Passive epidemiological surveillance in wildlife in Costa Rica identifies pathogens of zoonotic and conservation importance

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Abstract

Epidemiological surveillance systems for pathogens in wild species have been proposed as a preventive measure for epidemic events. These systems can minimize the detrimental effects of an outbreak, but most importantly, passive surveillance systems are the best adapted to countries with limited resources. Therefore, the present study aims to evaluate the technical and infrastructural feasibility to establish this type of scheme in Costa Rica targeting thedetection of pathogens of zoonotic and conservation importance in wildlife. Between 2018 and 2020, 85 carcasses of free-ranging vertebrates were admitted for post mortem analysis and complementary laboratory analysis, representing a solid basis for the implementation of a passive surveillance system for wildlife diseases in the country. However, we encounter during this research significant constraints that affected the availability of carcasses for analysis, mainly related to the initial identification of cases, detection biases towards events in populated- or easily accessible-areas with nearby located wildlife management centers, further associated with financial disincentives, and limited local logistics capacity. Thus resulting in the exclusion of some geographic regions of the country. This epidemiological surveillance scheme allowed us to estimate the general state of health of the country's wildlife, establishing the cause of death of the analyzed animals as follows: (i) 46 (54.1%) traumatic events, (ii) 23 (27.1%) infectious agents, (iii) two (2.4%) degenerative illness, (iv) three (3.5%) presumably poisoning, and (v) in 11 (12.9%)undetermined. It also allowed the detection of pathogens such as, canine distemper virus, *Klebsiella pneumoniae*, *Toxoplasma gondii*, *Trypanosoma* spp., *Angiostrongylus* spp., *Dirofilaria* spp., *Baylisascaris* spp., among others. As well as recognizing the circulation of these pathogens around national territory and also on those analyzed species. This strategy is crucial in geographical regions defined as critical for the appearance of diseases due to their great biodiversity and social conditions.

Introduction

Zoonotic diseases represent one of the major burdens to society (both locally and globally), and a direct threat to public health, conservation, and human welfare programs [1,2]. Moreover, zoonotic diseases are responsible for causing significant economic losses, distorting social patterns, and negatively impacting human development indices, with a strong impact on developing countries [3–6]. A current example is the COVID-19 pandemic, that evidences the inadequacy of the infrastructures and diagnostic facilities/techniques to ensure surveillance, for early detection of potentially zoonotic agents in wildlife and for establishing control measures and mitigation [7].

Wildlife populations act as reservoirs for numerous pathogens, many of which can affect both human and animal health though with different degrees. Wildlife play various roles within the epidemiology of diseases participating in their spread and maintenance [8–10]. These roles assign wildlife the important function of sentinels of the ecosystems' health, and allows the early detection of alterations in the environment; as well as, the distribution, the re-emergence or emergence of certain pathogens in a specific region [11,12].

Tropical countries (including Costa Rica) are among the areas of most extraordinary natural diversity with concomitant high diversity of pathogens and thus, a high potential for disease emergence [13,14]. The risk posed by wildlife as possible natural reservoirs, especially in geographic regions such as Costa Rica, a crossing point between North America and South America, is stressed by migratory movements that influence the appearance of diseases [15,16]. This risk has increased drastically because of anthropogenic pressures linked to over-exploitation of natural resources and increase of land use that in turn increases the possibility of contact between wildlife, domestic animals, and humans [17,18].

One of the preventive strategies against the risk of epidemic events, promoted by the World Organization for Animal Health (OIE) and World Health Organization (WHO) is to increase the efforts to establish early detection mechanisms for pathogens, of both zoonotic and conservation importance, via epidemiological monitoring systems, and emphasizing the need to develop robust surveillance and control programs for diseases in free-

ranging species [7,19,20]. As early as 2012, the World Bank stated that preventive investments in the "ONE HEALTH" approach through veterinary and public health services could mean significant savings in response to the zoonotic outbreaks the world faces annually [21]. This statement reinforces the idea that investment in programs of epidemiological surveillance using wildlife as sentinels may be an alternative measure to prevent or predict epidemic events, which could minimize the economic impact of an outbreak.

One of the first steps to know the health status of the wildlife in a region is monitoring through passive surveillance, which identifies the causes of morbidity and mortality in a range of species based on their pathological profiles through post-mortem examinations. This approach offers advantages such as profitability and the ability to carry out a convenience sampling by taking advantage of the established infrastructure and obtaining a sustainable tool that allows understanding the emerging potential of different pathogens. Furthermore, when these schemes are set in the long term, it has been proven to provide the core information for decision-making and policies, regulations, and strategies establishment, prioritizing disease prevention, even when the sampling is biased and with incomplete geographic coverage [22–25].

Significant efforts have been made in Latin American to improve epidemiological surveillance systems aimed at animal health, however, there is still the need to optimize these schemes [26,27]. For example, according to the U.S. Department of Agriculture, Costa Rica has the infrastructure and maintains adequate surveillance programs to detect and control zoonotic diseases in farm animals [28]. However, it does not contemplate local wildlife within its scheme as it should be [29].

Several pathogens such as zoonotic parasites, vector-borne diseases, and direct transmission viruses have been identified in Costa Rican wildlife [30–41]. Nonetheless, without being contemplated in a routine systematic wildlife surveillance program, this information cannot reveal the local distribution of these infectious agents or the general health status of wildlife. And it only reflects the urgency of establishing a constant and sustainable

surveillance diseases system, where aspects such as the pathogens zoonotic in wildlife are contemplated, and open the door to further research to know the eco-epidemiology of these agents at the local level.

Countries with limited resources (LMIC) such as Costa Rica, face severe financial and logistical restrictions to monitor the health and circulation of pathogens in wildlife. Nevertheless, the animal health system should, in the short term, establish at least a passive epidemiological surveillance in wildlife. Therefore, during our research a pilot scheme for surveillance of the general health status of wildlife was implemented, aiming to evaluate the technical and infrastructural feasibility to establish sustainably of this type of scheme in Costa Rica. Eighty-five carcasses of wild species were analyzed, detecting zoonotic pathogens, such us *Klebsiella pneumoniae*, *Toxoplasma gondii*, *Baylisascaris spp*. and pathogens of conservation importance in wildlife, such us canine distemper virus, among others. Furthermore, our research allowed to know the circulating pathogens in the analyzed species and their distribution in the national territory. This demonstrated that there is the logistical capacity to implement this surveillance program even when we experience some limitations.

Material and Methods

Statement of Ethics

All samples were obtained from dead wildlife (found dead in the field or euthanized after veterinary care in specialized centers). The study was approved by the Ministry of Environment and Energy (MINAE) (wildlife authority) through permit (R-SINAC-PNI-ACLAC-039), and with the support of the animal health authority, the National Animal Health Service through the office (SENASA-DG-0277-18).

