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Insights into the threats of toxoplasmosis for free-ranging black-tufted marmosets living in our neighborhood

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Toxoplasmosis is a globally significant zoonotic disease with the potential to severely impact wild animal populations. Neotropical non-human primates (NHPs), particularly callitrichids, are highly susceptible, often experiencing fatal outcomes. This study examines toxoplasmosis in free-ranging black-tufted marmosets (*Callithrix penicillata*) in anthropogenic environments of Central Brazil, analyzing epidemiological and pathological data from 2017 to 2022. A retrospective review of 1095 NHP deaths identified a 9.2% prevalence (101/1,095) of acute fatal toxoplasmosis (AFT) in black-tufted marmosets across Central Brazil and 10.3% (53/515) within the federal district (FD). Necropsied marmosets from the FD showed an estimated AFT prevalence of 50.7% and a lethality rate of 20.3%. AFT cases were linked to outbreaks and isolated incidents, with a likely seasonal peak during the dry season. Pathological findings included severe hepatic damage, splenitis, interstitial pneumonia, and myocarditis. Immunohistochemistry and qPCR confirmed *Toxoplasma gondii* infection, with the highest parasite loads in the spleen and liver. Given the anthropogenic pressures of habitat fragmentation, urbanization, and T. gondii exposure, this study advances the understanding of toxoplasmosis as an emerging disease in wild marmosets. Findings of this study establish a critical foundation for conservation strategies and insights into toxoplasmosis dynamics in free-ranging NHPs living in our neighborhood.

Keywords Non-human primate, Monkey, *Toxoplasma gondii*, Infection, Disease, Parasite, Conservation, Environment, Epidemiology, One health

Toxoplasmosis, a widespread zoonotic disease caused by the protozoan parasite *Toxoplasma gondii*, is a significant global public health concern affecting both humans and animals[1]. This obligate intracellular apicomplexan parasite infects warm-blooded animals, including non-human primates (NHPs). The life cycle of *T. gondii* comprises sexual reproduction in definitive hosts (Felidae family) and asexual reproduction in intermediate hosts[2,3].

Toxoplasmosis is regarded as one of the most lethal infectious diseases affecting neotropical captive NHPs[4–6], yet its ecological impacts on free-ranging NHP populations remain inadequately understood. The high susceptibility of platyrrhines to toxoplasmosis may originate from evolutionary and environmental factors that have not favored the development of an effective immune response[5]. Within this framework, the patterns of

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response to *T. gondii* infection appear to vary across species, with callitrichids identified as the most vulnerable NHP group, demonstrating an estimated mortality rate nearing 100%[5].

Due to the limited number of studies focusing on acute fatal toxoplasmosis (AFT) in neotropical free-ranging NHPs[7–10], comprehensive investigations and epidemiological studies are crucial to understanding the disease's dynamics within these populations. Furthermore, anthropogenic pressures on free-living NHPs, such as habitat destruction and fragmentation, increase their exposure to human-generated food waste, garbage[9–11], and environments contaminated with *T. gondii* oocysts[12–14].

Although the transmission dynamics of toxoplasmosis in these populations are not fully understood, the potential role of urbanized free-ranging marmosets as environmental sentinels for the disease has been previously proposed, emphasizing their significance within the One Health paradigm[10], similar to proposed roles for rodents, foxes, wild canids, chickens and sea otters[15–19]. The broad range of hosts for *T. gondii* and the uncertainty regarding the environmental factors critical for disease maintenance complicate the characterization and comprehension of toxoplasmosis epidemiology in NHPs within anthropogenic settings, impacting ecological integrity and public health.

To address the limited understanding of toxoplasmosis in anthropogenic environments, we investigated the disease in free-ranging marmosets from Central Brazil. This study provides a detailed analysis of the disease's key epidemiological characteristics, supported by robust pathological and molecular evidence. The research aims to elucidate the significance and actual impact of toxoplasmosis on wild marmosets inhabiting urbanized regions of Central Brazil, offering novel insights into the factors shaping the disease's dynamics in human-modified environments.

Results

Epidemiology and spatial distribution

Between 2017 and 2022, 1,346 records of free-ranging NHP deaths were reviewed. Only cases with confirmed geographical locations were included, representing 81.3% (1095/1346) of the dataset. Necropsies were performed on 515 NHPs from the federal district (FD), while formalin-fixed organs from 580 NHPs from Goiás (GO), Mato Grosso (MT), and Tocantins (TO) states were analyzed. The geographical distribution of AFT in free-ranging marmosets is illustrated in (Fig. 1).

The overall prevalence of AFT in Central Brazil was 9.2% (101/1,095), with regional prevalence rates of 10.3% (53/515) in the FD and 8.2% (48/580) in the other states: 5.3% (31/580) in GO, 0.3% (2/580) in MT, and 2.6% (15/580) in TO. Additionally, *T. gondii* DNA was detected in 45.0% (108/240) of NHPs with other causes of death tested in the FD. Extrapolating these findings, an estimated 208 marmosets that died of other causes in the FD (208/462, 45.0%) were likely non-fatally infected with *T. gondii*. Considering AFT cases and estimated non-fatal *T. gondii* infections, the overall estimated prevalence (*E-prevalence*) of toxoplasmosis in the FD was 50.7% (53 + 208/515) with an estimated lethality (*E-lethality*) rate of 20.3% (53/208 + 53).

A total of 61 AFT cases (60.3%, 61/101) were associated with 18 outbreaks, while 40 cases (39.6%, 40/101) were isolated incidents. The FD had the highest number of outbreaks (66%, 12/18), affecting 36 NHPs, followed by GO (27.7%, 5/18) with 20 NHPs, and TO (5.5%, 1/18) with 5 NHPs. Epidemiological investigations revealed that marmoset groups were often observed in trees, moving or feeding on the ground near death locations. Stray

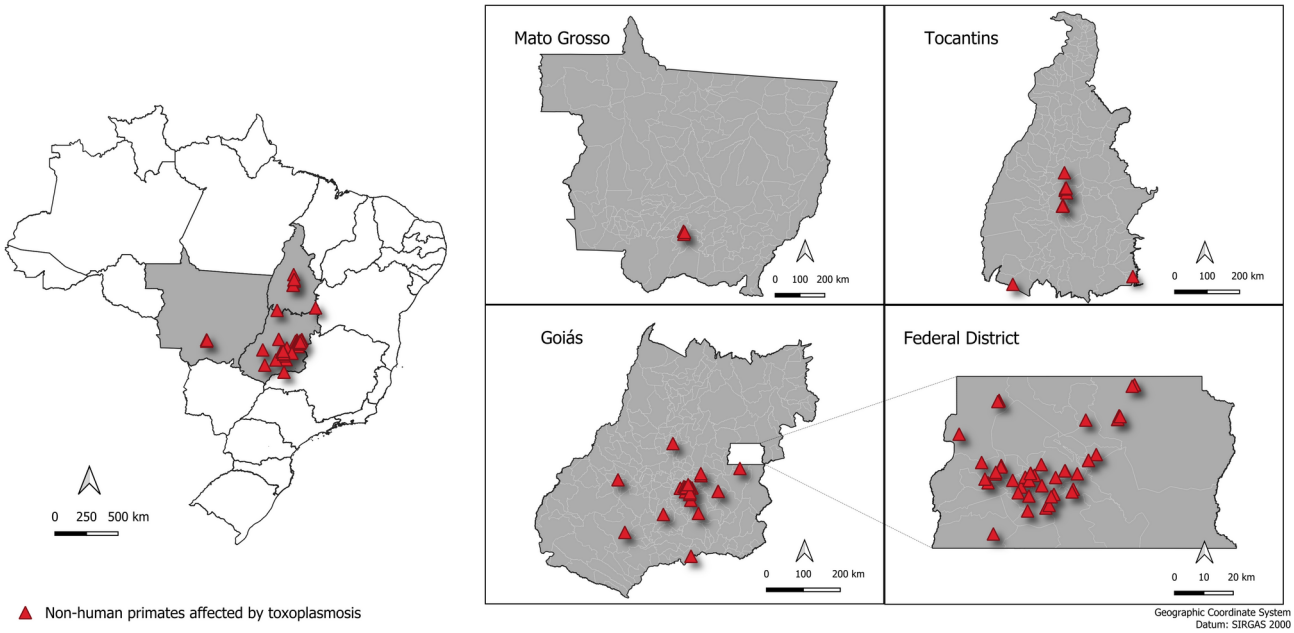

Fig. 1. Geographic distribution of acute fatal toxoplasmosis cases in free-ranging black-tufted marmosets from 2017 to 2022 in Central Brazil (Federal District, Goiás, Mato Grosso, and Tocantins States).

cats were consistently reported in the vicinity of all outbreak sites. Toxoplasmosis cases in the FD peaked during the dry season (June–September), rising from 8.3 to 19.3% ($p < 0.05$). The monthly prevalence of AFT over five years is shown in (Fig. 2).

