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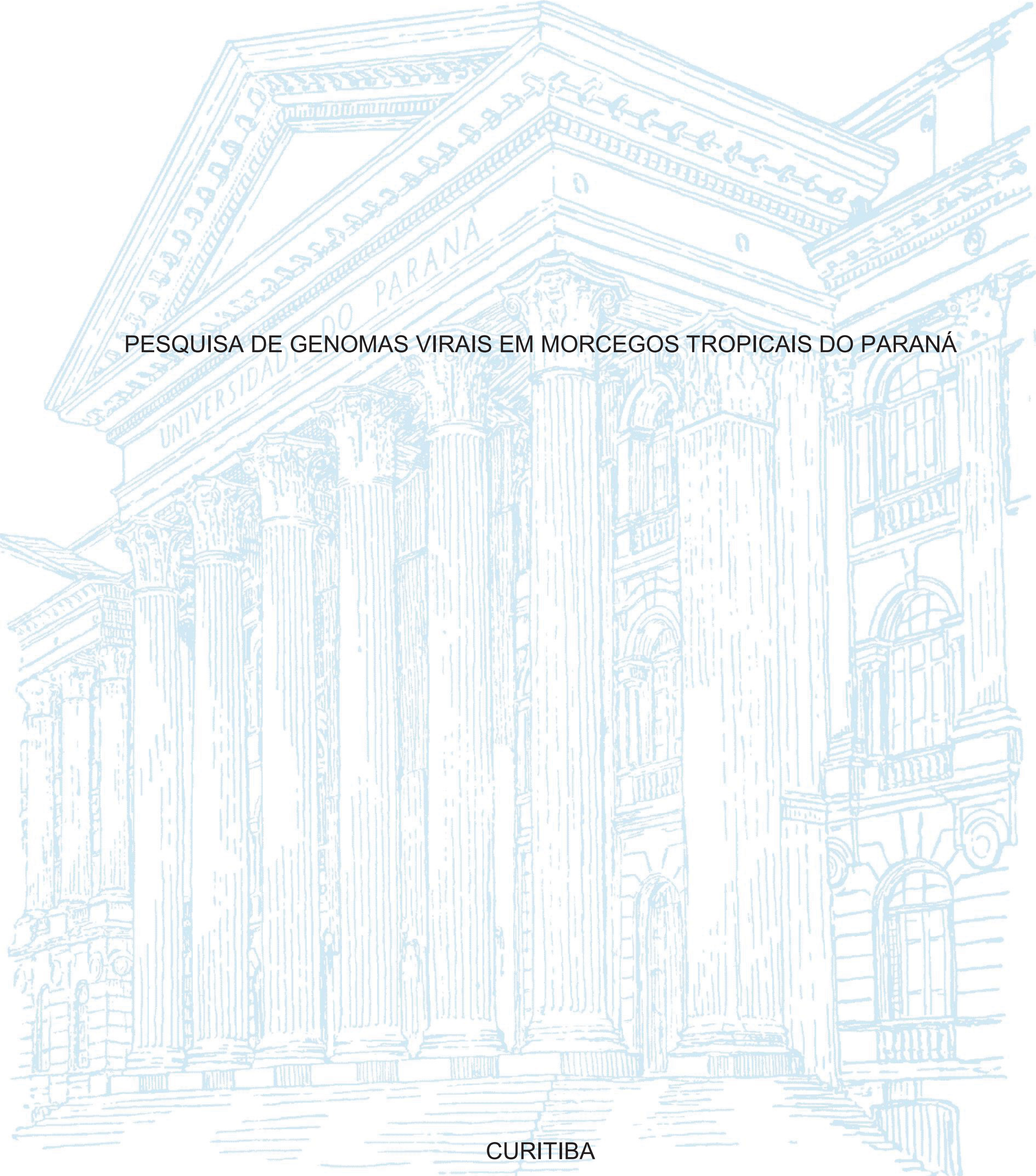

PESQUISA DE GENOMAS VIRAIS EM MORCEGOS TROPICAIS DO PARANÁ

CURITIBA
2019

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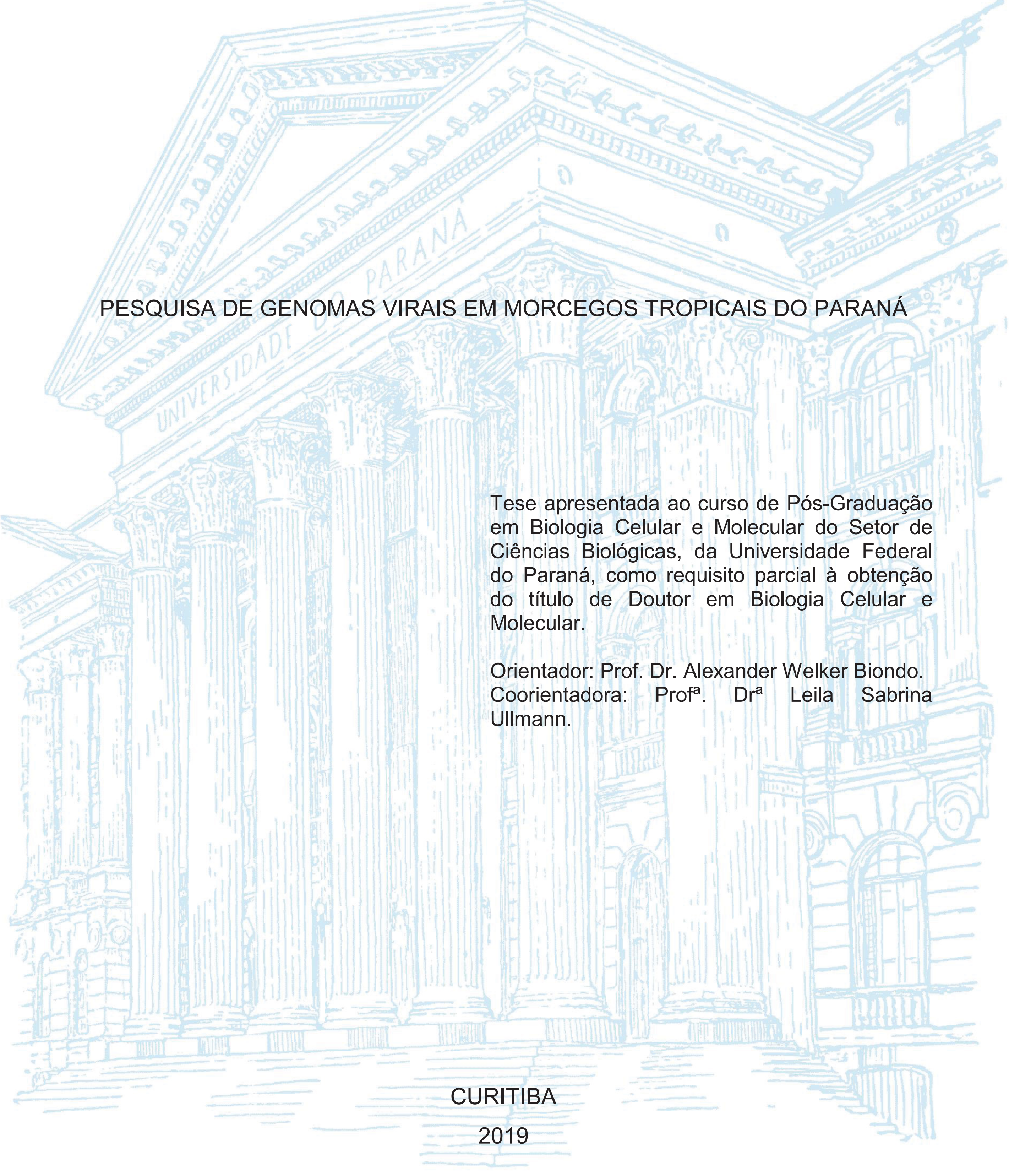

PESQUISA DE GENOMAS VIRAIS EM MORCEGOS TROPICAIS DO PARANÁ

Tese apresentada ao curso de Pós-Graduação em Biologia Celular e Molecular do Setor de Ciências Biológicas, da Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Biologia Celular e Molecular.

Orientador: Prof. Dr. Alexander Welker Biondo.
Coorientadora: Profª. Drª Leila Sabrina Ullmann.

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em BIOLOGIA CELULAR E MOLECULAR da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **JULIANO RIBEIRO** intitulada: **Pesquisa de genomas virais em morcegos tropicais do Paraná**, após terem inquirido o aluno e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 22 de Abril de 2019.

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“Aquele que ousa perder uma hora de
seu tempo não sabe o valor da vida."

Charles Darwin

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RESUMO

As últimas doenças infecciosas emergentes originaram-se de espécies de animais selvagens e os morcegos têm desempenhado um papel muito importante como reservatórios virais. Além da sua habilidade de voar, os morcegos apresentam características interessantes quanto a sua dinâmica populacional, hibernação, migração, alimentação e habitat. Devido à perda de habitat e desmatamento, os morcegos, os animais domésticos e o homem compartilham do mesmo ambiente, aumentando a chance de transmissão de vírus. Esta tese está dividida em dois capítulos. O primeiro capítulo apresenta dados da vigilância em morcego e fatores de risco para o "spillover" da raiva na região urbana da cidade de Curitiba a qual não apresenta casos de raiva em humanos, neste capítulo foram avaliados dados entre os anos de 2010 a 2015. No período do estudo não foram coletados morcegos hematófagos. Entretanto, as coletas de morcegos insetívoros e frugívoros ocorreram com maior frequência na região centro-norte da cidade. Foi evidenciado que o maior risco para transmissão da raiva por morcegos pode ser mais provável em gatos devido à exposição associada destes animais pelos hábitos de caça inatos, predispondo a um contato ainda mais próximo com morcegos potencialmente infectados. Portanto, os gatos devem ser sempre incluídos nos programas de vigilância e vacinação contra a raiva. O segundo capítulo avaliou a distribuição epidemiológica e espacial de abrigos de morcegos e sua relação com a transmissão da raiva nos herbívoros no Estado do Paraná, entre os anos de 2011 a 2017. Apesar do contínuo mapeamento e do controle de abrigos de morcegos hematófagos, a raiva nos herbívoros no estado do Paraná tem estado estável, e está diretamente associada a abrigos de morcegos hematófagos. Assim, as medidas preventivas como a vacinação antirrábica deve ser mantida para aumentar a eficácia da proteção do rebanho, particularmente nas áreas que possuem predisposição geográficas para presença de inúmeros abrigos de morcegos hematófagos e, em áreas localizadas próximas a feições de enormes bacias hidrográficas.

Palavras-chave: Morcego não hematófago, raiva, herbívoro, morcego hematófago, *Desmodus rotundus*, morcego-vampiro comum, abrigos de morcegos.

ABSTRACT

The latest emerging infectious diseases originated from wild animal species and bats have played a very important role as viral reservoirs. In addition to their ability to fly, bats have interesting characteristics regarding their population dynamics, hibernation, migration, feeding and habitat. Due to loss of habitat and deforestation, bats, domestic animals and man share the same environment, increasing the chance of virus transmission. This thesis is divided into two chapters. The first chapter presents data on bat surveillance and risk factors for rabies spillover in the urban area of the city of Curitiba, which does not present cases of rabies in humans, in this chapter data were evaluated between the years 2010 and 2015. In the study period there was no were collected hematophagous bats. However, collections of insectivorous and frugivorous bats occurred more frequently in the north-central region of the city. It has been shown that the increased risk of transmission of rabies by bats may be more likely in cats due to the associated exposure of these animals to inbred hunting habits, predisposing to closer contact with potentially infected bats. Therefore, cats should always be included in the surveillance and vaccination programs against rabies. The second chapter evaluated the epidemiological and spatial distribution of bat shelters and their relationship with the transmission of rabies in herbivores in the Paraná State between the years 2011 to 2017. Despite the continuous mapping and control of hematophagous bat shelters, rabies in the herbivores in the Paraná State has been stable and is directly associated with hematophagous bats shelters. Thus, preventive measures such as anti-rabies vaccination should be maintained to increase the effectiveness of herd protection, particularly in geographically predisposed areas for the presence of numerous hematophagous bats shelters and features of large river basins.