Notification and receptionof cases

Between 2018 and 2020, officials from the wildlife management centers, veterinarians from the National Animal Health Service (SENASA), and wildlife officials from the National Wildlife Service (SINAC) reported cases, and voluntarily sent specimens from different localities in the country to the Wildlife Program of SENASA or directly

to Pathology Department of the Escuela de Medicina Veterinaria, Universidad Nacional. The carcasses used in this study were all from free-ranging vertebrates with any apparent clinical condition or after death due to any associated disease or trauma. Basic information was requested for every sample submission: geographic location, the standard and scientific name of the animal, clinical signs, and any information considered relevant to the case, following the scheme recommended by the OIE for the notification of cases for disease surveillance system in wild animals [20,42]. All carcasses were shipped fresh under refrigerated conditions or stored at -20 °C for a maximum period of one week before shipping.

Pathological analysis

The carcasses received were classified by their autolysis degree according to an established scale of one to five [43]. Thus, ranging from a fresh carcass or recently dead animal (grade 1) to advanced decomposition (grade 4) and partial, mummified carcasses or skeletal remains (grade 5). Only carcasses with grades 1 to 3 were included in the study for post-mortem analysis and tissue sampling [44]. Therefore, 96 specimens were received, of which, 85 were admitted in the study. These were divided by taxonomic class into birds and mammals. The mammal class was subdivided into taxonomic groups. The geographical distribution and the taxa admitted are shown in S1 Fig.

All morphological findings were recorded. In addition, tissue samples were taken for routine histopathological and microbiological analysis as required. Tissue samples for histopathology were processed based on standard routing protocols [44].

Detection of different infectious agents

Virus Detection: Molecular methods were used for the detection of different viral agents. All molecular methods were done in the presence of a positive and a negative control. The samples analyzed were fresh tissues collected in a sterile manner during post-mortem analysis. In addition, we performed RNA extraction using the

commercial kit DNeasy Blood and Tissue (QIAGEN, Venlo, The Netherlands), following the manufacturer's recommendations. The methods used and the samples collected are specified in Table 1.

Protozoa Detection: Confirmation was performed using molecular techniques for pathogen identification when a previous presumptive protozoa presence was established in the histological study. All molecular methods were done in the presence of a positive and a negative control. Tissue samples previously embedded in paraffin were used for this purpose. The deparaffinization procedure was done using xylol washes following the method recommended to perform DNA extraction from the tissue [45]. According to the manufacturer's instructions, we performed DNA extraction using the commercial kit DNeasy Blood and Tissue (QIAGEN, Venlo, The Netherlands). The methods used and the samples collected are specified in Table 1.

Table 1. Molecular techniques for the detection of viral agents and protozoa.

Infectious agent	Target region	Method	Primer	Sequence	Reference protocol	Used material
Canine Distemper Virus (CDV).	N gene	Nested RT-PCR	First round: CDV-1F	*5'- ACT GCT CCT GAT ACT GC-3'*	Da Budaszewski et al., 2014. [46]	Tissue[a]
			CDV-2R	*5'- TTC AAC ACC RAC YCC C-3'*		
			Second round: CDV-3F	*5'- ACA GRA TTG CYG AGG ACY TRT-3'*		
			CDV-4R	*5'- CAR RAT AAC CAT GTA YGG TGC-3'*		
Alphaviruses.	nsP4	Nested RT-PCR	First round: --	*5'- TTT AAG TTT GGT GCG ATG ATG AAG TC-3' (500 nM)*	Grywna et al., 2010. [47]	Tissue[a]
				5'- GCA TCT ATG ATA TTG ACT TCC ATG TT-3' (500 nM)		

			Second round: --	*5'-GGT GCG ATG ATG AAG TCT GGG ATG T-3' (200nM)*		
				5'- CTA TGA TAT TGA CTT CCA TGT TCA TCC A-3' (100 nM)		
				5'-CTA TGA TAT TGA CTT CCA TGT TCA GCC A-3' (100 nM)		
Flaviviruses.	NS5 gene	Semi-nested RT-PCR	First round: MAMD	*5'- AAC ATG ATG GGR AAR AGR GAR AA-3'*	Scaramozzino et al., 2001. [48]	Tissue[a]
			cFD2	5'-GTG TCC CAG CCG GCG GTG TCA TCA GC-3'		
			Second round: FS 778	*5'-AAR GGH AGY MCD GCH ATH TGG T-3'*		
			cFD2	*5'-GTG TCC CAG CCG GCG GTG TCA TCA GC-3'*		
Avian Influenza virus (AI).	matrix (M) gene	qRT-PCR	M + 25	*5'-AGA TGA GTC TTC TAA CCG AGG TCG-3'*	Spackman et al., 2002. [49]	Tissue[b]
			M 124	*5'-TGC AAA AAC ATC TTC TTC AAG TCT CTG-3'*		
			M + 64	*5'-FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA-3'*		
Rabies virus.	Nucleoprotein	RT–PCR	RAB504	*5'-TAT ACT CGA ATC ATG AAT GGA GGT CGA CT-3'*	Primers: Oliveira et al. 2010. [50] Protocol: Carnieli et al. 2008 [51]	Tissue[c]
			RAB304	*5'-ACG CTT AAC AAC AAR ATC ARA G-3'*		
Newcastle virus.	Fusion gene, F0	RT-PCR	NCD3	*5'-GTC AAC ATA TAC ACC TCA TC-3'*	STAUBER, 1995. [52]	Tissue[b]

			NCD4	*5'-GGA GGA TGT TGG CAG CAT T-3'*		
Toxoplasma gondii.	529bp repetitive segment	PCR	Tox-8	*5'-CCC AGC TGC GTC TGT CGG GAT-3'*	Homan et al., 2000. [53]	FFPE[d]
			Tox-11	*5'-GCG TCG TCT CGT CTA GAT CG-3'*	Reischl et al., 2003. [54]	
Trypanosoma cruzi.	18S rRNA gene	Nested PCR	First round: SSU4_F	*5'-GTG CCA GCA CCC GCG GTA AT-3'*	First round primer: Pinto et al., 2015. [55]	FFPE[e]
			18Sq1R	*5'-CCA CCG ACC AAA AGC GGC CA-3'*		
			Second round: SSU561F	*5'-TGG GAT AAC AAA GGA GCA-3'*	Second round primer: Noyes et al., 1999. [56]	
			SSU561R	*5'-CTG AGA CTG TAA CCT CAA AGC-3'*	Protocol: Aleman et al., 2017. [57] Murphy & O'Brien, 2007.[58]	
***Leishmania* spp.**	Kinetoplast	PCR	13A	*5'- GTG GGG GAG GGG CGT TCT-3'*	Medeiros et al. 2002. [59]	FFPE[f]
			13B	*5'-ATT TTA CAC CAA CCC CCA GTT-3'*	Sosa-Ochoa et al. 2015. [60]	

FFPE: formalin-fixed paraffin-embedded;

[a] brain and lung;

[b] Lung and Trachea and cloacal swab;

[c] hippocampus, cerebellum, and medulla oblongata;

[d] spleen, lung, and liver;

[e] heart;

[f] spleen

Bacteriological detection: Tissue samples from animals with inflammatory processes (suppurative or abscesses) were cultured following standard bacteriological procedures. For bacterial isolation, samples were inoculated on non-selective and selective agar media. Significant bacterial growth was identified using the automated VITEK-2 Compact system, software version 8.02 (bioMérieux, Marcy l'Etoile, France). VITEK test cards for Gram-negative [GN], Gram-positive [GP], and anaerobes [ANC] were used for identification according to the manufacturer's instructions.