Black-tufted marmosets (*Callithrix penicillata*) accounted for 97.1% (101/104) of AFT cases, with a few cases in *Alouatta spp.* (0.96%, 1/104) and *Aotus spp.* (1.92%, 2/104), which were excluded from analyses due to their low numbers. Among the marmosets, 47.5% (48/101) were male, 42.5% (43/101) female, and 9.9% (10/101) of undetermined sex. Adults constituted the majority of cases (42.6%, 43/101), followed by juveniles (21.8%, 22/101), and age unspecified represented 35.6% (36/101).

Pathology

Gross findings in NHPs with AFT included hepatomegaly, pale hepatic areas, and diffuse lobular pattern aspect (Fig. 3A). Spleens showed moderate to severe splenomegaly and congestion (Fig. 3B), and lungs were congested, dark red mottled, and moist (Fig. 3C) with frothy fluid in the bronchi. Hearts occasionally exhibited moderate hyperemia and multifocal pale myocardial areas (Fig. 3D).

The semiquantitative histopathological evaluation of affected organs is summarized in (Fig. 4). The liver showed multifocal random necrosis (Fig. 5A) in all AFT cases (101/101), with an average of 1.65 necrotic foci per 20x field in 82% of cases (83/101). Macrophages (64%, 65/101), followed by neutrophils (28%, 29/101) and lymphocytes (7%, 7/101) predominated within necrotic foci. Associated findings within hepatic lesions included *T. gondii* cysts (93.1%, 94/101), multifocal hemorrhage (84.1%, 85/101), and multinucleated syncytial hepatocytes (12.8%, 13/101).

Mild to severe histiocytic splenitis (96%, 97/101) (Fig. 5B) and germinal center necrosis were observed in 71.3% (72/101) of cases. Splenic white pulp hyperplasia occurred in 67.3% (68/101) of AFT cases.

Lung lesions included interstitial lymphohistiocytic pneumonia (73.2%, 74/101), mild to severe alveolar edema (74.2%, 75/101) (Fig. 5C), hemorrhagic foci (51.5%, 52/101), and occasional *T. gondii* cysts (6.9%, 7/101). Less frequent findings included type II pneumocyte hyperplasia (17.8%, 18/101) (Fig. 5C) and hyaline membrane formation (3.9%, 4/101). In the heart, mild to moderate multifocal lymphohistiocytic myocarditis was the predominant lesion (84.1%, 85/101) (Fig. 5D), followed by myocardial necrosis (68.3%, 69/101), hemorrhage (71.2%, 72/101), and occasional *T. gondii* cysts (8.9%, 9/101).

Immunohistochemical quantification of *T. gondii* and parasite load

The spleen exhibited the highest number of *T. gondii* cysts and zoites quantified in this study, based on counts from samples subjected to IHC. The red pulp was the region with the greatest concentration of cysts, with an

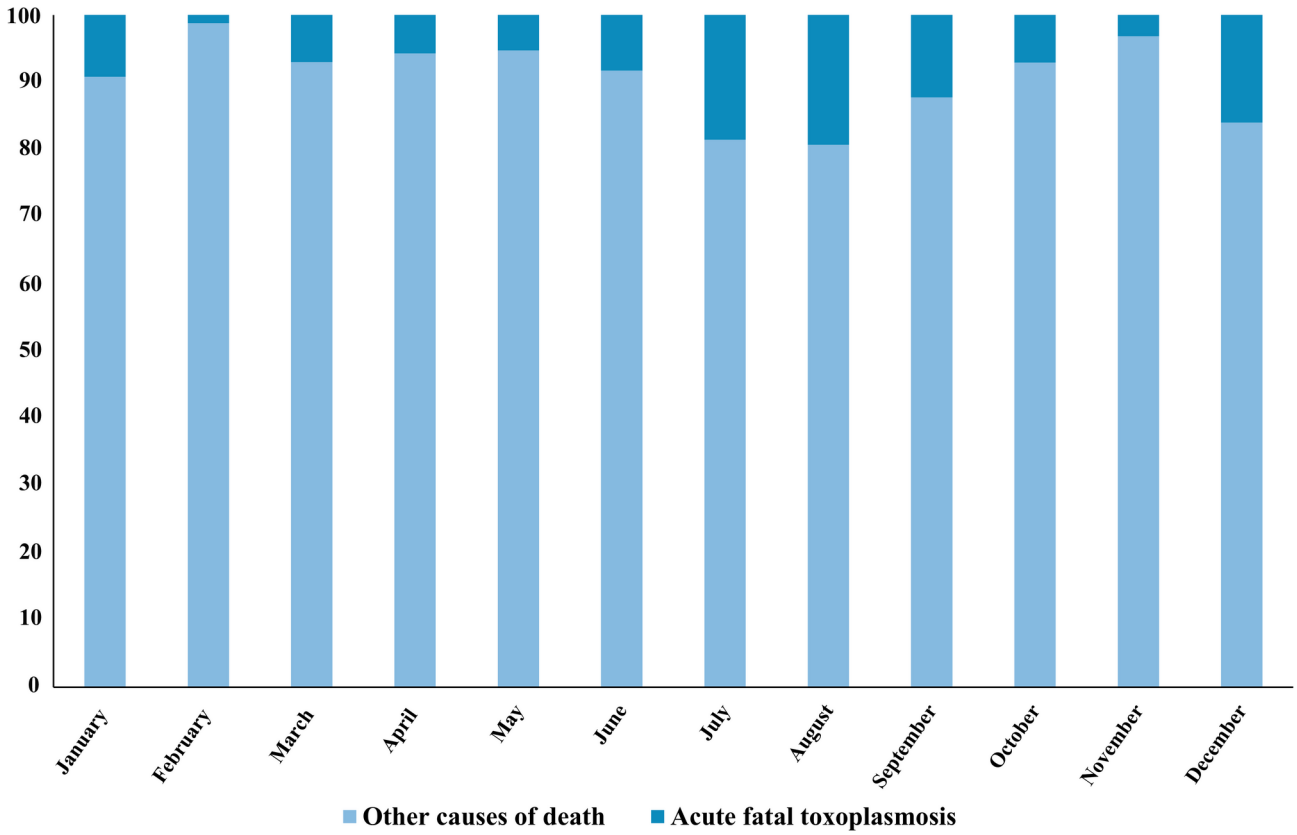

Fig. 2. Monthly prevalence (%) of acute fatal toxoplasmosis cases and free-living black-tufted marmosets between 2017 and 2022.