Keywords: non-hematophagous bat, rabies, herbivorous, hematophagous bat, *Desmodus rotundus*, common vampire bat, bats shelters.

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LISTA DE ABREVIATURAS

UFPR	Universidade Federal do Paraná
RABV	Rabies virus
RNA	Ribonucleic acid
SPILLOVER	Ocorre quando uma população de reservatório com alta prevalência de patógenos entra em contato com uma nova população hospedeira. A infecção é transmitida da população do reservatório e pode ou não ser transmitida dentro da população hospedeira.
AgV3	Variante RABV compatível com morcego hematófago
CCZV	Centro de Controle de Zoonoses e Vetores
LACEN-PR	Laboratório Central do Estado do Paraná
FAT	Teste de anticorpos fluorescentes
RDSTK	R and the Data Science Toolkit
IPPUC	Instituto de Pesquisa e Planejamento Urbano de Curitiba
ADAPAR	Agência de Defesa Agropecuária do Paraná
SEAB-PR	Secretaria da Agricultura e do Abastecimento do Paraná
UTM	Universal Transversa de Mercator
URS	Unidade Regional de Sanidade Agropecuária
ULSA	Unidade Local de Sanidade Agropecuária

SUMÁRIO

1. INTRODUÇÃO

Morcegos pertencem à ordem Chiroptera, palavra derivada do grego *cheir* (mão) e *pteros* (asa). Quirópteros são um dos grupos de mamíferos mais diversificados do mundo, com 18 famílias, 202 gêneros e 1120 espécies (SIMMONS, 2005). Isso representa aproximadamente 22% das espécies conhecidas de mamíferos, que hoje totalizam 5416 espécies (WILSON; REEDER, 2005). Tradicionalmente os Chiropteros são divididos em duas subordens, os Megachiroptera sem ocorrência no Brasil e a Microchiroptera composta por 17 famílias e 930 espécies no mundo, no Brasil são conhecidas 9 famílias, 64 gêneros e 167 espécies (DOS REIS et al., 2007). A presença de morcegos têm sido relatadas em todas as áreas geográficas do mundo, exceto Ártico, Antártida, áreas desérticas extremas e algumas ilhas oceânicas isoladas (POKHREL; BUDHA, 2015), a maioria se alimenta de insetos e outros artrópodes, mas algumas espécies se alimentam de vertebrados, peixe, sangue, frutas, néctar ou pólen. (DACHEUX et al., 2014).

Devido as características ímpares entre os mamíferos, os morcegos têm um papel fundamental na transmissão e emergência de doenças (CALISHER et al., 2006), embora pouco se saiba sobre a diversidade viral (viroma) que eles albergam (DACHEUX et al., 2014). Os morcegos podem ser considerados reservatórios potenciais perfeitos para doenças infecciosas emergentes devido à sua longevidade, as características populacionais e por poderem disseminar os vírus em longas distâncias pela sua capacidade de vôo (CALISHER et al., 2006; SMITH; WANG, 2013; WYNNE; WANG, 2013).

Algumas espécies de morcegos hibernam durante o inverno, reduzindo seu metabolismo para economizar energia, o que pode suprimir o sistema imunológico e/ou favorecer a replicação e manutenção viral nos mesmos. A interação entre as diferentes espécies de morcegos pode gerar novos vírus capazes de transpor a barreira entre as espécies de mamíferos (WONG et al., 2007)).

O aumento das interações entre homem, morcegos e animais domésticos e silvestres resultante de atividades antropogênicas levam a perda de habitat e fonte de alimentos dos morcegos (SMITH; WANG, 2013). Nas Américas, os morcegos são reservatórios significativos do vírus da raiva e, embora o vírus da raiva possa afetar qualquer espécie de morcego, o morcego vampiro comum *Desmodus rotundus* é considerado o maior transmissor do vírus da raiva devido à sua natureza hematófica

(SEETAHAL et al., 2017). O morcego vampiro comum está amplamente distribuído em toda a região neotropical, do México a América do Sul, sendo encontrado em todo o território brasileiro (PERACCHI, A. L.; LIMA, I. P.; REIS, N. R. & ORTENCIO-FILHO, 2011; SIMMONS, 2005). Entretanto a transmissão do vírus da raiva tem sido descrita não somente por morcegos hematófagos, mas também entre os morcegos não hematófagos, os quais mantêm a circulação do vírus da raiva entre animais domésticos de estimação cães e gatos (KOTAIT, I.; CARRIERI, M.L; TAKAOKA, 2009)

A raiva é uma doença viral que afeta o Sistema Nervoso Central, causa encefalomielite aguda, com uma taxa de letalidade de quase 100% (GARCÉS-AYALA et al., 2017; VELASCO-VILLA et al., 2008). O vírus pertencente ao gênero *Lyssavirus* da família *Rhabdoviridae*. O genoma do RABV é um RNA de sentido negativo simples, não-segmentado e que codifica para cinco proteínas: nucleoproteína (N), fosfoproteína (P), proteína de matriz (M), glicoproteína (G) e proteína “Large” (L) (MASATANI et al., 2011). RABV é eliminado na saliva de animais doentes e pode ser transmitido para outros animais através da mordida (DIAS et al., 2011; VELASCO-VILLA et al., 2008). A raiva é endêmica em muitos países ao redor do mundo onde os mamíferos selvagens ou domésticos podem atuar como hospedeiros, reservatórios e transmissores da doença (PEREIRA et al., 2017).

O ciclo da raiva pode ser dividido em dois ciclos principais, o ciclo urbano, com o cão como reservatório principal e o ciclo silvestre, com diferentes espécies de vida selvagem que atuam como reservatórios ou transmissores; no entanto, a cadeia epidemiológica da raiva no Brasil pode ser dividida em quatro ciclos de transmissão: urbano, rural (animais de produção), selvagem aéreo e terrestre selvagem (FAVORETTO et al., 2013; KOTAIT, I.; CARRIERI, M.L; TAKAOKA, 2009; ROCHA et al., 2017)

Os morcegos *D. rotundus* podem causar grandes perdas econômicas para a indústria pecuária na América Latina, devido ao seu papel na transmissão da raiva em espécies herbívoras (ALBAS et al., 2011; MIALHE, 2013). Outro fato relevante quanto a participação deste animal na transmissão da raiva é o seu papel no ciclo silvestre e aéreo, devido aos hábitos sinantrópicos, os quais alcançaram áreas de transição e urbanas pela maior oferta de alimentos e abrigos existente nestas áreas, bem como, pelo impacto ambiental provocado pela ação humana em seus habitats naturais (KOTAIT et al., 2007)

Embora os casos humanos em países em desenvolvimento tenham sido associados principalmente a mordidas de cães. Espécies de morcegos também podem estar infectadas pela RABV, fatalidades humanas na América Latina foram recentemente relacionadas ao “spillover” de morcegos hematófagos, insetívoros e frugívoros (ELLISON et al., 2014; STREICKER et al., 2012). Não surpreendentemente, os maiores surtos registrados de raiva no Brasil foram transmitidos por morcegos e ocorreram em áreas rurais do norte do Brasil (21 óbitos) e áreas remotas da floresta amazônica (16 mortes) devido ao vírus da raiva variante 3 (AgV3), encontrado principalmente em *Desmodus rotundus* (DA ROSA et al., 2006; MENDES et al., 2009).

Mudança nos habitats de morcegos não hematófagos tem forçado a migração destes animais das áreas rurais para áreas urbanas, provavelmente devido ao aumento da oferta de alimentos e de abrigos artificiais encontrados nos centros urbanos, esta situação tem aumentado a probabilidade de contato direto de morcegos raivosos com seres humanos e com animais domésticos (KOTAIT et al., 2007; SHI, 2010). Registros de espécimes de morcegos positivos para raiva no Brasil foram relatados, sendo: 20/41 (49,1%) foram de espécies não hematófagas, seguidos por 12/41 (29,0%) de hematófagos e 9/41 (21,9%) morcegos não identificados (ALBAS et al., 2011; SODRÉ; GAMA; ALMEIDA, 2010). Contudo, apesar da diminuição da raiva humana e canina no Brasil, os casos de raiva em humanos (78,0%) ocorreram principalmente por variantes de morcego não hematófago, dados observados entre 2000 e 2009 (WADA; ROCHA; MAIA-ELKHOURY, 2011).

Em áreas urbanizadas os gatos também têm sido considerados uma espécie de animal de alto risco para a transmissão da raiva para humanos, principalmente devido aos hábitos de caça, particularmente em relação a animais voadores, incluindo morcegos, que podem transmitir o vírus da raiva do ciclo silvestre-aéreo para os ambientes urbanos (FRYMUS et al., 2009). Tal cenário pode similarmente ocorrer nas principais cidades do Brasil, como Curitiba, a nona maior cidade brasileira, onde um gato foi diagnosticado com raiva em 2010, por variante 4 compatível de isolados de morcegos insetívoros *Tadarida brasiliensis*, sendo este um caso de raiva em animal doméstico após quase 30 anos sem casos de raiva de animais de estimação (MORIKAWA et al., 2012).

Nesse sentido, estudos utilizando dados dos órgãos governamentais da Saúde, Agropecuária, Meio Ambiente podem oferecer novas informações para estudos da ciência e podem ser revertidos para elaboração de estratégias de trabalhos e ações para os setores de controle e monitoramento da raiva no Brasil.

1.1 OBJETIVO GERAL

Descrever a epidemiologia da raiva e determinar as áreas de circulação do vírus da raiva em morcegos hematófagos e não hematófagos no Estado do Paraná.

1.2 OBJETIVOS ESPECÍFICOS

Descrever a epidemiologia da raiva em morcegos não hematófogos no município de Curitiba, no período de 2010-2015.

Levantar dados, analisar e descrever a distribuição espacial e temporal dos casos de raiva em herbívoros no período de 2011-2017 no Estado do Paraná.

Delinear o perfil epidemiológico e sazonalidade da raiva em herbívoros no período de 2011-2017 no Estado do Paraná.

Estruturar um modelo de risco para transmissão do vírus da raiva em herbívoros no Estado do Paraná.

1.3 MATERIAL E MÉTODOS

Para realização do **primeiro estudo** deste projeto foi utilizado os registros oficiais da cidade Curitiba das reclamações registradas nos “sistema 156” referente a morcegos de janeiro de 2010 a dezembro de 2015.

Os registros de morcegos, cães e gatos enviados para testes de raiva foram obtidos na Coordenação de Controle de Zoonoses e Vetores (CCZV) de janeiro de 2007 a dezembro de 2015.

Os morcegos foram individualmente identificados com base na referência de taxonomia padrão brasileira atual (GARDNER, 2008; GREGORIN; TADDEI, 2002).