Parasites identification: All parasites recovered from animals of the two investigated classes were washed with physiological saline, preserved in alcohol, acetic acid, and formalin (AFA) solution, and subjected to identification to genus level [61]. Physical and morphometric characteristics were recognized after fixation and clarification with Hoyer's solution [62–64]. In addition, processed cestodes were stained with dilute Harris' hematoxylin solution.

Geocoding and spatial analysis

Each case was geocoded using the latitude and longitude generated by GPS of the point where the specimen was found by field personnel. When the GPS was not available, they were geocoded using the latitude and longitude of the approximate location where they were found, and this was generated by Google Earth Pro v7.3 (2021, Google Inc.). The georeferenced points of each sample admitted created a map using ArcGIS 10.7 (ERSI) (S1 Fig).

Results

Distribution of cases for age, sex, taxonomic classification and sender

Of the 85 specimens admitted to the study, there was an age distribution of 23 (27.1%) young animals and 62 (72.9%) adults. The sex distribution was 48 (56.5%) males and 37 (43.5%) females. According to the taxonomic class, we received nine (10.5%) specimens of the birds and 76 (89.5%) of the mammals. The notification of cases was made by SINAC and the Animal Health Service SENASA, with 21 (24.7%) of the cases and 64 (75.3%) by wildlife rescue centers. The biological data of the admitted specimens are shown in S2 Table. Furthermore, cases of two mass fatality events were received. In the first case, field officials reported the death of hundreds of pelicans, of which we received two specimens and they were found positive for flaviviruses. The second case was the death of several carnivores who tested positive for the canine distemper virus.

Distribution of causes of death and injuries according to etiology

The distribution of the presumptive cause of death according pathological findings corresponded to 46 (54.1%) associated with traumatic events (mainly roadkill and electrocution), 23 (27.1%) with fatalities directly related to infectious agents, and 2 (2.4%) with degenerative disease. Additionally, in 3 (3.5%) cases, the death was presumptively associated with intoxication, and in 11 (12.9%), the cause of death was not determined. Of the individuals with a cause of traumatic death, 31 (67.4%) concomitantly presented lesions associated with an infectious etiology (24 with parasites, three with bacteria, one with protozoa and four with multiple microorganisms). Of the specimens with an infectious cause of death, ten presented lesions associated with viruses, five with parasites, two with protozoa, one with bacteria, and five presented lesions associated with multiple etiologies.

Of the identified lesions by analyzed organs (gross and microscopic injuries), 199 (74.8%) were associated with infectious processes, and 67 (25.2%) were associated with non-infectious processes (46 traumatic, five toxic, four degenerative diseases, and twelve non-relevant focal lesions). According to the predominant inflammatory infiltrate of the infectious processes, presumptively 69 (34.7%) lesions were associated with protozoa and

parasites (histiocytic and eosinophilic infiltrate, respectively), 55 (27.6%) were linked with viral infections (lymphoplasmacytic infiltrate), and 36 (18.1%) were related to bacterial processes (suppurative infiltrate). Examples of these inflammatory lesions is observed in Fig 1 (see legend). In 39 lesions, the infectious etiology was not investigated because they were considered non-relevant focal lesions. The distribution of lesions identified by taxon and etiology are described in Table 2. The description of the pathological findings identified by species is detailed in S2 Table.

Of the lesions associated with infectious etiology, in 44 (22.1%), the causal agent was not possible to be determined. In 21 of these lesions, a predominantly lymphoplasmacytic inflammatory infiltrate was observed, suggestive of viral etiology (11 pneumonia, five hepatitis, four encephalitis). In 18 lesions, the inflammatory infiltrate was suppurative, suggestive of bacterial etiology (five pneumonia, four nephritis, four myositis, one dermatitis). Five lesions had a predominantly histiocytic infiltrate suggestive of the presence of protozoa (n=2 hepatitis, n=2 colitis, n=1 splenitis). All parasites associated with lesions were identified in the complementary analysis.

Table 2. Main morphological diagnoses identified in free-ranging wild animals analyzed in the years 2018-2020.

Cause of morbidity or mortality	Taxa								
	Primate (n=25)	Carnivora (n=25)	Pilosa (n=11)	Marsupial (n=5)	Rodentia (n=4)	Ungulate (n=4)	Cingulate (n=2)	Bird (n=9)	Total
Infectious[1]									**199**
Viral									**55**
Meningoencephalitis	1	12	0	0	1	0	0	0	14
Pneumonia	4	12	1	3	1	0	0	3	24
Enteritis	1	3	0	0	0	0	0	0	4
Hepatitis	3	5	0	1	0	0	0	2	11
Nephritis	0	0	0	0	0	0	0	2	2

Bacterial									**36**
Pneumonia	3	4	3	1	2	0	1	0	14
Enteritis	2	0	0	0	0	0	0	0	2
Hepatitis	2	0	0	0	0	0	0	0	2
Nephritis	2	4	0	1	0	0	0	0	7
Myositis	1	1	2	0	2	1	1	0	8
Osteomyelitis	1	0	1	0	0	0	0	0	2
Dermatitis	1	0	0	0	0	0	0	0	1
Protozoan-parasitic									**69**
Meningoencephalitis	2	0	0	1	0	0	0	0	3
Pneumonia	7	3	2	0	0	0	0	0	12
Myocarditis	0	0	0	3	0	0	0	0	3
Vasculitis	0	5	0	0	0	0	0	0	5
Colitis	15	5	0	0	0	0	0	0	20
Enteritis	2	2	0	1	0	0	0	0	5
Hepatitis	8	0	0	0	0	0	0	0	8
Gastritis (ventriculitis)	0	1	0	1	0	0	0	3	5
Nephritis	2	0	0	0	0	0	0	0	2
Splenitis	3	0	0	0	0	0	0	0	3
Dermatitis	0	1	0	1	1	0	0	0	3
Miscellaneous[2]	9	17	7	0	2	2	0	2	**39**
Non-infectious									**67**
Traumatic[3]									**46**
Cranioencephalic trauma	2	5	1	2	0	2	0	0	12
Laceration of internal organs	5	4	1	0	0	0	0	0	10