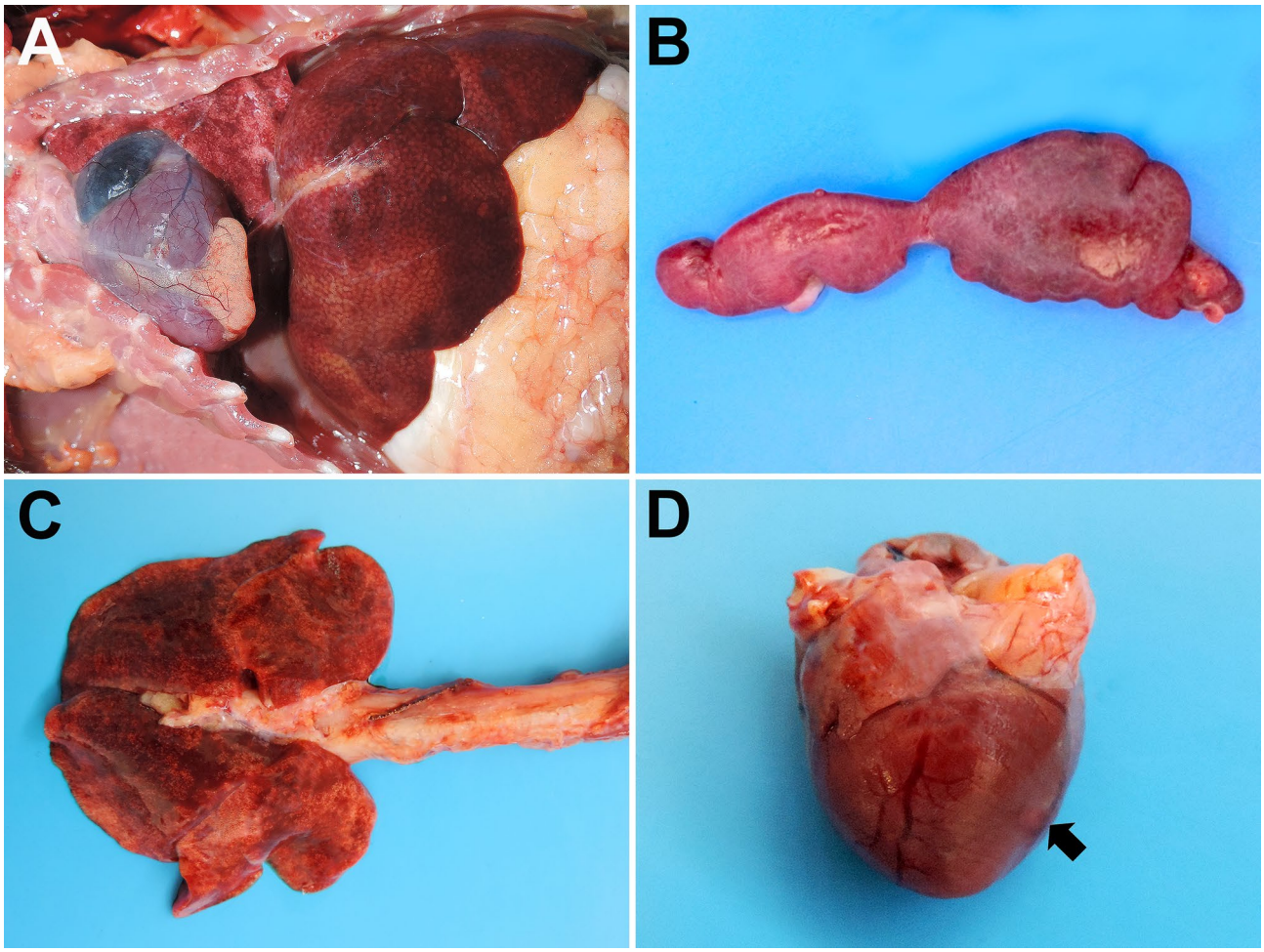

Fig. 3. Gross findings in free-ranging black tufted marmosets (*Callithrix penicillata*) with acute fatal toxoplasmosis. (**A**) Liver with marked hepatomegaly, some areas of paleness and marked lobular pattern. (**B**) Spleen markedly enlarged, congested, and with an irregular surface. (**C**) Lungs mottled with dark red areas and moist surface. (**D**) Heart with a pale area surrounded by a hyperemic halo in the ventricle (arrow head).

average of 1.75 cysts per HPF and 25.1 zoites per HPF (Fig. 6B). In the splenic white pulp, there was an average of 0.4 cysts per HPF and 7.2 zoites per HPF.

The cyst and tachyzoite of *T. gondii* count performed on the IHC-stained sections demonstrated that the liver was the second organ with the highest number of cysts and zoites in marmosets with AFT (Fig. 6A). In the liver, there was an average of 0.85 cysts per HPF and 13.0 zoites per HPF.

The lungs and heart generally showed lower quantities of *T. gondii* cysts and zoites than those found in the spleen and liver. The lungs had an average of 1.2 cysts per HPF and 3.0 zoites per HPF (Fig. 6C). The heart showed 0.3 cysts per 40x microscopic field and 1.75 zoites per HPF (Fig. 6D).

The comparison of mean values and standard deviations (m ± sd) for cyst and tachyzoite counts across the evaluated organs, the parasite loads (Table 1), alongside the correlations between these parameters and the number of hepatic necrotic foci, are presented in (Fig. 7).

Preliminary analyses revealed weak or no correlation between histiocytic splenitis, germinal center necrosis, white pulp hyperplasia, and the number of cysts or tachyzoites in the spleen. Similar findings were observed for pulmonary and cardiac lesions. Hepatic necrotic foci and inflammatory cell counts also demonstrated insignificant correlations with the number of cysts and tachyzoites in the liver. Consequently, these parameters were excluded from the primary correlation analysis (Fig. 7).

The mean cyst and tachyzoite counts detected via immunohistochemistry were significantly higher in the spleen than in other evaluated organs ($p < 0.01$). The liver exhibited the second-highest mean cyst count, while the heart showed the lowest mean tachyzoite count ($p < 0.01$). Additionally, our findings revealed a strong correlation between cysts and tachyzoite counts in the liver, which was similarly observed in the spleen.

Free-ranging marmosets with AFT displayed significantly higher parasite loads in the liver, spleen, and lungs compared to the heart ($p < 0.01$) (Fig. 8). One of the most striking findings in this study was the significant difference ($p < 0.0001$) in parasitic loads (10^3 parasite [par.] equivalent [eq.]/mL) in the liver when comparing *T. gondii* acute fatal infections (169.777×10^3 par.eq./mL ± 189.913×10^3 par.eq./mL, $n = 40$) with non-fatal infections (7.244×10^3 par.eq./mL ± 8.689×10^3 par.eq./mL, $n = 208$).

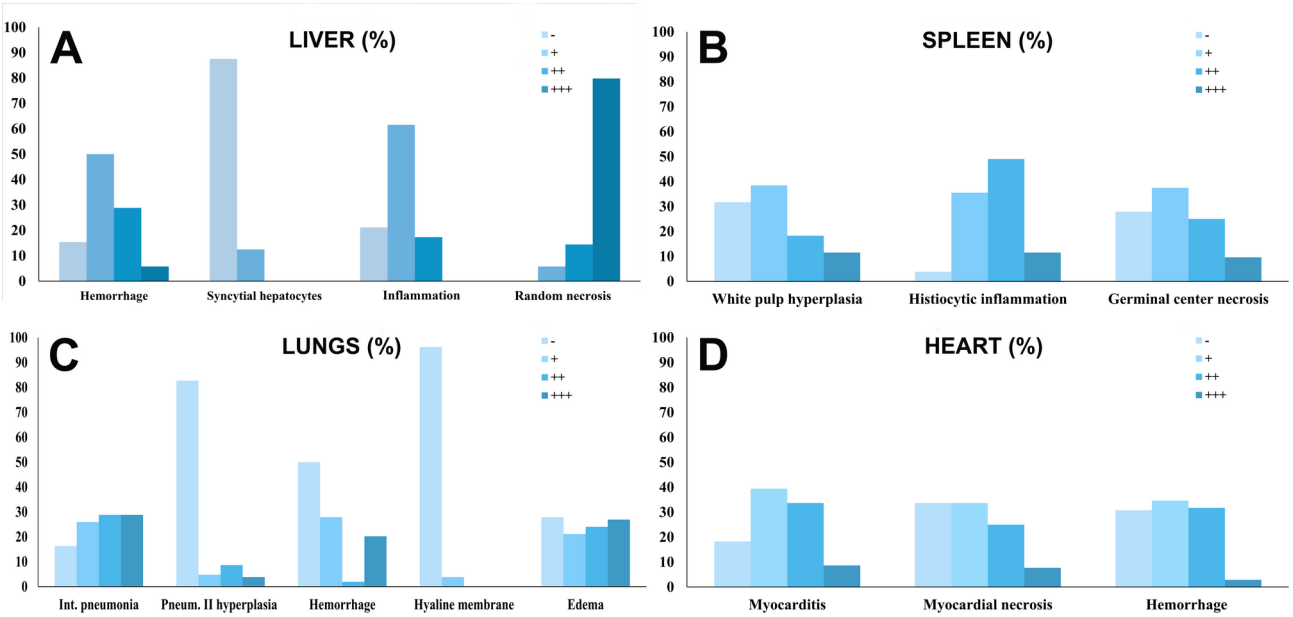

Fig. 4. Semiquantitative histopathological evaluation of the liver (**A**), spleen (**B**), lungs (**C**), and heart (**D**) from black-tufted marmosets with AFT. The charts illustrate frequencies of histopathological findings graded as absent (−), mild (+), moderate (++), or severe (+++).

Discussion

Surveillance of infectious diseases in wildlife, particularly free-ranging NHPs, is crucial for identifying zoonoses and emerging or neglected diseases that pose risks to public health. Monitoring these diseases also enhances our understanding of ecological dynamics, assesses their impact on NHP populations, and aids in biodiversity conservation in natural and urban settings.

This study offers a groundbreaking overview of toxoplasmosis in free-ranging marmosets in Central Brazil. Our findings reveal a relevant incidence of fatal *T. gondii* infections (9.2%) in marmosets within the Brazilian Cerrado biome, rising to over 10% among free-ranging marmosets in the FD, with no apparent discernible influence from sex, age, or location. Variations in AFT prevalence across states may result from limited sample sizes in some regions (e.g., Mato Grosso State) or nonspecific local factors that influence diagnostic rates. Additionally, we estimated an overall prevalence of 50.7% of non-fatal *T. gondii* infections and a lethality rate of 20.3% for toxoplasmosis in free-ranging black-tufted marmosets in the FD, which are key findings that underscore the study's significance.