Todos os testes de raiva foram realizados pelo Laboratório Central do Estado do Paraná (LACEN-PR) seguindo diretrizes internacionais para técnicas laboratoriais e de diagnóstico usando o teste de anticorpos fluorescentes (FAT) com um painel de anticorpos monoclonais e inoculação intracerebral em camundongos de 21 dias (DEAN DJ, ABELSETH MK, 1996; KOPROWSKI, 1996).

Um banco de dados foi estruturado com um pacote estatístico comercialmente disponível (Microsoft Excel 2007, Microsoft Company, Edmond, WA, EUA) e incluiu a data da coleta, situação em que o animal foi coletado ou capturado, número de animais, gênero e espécie de animais, procedimentos na CCZV, data e resultado da raiva.

A estatística descritiva foi realizada pelas frequências e distribuições, seguida de uma análise sazonal da decomposição aplicando o cálculo dos índices sazonais e um modelo de regressão com significância de 5%. Um modelo linear simples foi realizado após testes instalados para distribuição normal de dados.

Uma abordagem de georreferenciamento foi aplicada nos dados de endereços, usando o pacote "RDSTK" no ambiente R. Um mapa foi construído em um software comercial com pontos de morcego (positivos / negativos), informações de urbanização e limites de bairro com arquivos de forma obtidos com os Serviços de Geografia da Cidade (Instituto de Planejamento Urbano e Pesquisa de Curitiba, IPPUC).

Análise de densidade de kernel foi realizada com o pacote "stats" no ambiente R.

Para o **segundo estudo** foi utilizado dados disponibilizados pela Agência de Defesa Agropecuária do Paraná (ADAPAR), vinculada à Secretaria do Estado da Agricultura e do Abastecimento do Paraná (SEAB-PR), através de registros dos casos de raiva diagnosticados em herbívoros no estado do Paraná.

Todos os testes de diagnóstico da raiva em morcegos hematófagos foram realizados pelo Centro de Diagnóstico "Marcos Enrietti", laboratório de referência para o diagnóstico da Raiva no estado do Paraná, seguindo as diretrizes internacionais para técnicas laboratoriais e diagnósticas. Tanto o teste de anticorpo fluorescente (FAT), com um painel de anticorpos monoclonais, bem como a inoculação intracerebral em testes de camundongos com 21 dias de idade foram usados para o diagnóstico (DEAN DJ, ABELSETH MK, 1996; KOPROWSKI, 1996).

Os morcegos encaminhados para diagnóstico foram identificados como morcegos hematófagos pelo Centro de Diagnóstico Marcos Enrietti através da "Chave de Determinação dos Morcegos do Brasil" (GREGORIN; TADDEI, 2002).

Um banco de dados dos casos positivos foi construído com um pacote estatístico (Microsoft Excel 2007, Empresa Microsoft, Redmond, WA, EUA) e incluiu: ano de coleta, número do protocolo ADAPAR, data de coleta do material, nome do proprietário, espécie animal afetada, município e Latitude / Longitude da propriedade (em Grau, Minuto e Segundo, Decimal Grade e UTM).

Um banco de dados de abrigos de morcegos foi construído com um pacote estatístico (Microsoft Excel 2007, Empresa Microsoft, Redmond, WA, EUA) e incluiu: Unidade Regional de Saúde Agrícola - URS, Unidade de Saúde Agrícola Local - ULSA, município, período de captura (dia ou noite), tipo de abrigo, situação de abrigo (ativo ou inativo) e Latitude / Longitude de abrigos (em Grau, Minuto e Segundo, Grau Decimal e UTM).

A decomposição da série histórica foi avaliada mês a mês de: Todas as amostras encaminhadas, todas as amostras positivas, todos os bovinos encaminhados, todos os bovinos positivos, todos os cavalos encaminhados e todos os cavalos positivos.

Para avaliar a sazonalidade e tendência, o índice sazonal de cada mês no período e a avaliação de tendência com modelos aditivos de decomposição de séries históricas foram calculados no MiniTab 17.0.

Análises espaciais de dados positivos de animais e abrigos registrados no período foram distribuídas em um mapa com o software ArcGIS 10.0 (ESRI, 2011).

Como a série compreendeu o período de janeiro de 2011 a dezembro de 2017, uma análise de cluster espaço-temporal foi realizada com um modelo de permutação no espaço-tempo (KULLDORFF et al., 2005) foi realizada no SatScan (KULLDORFF et al., 2006).

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CAPÍTULO I

BAT RABIES SURVEILLANCE AND RISK FACTORS FOR RABIES SPILLOVER IN AN URBAN AREA OF SOUTHERN BRAZIL

2. Bat-rabies surveillance and risk factors for rabies spillover in an urban area of southern Brazil

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2.1 ABSTRACT

Background: Bat-rabies surveillance data and risk factors for rabies spillover without human cases have been evaluated in Curitiba, the ninth biggest city of Brazil, during a 6-year period (2010-2015). A retrospective analysis of bat complaints, bat species identification and rabies testing of bats, dogs and cats have been performed using methodologies of seasonal decomposition, spatial distribution and kernel density analysis.

Results: In overall, a total of 1,003 requests for bat removal have been attended and 806 bats were collected in 606 city points. Bat species were identified among 13 genera of three families, with higher frequency of *Nyctinomops* in the central-northern region and *Molossidae* scattered throughout city limits. Out of bats captured alive, 419/806 (52.0%) healthy bats were released due to no human or animal contact. The remaining 387/806 (48.0%) bats were sent for euthanasia and rabies testing, resulting in 9/387 (2.32%) positives. Temporal statistical analyses have shown a linear tendency (regression: $y = 2.02 + 0.17x$; $p<0.001$ and $r^2 = 0.29$) for tested bat samples, and significant seasonality with increase in January and decrease in May, June, and July. The Kernel density analysis has shown the center-northern city area as statistically important and the southern region with no tested samples within the period. In addition, a total of 4,769 random and suspicious samples were sent for rabies diagnosis including dogs, cats, bats and others from 2007 to 2015. While all 2,676 dog brains tested negative, only 1/1,136 (0.088%) cat brain tested positive for rabies.

Conclusions: Only non-hematophagous bats were collected during the study and the highest frequency of collections occurred in the city center-northern region. Rabies spillover from bats may be more likely to cats due to registered exposure associated to innate hunting habits, predisposing even closer contact with potentially infected bats. Although with very low frequency, cats should be always included in rabies surveillance and vaccination programs.

Key words: Non-hematophagous bat. Dog. Cat. Rabies. AgV-3. Geo-referencing. Kernel. Seasonal decomposition.

2.2 BACKGROUND

Bats (Order Chiroptera) have been considered one of the most diverse worldwide mammal groups, accounting for 20.7% out of 5,416 currently known mammal species, with 18 families and 1,120 species [1, 2]. The presence of bats has been reported in all geographic areas of the world except the Arctic, Antarctic, extreme desert areas, and some isolated oceanic islands [3]. Brazil has been ranked as the second highest country in bats species, harboring 178 (15.9%) of known worldwide species [4, 5].

Of the species of bats identified worldwide, only three feed exclusively on blood, *Desmodus rotundus*, *Diphylla ecaudata* and *Diaemus youngi*. *D. rotundus* is known as the common vampire bat, being the only one that feeds on mammalian blood, while the other two species are spared from bird blood. Vampire bats are distributed from Mexico to South America [6]. The deforestation, drastically reduced the number of natural prey *D. rotundus*, faced with this ambivalent change vampire bats found in cattle, introduced by man at the South America, a great source of food. This has given rise to the species of vampire bats and their contact with cattle and man causing a direct impact on human and animal health by the transmission of the rabies virus [7].

Rabies virus (RABV) can affect all mammals; however, the orders Carnivora and Chiroptera act as reservoirs for the virus (Rupprecht et al., 2002). RABV has been divided into two main variants: the first associated with carnivores, mostly dogs, on urban cycle; and the second, associated with bats, raccoons, and skunks, on sylvatic cycle [6, 7, 8]. Although human cases in developing countries have been mostly associated to dog bites, bat species may also be infected by RABV and human fatalities in Latin America have been recently connected to spillover from hematophagous, insectivorous and frugivorous bats [8, 9]. Not surprisingly, the highest recorded rabies outbreaks in Brazil were bat-transmitted and occurred in Brazilian northern rural (21 deaths) and remote areas of the Amazon forest (16 deaths) due to rabies virus variant 3 (AgV3), mainly found in *Desmodus rotundus*, a vampire-bat species [10, 11].

Meanwhile, a switch on habits of non-hematophagous bats has also been recently observed, with migration from rural to urban areas, probably due to increased food supply in urban centers and environmental impact in their natural habitats, increasing potential contact towards domestic and wild animal populations, and human beings [12, 13]. As a result, 20/41 (49.1%) positive bat specimens currently reported for rabies in Brazil were from non-hematophagous species, followed by 12/41 (29.0%) hematophagous, and 9/41 (21.9%) unidentified species [14]. In addition, despite a decrease in human and canine rabies in Brazil, human cases have mostly (78.0%) occurred by bat variants between 2000 and 2009 [15].

Cats have been considered a high-risk species for rabies transmission to humans in some European countries mainly due to hunting habits, particularly toward flying animals including bats, which may connect rabies from the sylvatic-aerial cycle to urban settings [16]. Such scenario may similarly occur in major cities of Brazil such as Curitiba, the ninth biggest Brazilian city where a cat has been diagnosed with bat variant rabies after almost 30 years of no pet rabies cases [17]. Accordingly, this study has aimed to analyze the bat-rabies surveillance and risk factors for rabies spillover in an area without human cases in southern Brazil during a 6-year period (2010-2015). In addition, a retrospective analysis of bat complaints, bat species identification and rabies testing of bats, dogs and cats in the same area have been performed using methodologies of seasonal decomposition, spatial distribution and kernel density analysis.

2.3 METHODS

Curitiba (25º25'48'' S, 49º16'15'' W), capital of Paraná state, southern Brazil, has been currently ranked as the ninth biggest Brazilian city with approximately 1.8 million habitants [18]. Although categorized as 100% urban area, Curitiba city has been considered as an environment-friendly and the first in sustainability and life quality in Brazil, with a high green-area ratio distributed in more than 40 city parks and preservation areas [19].

Since 1984, an official central telephone system 156 has been used in Curitiba as a communication channel between the population and public managers, this system allows the population to request various services performed by the various Secretariats of the Municipality of Curitiba, among the services performed we highlight requests such as: collection of dead animals for examination of rabies (dogs and cats), removal of fallen bats and / or inside the house and removal and observation of aggressive animals. Complaints of dead animals have been used as a source of brain samples from dogs and cats, mostly sent for rabies diagnosis at the Parana State Reference Laboratory (LACEN) and used for monitoring rabies virus circulation. In addition, complaints of bats have followed another specific protocol: local inspection by professionals from the Curitiba Zoonosis Control Center (ZCC), capture or collection of bats, epidemiological questionnaire and bat health status. If bats were healthy and had no human or pet contact, they were released using an open box at sunset of the same day at the ZCC, which was located nearby preserved areas at the time. If bats were dead, had contact with pets or human beings, or unhealthy (no flying, neurological signs, injuries), they were euthanized and their brains sent for rabies testing at the LACEN.

Official city records of bat complaints, local inspections and bat destinations were obtained from January 2010 to December 2015. Additionally, records of bats, dogs and cats sent for rabies testing were obtained at the ZCC from January 2007 to December 2015. Bats were individually identified based on the current Brazilian standard taxonomy reference [20, 21]. All rabies tests at the LACEN were performed following international guidelines of laboratory techniques and diagnosis [22, 23].