Electrothermal burns	3	0	4	0	1	0	0	0	7
Multifocal perforating wounds	1	0	1	1	0	0	2	0	6
Multiple exposed fracture	1	1	0	0	0	0	0	2	4
Hip fracture	2	1	0	0	0	1	0	0	4
Central nervous system haemorrhages	1	0	2	0	0	0	0	0	3
Degenerative disease									**4**
Neoplasm	0	1	0	0	0	0	0	0	1
Myxomatous valve degeneration	0	1	0	0	0	0	0	1	2
Crystal deposits	0	0	0	0	0	0	0	1	1
Toxic									**5**
Hepatic necrosis	0	1	0	0	0	0	0	2	3
Haemorrhages generalized	0	0	0	0	0	0	0	2	2
Miscellaneous[4]	4	6	2	0	0	0	0	0	**12**

[1]infectious etiologies were inferred based on inflammatory infiltrate;

[2]miscellaneous infectious lesions are non-relevant focal lesions;

[3]main injury associated with the cause of traumatic death was recorded;

[4]miscellaneous non-infectious alterations are non-relevant focal lesions

Fig 1. Frequent pathological findings associated with inflammatory processes in wild animals. A) Brain (*Procyon lotor*-raccoon). Perivascular lymphoplasmacytic encephalitis associated with CDV infection (Arrowhead; H&E 200x). B) Trachea (*Pelecanus occidentalis*-brown pelican). Ulcerative haemorrhagic pyogranulomatous tracheitis with pseudomembrane formation (arrowhead: H&E 200x). C) Heart (*Didelphis marsupialis*-opossum). Lymphoplasmacytic myocarditis associated

with *Trypanosoma cruzi* amastigotes (Arrowhead; H&E 200x). D) Liver (*Pelecanus occidentalis*-brown pelican). Lymphoplasmacytic and necrotizing hepatitis associated with flavivirus infection (arrowhead; H&E 200x) E) Kidney (*Canis latrans*-coyote). Lymphoplasmacytic interstitial nephritis (arrowhead; H&E 200x). F) Kidney (*Jabiru mycteria*-jabiru). Necrotizing granulomatous glomerulonephritis with crystal formation (arrowhead; H&E 100x).

Identification of viral, protozoan, bacterial and parasitic infectious agents by taxonomic group

Ten virus, seven protozoa, and seven bacteria were identified in mammalian specimens. In 22 cases, these pathogens were involved with lesions or systemic disease, of which, 19 were directly associated with the cause of death of mammals. Only *Sarcocystis* spp. detected in two cases was an incidental finding. Additionally, 38 mammals had internal parasites (23 different genera). Multi-parasitosis was observed in 13 (17.1%) of the cases. Parasites such as *Prosthenorchis* spp. (n=15), *Angiostrongylus* spp. (n=6), and *Cilycospirura* spp. (n=1) were responsible for severe parasitosis with systemic disease. Some of the lesions such as pyogranulomatous and eosinophilic meningoencephalitis, pyogranulomatous abscessing bronchopneumonia and eosinophilic gastritis associated with infectious agents are observed in Fig 2 (see legend). In 69% (n=43) of the mammalian cases, infectious agents with zoonotic potential such as *Klebsiella pneumoniae, Toxoplasma gondi*i, *Angiostrongylus* spp. were identified. The etiological agents identified by taxonomic groups and the number of specimens analyzed are specified in Table 3.

Table 3: **Number of infectious agents tested and positive in mammals according to etiology.**

Etiological agent		Mammalian taxonomic groups (positives)						
		Primate	**Carnivora**	**Pilosa**	**Marsupial**	**Rodentia**	**Ungulate**	**Cingulate**
Viral	Canine Distemper Virus (n=18)	0	10	0	0	0	0	0
	Alphaviruses (n=9)	0	0	0	0	0	0	0
	Flaviviruses (n=9)	0	0	0	0	0	0	0
	Influenza virus (n=8)	0	0	0	0	0	0	0
	Rabies virus (n=76)	0	0	0	0	0	0	0
Bacterial	*Clostridium perfringens* (n= 18)	0	0	0	0	0	1	0

	Escherichia coli (n=18)	1	0	0	0	0	0	0
	Klebsiella pneumoniae (n=18)	1	0	0	0	0	0	0
	Trueperella pyogenes. (n=18)	0	0	0	0	1	0	0
	Staphylococcus aureus (n=18)	1	1	1	0	0	0	0
	Mycobacterium spp. (n=18)	0	0	0	0	0	0	0
Protozoan	*Toxoplasma gondi*i (n=4)	2	0	0	0	0	0	0
	Trypanosoma spp. (n=14)	0	0	0	3	0	0	0
	Leishmania spp. (n=8)	0	0	0	0	0	0	0
	Sarcocystis spp. (n=5)	0	1	0	0	0	1	0
Parasitic[1]	*Angiostrongylus* spp.	0	5	0	1	0	0	0
	Dirofilaria spp.	0	4	0	0	0	0	0
	Dipetalonema spp.	5	0	2	0	0	0	0
	Gnathostoma spp.	0	0	0	1	0	0	0
	Baylisascaris spp.	0	1	0	0	0	0	0
	Ancylostoma spp.	0	1	0	0	0	0	0
	Cylicospirura spp.	0	1	0	0	0	0	0
	Prosthenorchis spp.	10	5	0	0	0	0	0
	Macracanthorhynchus spp.	0	1	0	0	0	0	0
	Spirometra spp.	0	2	0	0	0	0	0

n= Number tested;

[1] Only zoonotic parasites are shown

Fig 2. Infectious agents in lesions identified in wild animals. A) Brain (*Alouatta palliata*-howler monkey). Presence of protozoan pseudocysts in the blood vessel, morphology compatible with *Toxoplasma gondii* (arrowhead; H&E 600x). B) Brain (*Didelphis marsupialis*-opossum). Pyogranulomatous and eosinophilic meningoencephalitis associated with *Angiostrongylus* spp. (arrowhead; H&E 400x). Inset: Nematode magnification (H&E 200x & 400x). C) Lung (*Alouatta palliata*-howler monkey). Pyogranulomatous abscessing bronchopneumonia associated with *Klebsiella pneumoniae* (arrowhead; H&E 200x). Inset: Bacterial aggregates magnification (H&E 600x). D) Lung (*Cebus imitator*-white-faced

monkey). Pyogranulomatous and eosinophilic pleuro-bronchopneumonia associated to multiple *Filariopsis* spp. nematodes (arrowhead; H&E 40x). Inset: Nematode magnification (H&E 200x). E) Ventricle (*Pelecanus occidentalis*- brown pelican). Pyogranulomatous ulcerative and eosinophilic ventriculitis associated with multiple *Contracaecum* spp. nematodes (arrowhead; H&E 40x). F) Jejunum (*Ateles geoffroyi*-spider monkey). Pyogranulomatous and necrotizing jejunitis associated with *Staphylococcus aureus* infection (arrowhead; H&E x200). G) Stomach (*Herpailurus yagouaroundi*-jaguarundi). Eosinophilic gastritis associated with multiple *Cylicospirura* spp. nematodes (arrowhead; H&E 40x). H) Skeletal muscle (*Nasua narica*-coati). Presence of protozoan cyst, morphology consistent with *Sarcocystis* spp (arrowhead; H&E 200x). I) Skin (*Sphiggurus mexicanus*-porcupine) Pyogranulomatous and eosinophilic dermatitis associated with massive infestation of *Sarcoptex* spp. (arrowhead; H&E 400x). Inset: Mites magnification (H&E 100x).