While AFT is well-documented in captive neotropical NHPs in South America[20–22], reports of AFT in free-ranging neotropical NHPs remain limited to Brazil and may not accurately reflect the disease's prevalence in wild populations[7–10]. Most studies on AFT in wild NHPs estimated seroprevalence[23–27], but acute toxoplasmosis can cause death in susceptible animals before seroconversion, complicating its detection through serological methods such as the modified agglutination test (MAT)[28]. This limitation underscores the challenges of determining AFT prevalence in free-ranging NHPs in Brazil, which remains largely unknown.

Similar to the high infection rate reported in this study, previous research detected *T. gondii* DNA in 17.9% of heart samples from 38 free-ranging common marmosets (*Callithrix jacchus*) with undetermined causes of death in Northeastern Brazil[29]. AFT accounted for 25% of fatal infectious diseases and 4.6% of mortality causes in free-ranging NHPs in Rio Grande do Sul state[8]. Additionally, a retrospective analysis of 1,001 deaths among free-ranging callitrichids from the Brazilian Atlantic Forest reported a 1.6% prevalence of AFT in Rio de Janeiro[9]. Comparing toxoplasmosis prevalence across NHP populations reveals notable variations, likely influenced by differences in sample size, diagnostic methods, and ecological characteristics of regions such as the Atlantic Forest, Southern Brazil, Northeastern Semiarid, and Cerrado Biome in Central Brazil.

Similarly, the lethality of toxoplasmosis in Brazilian wild neotropical NHPs remains uncertain. Our study estimated a lethality rate of roughly 20% by analyzing both fatal and non-fatal cases of *T. gondii* infection in the overall studied population in FD. This finding underscores the disease's significance as a cause of death in free-ranging NHPs. Despite potential biases, such as variations in the collection rate of dead animals and environmental and ecological variables, this study offers a novel perspective on toxoplasmosis in predominantly urbanized marmoset populations in Central Brazil.

In Central Brazil, AFT was diagnosed in both outbreaks and isolated cases, consistent with reports from captive neotropical NHPs and, more recently, free-ranging callitrichids in Rio de Janeiro[4,9,22,30]. While most cases in our study involved single AFT diagnoses, it is possible that other family group members also died, but their carcasses were not found to be recovered for necropsy and sampling.

Our findings indicated a seasonal trend, with an increase in AFT cases during the dry season (May to September) and extending into the early rainy season (October to April) in Central Brazil. Seasonal variations in

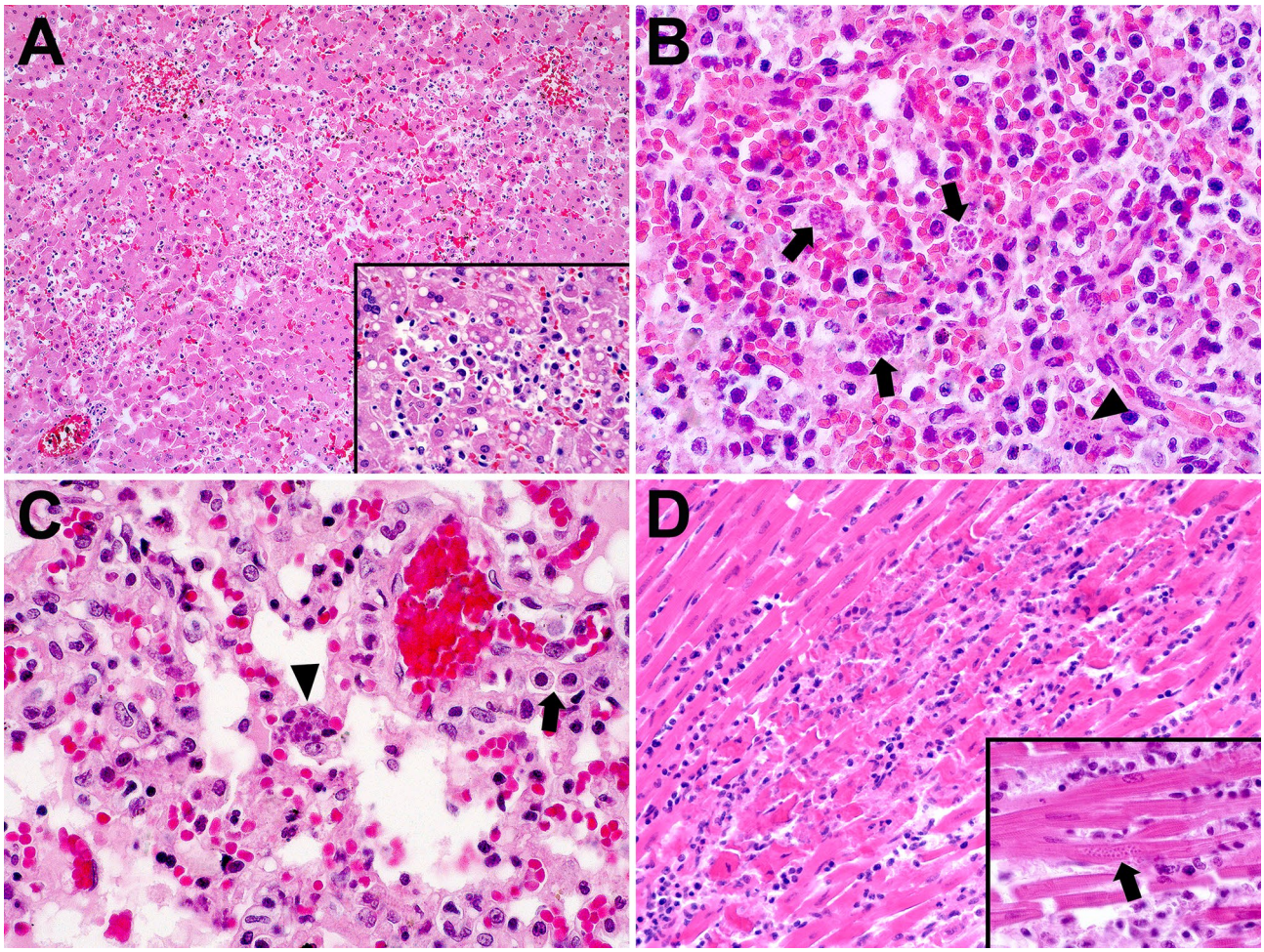

Fig. 5. Histological lesions in free-ranging black tufted marmosets (*Callithrix penicillata*) with acute fatal toxoplasmosis. (**A**) Liver. Multifocal random hepatocellular necrosis (H&E, original magnification 20X). Inset: higher magnification image of mononuclear inflammatory infiltrate within a focus of necrosis (H&E, original magnification 40X). (**B**) Spleen. Histiocytic splenitis with *T. gondii* cysts (arrows) and zoites (arrowhead) (H&E, original magnification 40X). (**C**) Lung. Thickening of the interalveolar septa due to histiocytic and lymphocytic infiltration with intralesional *T. gondii* zoites (arrowhead), congestion, and type 2 pneumocyte hyperplasia (arrow) (HE, original magnification 40X). (**D**) Heart, ventricle, myocardium. Focal area of myocarditis with intralesional histiocytes, lymphocytes, and necrosis of cardiomyocytes (H&E, original magnification 40X). Inset: higher magnification image of an intramyocardial *T. gondii* cyst (arrow) (H&E, original magnification 40X).

food availability and rainfall likely create environmental pressures influencing mortality patterns in primates[31]. Reduced food availability during the dry season[32] may drive marmosets to forage more intensively, increasing their exposure to contaminated environments and food sources (e.g., domestic waste) with *T. gondii* oocysts, thereby elevating infection risk. However, the role of seasonality in AFT patterns among free-ranging NHPs remains poorly understood, warranting further research to clarify its impact on mortality trends and disease persistence in NHP populations.

The susceptibility of neotropical NHPs to toxoplasmosis is well-documented, and arboreal behavior is considered an evolutionary factor contributing to low resistance against soil-borne pathogens like *T. gondii*[5,6]. Urban expansion and increased human activity in natural environments amplify interactions between urbanized marmosets and other threatening conditions such as electrocutions, leptospirosis, and Alphaherpesvirus infections, including *T. gondii*-contaminated environments and food sources[33–36]. Urbanized free-ranging marmosets with AFT may also act as environmental sentinels, signaling geographical areas of potential risk for human infection[10], and highlights the importance of diagnosing toxoplasmosis in urbanized free-ranging NHPs.