A database was structured with a commercially available statistical package (Microsoft Excel 2007, Microsoft Company, Edmond, WA, USA) and included collection site, situation in which the animal was collected or captured, number of animals, animal genus and species, procedures at ZCC, date, and rabies result. Descriptive statistical were conducted with frequencies and distributions, followed by a seasonal decomposition analysis applying seasonal indices calculation and a regression model with significance of 5%. A simple linear model was performed after fitted tests to normal distribution of data.

A geo-referencing approach was applied on the addresses data, using “RDSTK” package [24] in R environment [25]. A map was built in a commercial software [26] with bat points (positives/negatives), urbanization information, and neighborhood boundaries with shape files obtained with the City Geography Services (Institute of Urban Planning and Research of Curitiba, IPPUC). Finally, a kernel density analysis was performed with “stats” package in R environment [26].

2.4 RESULTS

In overall, a total of 4,769 samples were sent for rabies diagnosis including dogs, cats, bats and other animal species from 2007 to 2015 (Table 1). The highest number of brain samples among these animals were dogs (2,676; 56.1%), followed by cats (1,136; 23.8%), bats (940; 19.7%), and other animals (17; 0.35%) which included three rabbits (*Oryctolagus* sp.), three bush dogs (*Speothos venaticus*), two ferrets (*Galictis* sp.), two horses (*Equus ferus cabales*), a non-human primate (*Cebus* sp.), a squirrel (*Sciurus ingrami*), an opossum (*Didelphis albiventris*), a deer (*Cervus* sp.), a raccoon (*Procyon* sp.), a marmoset (*Callithrix* sp.), and a gerbil (*Meriones* sp.). Out of the tested samples, only 9/4,769 (0.18%) bats and 1/4,769 (0.02%) cats have been positive to rabies virus.

Table 1. Animal samples sent for rabies surveillance in Curitiba, Parana, Brazil from 2007 to 2015.

YEAR	DOGS	CATS	BATS	OTHER	TOTAL
2007	93	8	52		153
2008	49	3	37	1 (ferret)	90
2009	26	1	45		72
2010	38	119	54		211
2011	21	116	64	2 (non-human primate and rabbit)	203
2012	250	173	86	2 (rabbit and horse)	511
2013	911	235	66	2 (bush dog)	1,214
2014	916	230	351	5 (rabbit, horse, bush dog, squirrel and opossum)	1,502
2015	372	251	185	5 (deer, raccoon, ferret, marmoset, gerbil)	813
Positives*	0	1	9	0	10
TOTAL	**2,676**	**1,136**	**940**	**17**	**4,769**

*Values not added to avoid overlapping

The central 156 phone system has registered 1,003 bat removal requests from 2010 to 2015 (Table 2), resulting in a total of 806 captured or collected bats. Due to environmental preservation and no evident risk of rabies transmission, 419 healthy bats with no observed contact to other animal species or human beings were systematically released at within-city preserved areas. The remaining 387 bats were immediately submitted for euthanasia and rabies testing, resulting in 9/387 (2.32%) positive bats.

Table 2. Bats complaints and proceedings for rabies surveillance in Curitiba, Parana, Brazil, 2010 to 2015.

Year	Complaints	Collected	Released	Rabies test	Positive
2010	129	54	27	27	1
2011	72	64	21	43	3
2012	139	86	28	58	1
2013	140	66	22	44	0
2014	250	351	267	84	2
2015	273	185	54	131	2
TOTAL	**1,003**	**806**	**419**	**387**	**9**

The overall 806 captured or collected bats were individually identified and resulted in 13 genera divided into three families (*Molossidae*, *Vespertilionidae* and *Phylostomidae*). The family *Molossidae* was the most frequent with 658/806 (81.5%) bats, followed by *Vespertilionidae* with 57/806 (7.1%) bats and *Phyllostomidae* with 45/806 (5.6%) bats; 46/806 (5.8%) bats were not identified (Table 3).

Table 3. Families and genus of bats collected for rabies surveillance in Curitiba, Parana, Brazil from 2010 to 2015.

Family	Genus	n	tested	Positives*	Genus (%)	Families (%) (Positives, %)
Molossidae (Total: 658)	*Molosus*	241	136	2 (1.47%)	29.9	81.6 (7/283, 2.47%)
	Promops	61	50	2 (4.00%)	7.5	
	Tadarida	19	10	-	2.3	
	Nyctinomops	336	86	3 (3.48%)	41.6	
	Eumops	1	1	-	0.12	
Vespertilionidae (Total: 57)	*Eptesicus*	13	10	-	1.6	7.1 (1/43, 2.32%)
	Myotis	23	16	1 (6.25%)	2.8	
	Histiotus	7	5	-	0.86	
	Lasiurus	14	12	-	1.7	
Phyllostomidae (Total: 45)	*Artibeus*	27	18	-	3.3	5.6 (1/32, 3.12%)
	Sturnira	14	12	1 (8.33%)	1.7	
	Glossophaga	3	1	-	0.37	
	Pygoderma	1	1	-	0.12	
Not identified		46	29	-	5.7	5.7
Total		806	387	9 (2.32%)	100	100

*Values not added to avoid overlapping, it tested were used how based percentage positives.

The case distribution map has shown all the points where bats were captured or collected in Curitiba from 2010 to 2015, including the nine positive cases (Figure 1). A seasonal decomposition was made for the same period to identify in which part of the year more captures or collections have occurred (Figure 2). The kernel density for negative cases has presented a homogeneous distribution, despite the aggregation observed in downtown Curitiba (Figure 3a). The kernel density estimation for positive bats has shown an aggregation of bat points at north Curitiba (Figure 3a).

2.5 DISCUSSION

Although the National Program for Rabies Control and Prevention has recommended an arbitrary 0.2% dog sampling based on city estimative of canine population [27], all animal sampling submitted for rabies diagnosis have been yearly increased, particularly between 2012 and 2014 (Table 1). Moreover, dogs were by far the highest number of samples sent for rabies diagnosis and all resulted negative. Important to point out that, despite Curitiba has been free of canine rabies since 1975 [17], dog sampling has not been based on suspicious nervous clinical signs or critical bat rabies areas, which may have lowered the surveillance sensitivity.

On the other hand, one cat tested positive for rabies virus variant 4 in 2010, compatible with isolates from insectivorous bat *Tadarida brasiliensis* [17], which may suggest that a direct contact between bat and cat have been occurred [28]. The predatory behavior of cats may induce bat hunting, which can raise the risk of cat rabies infection, making cats a potential rabies source for other animals species and human beings [29]. The last human case of rabies in the nearby São Paulo state was recorded in 2001, when a woman was likely infected by her cat's biting with the variant 3, commonly found in vampire bats (*Desmodus rotundus*) [30]. In 2008, at the city Santander de Quilichao, Colombia, rabies transmission was recorded from a cat leading to the death of two people; the virus-typing was AgV3 in both cases, mostly associated with hematophagous bats [31]. Colombia recorded another human case of rabies in 2013, with the owner bitten by a cat described as bat hunter; rabies typing was identified as variant 4, associated to insectivorous bats [32].

A recent study has shown the importance of rabies spillover by bats involving other animal species and the likelihood of rabies transmission by the bat-cat-human chain, despite not estimating the risk of bat-dog and bat-cat transmission [33]. Recipient hosts have been exposed to virus source in a sufficient amount to establish an infection, showing susceptibility to the virus [33, 34]. The positive cat rabies case from Curitiba reported in 2010 [17], associated to the data presented herein, may emphasize the importance of the surveillance service and monitoring to suspect bats for rabies, providing substantial information to authorities to establish strategies and actions.

The record analysis of bat capture and collection from 2010 to 2015 (Table 2) and family and genus data of positive bats collected during the same period (Table 3) has shown the importance of quantitative and qualitative information on bats collected, particularly due to specific bat behavior. Some bat species such as *Tadarida brasiliensis*, *Molossus rufus*, *Molossus molossus*, among others bats normally form maternal colonies [35, 36], which may push males to competition and segregation, and make females with more body contact. All these intraspecific and interspecific behaviors may have direct impact or influence on rabies transmission and dispersion. [14, 37].

Requests to remove the bats were higher than the number of animals collected since requests have occasionally involved bat colonies, which were not considered as imminent risk for rabies transmission by the Curitiba ZCC (Table 2). However, identification of bat colony genus and geo-referencing have been provided priorities for the ZCC on rabies sanitary blocking, preventive informative and pet vaccination programs [17, 38].

As expected, bats captured or collected, as well as positive for rabies, were mostly from the *Molossidae* family, since specimens from this insectivorous family may be attracted to insects near urban artificial lights and find artificial shelters in buildings such as roofs, ceilings, arctic, expansion joints that big cities offer [37, 38]. Although bats were captured or collected throughout the Curitiba city limits during the study, highest bat capture and collection at the central-northern region may have been influenced by high human population density (Fig.1), particularly downtown, providing artificial shelters and food supply [41, 42]. On the other hand, bats may be evenly distributed and dispersed throughout the city and more densely housed areas may generate more likelihood conditions for human-bat encounters. Further studies should focus on natural bat species distribution in all city regions, fully establishing the true causes for bat capturing or collection (and positivity for rabies).