All birds submitted were evaluated for the presence of the (n=9) virus, two of the birds, which were involved in an episode of high mortality during the study period, were positive for flaviviruses. Additionally, three birds had internal parasites (2 different genera). Most of the pathogens identified were directly associated as the cause of the death of birds and linked with lesions illustrated in Fig 2. Only *Procyrnea* spp. identified in one case was an incidental finding. In 22.2% (n=2) of the birds cases, infectious agents with zoonotic potential such as *Contracaecum* spp. were identified. The etiological agents identified in birds and the number of samples analyzed are specified in Table 4.

Table 4: **Number of infectious agents tested and positive in birds according to etiology.**

Etiological agent		Birds
		Positive
Viral diseases	Alphaviruses (n=3)	0
	Flaviviruses (n=3)	2
	Influenza virus (n=9)	0
	Newcastle virus (n=9)	0
Bacterial diseases	*Clostridium perfringens* (n=1)	0
	Escherichia coli (n=1)	0

	Klebsiella pneumoniae (n=1)	0
	Salmonella spp. (n=1)	0
	Staphylococcus aureus (n=1)	0
Parasites diseases	*Contracaecum* spp.	2

n= number of tested;

[1] only zoonotic parasites are shown

Geospatial distribution of detected infectious agents and their accumulation by geographic region

We established a distribution of the most frequently identified infectious agents in the analyzed specimens (Fig 3). First, a wide distribution of zoonotic parasites was evidenced in the country. Then, there was an accumulation in the Central Pacific region of specimens with acanthocephaliasis (12 with *Prosthenorchis* spp., one with *Macracanthorhynchus* spp.), and an accumulation of specimens with gastrointestinal nematodes in the great metropolitan area and tourist areas of Guanacaste (six with *Angiostrongylus* spp., one with *Baylisascaris* spp., one with *Ancylostoma* spp.). Additionally, vector-borne diseases occurred exclusively in specimens from coastal regions and altitudes less than 300 meters above sea level (11 with filariae, two with flaviviruses). The CDV present in carnivores from various areas of the country did not show a specific pattern of distribution (n=10). It is important to note that the Caribbean-south and southern part of the country do not present any pattern because there was no shipment of samples from this area, a caveat of this scheme of passive surveillance. The analyzed specimens associated with these infectious agents can be observed in S1 Fig.

Fig 3. Geographical distribution of the most frequently identified infectious agents in the referred specimens. The individuals reported as negative were depicted even though the infectious agent was not detected in the complementary analyzes or no lesions suggestive of the disease were found in the pathological analysis.

Discussion

The schemes aimed to develop epidemiological surveillance of infectious agents in free-living vertebrates have proven to be a fundamental tool in monitoring pathogens of zoonotic importance [65–67]. These surveillance systems are even more critical in geographical areas where high rates of biodiversity are prominent [7]. For example, Costa Rica is economically dependent on its ecotourism services, and its fauna is one of its most important assets [68]. However, currently, there is no epidemiological surveillance system directed to wildlife and to study outbreaks or any other health event involving them.

Evaluating the viability of implementing a passive surveillance system should be essential for a country considered a "hotspot" for the appearance or emergence of new infectious agents associated with its biodiversity and fauna characteristics [14,18]. However, some authors have established obstacles to implementing this type of system. These obstacles are mainly related to bureaucratic restrictions, financial disincentives, lack of legislation for data collection, and willingness to cooperate between agencies [69,70]. We encounter during this research those same obstacles for implementing surveillance systems in wildlife animals in Costa Rica.

Centers specialized in wildlife disease surveillance and health have shown that it is possible to establish robust and sustainable disease surveillance systems with broad coverage and diagnostic capacity [25,65,66,71]. However, factors such as logistics, rescue centers location, and dependence on voluntary staff excluded the participation of some geographic regions of the country (remote or difficult-to-access areas), affecting the efficiency and sustainability of surveillance system implemented in our research. These findings are consistent with other studies, where significant constraints hindered the availability of carcasses for analysis [72,73].

Reporting and dispatching of carcasses by national wildlife and animal health authorities were lower than rescue centers. These patterns agree with previous reports indicating that notification of wildlife mortality or morbidity generally depends on the initial detection of cases by the general public [22,74–76]. As a result, cases are biased

towards events in populated or easily accessible areas with nearby wildlife management centers, this added to the logistic capacity (storage, packaging, and shipping) of some of these centers, would explain the higher dispatch of cases. An example of these biases is the accumulation of reported cases in the Central Pacific region.

Wildlife management centers often report data with similar mortalities between birds and mammals [67,76]. However, in our study, there is a higher number of mammalians received. This difference is probably related to the fact that the surveillance systems in those countries actively include avian influenza surveillance, leading to a higher reporting of birds than our study. In our case, there is no surveillance system for avian influenza in wild birds by the local veterinary authority, and the suspected cases of this virus are confined to the local poultry production systems [71]. On the other hand, most of the birds found were no longer suitable for the study, thus underestimating the number of birds that die for various reasons [77].

Regarding the mammals, two taxa presented a higher representation (carnivores and primates). These data can be associated with the fact that they are medium to large-sized animals and with a more significant contact of these species with human environments, facilitating the recognition of morbidities and mortalities [23]. Human proximity with these mammals enabled the detection of infectious diseases, which is highly represented in these groups [78,79].

The distribution of the causes of death established in our research is consistent with most studies, highlighting traumatic events (anthropogenic effect) as the leading cause of death in these wild species [73,80–82]. Most of the traumas correspond to vehicular collisions, with a high percentage reported in urban areas or roads that cross or pass near forest areas. However, the number of deceased animals may be underestimated since not all cases are reported or recorded, much less referred to the laboratory since the condition of the corpses is not suitable for the study [83,84].

In addition, most of the traumatic cases presented some pre-existing infectious pathology. Free-living animals are frequently exposed to infectious agents naturally, so it is common in post-mortem analysis to find incidental lesions associated [82,85,86]. In wild animals highly exposed to human conditions, factors such as climate change and anthropogenic impact could become stressful stimuli, which facilitate these infectious agents to evolve into severe disease and increase the risk of suffering a traumatic event [82,87]. However, it is difficult to establish a clear association [88,89].