In this study, no clinical signs were observed in NHPs diagnosed with AFT, as the animals were found dead. Clinical reports of toxoplasmosis in free-ranging marmosets are rare, with nonspecific symptoms like depression, lethargy, tachypnea, and fever leading to death within 12 h[7]. Additional clinical signs have been described in captive neotropical NHPs, including abdominal distension, cough, and neurological symptoms such as tremors, incoordination, and paralysis[20,22,30,37].

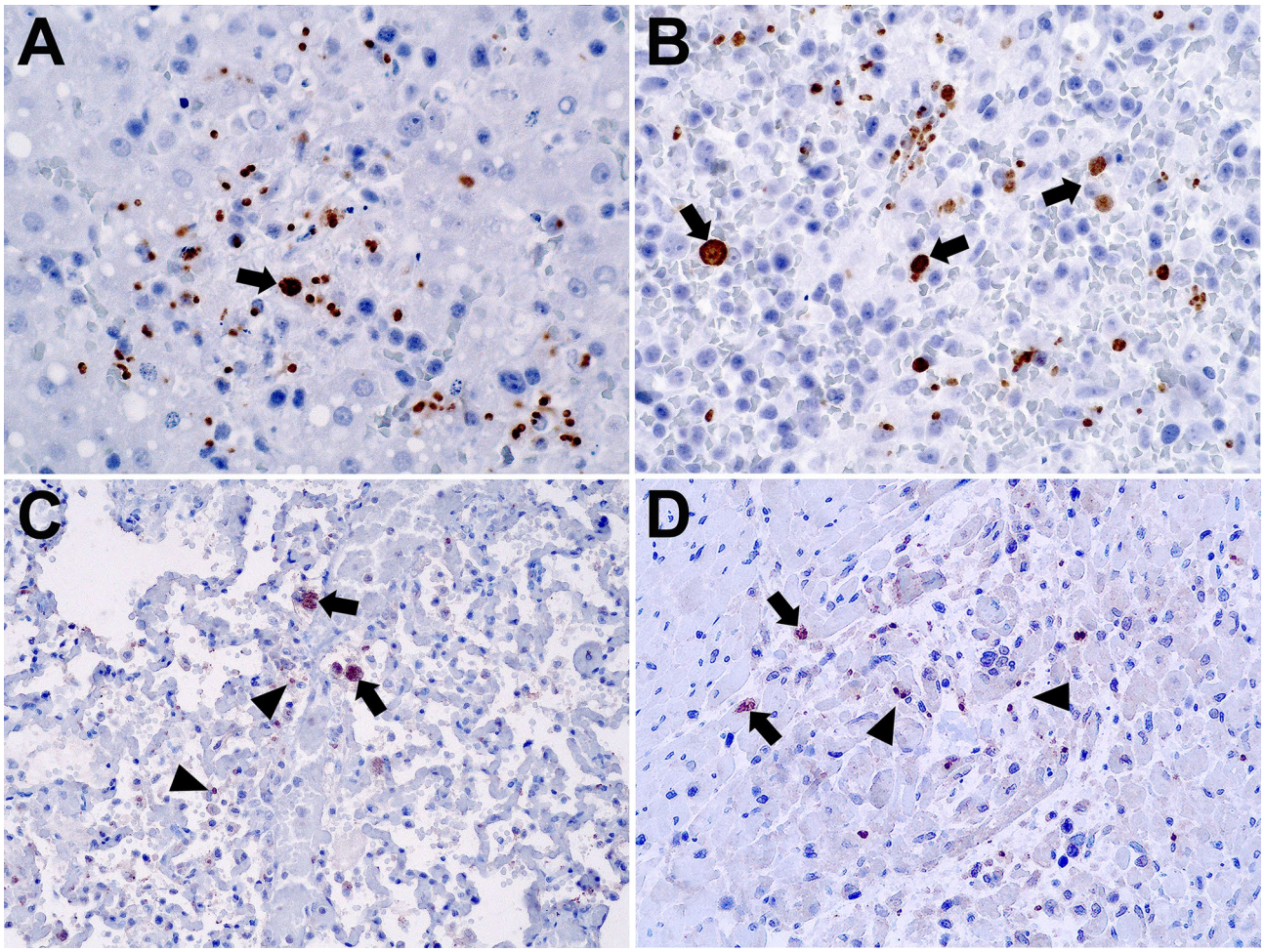

Fig. 6. (**A**) Immunostaining for *Toxoplasma gondii* within lesions in free-ranging black tufted marmosets (*Callithrix penicillata*) with acute fatal toxoplasmosis. (**A**) Liver. Intralesional protozoan cysts (arrow) and numerous zoites within a necrosis focus (IHC, immunoperoxidase, original magnification 40x). (**B**) Spleen. Protozoan cysts (arrows) and a number of zoites immunostained in the splenic red pulp (IHC, immunoperoxidase, original magnification 40x). (**C**) Lung. A few protozoan zoites (arrowheads) and cysts (arrows) immunostained in the pulmonary septa (IHC, immunoperoxidase, original magnification 40x). (**D**) Heart. Focus of myocardial necrosis with a few immunostained zoites (arrowheads) and cysts (arrows) (IHC, immunoperoxidase, original magnification 40x).

The clinical course of toxoplasmosis varies with species susceptibility and treatment duration, with death reported in captive NHPs within 2 to 15 days after symptom onset[6,22,37,38]. The acute and frequent fatal nature of toxoplasmosis in free-ranging callitrichids, combined with the rapid progression of the disease and lack of population monitoring, posed significant challenges in identifying clinical signs in affected animals in this study.

The primary gross findings in NHPs with AFT in Central Brazil included hepatomegaly with an accentuated lobular pattern, splenomegaly, pulmonary congestion, hemorrhage, and edema. Hepatomegaly with an accentuated lobular pattern was the main gross lesion in free-living marmosets with AFT. A free-living *Brachyteles arachnoides* with AFT showed similar gross findings and also hemoperitoneum, petechiae, ecchymoses in the lungs, kidneys, adrenal glands, hepatic lipidosis, and brain congestion[7]. Comparable lesions, especially involving the liver and lungs, have also been documented in captive neotropical NHPs across various countries[21,22,30,38–40].

Less commonly observed gross lesions, such as enteritis, ulcerative jejunitis[41,42], and mesenteric lymphadenopathy[39,42], were noted in captive NHPs but were absent in this study. Severe gross central nervous system (CNS) lesions are rare, with only one report of diffuse submeningeal hemorrhage in the cerebral cortex of a captive *Aotus nigriceps* with AFT[37]. The lack of significant CNS macroscopic changes in AFT cases in our study underscores the low prevalence of such alterations, although the underlying pathogenesis remains unclear. Further research is warranted to characterize necropsy findings fully, as well as their clinical progression and fatal outcomes, particularly in free-ranging marmosets.

In all evaluated AFT cases, random hepatocellular necrosis with inflammatory infiltrate, predominantly histiocytic, was the primary microscopic hepatic lesion. Diffuse histiocytic splenitis was the second most frequent lesion. Necrotizing liver lesions and histiocytic spleen inflammation are hallmark findings in fatal *T. gondii* infections and have been consistently observed in both captive and free-living neotropical NHPs[7,9,10,22,30,40].

ANALYSIS	ORGAN	MEAN ± SD
Parasite load (qPCR*)	Spleen	176,673 ± 176,182[a]
	Liver	169,777 ± 189,913[a]
	Lung	109,577 ± 82,573[a]
	Heart	52,138 ± 63,054[b]
Counts of protozoan forms,IHC$^{\Delta}$	Cysts	
	Spleen	67,9 ± 27,6[a]
	Liver	22,6 ± 9,33[b]
	Lung	11,3 ± 11,6[c]
	Heart	7,2 ± 10,2[c]
	Zoites	
	Spleen	1043 ± 438,7[a]
	Liver	334 ± 133,5[b]
	Lung	473,8 ± 426,4[b]
	Heart	68,4 ± 86,7[c]

Table 1. Parasite load and quantitative analysis of immunostained *Toxoplasma gondii* cysts and Zoites in tissues of free-ranging marmosets with acute fatal toxoplasmosis. Different lowercase letters in the same variable are significant ($p < 0.01$). *Par. eq. $x10^3$/mL /50ng of DNA. $^{\Delta}$Cysts/zoites per 20 microscopic HPF.

Severe cellular damage and host cell membrane rupture during parasite invasion and replication are closely associated with cell death and tissue necrosis[43], supporting the hepatic lesions detected in this study. Simultaneously, macrophages and antigen-presenting cells recognize *T. gondii*, triggering IL-12 and TNF-α production, which activates the Th1 (CD4 T cell) IFN-γ-dependent immune response[44–47]. These immune mechanisms likely contributed to the observed microscopic lesions, with macrophages predominating in the liver and spleen of NHPs with AFT.