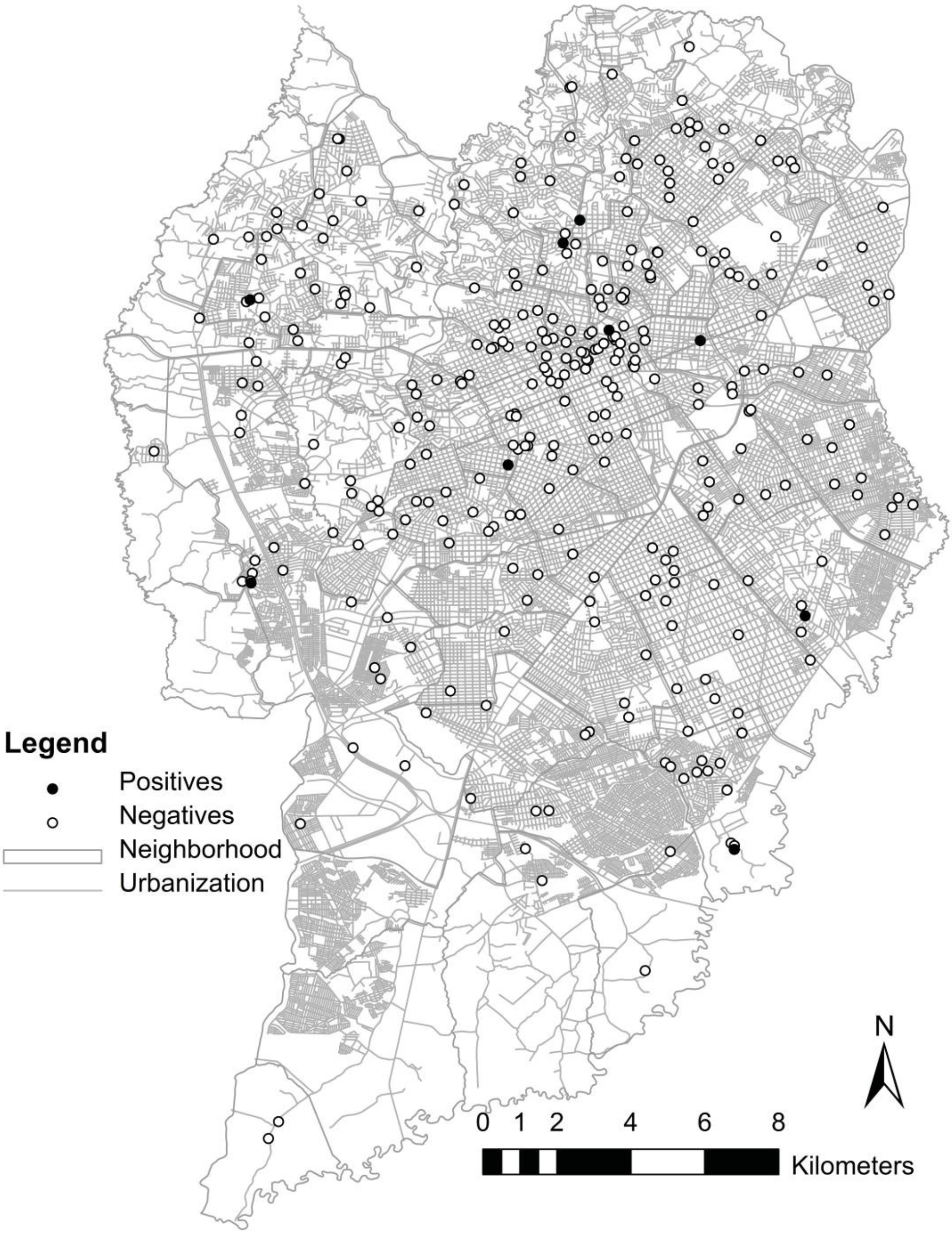


Figure 1. Urbanization map of the city of Curitiba showing the site where were collected bats at period 2010-2015. The dark circles indicate bats collected positive for the virus of rabies (9 bats); the white circles indicate bats collected not positive for for the virus of rabies.

The analysis of seasonal distribution (Fig. 2) has shown a close relationship between the warmer tropical months with the number of requests made by citizens for bat removal. Studies about *Tadarida brasiliensis* bats made in Argentina showed that the weather conditions directly influence the bats behavior, on very hot days (temperatures > 27°C) they were more active [35]. Higher temperatures were recorded from December to March in the study area, which may have led to increasing food supply for non-hematophagous bats, mainly insectivorous.

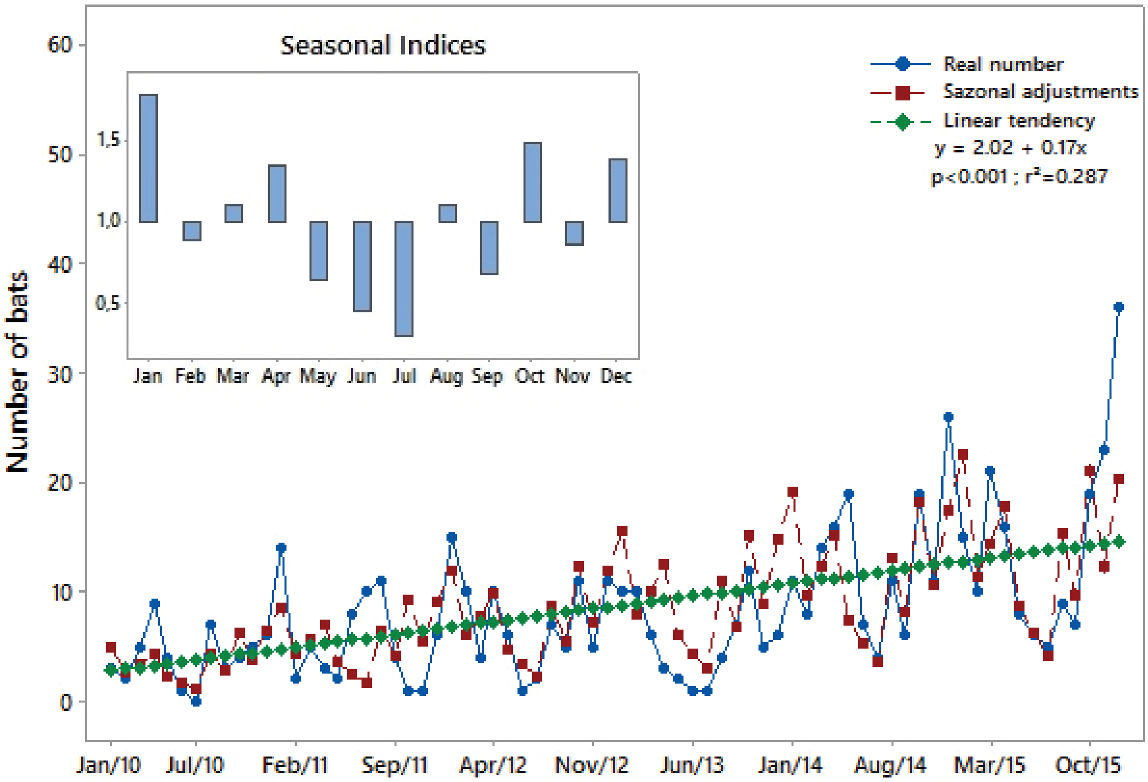
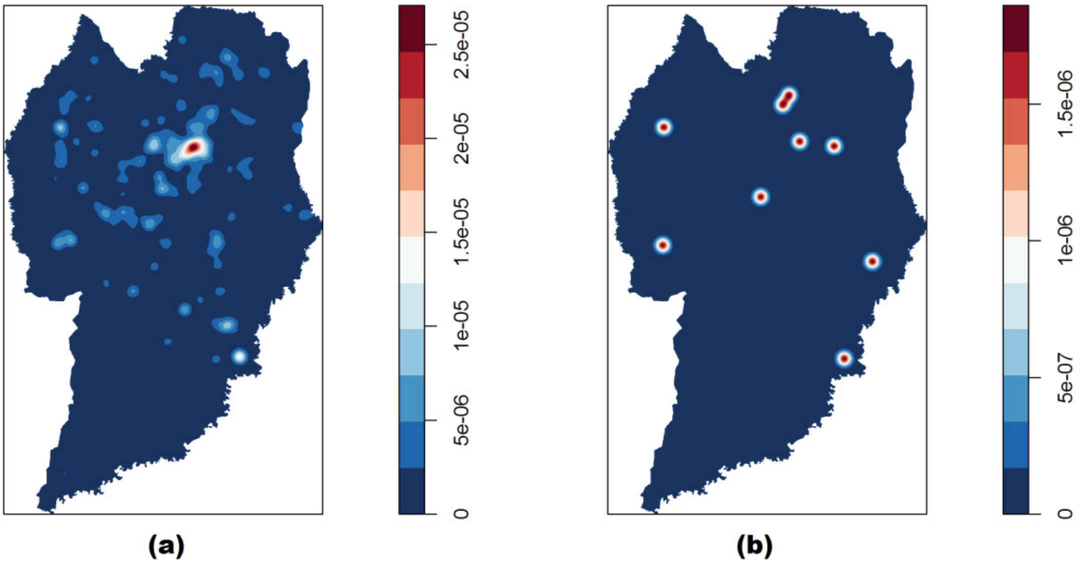


Figure 2. Analysis of seasonal decomposition, which showed bats capture pattern with random variation, but with a tendency to occur in the warmer periods of the year.

Insectivorous bats were collected throughout the study area but more frequent in the central region, same was observed for fruit bats, probably due to the abundance of food and shelter for bats in the region. Food source may be a key factor influencing the bat activity during periods of high temperatures, which may increase the activity of flying insects and consequently attract bats to areas of high concentration of insects due to food availability [41, 42]. Another important point on higher temperature periods has been the habit of leaving the windows opened, which may facilitate bat entry overnight, visualization on the next day and 156 complaint, accounting for most requests for bat removal by the surveillance service (Fig. 3a). The area with the highest concentration observed in Figure 3 corresponds to the Matriz sanitary district, which houses the most populous region of the city. In this sector, the ZCC technical staff identified several artificial shelters, such as: celling, arctic, expansion joints, air conditioning, shutters boxes, chimneys among others (internal information not published)

The kernel density estimation (Fig. 3b) has shown that the city center-northern area may be characterized as a particular area to bats affected by rabies virus. Such finding may point out the importance of monitoring service and local capturing of bats, since this service may prevent an accidental contact between an infected bat with a pet or human being.



(a) **(b)**

Figure 3. Kernel maps showing the frequency of collected and positive bats for rabies surveillance at Curitiba city among 2010-2015. (a) bats collected showing highest densities at center-northern area of the city. (b) positive bats for rabies virus (9 bats).

The geo-referencing may be an important tool used to identify the places where the bats were collected, providing a bat distribution overview throughout a region or city, which may be crossed with the geo-referencing of either rabies positive or negative pets, allowing health authorities to spatially combat the disease spreading, particularly to other animal species and human beings.

2.6 CONCLUSION

Data analysis have demonstrated that insectivorous bats of *Molossidae* family (658) were the most important for rabies monitoring in a large urban center. In addition, 8/9 positive bats for rabies during the studied period were insectivores and the rabies positive cat in 2010 was positive for the variant 4, compatible with insectivorous bats. The observed likelihood of rabies spillover from bats to cats have indicated that cat samples should be always sent for rabies diagnosis, particularly when presenting nervous or other characteristic symptoms. Likelihood of cat infection by bats spillover due to hunting habits may post a threat to public health services, which should re-evaluate the higher importance of mass vaccination of cats in large urban centers when compared to dogs. Health services should maintain and improve the monitoring of non-vampire bats in diurnal or unusual locations, collection of information on how the animal have been found, sex, age, animal health conditions, species, general, and quality of georeferencing information may improve the understanding of how the dynamics of rabies occur in bats on large cities of tropical countries. Finally, this paper showed the importance of the partnership between public health agencies and research institutions, since many data collected have been used only to compose internal reports. However, these partnerships allow the discussion and publication of these information, which may help in understanding the dynamics of rabies virus not only in bats, but as the rabies spillover can affect other animals and humans.

Competing interests: The authors declare that they have no competing interests.

Authors' contributions: JR participated in the study design, analysis and manuscript preparation. CS participated in bat collection, identification, and sent of sample for rabies testing. CMM and FF participated with discussion and analysis of the project about data geo-referencing and kernel density. JP and LSU participated in the study design and manuscript preparation. AWB participated in the study design, coordination and supervision. All authors read and approved the final manuscript.

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and material: the dataset(s) supporting the conclusions of this article is (are) available in the ZCC repository, solicited can be to health secretary of Curitiba city.

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CAPÍTULO II

SPATIAL DISTRIBUTION OF BAT SHELTERS AND HERBIVOROUS RABIES IN SOUTHERN BRAZIL

3. Spatial distribution of bats shelters and herbivorous rabies in southern Brazil

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3.1 ABSTRACT

Background: Common vampire bats (*Desmodus rotundus*) are considered major reservoirs for livestock and wildlife rabies, no study has focused on the spatial distribution of bat shelters in relation to animal rabies. Accordingly, the present study has aimed to characterize the epidemiological and spatial distribution of bat shelters and herbivorous rabies in the state of Parana, southern Brazil, from 2011 to 2017. In addition, a decomposition of the month-to-month seasonality series and a spatiotemporal cluster analysis were performed based on the data of all rabies-positive animals and hematophagous bat shelters.