Some of these identified infectious agents cause foodborne illness in human populations. Examples of these are *Clostridium* spp., *Toxoplasma* sp. and *Sarcocystis* spp. Although the law prohibits hunting in Costa Rica, there is the illegal consumption of wild animal meat. Consequently, this type of practice might favor the transmission of infectious agents and lead to local outbreaks or maintain circulating virulent strains in local human populations [90–93].

Our study shows the presence of potentially zoonotic bacterial infectious agents classified as emerging diseases in some regions [94–96]. The most relevant are *Klebsiella pneumoniae*, *Escherichia coli*, and *Staphylococcus aureus*, which were associated with primary disease in some of the analyzed specimens. The direct or indirect contact occurs through the handling of these wild animals, thus facilitating the transmission, which evidences a latent risk. In addition, these bacteria currently top the list of infectious agents with antibiotic resistance genes, making them considered within antimicrobial surveillance schemes [97,98]. Furthermore, cases of antimicrobial resistance have already been demonstrated in Costa Rica with other bacteria in wild animals in urban environments, thus reflecting the need for a wildlife pathogen surveillance scheme to consider active antimicrobial resistance monitoring [31,99].

Zoonotic vector-borne diseases arise when there is a conjunction of spatial-temporal factors and other variables (reservoirs, climate, susceptible population, among others) [100]. Environmental conditions in tropical regions favor these diseases that significantly impact public health and are recognized as agents with epidemic potential

in Latin America [101–103]. We identified many primates and carnivores with infectious agents of vector transmission, for example, *Dirofilaria* spp. and *Dipetalonema* spp. mainly present in low-lying areas (CoastLine). These regions are already defined as endemic areas for these parasites in domestic animals [104,105]. Nevertheless, detecting this type of agent in a jungle cycle reveals a potential risk to public health in places with a high rate of visiting tourists in Costa Rica. This risk is reinforced by reports of the health system, which showed at least three disease cases in humans associated with *Dirofilaria immitis* and isolated cases of subcutaneous filariasis [106–108].

Similarly, several Latin American countries are considered endemic to various diseases caused by arboviruses [109]. In Costa Rica, other studies have identified the stationary circulation of this type of agent in humans and animals in various regions [33,110,111]. The cases of arbovirus-flaviviruses detected in our research are consistent with the high circulation of this type of virus in Costa Rica [33,111]. Furthermore, this agent was associated with a mass mortality of pelicans during the conduct of our research. Detecting virus-related mortalities such as West Nile in wild birds (as it was possibly our case) allows early alerts. It has been shown that there is a higher risk of exposure for human populations close to the regions where mortalities of wild birds occur [112,113].

The canine Distemper virus (CDV) (genus: morbillivirus) is a pernicious infectious agent with a global distribution that affects at least 20 families of mammals. Especially susceptible are carnivores of all species [114,115]. Endemic CDV outbreaks have been reported anecdotally throughout Costa Rica and America in dog populations and, more recently, sporadic outbreaks in wild carnivores of urban and suburban areas in the country have been recorded [116,117]. CDV was identified in our studies, reflecting the relevance of this virus in the role of spillover towards carnivore species and possibly the implications of a spillback towards susceptible or non-vaccinated domestic canines [117–119]. Costa Rica has a domestic dog population of ~250 thousand animals, mainly

356 located in urban areas. Herd immunity data in this population is uncertain, especially for dogs without an owner
357 or in non-urban areas, where owners neglect these vaccines. Indeed, this poses a risk to wild carnivores,
358 especially in urban areas with susceptible canine populations. Furthermore, the possibility of transmission of
359 this virus to other species beyond carnivores is a hypothesis that has been investigated [120]. Given the high
360 diversity of vertebrates present in Costa Rica, this virus should be considered within epidemiological surveillance
361 programs.
362 This study did not detect rabies virus infections. This findings are supported by previous studies in wild animals
363 in Costa Rica [32]. However, human and productive animal fatalities have been reported associated with rabies
364 infections, which stresses the relevance of its continuous monitoring [38,121]. A similar situation applies to
365 Newcastle and Influenza virus. In our samples, none of the birds showed evidence of disease or associated
366 clinical signs. However, due to the sanitary status (declared itself free) of Costa Rica for these avian viral agents
367 and the risk for national poultry production, it is advisable to establish monitoring in any event of mortality of
368 wild birds in the country [122,123].
369 The gastrointestinal and pulmonary parasites detected in this study are relevant for public health and wildlife
370 conservation programs. For instance, the nematodes *Baylisascaris* spp., *Ancylostoma* spp., and *Cylicospirura*
371 spp. were detected in mammalian species located in densely populated areas. Furthermore, we identified
372 parasites transmitted by water or food of aquatic origin (such as the cestode *Spirometra* spp. and the nematodes
373 *Contracaecum* spp. and *Gnathostoma* spp.) mainly in rural areas of the country's northern region. In this region,
374 fishing and rivers for recreational, irrigation, and consumption purposes are common, showing possible
375 contamination in both ways [124,125].

376 The last two reports of human angiostrongyliasis in Costa Rica have shown 12.9% seropositivity in the screening
377 test, the majority were children under the age of ten who reside in San José, which is the province with the
378 highest number of human samples [126,127]. In this study, six specimens of mammals infected by

379 *Angiostrongylus* spp. were identified, mainly in the *Nasua narica* species, from recreational parks in cities in the
380 country's northern region. This species has been established as the definitive host in the parasite's life cycle
381 [128]. The high degree of positive cases suggests a high prevalence of the parasite in the mammalian reservoir.
382 This result evidences the urgency of expanding sampling with better diagnostic techniques in children and wild
383 carnivores from these regions. The same situation happens with the number of cases detected with
384 acanthocephalans (*Prosthenorchis* spp., *Macracanthorhynchus* spp.) in the Central Pacific region. There is no
385 information on the real prevalence in animal populations, nor are there samplings that allow detecting cases in
386 humans in this region, despite the zoonotic risk previously mentioned [129,130].
387 Finally, we could not identify the causative agent of lesions in some of the samples analyzed. However,
388 histological changes suggest the presence of an infectious agent. Although we analyzed samples for the main
389 circulating infectious agents in Costa Rica, no conclusive data was obtained for some of them. Ranges of 17-22%
390 have been reported in pathological studies in wild species, where the causative agent of the disease cannot be
391 determined, mainly associated with the degree of autolysis and the diagnostic complexity [25,73,82,85]. These
392 results are consistent with the percentages of an absence of identification of the etiological agent in our
393 samples. These results show that further work is necessary to develop robust diagnostic techniques for wild
394 animals and efforts and incentives financed by government authorities in the surveillance of pathogens in
395 wildlife through the consistent implementation of new generation metagenomics [131–134].