Pulmonary lesions, including lymphohistiocytic interstitial pneumonia and edema, were prominent in marmosets with AFT. In some cases, additional findings, such as type II pneumocyte hyperplasia and hyaline membrane formation, were indicative of diffuse alveolar damage (DAD)[48]. Similar pulmonary lesions have been documented in 94% of free-ranging callitrichids with AFT in Rio de Janeiro State, as well as in individual cases involving *B. arachnoides* and *C. penicillata*[7,9,10]. Captive NHPs worldwide have shown comparable pathological findings [39,42,40,30.22]. Type II pneumocyte hyperplasia and hyaline membrane formation associated with DAD[48] have also been described in severe AFT-associated pneumonia in captive neotropical NHPs[22,28,30].

Acute respiratory disease due to experimental *T. gondii* infection in cats begins with minimal pathological changes during tachyzoite invasion of respiratory epithelial and endothelial cells. However, significant pulmonary function impairment follows due to severe damage, including respiratory basement membrane denudation, fibrin production, and pneumocyte necrosis[49]. Non-inflammatory lesions indicative of DAD may occur during the early stages of infection, but these changes are often visible only through electron microscopy, complicating the correlation between observed lesions and stages of pulmonary injury via light microscopy[49]. It is plausible to hypothesize that the pulmonary lesions identified in free-ranging marmosets with AFT may share pathogenic mechanisms similar to those observed in experimentally infected cats.

Cardiac involvement was less frequent in this study, with myocarditis and cardiomyocyte necrosis being the most significant lesions observed. Cardiac lesions are generally less common in humans and NHPs with toxoplasmosis, and inflammation with or without cardiomyocyte necrosis is the most prevalent manifestation[9,50], as also noted in our findings. Cardiac involvement was reported in approximately 44% of free-ranging callitrichids in Rio de Janeiro State[9], 33% of captive neotropical NHPs in the Belo Horizonte Zoological Garden[9,28], and in isolated cases among captive NHPs[21,37,38].

Severe inflammation plays a critical role in developing cardiac lesions caused by *T. gondii*. Experimental studies in mice have shown elevated levels of IFN-γ and TNF-α in the heart during the acute phase of infection and oxidative stress-related proteins such as COX2 during the chronic phase[51]. The invasion and rupture of cardiomyocyte membranes by *T. gondii* initiate the recruitment of antigen-presenting cells, leading to inflammation and cardiomyocyte necrosis[50,51]. While these studies suggest a strong association between intramyocytic protozoans and myocarditis in humans and mice, the absence of data on inflammatory mediators like IFN-γ, TNF-α, and COX in the heart represents a key gap in understanding AFT pathophysiology in free-ranging NHPs.

IHC revealed organ-specific differences in cyst and zoite counts. The spleen showed the highest number of cysts and zoites, followed by the liver. Statistically, the spleen had significantly higher mean counts of cysts and zoites than any other organ, while the liver ranked second in zoite counts. These results underscore the importance of the spleen and liver in detecting *T. gondii* infection in free-ranging marmosets. Previous research on free-ranging marmosets also highlighted the liver's role as a key organ for histopathological diagnosis of toxoplasmosis[9].

Although AFT causes notable liver damage, the substantial presence of *T. gondii* forms in the spleen, as confirmed by IHC and histopathology, suggests that the spleen is critical for diagnosing toxoplasmosis.

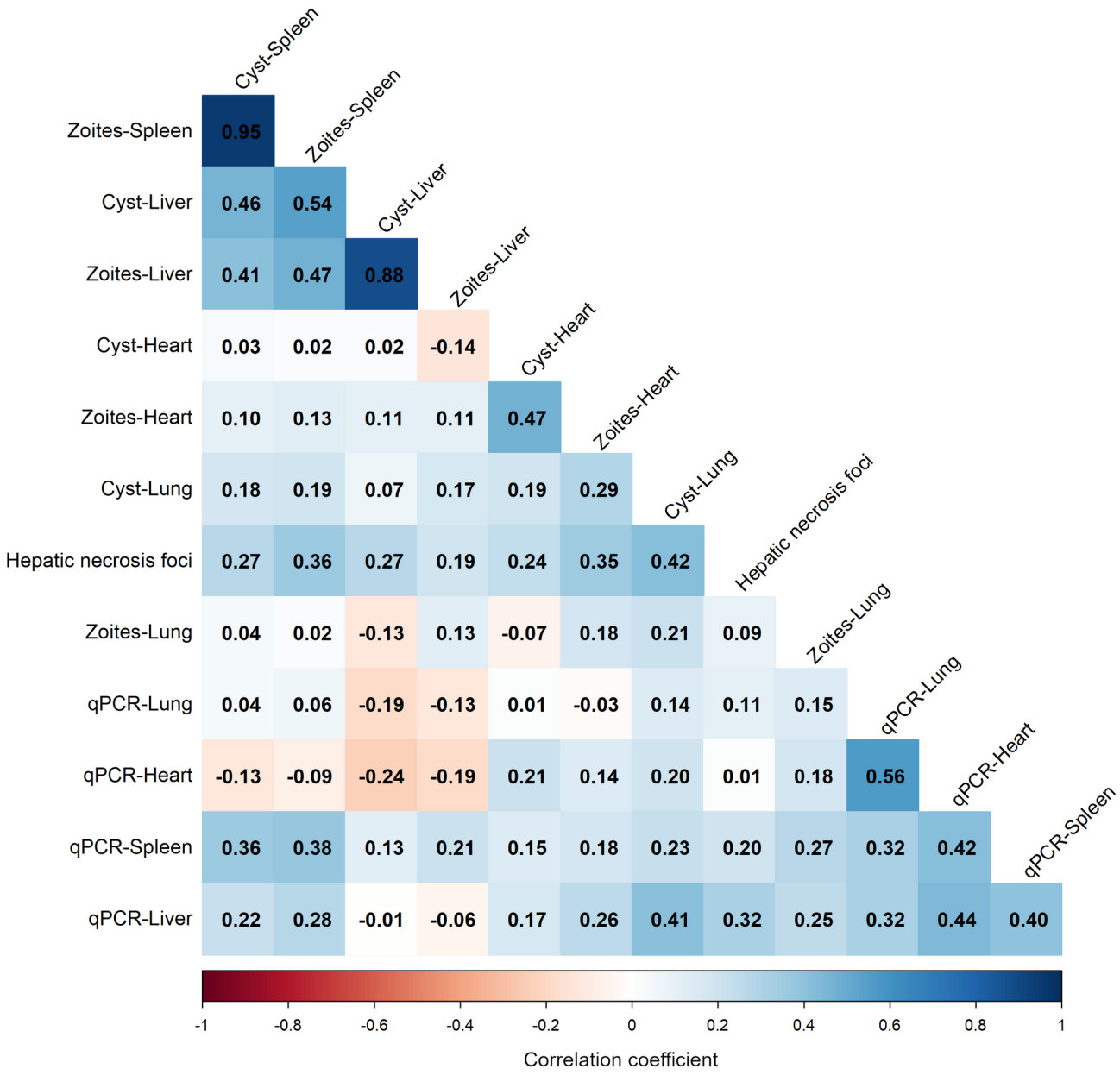

Fig. 7. Spearman correlation analysis illustrating the relationships among tissue parasitism (cyst and zoite counts), parasite loads (qPCR) in the liver, spleen, lungs, and heart, and hepatic necrosis foci counts in free-ranging black-tufted marmosets with acute fatal toxoplasmosis in the federal district.

Furthermore, the strong correlation between cyst and tachyzoite counts in the spleen and liver supports their designation as preferential target organs for AFT diagnosis in free-ranging marmosets.

In contrast, the heart displayed the lowest cyst and zoite counts across histopathological and IHC evaluations, with mean zoite levels significantly lower than in other organs. This finding contrasts with a study of 16 AFT cases in marmosets, where *T. gondii* was readily detectable in cardiac tissue, with high concordance between zoites identified via histopathology and IHC[9]. While toxoplasmosis is recognized as a systemic disease in neotropical NHPs, these discrepancies may result from variations in host susceptibility, the virulence of pathogenic strains, sample sizes, or the number of confirmed AFT cases analyzed. Such factors likely influence the detectability of *T. gondii* in cardiac tissue, explaining the observed differences.

The high parasite load observed in free-ranging marmosets with AFT is a significant finding of this study. Comparative analysis revealed that the mean parasite loads in the spleen, liver, and lungs were notably higher than those in the heart. Interestingly, despite no correlation between parasitic load and cyst or zoite counts identified via IHC, organs with the highest and lowest cyst and zoite counts (spleen and heart, respectively) also exhibited the highest and lowest mean parasite loads. This pattern suggests reduced detectability of *T. gondii* in the heart compared to other organs.