Results: 1,742 animal samples were sent for rabies diagnosis within the period, with 481 (27.92%) positive and 1,261 (72.08%) negative results by direct immunofluorescence and biological testing in mice. Out of the 481 positive samples, 413 (85.8%) were bovine, 44 (9.1%) were equine, 6 (1.2%) were sheep, 5 (1.0%) were bubaline and 14 (2.9%) were bats. Out of 22 Regional Units of Agricultural Health (RUAH), the northeastern and central units had the highest rates of positive returns, with 157 (24.76%) and 123 (19.40%) cases. Only 1/22 RUAH did not present rabies-positive cases. The state was continuously endemic for herbivore rabies, with the highest number of cases found within the south central region, a result that is positively correlated with the highest number of hematophagous bat shelters and predisposed geological formation. A significant decrease in cases of herbivore rabies was observed in 2013 and 2014 but remained steady in subsequent years, probably due to continuous control and surveillance.

Conclusions: Despite continuous pinpoint mapping and control of hematophagous bat shelters, herbivore rabies in southern Brazil has been steady and statistically correlated with hematophagous bat shelters. Thus, the application of rabies vaccinations should be based on the spatial distribution of bat shelters to increase the effectiveness of preventive measures, particularly in predisposing geographical areas.

Keywords: Rabies, herbivorous, hematophagous bat, *Desmodus rotundus*, common vampire bat, bats shelters.

3.2 IMPACTS

1. Hematophagous bats have been considered the main rabies transmitters for livestock in Latin America, causing great economic losses and public health risks.
2. Areas with a high density of hematophagous bat shelters should receive special attention in epidemiological surveillance and control of the common vampire bat.
3. Monitoring and prevention strategies should be paired with intensified vaccination in livestock living in risky bat shelter areas.

3.3 INTRODUCTION

Rabies has been described as an important zoonotic viral disease affecting the central nervous system, causing acute encephalomyelitis and a case fatality rate of nearly 100% (Garcés-Ayala et al., 2017; Velasco-Villa et al., 2008). The rabies virus (RABV) belongs to the *Lyssavirus* genus of the *Rhabdoviridae* family. The virus, a negative-sense (NNS) single-stranded RNA (ssRNA)(Masatani et al., 2011), is found in the saliva of infected animals and transmitted mostly by bite to other species (Dias et al., 2011; Velasco-Villa et al., 2008). Rabies is endemic throughout the world, with both wild and domestic mammals serving as viral hosts, reservoirs and transmitters (Pereira et al., 2017).

Despite that rabies has been traditionally divided in to urban and sylvatic cycles, with dogs and wildlife species as the main reservoirs, respectively (Kotait et al., 2009), the disease has shown a different pattern in the Americas and may be divided into urban (dogs), rural (livestock), aerial (bats) and terrestrial wildlife cycles (Favoretto et al., 2013; Ribeiro et al., 2018; Rocha, de Oliveira, Heinemann, & Gonçalves, 2017).

Although any bat species may be susceptible to rabies, the common vampire bat (*Desmodus rotundus*) has become the major virus reservoir and transmitter due to its hematophagous nature (Seetahal et al., 2017), and this species is widely distributed from Mexico throughout the Neotropical region of South America (Simmons, 2005; Reis, 2013). Due to their role in rabies transmission to several herbivorous species, hematophagous bats have in directly caused great economic losses to the Latin American livestock and meat industry (Albas et al., 2011; P. Mialhe, 2013).

Geology and climate, in addition to blood availability, may favor high populations of *D.rotundus*, which often inhabit bat shelters near creeks and rivers (Lord, 1988). Unsurprisingly, the main rivers in southeastern Brazil are often associated with geographical features directly related to bat presence (P. Mialhe, 2013), and the cities within these hydrographic basins have been those most populated by *D. rotundus* (Taddei et al., 1991; Gomes, Monteiro, & Filho, 2007).

Although common Brazil vampire bats (*Desmodus rotundus*) are considered major reservoirs for livestock and wildlife rabies, no study has focused on the spatial distribution of bat shelters in relation to the presence of rabies. Accordingly, the present study has aimed to characterize the epidemiological and spatial distribution of bat shelters and herbivorous rabies in the state of Paraná, southern Brazil, from 2011 to 2017. In addition, a decomposition of the month-to-month seasonality series and a spatiotemporal cluster analysis were performed based on data of all rabies positive animals and hematophagous bat shelters.

3.4 MATERIAL AND METHODS

The state of Parana (22°29'30" to 26°43'00" S, 48°05'37" to 54°37'08" W), southern Brazil, is primarily located within the Atlantic Forest biome and is characterized by subtropical (Cfa) northern, western/southwestern and coastal, and southern/southeastern temperate (Cfb) climates (Waltrick et al., 2015). Parana has been ranked as the largest meat-producing state in Brazil (first in poultry, second in swine and ninth in beef cattle), accounting for 21.0% of the total national meat production (IBGE, 2018).

Suspicious animal samples have been received for rabies testing in the state between 2011 and 2017 through the Agency of Agricultural Defense of Parana State (ADAPAR), which has historically managed the State Program of Rabies Prophylaxis and Control in (Livestock) Herbivores. Divided into 22 Regional Units of Agricultural and Livestock Health (RUAH), the program has aimed to establish and execute rabies prevention and control measures, particularly rabies transmitted by hematophagous bats to livestock. In addition, ADAPAR has continuously promoted farmer training for the notification of potential hematophagous bat colonies, rabies prevention, vaccination for livestock animals, and animal sample shipment for rabies diagnosis (ADAPAR, 2018).

All rabies tests were performed by the Marcos Enrietti Diagnostic Center (MEDC), a state reference laboratory for rabies diagnosis, following international guidelines for laboratory and diagnostic techniques. Both a fluorescent antibody test (FAT), with a panel of monoclonal antibodies, and a test using intracerebral inoculation in 21-day-old mice were routinely employed for diagnosis (Dean 1996; Koprowski 1996). Bats referred for diagnosis were also identified based on the "Brazilian Bat Determination Key", as previously established (Reis, Peracchi, Pedro, & Lima, 2007).

A database of positive cases and bat shelters was constructed to track positive cases, sampling dates, animal species, owner names, property locations and latitude/longitude. Information collected on bat shelters included the Regional Unit of Agricultural Health (RUAH) data, catching period (day or night), shelter type (cave, tree, or other), shelter situation (active or inactive) and latitude/longitude.

The decomposition of the historical series was evaluated in a month-by-month fashion and included sample data on shipment, positivity, and animal source. Seasonality, trend evaluation of seasonal monthly index and trend evaluation with additive models of historical series decomposition were calculated by the MiniTab 17.0 (Minitab, 2010).

Spatial analysis of positive samples and bat shelters was distributed on a map with ArcGIS 10.0 software (ESRI, 2011). Since the series comprised the period from January 2011 to December 2017, a space-time cluster analysis with a space-time permutation model (Kulldorff, Heffernan, Hartman, Assunção, & Mostashari, 2005) was also performed, using SatScan (Kulldorff, 2006).

3.5 RESULTS

Overall, a total of 1,742 animal samples were sent for rabies diagnosis from 2011 to 2017, of which 481 (27.92%) tested positive, and 1,261 (72.08%) tested negative (Table 1). Out of the positive samples, 413 (85.9%) were cattle, 44 (9.1%) were horses, 6 (1.2%) were sheep, 5 (1.0%) were buffalos, and 14 (2.9%) were hematophagous bats; no positive samples were found for asinine, caprine, mule or swine species.

Table 1. History of rabies samples submitted for rabies diagnosis, in the State of Paraná – Brazil from 2011 to 2017.

Groups of animals	**Result**	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**	**2017**	**Total**
Asinine	P	0	0	0	0	0	0	0	0 (0.0%)
	N	0	0	1	0	0	0	0	1
Bovine	P	95	98	79	32	34	34	41	413 (85.8%)
	N	170	196	179	104	112	126	85	972
Bubaline	P	1	0	0	0	0	4	0	5 (1.0%)
	N	0	0	0	1	0	0	0	1
Caprine	P	0	0	0	0	0	0	0	0 (0.0%)
	N	1	2	3	0	1	2	0	9
Chiroptera	P	2	2	2	1	4	1	2	14 (2.9%)
	N	0	0	0	0	2	0	0	2
Equine	P	5	9	17	2	3	4	4	44 (9.1%)
	N	28	27	36	24	27	20	21	183
Mules	P	0	0	0	0	0	0	0	0 (0.0%)
	N	5	4	0	0	0	2	0	11
Sheep	P	0	0	4	1	1	0	0	6 (1.2%)
	N	7	13	6	13	7	3	6	55
Swine	P	0	0	0	0	0	0	0	0 (0.0%)
	N	2	6	4	4	3	4	4	27
Total	**P**	**103**	**109**	**101**	**36**	**42**	**43**	**47**	**481 (27.92%)**
	N	**213**	**248**	**229**	**146**	**152**	**157**	**116**	**1261 (72.08%)**

P = Positive; N = Negative. Only the percentages of the positive samples were inserted

Among the 22 Regional Units of Agricultural and Livestock Health (RUAH) (Figure 1), the northeastern (Ponta Grossa) and central (Guarapuava) RUAH presented the highest number of positive cases, with 157 (24.76%) and 123 (19.40%) animals, respectively. Eleven RUAH presented between 10 and 100 cases, and in 8 RUAHs, less than 10 cases of animal rabies were identified within the period (Figure 2). Only one RUAH, Cianorte (located in northwest Parana), did not present a single case of animal rabies.

Figure 1. Map of the state of Paraná, Brazil, subdivided in the 22 Regional Units of Agricultural and Livestock Health (URS). Source: From the authors themselves.

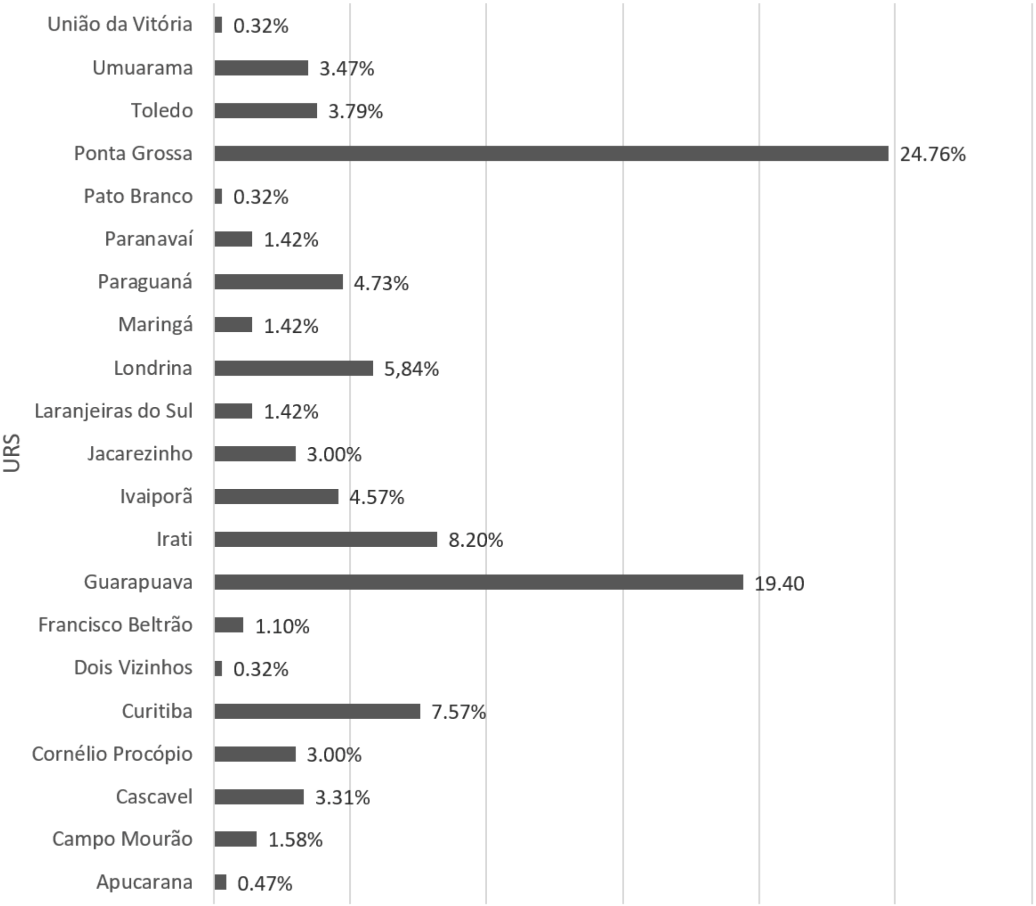


Figure 2. Percentage of rabies cases by Regional Units of Agricultural and Livestock Health (URS), 2011 to 2017. *URS of Cianorte was not included in the graph because it did not present cases of rabies during the study period. Source: From the authors themselves.