396 Most of the pathogens detected in our study have already been previously identified in wild animals in Costa
397 Rica, and the detection of an infectious agent in a wild specimen does not necessarily imply disease or affect
398 wild populations [30,33,41,128]. However, monitoring the general health status of wild animals over time allows
399 us to know the circulation and behavior of these pathogens, as well as to provide an early warning of epidemic
400 events. This information can be used by health authorities together with a preventive strategy and a ONE

HEALTH approach to address zoonotic diseases, facilitating more specific public health interventions, implementing measures to reduce the risk of spread [25,66,73] .

This study was performed as a pilot and was the first structured attempt to test the establishment of a passive epidemiological surveillance scheme for diseases in wild vertebrates. However, it highlights the necessity of an inter-institutional and trans-institutional commitment with the sustainability over time of this surveillance scheme focused on the benefits beyond the economic part. For example, this work allowed us to estimate the general health status of the country's wildlife and know the distribution of pathogens in the national territory. This information is critical in regions established as hotspots for the emergence of infectious diseases due to their great biodiversity and social conditions [18].

Acknowledgments

The authors express deep appreciation to the National Animal Health Service (SENASA) and National Wildlife Service (SINAC) for their support and cooperation provided in this study and the wildlife management centers for the notification of specimens, especially DVM. Isabel Hagnauer Barrantes from Zooave rescue center, DVM. Martha Cordero Salas from Las Pumas rescue center and DVM. Carmen Soto Valverde from the Kids Saving the Rainforest rescue center, for their particular interest and participation. Also, the collaboration provided by the technicians of the laboratories of the Escuela de Medicina Veterinaria of the Universidad Nacional is appreciated.

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All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest: the authors declare no conflict of interest.

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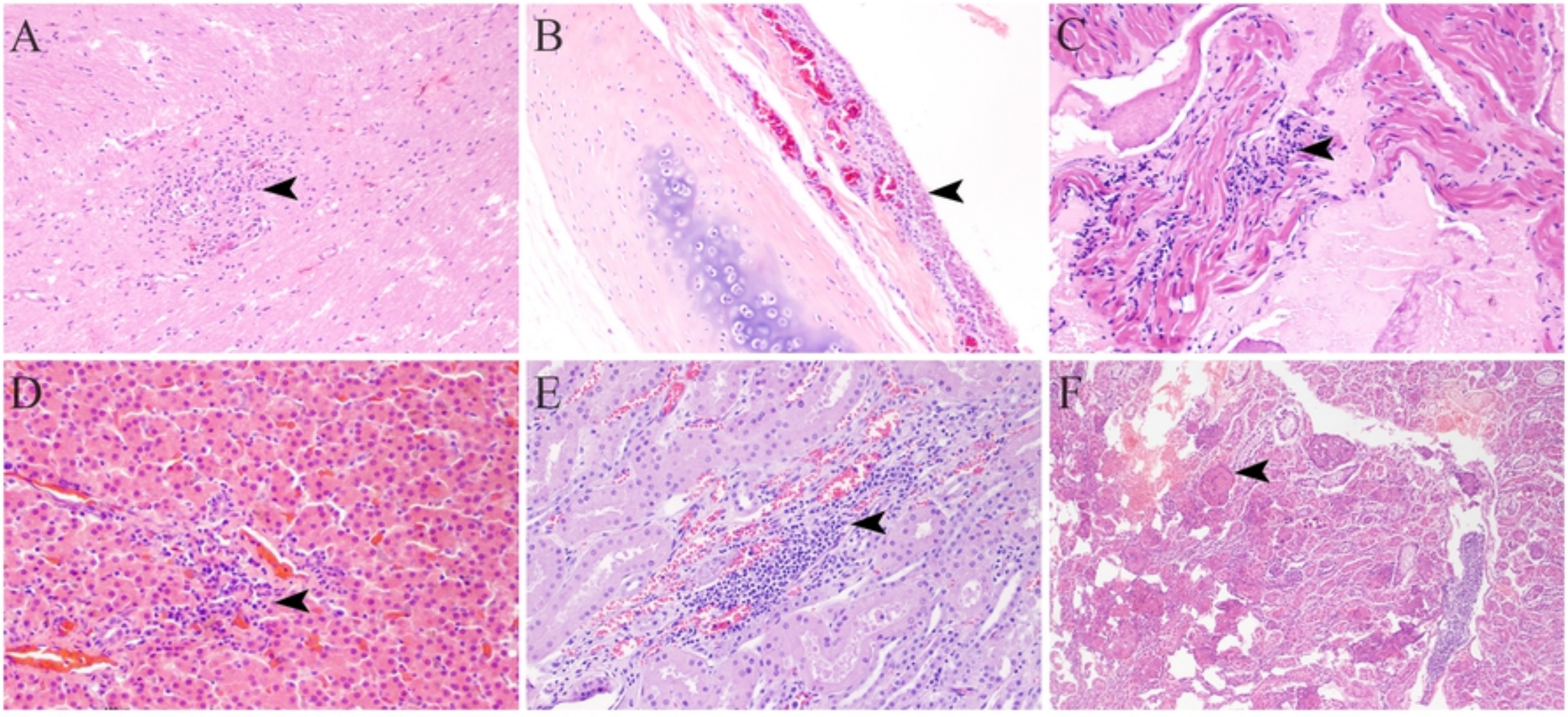
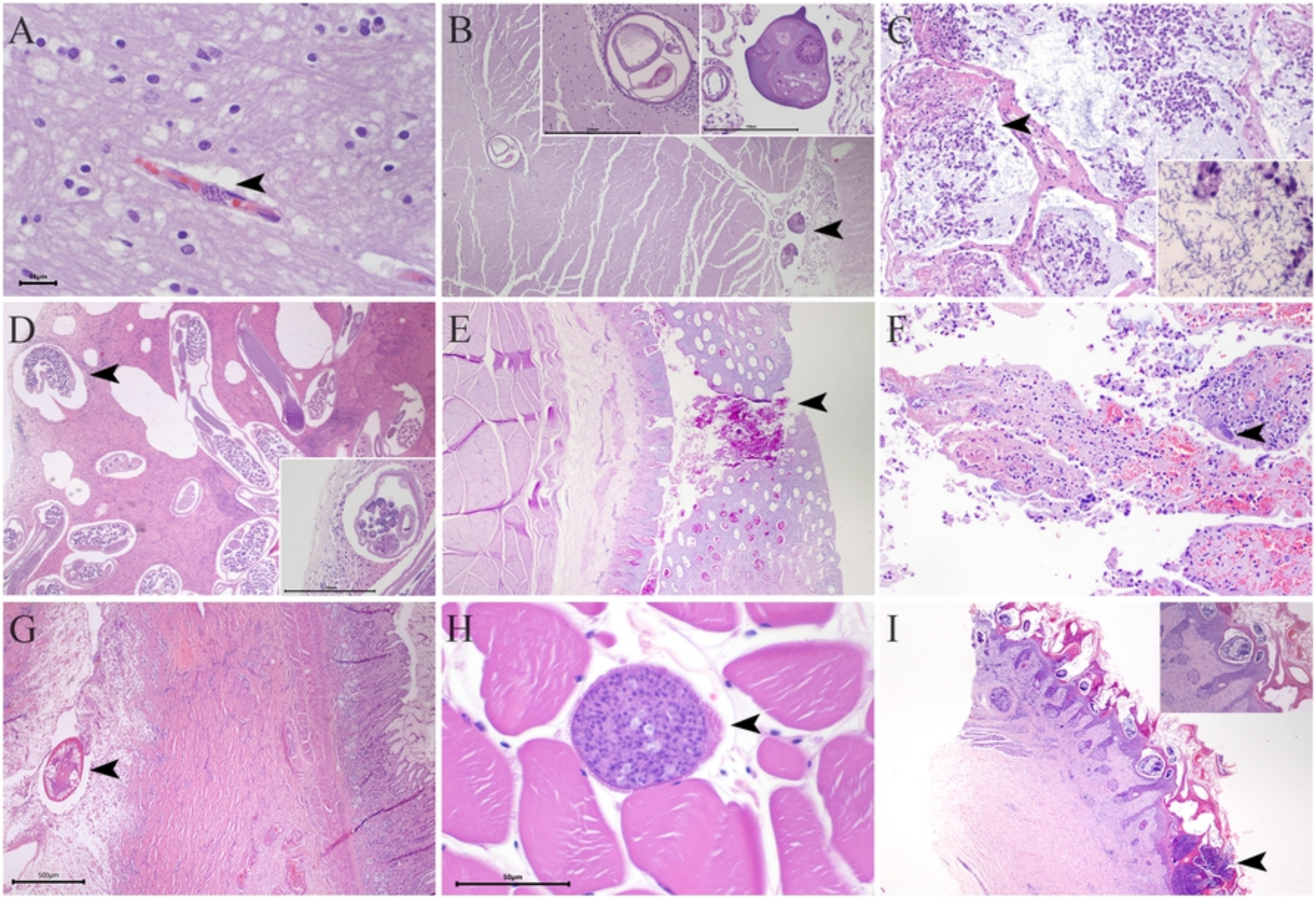
807 Supporting information