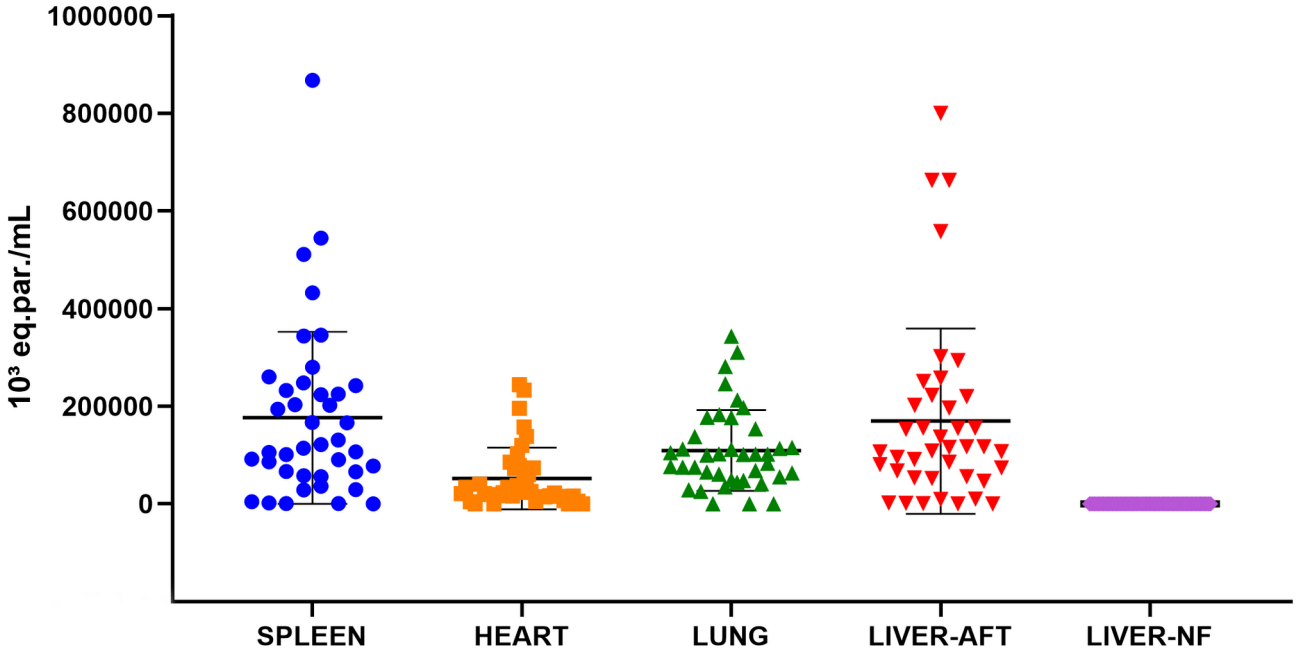

Fig. 8. Parasite loads in the liver, spleen, and lungs of free-ranging marmosets with AFT and in the liver of cases with non-fatal toxoplasmosis x 10^3 parasites equivalent/mL (x 10^3 par.eq./mL).

Contrastingly, an AFT outbreak in squirrel monkeys (*Saimiri sciureus*) in Japan reported higher parasitic loads in the lungs, liver, and heart compared to the spleen[30], although the overall loads were much lower than those observed in our study. Quantifying parasitic load in NHPs with toxoplasmosis remains underexplored. Variability in parasite loads across organs may stem from differences in DNA quality from formalin-fixed paraffin-embedded tissues, smaller sample sizes, and individual susceptibility among NHPs.

Our findings also revealed significantly higher parasite loads in fatal cases of toxoplasmosis compared to non-fatal cases. This represents a novel insight, emphasizing the high lethality of the disease in free-ranging black-tufted marmosets and underscoring the severe threat toxoplasmosis poses to this population. Approximately one-fifth of infected marmosets experienced fatal outcomes, characterized by systemic lesions most prominently affecting the liver, spleen, lungs, and heart. However, it was not possible to determine specific parasite load thresholds predictive of fatal outcomes in this study.

AFT in urbanized free-ranging NHPs represents a significant ecological and public health challenge. Similar to the role of terrestrial and aquatic species as environmental sentinels for toxoplasmosis[15–19], the pronounced susceptibility of marmosets to AFT suggests they could serve as reliable indicators of the extent and risks associated with environmental contamination by *T. gondii* in anthropogenic environments[10]. Consequently, NHPs may act as valuable environmental sentinels, providing early warnings of human exposure risk, particularly for acquired, gestational, and congenital toxoplasmosis.

Multiple ecological and anthropogenic factors influence the transmission and persistence of *T. gondii* in the environment[52,53], particularly affecting free-ranging marmoset populations in this study. Environmental contamination by oocysts shed by domestic cats is a major driver of infection risk and the maintenance of toxoplasmosis[54]. The high prevalence of the disease in marmosets suggests that anthropogenic landscapes in Central Brazil may provide favorable conditions for transmission. Despite a limited understanding of spatial and temporal variations in toxoplasmosis, urban expansion and increasing human activity may create yet unidentified conditions that sustain the parasite's life cycle. These factors could also intensify interactions between marmosets and contaminated sources, such as domestic waste carrying *T. gondii* oocysts. Consequently, the growth of non-human primate (NHP) populations in these environments likely elevates the cumulative risk of infection as individuals traverse areas with varying environmental parasite loads.

Conclusion

Despite the significant insights provided, research on wild free-ranging marmosets in urbanized regions of Brazil remains limited. This study contributes to the understanding of toxoplasmosis as an emerging and neglected disease in these populations. These findings form a critical foundation for developing targeted conservation strategies for free-ranging NHPs and may integrate toxoplasmosis management into public health initiatives.

Furthermore, our findings contribute to understanding the role of toxoplasmosis in NHP mortality, offering valuable insights for developing diagnostic algorithms and enhancing the role of NHP surveillance in public health efforts. However, the broader epidemiological impact on other NHP populations and species across Brazil remains poorly studied. Additional research is critical to addressing these knowledge gaps and advancing a more comprehensive understanding of the disease's ecological and public health implications.