A map of the state of Parana showing the hydrography of the main rivers, the 481 rabies cases and the 921 hematophagous bat shelters identified by ADAPAR was constructed (Figure 3). Shelters were classified as cave, grotto, land hole, water box, house, corral, sewer, shed, tree, storehouse, water well, ravine, culvert and tunnel. Out of all bat shelters, 845 (92.0%) were active, and the remaining 76 (8.0%) were inactive.

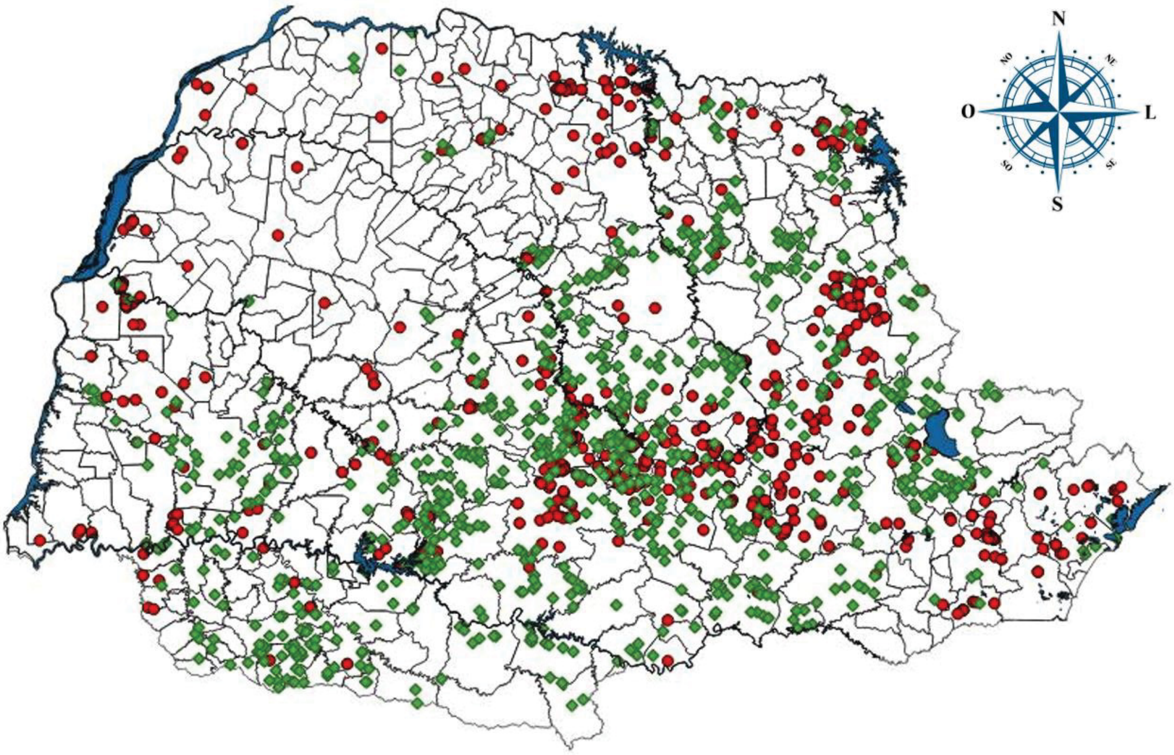
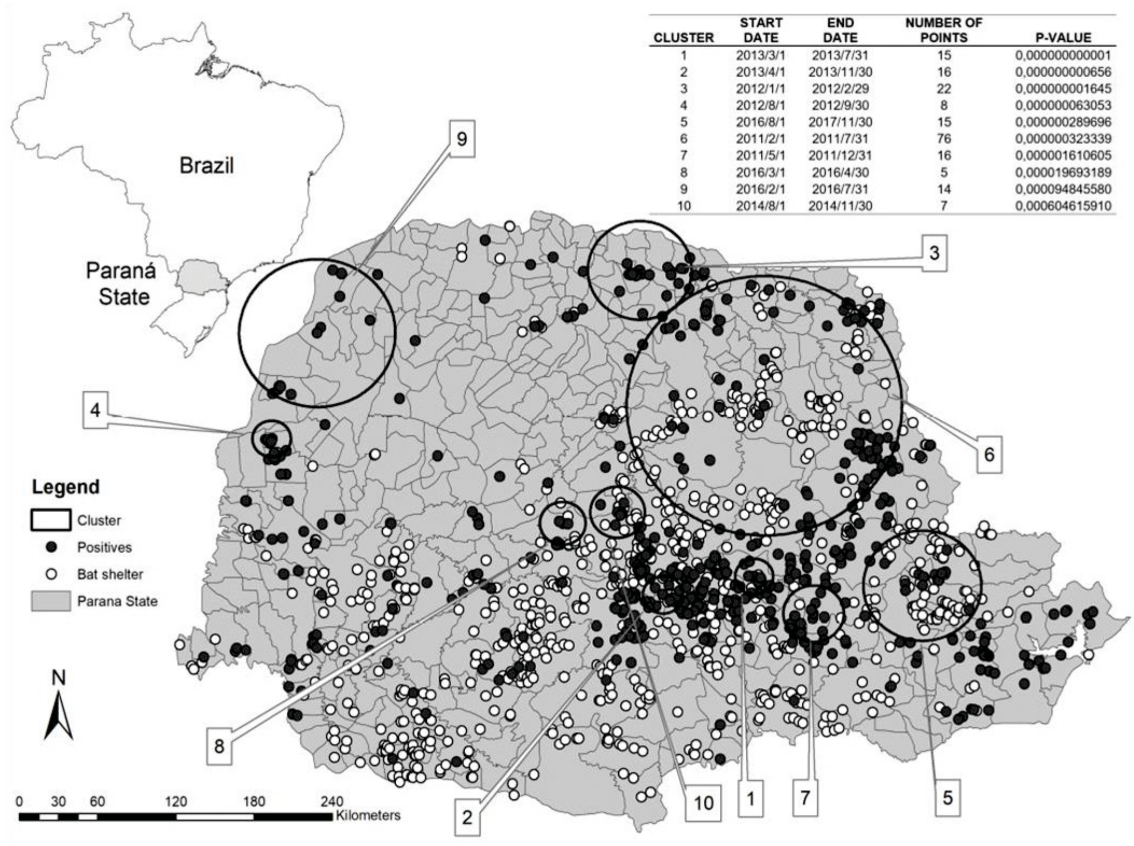


Figure 3. Map of the State of Paraná showing the main rivers that cut the State (blue); the balls show the positive cases of animal rabies recorded by ADAPAR between 2011 to 2017 (red), lozenges show the shelters of hematophagous bats identified by ADAPAR (green). Source: From the authors themselves.

A spatiotemporal cluster analysis for rabies cases and hematophagous bat shelters was performed using a permutation model, as previously reported (Kulldorff, 2005; Kulldorff, 2006), producing a map with 10 identified clusters (Figure 4). The decomposition of the historical series of seasonality and tendency with additive models has been analyzed month-by-month (Figure 5), with all bovine (Figure 5a) and positive bovine samples (Figure 5b), all equine (Figure 5c) and all positive equine samples (Figure 5d), and all samples (Figure 5e) and all positive samples (Figure 5f) presented.



CLUSTER	START DATE	END DATE	NUMBER OF POINTS	P-VALUE
1	2013/3/1	2013/7/31	15	0,000000000001
2	2013/4/1	2013/11/30	16	0,000000000656
3	2012/1/1	2012/2/29	22	0,000000001645
4	2012/8/1	2012/9/30	8	0,000000063053
5	2016/8/1	2017/11/30	15	0,000000289696
6	2011/2/1	2011/7/31	76	0,000000323339
7	2011/5/1	2011/12/31	16	0,000001610605
8	2016/3/1	2016/4/30	5	0,000019693189
9	2016/2/1	2016/7/31	14	0,000094845580
10	2014/8/1	2014/11/30	7	0,000604615910

Figure 4. Maps of Brazil with the state of Paraná highlighted, where positive cases of animal rabies were scored between 2011 to 2017 (black spots) superimposed on the hematophagous bat shelters cataloged by ADAPAR (white dots). Circles 1 through 10 identify 10 space-time permutation clusters, and the table above the map shows details of the identified cluster. Source: From the authors themselves.

