathology of wild animals has proven to be an extremely important tool in the diagnosis of mycobacterial diseases in wild animals. This contributes to the preservation of human life and that of other animals, mainly due to the zoonotic nature of the disease.

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