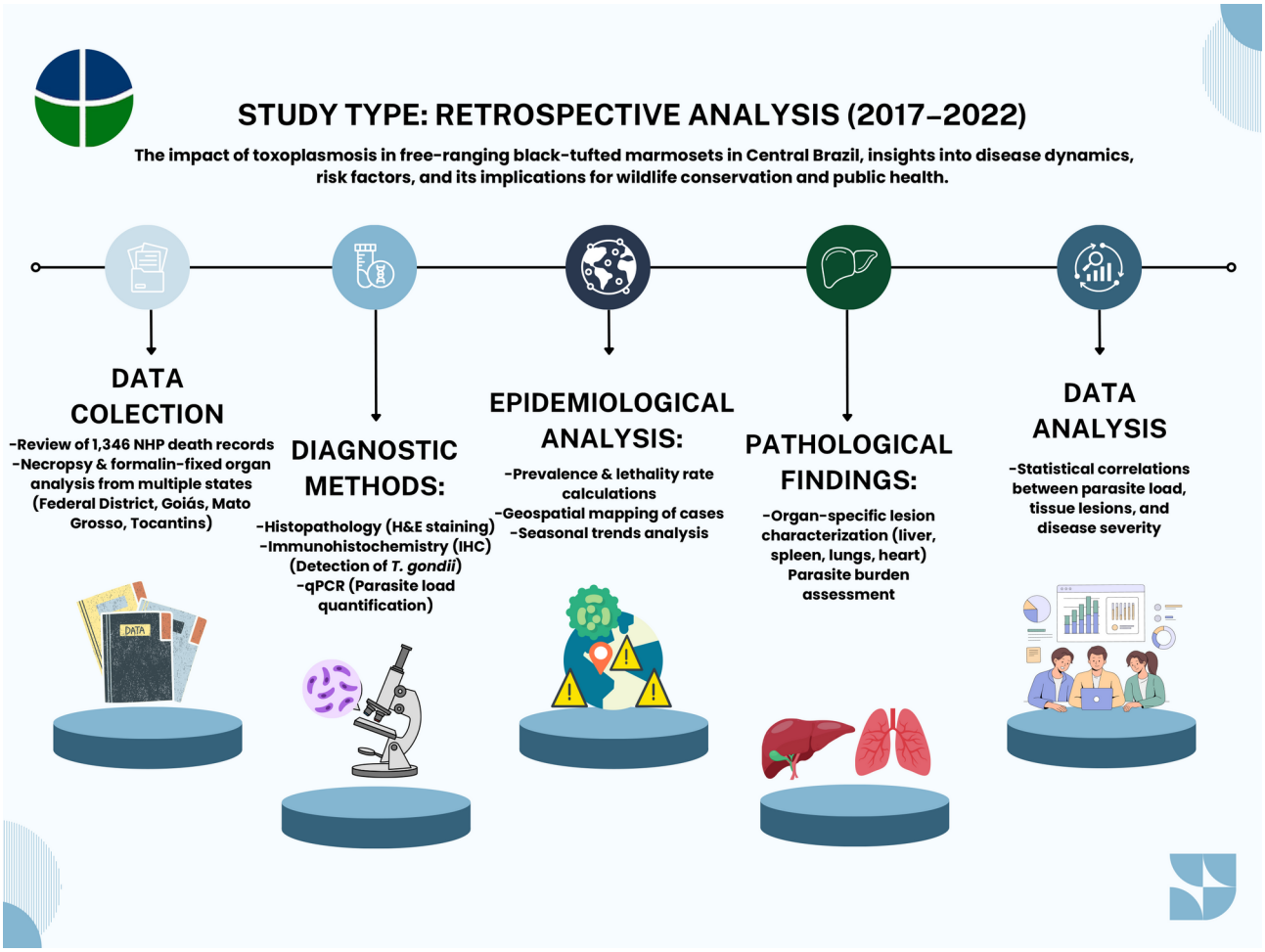

Fig. 9. Diagrammatic representation of the study design investigating acute fatal toxoplasmosis in NHPs in Central Brazil from 2017 to 2022.

Methods

The design of this study is summarized in (Fig. 9).

Animals and epidemiological data

The Veterinary Pathology and Forensics Laboratory at the University of Brasília (VPFL-UnB) is one of the Official Regional Laboratories designated by Brazil's Ministry of Health to diagnose Yellow Fever and other diseases in deceased NHPs across the Midwest Region. The VPFL-UnB receives deceased NHPs for necropsies and yellow fever examinations. Additionally, VPFL-UnB receives formalin-fixed organ fragments from both captive and free-ranging NHPs, and these specimens are collected by State Health Surveillance Services (Federal District and the states of Goiás, Mato Grosso, and Tocantins) as part of the Brazilian Yellow Fever Control Program. This study utilized the archives of NHP necropsy records at the VPFL-UnB, including data and samples. Therefore, ethical approval was not required for the study.

A retrospective study on AFT in free-ranging marmosets was conducted using VPFL-UnB archival data (from January 2017 to December 2022). Data from necropsy and epidemiological records included sex, age group, date of death, and geolocation. Cases without a location or incomplete data were excluded from the study. All AFT cases exhibited random necrotizing hepatitis and/or histiocytic splenitis with intralesional zoites and cysts, confirmed via immunohistochemistry (IHC) for *T. gondii*.

Yellow Fever Virus infections were ruled out in all cases using IHC and polymerase chain reaction (PCR) of liver samples. Additionally, liver samples from 240 NHPs randomly selected with other causes of death were tested using quantitative polymerase chain reaction (qPCR) for *T. gondii* estimated prevalence and lethality calculation. Monthly prevalence was assessed by correlating the monthly distribution of AFT cases with Central Brazil's dry (April–September) and rainy (October–March) seasons[55].

Data analysis

Prevalence and lethality rates of AFT were calculated relative to the total NHP necropsies during the study. The estimated overall prevalence (*E-prevalence*) and lethality (*E-lethality*) rates of toxoplasmosis were determined

based on the results of qPCR analyses performed on samples collected from necropsied marmosets in the federal district (FD).

E-prevalence

- total of necropsied NHPs in FD (X) - total number of NHPs with AFT (Y) = number of NHPs with other causes of death (Z);
- total of *T. gondii*-positive cases in tested NHPs with other causes of death : total of *T. gondii*-tested in NHPs with other causes of death = prevalence of toxoplasmosis in *T. gondii*-tested NPHs in FD (K);
- K x Z = estimated number of NHPs with other causes of death and *T. gondii*-infected (W);
- (W + Y) : X = *E-prevalence*.

E-lethality

Y : (W + Y) = *E-lethality*.

Frequencies were analyzed using Chi-square or Fisher's exact tests, while quantitative data and parasite loads were compared using ANOVA and the Mann-Whitney test in GraphPad Prism® 8.01. Spearman correlation analysis assessed the relationships between hepatic necrotic foci, parasitic load, cyst counts, and zoite counts. Normality was tested with the Shapiro-Wilk method, and correlation analyses were conducted using R software (version 4.2.1) with the "corrplot" package.

Spatial distribution

The spatial distribution of AFT cases was mapped using geolocation data from free-ranging marmoset deaths with QGIS 3.16.

Pathology and immunohistochemical parasite quantification

Organ samples were collected during necropsies, fixed in 10% buffered formalin, processed for histological analysis, stained with hematoxylin and eosin (H&E), and evaluated microscopically, with *T. gondii*-related lesions graded as absent (−), mild (+), moderate (++), or severe (+++). Additionally, a quantitative analysis of the number of liver necrosis foci was performed with a calibrated graticulate eyepiece at 20x magnification (0.25×10^6 μm^2) in 20 fields. Fresh liver, spleen, lungs, and heart samples were frozen at −80 °C.

IHC staining for *T. gondii* utilized the biotin-peroxidase-streptavidin method (ImmunoDetector DAB, HRP, BioSB Inc., Santa Barbara, CA, USA) with antigen retrieval conducted in a pressure cooker and citrate buffer solution (pH 6.0) for 3 min at 125 °C. The histological slides containing the tissue sections were then incubated overnight with the polyclonal anti-*T. gondii* antibody (VMRD Inc., Pullman, WA, USA) at a 1:400 dilution[30].

Quantitative analysis of *T. gondii* in the liver, spleen, lungs, and heart samples was conducted using a calibrated graticulate eyepiece at 40x magnification (4×10^6 μm^2). Twenty microscopic high-power fields (HPF, objective 40x) were randomly selected for analysis, and cysts were counted on H&E-stained slides, while cysts and zoites were counted on IHC anti-*T. gondii* stained slides only.

DNA extraction and parasite load

Randomly selected frozen liver, spleen, lung, and heart samples of 40 NHPs with AFT (40/101, 39.6%) and frozen liver samples from 240 NHPs (240/462, 51.9%) that died from other causes unrelated to toxoplasmosis in the FD were submitted for DNA extraction followed by qPCR for absolute quantification of *T. gondii*. DNA extraction was performed using the commercial PureLink Genomic DNA Mini Kit (Invitrogen™, Massachusetts, USA) using 25 to 50 mg of frozen tissues and according to the manufacturer's instructions. The concentration and purity of the eluted DNA were determined, as well as the integrity of the extracted genetic material and the absence of PCR inhibitors[56].

The parasite load in the tissues was determined by real-time PCR (qPCR) through the absolute quantification of *T. gondii* DNA. A standard calibration curve was generated using serial dilutions (1:10) of *T. gondii* DNA extracted from cell culture (from 10^6 to 10^2 parasites) with an efficiency of 109% and used to quantify the parasite load based on the linear regression line. To minimize variations between multi-plate measurements from different runs, a *T. gondii* DNA sample was included in each qPCR and used as a calibrator, applying a correction factor between assays[57].

The reaction mix consisted of 50 ng of template DNA, 0.2 μM of each primer[58], and 10 μL of Power SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific/Fermentas, CA, USA), in 20 μL final volume. The qPCRs were carried out in 96-well plates (Optical 96-Well Reaction Plate, MicroAmp®) in duplicate on a QuantStudio 3 thermocycler (Applied Biosystems, CA, USA): 2 min at 50 °C (due to the presence of dUTP in the master mix), 10 min at 95 °C, 15 s at 95 °C, 50 s at 58 °C, and 10 s at 72 °C (the last three steps repeated for 40 cycles). A dissociation step was performed at the end of thermal cycling from 60 to 95 °C. The specificity of the amplified products was checked by analysis of the melting curve. All reactions included two negative controls (no-template control and a negative sample for *T. gondii*) and a *T. gondii* DNA sample used in the standard curve.

Data availability

All data generated or analyzed during this study are included in this manuscript.

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Author contributions

Conceived the study: ASSR, ILM, LAC, LM, MJ, MM, MO, VFSP. Supervised the study: APMR, CBM, EMML. DGR, GRTC, LH, PABJ, PHOP. Formal analysis and investigation: LM, VFSP, PHOP, DGR, APMR; PABJ. Writing original draft: DERS, MBC. Writing review and editing: ASSR, CBM, DERS, EMML LH, ILM, LAC, LM, MBC, MJ, VFSP, MO, MM. All authors have read, reviewed and approved the final version of the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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