808 **S1 Fig. Geographical distribution of the analyzed specimens and number of individuals by taxon.**

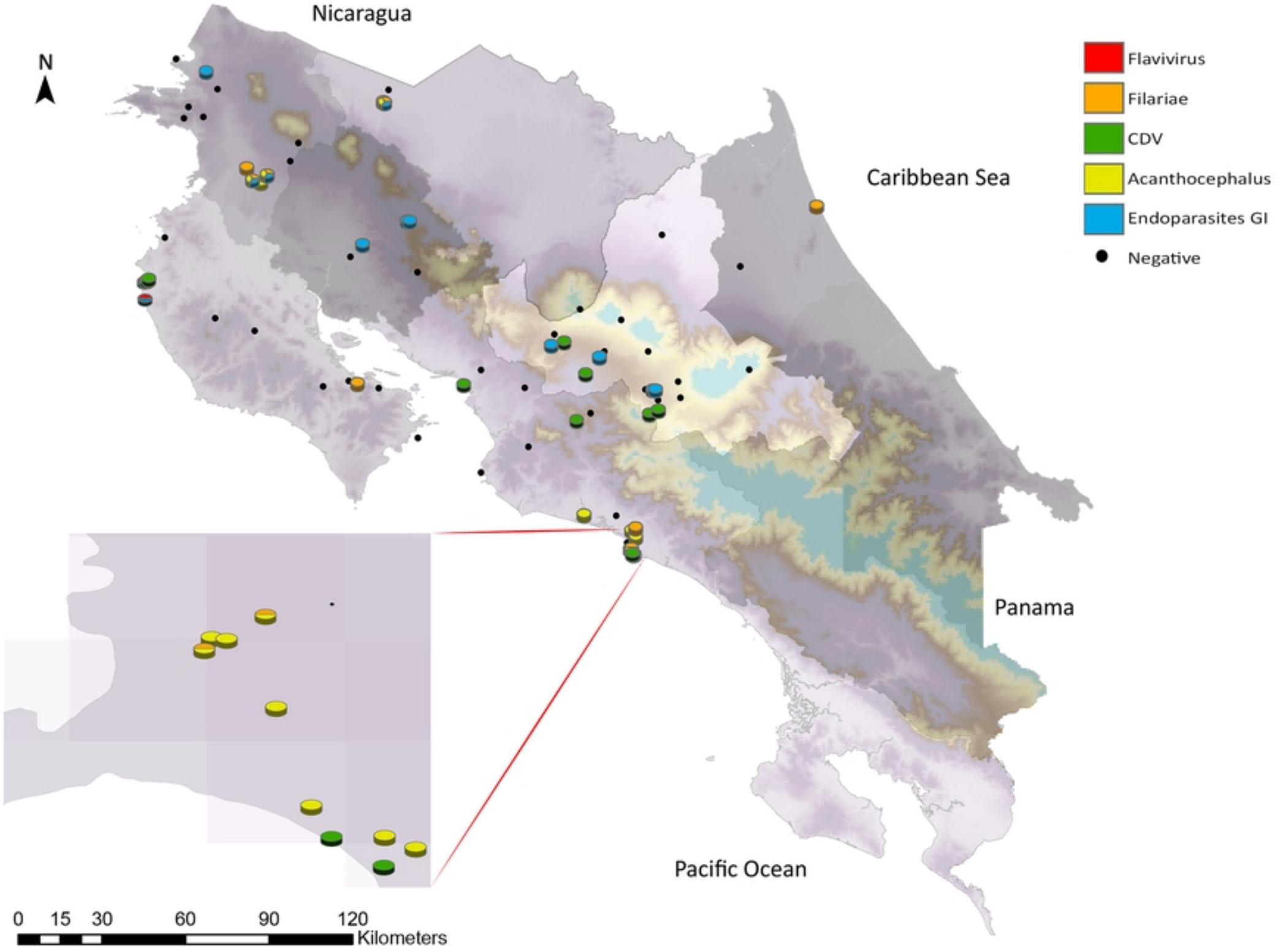
809 **S2 Table. Biological data and more representative pathological findings of the analyzed specimens.**



Figure



Figure


Nicaragua
N
Caribbean Sea
Flavivirus
Filariae
CDV
Acanthocephalus
Endoparasites GI
Negative
Panama
Pacific Ocean
0 15 30 60 90 120
Kilometers

Figure