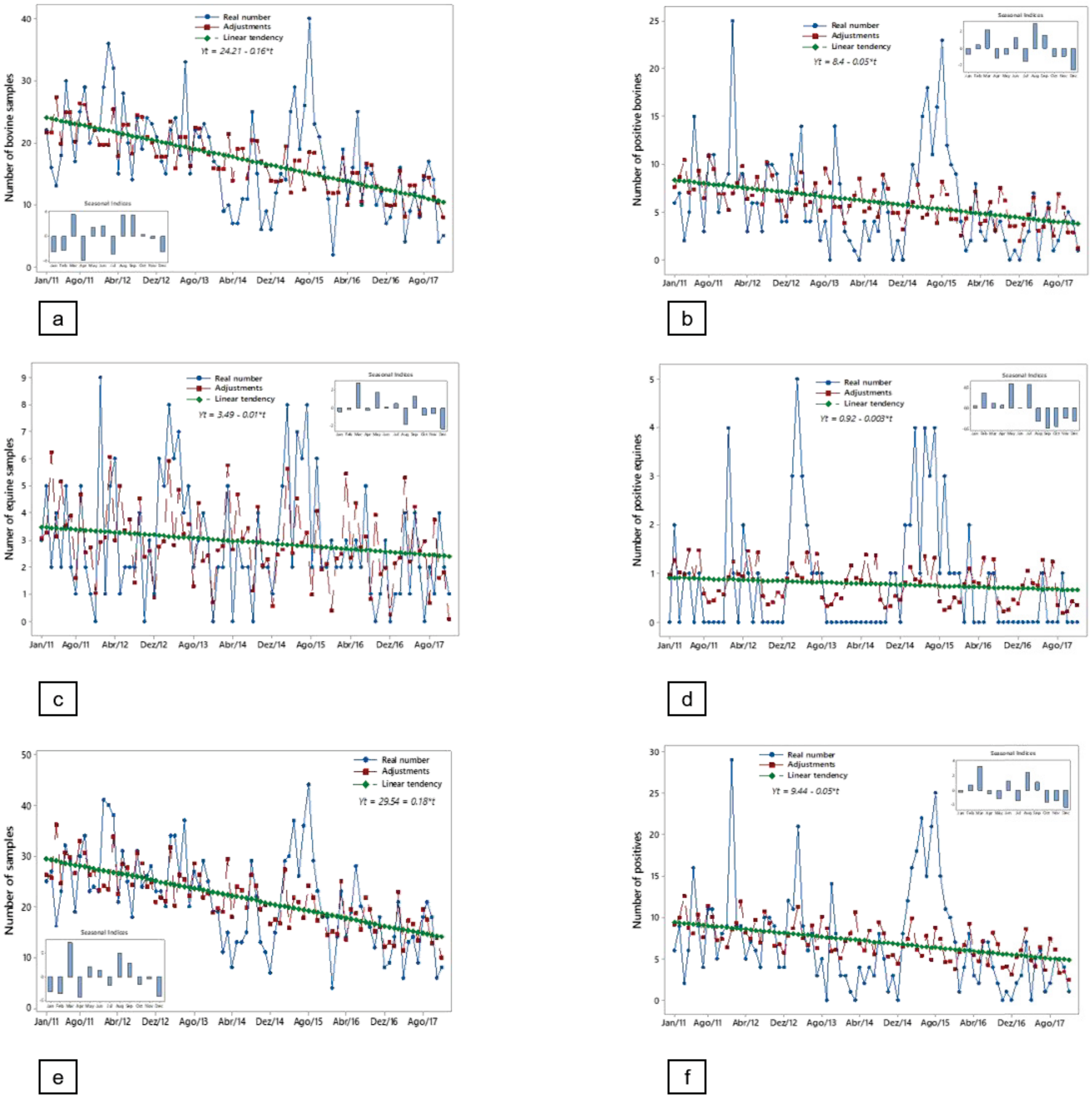


Figure 5. Decomposition of the month-by-month series of seasonality and tendency: (a) all bovine samples forwarded; (b) all positive bovine samples; (c) all samples of equine forwarded; (d) all samples of positive equine; (e) all samples forwarded; (f) all positive samples. Source: From the authors themselves.

3.6 DISCUSSION

In the present study, it was found that positive cases of herbivorous rabies in the state of Parana have decreased overtime, despite a previous report of endemic rabies in herbivores in which occurrence varied between years, no seasonal patterns were identified and occurrence fit into an 18 year cycle, as previously reported (Dognani et al., 2016).

The rabies cases in livestock found here have shown that beef cattle (85.8% of cases) was the most affected species throughout the years, probably due to the number of exposed heads, particularly in proximity to bat shelters. A study of vampire bat phylodynamics has demonstrated that the viral population may present a temporal and geographic structure consistent with the behavior of the common vampire bat (*Desmodus rotundus*), suggesting that the dispersion of the RABV lineages would be better explained by the ecology of bat populations (Torres et al., 2014). Out of the 44 positive horse samples for rabies, 17 were part of a 2013 outbreak, where two south central cities 25 km apart were alone responsible for 8 of the cases during a 4-month period; all 8 cases were located near six active hematophagous bat shelters. Similar results were found in southeastern Brazil, where two clusters 30 km apart and at a similar altitude was the shortest sample distance and is close to what *D. rotundus* may travel to feed (Vieira, Pereira, Carnieli, Tavares, & Kotait, 2013).

South central and southeastern Parana (Figure 1) accounted for more than half of the cases within all 22 RUAHs, with 52.36% of the rabies cases found in herbivores (Figure 1). Such regions were located on the second state plateau, bounded to the east by the Devonian escarpment, flowing into the crystalline plateau, and to the west by the basaltic plateau (MINEROPAR, 2001; Silva, 2008). The presence of large areas of rural vegetation in shallow soils, favorable to cattle farming, in association with intrusive elements such as dams and sills, along with great amplitudes, escarpment abruptness, slopes, canyons and stretches of embedded rivers, favors bat shelters (Maack, 2001).

A risk model of hematophagous bats attacking cattle, which considered the factors of vulnerability and receptivity, has been recently validated in the neighboring Sao Paulo state. The environmental vulnerability to rabies may correspond to a set of factors related to the ability of rabies transmitters to enter an area, while viral circulation and receptivity may be related to food availability (herbivore density/area) and hematophagous bat shelters (P. J. Mialhe, 2018; A. D. Rocha & Bichuette, 2016; Torquetti, Silva, & Talamoni, 2017).

In addition, the distance from the hematophagous bat shelters to the herds and the number of bats in these shelters have been more relevant in defining attack areas than the population density of domestic herbivores in the area (P. J. Mialhe, 2018). In the present study, a deadly overlapping of blood source from cattle farming and hematophagous bats from geographically favorable shelters has probably occurred in south central and southeastern state regions.

Similarly, several predisposing factors for *Desmodus rotundus* such as landscape, topography, hydrography, watersheds, animal production and land use (Estévez Garcia et al., 2014; Grenfell et al., 2004) were present in northeast Parana state, close to the borders with Sao Paulo state and Paraguay. In addition, that area is crossed by the Paranapanema River, which merges into the Parana River, which at approximately 2,450 miles in length is the second longest river in Brazil and the eighth longest in the world.

In contrast, the constructed maps have pointed towards a lack of rabies cases in herbivores (index = 4.89%) in the northwest portion of the state (Figures 3 and 4), which comprised 3 of the lowest incidences of rabies cases across the RUAH (Figure 1). This region is considered the most environmentally degraded, with only a few environmentally protected remnants (Gallassi, Santana, Londrina, Camargo, & Raminelli, 2017). The area lacks most of the rabies predisposing factors. Furthermore, the area has been characterized by the predominance of pastures, significant bovine livestock, lack of forests and little modernization (Fuentes et al., 2006). As expected, few hematophagous bats shelters were present throughout this region.

The northwest region (Figures 3 and 4) has shown higher rates of rabies cases and 282/922 (30.1%) bat shelters, fitting into the vulnerability and receptivity factors described above, since most (229/282; 81.2%) were identified as natural bat shelters such as caves, crevices, holes, caves, galleries and walls, along with a high density of beef and dairy cattle. On the other hand, despite similar high cattle density, the northwestern state was characterized by a low density of hematophagous bats shelters (0.97%, 9/922), resulting in lower positive cases of animal samples.

Out of the 10 clusters obtained in the present study (Figure 4), the largest was cluster 6, with 76 positive cases and the largest involved area, comprising the second and third state plateaus; followed by cluster 3, with 22 positive cases in a 2-month outbreak, all within the third plateau and nearby Tibagi river, an affluent of the Paranapanema River, mouthing into Paraguayan border; cluster 5, with 15 cases in a 16-month period, all within the coastal first state plateau, a mountainous region towards the seashore; cluster 9, with 14 cases in a 6-month period; and nearby cluster 4, with 8 cases in the western state, aside two major affluent rivers of the Parana River, which borders Brazil and Paraguay on the western side of Parana state. The other remaining clusters were located in the central state region between the second and third plateau, near freshwater springs and hydrographic basins, confirming an vantage as important routes for *Desmodus rotundus* in tracking herbivores for daily feeding (Castro & Michalski, 2015; P. Mialhe, 2013; P. J. Mialhe, 2018).

A decrease in rabies cases (Figure 5a, 5c and 5e) was observed in all decomposition analyses of the seasonal month-by-month series, positive cases of bovine rabies (Figure 5b) and all rabies positive animals (Figure 5e). Graphics showing a decrease in animal sample shipment in all species have been directly influenced by cattle rabies cases, verified by the analysis of the tendency line of positive rabies cases, which presented little variation during the study period and had no influence on the total positive cases of all evaluated animals. These results may have been the consequence of continuous state surveillance of livestock rabies, since the National Program for the Control of Rabies of Herbivores (NPCRH) has coordinated actions for hematophagous bat population control and livestock herd vaccination, along with animal health education throughout the Brazilian states (BRASIL/PNCRH, 2018). The NPCRH has aimed to establish and execute control and prevention measures for hematophagous bat rabies, including livestock animals and human beings (Dognani et al., 2016).

Finally, the Agency of Agricultural Defense of Parana State (ADAPAR), which has the federal responsibility of executing the PNCRH in the state of Parana, has already accepted the results obtained here as a basis for more effective measures against livestock rabies. In addition, ADAPAR has contacted other southern Brazilian states to share this ecological approach for controlling and preventing hematophagous bat rabies.

3.7 CONCLUSION

Despite continuous pinpoint mapping and control of hematophagous bat shelters, herbivore rabies in southern Brazil has been steady and statistically correlated with hematophagous bat shelters. Thus, the application of rabies vaccinations associated with the identification of bat shelters and hematophagous bat control should be established to increase the effectiveness of preventive measures, particularly in predisposing geographical areas.

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4. CONSIDERAÇÕES FINAIS

As análises demonstraram a importância de se manter estruturado e ativo o monitoramento de morcegos, a coleta adequada de dados, como: local e hora onde o animal foi encontrado, sexo, idade, condições de saúde, gênero e espécie, coordenadas de georreferenciamento e se houve contato com humanos e/ou com outros animais. Nos grandes centros urbanos há de se considerar a importância da coleta de morcegos insetívoros, uma vez que 8/9 casos de morcegos positivos para raiva em Curitiba foram pertencentes a família *Molossidae*, indiscutivelmente a circulação do vírus da raiva entre morcegos insetívoros e destes para outros animais tem sido relatada com alta frequencia, ressaltamos o caso do gato diagnosticado com raiva em Curitba em 2010, onde o vírus da raiva tipificado foi a variante 4, compatível com morcegos insetívoros, fato este que comprovou a grande possibilidade de spillover do vírus da raiva para seres humanos e/ou outros animais pelos felinos, ou seja, reafirmamos a importância do envio de amostras de gatos para diagnóstico de raiva, particularmente quando estes apresentam sintomas nervosos ou outros sintomas compatíveis a doença.

O Estado do Paraná permanece endêmico para raiva em herbívoros, apesar de que nos últimos anos foi observada uma tendência na redução no número de casos, esta diminuição provavelmente está ligada aos trabalhos e atividades de vigilância epidemiológica e controle da raiva dos herbívoros desenvolvida pela Agência de Defesa Agropecuária do Paraná (ADAPAR). Contudo, a ADAPAR deve aumentar a busca e identificação dos abrigos naturais (cavernas, fendas, buracos, galeria, gruta, árvore oca) de morcegos hematófagos em todas as regiões do Estado do Paraná, pois, foi observado elevado número de casos de raiva nos herbívoros próximos as localidades que apresentam grande número de abrigos naturais disponíveis para morcegos hematófagos. Manter e incrementar as ações de vacinação anti-rábica, particularmente em áreas geográficas predisponentes a abrigos naturais, deve ser estabelecido como estratégia para aumentar a eficácia das medidas preventivas.

Por fim, os programas de controle e monitoramento da raiva nos níveis Federal, Estadual e Municipal devem aprimorar a coleta e registro das atividades, bem como estabelecer parcerias com universidades e outras instituições de ensino e pesquisa para que os mesmos possam ser analisados, discutidos e disponibilizados para melhorias de estratégias de trabalho pelos programas de controle da raiva.

5. ANEXOS

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RESEARCH ARTICLE Open Access

Bat rabies surveillance and risk factors for rabies spillover in an urban area of Southern Brazil

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Abstract

Background: Bat rabies surveillance data and risk factors for rabies spillover without human cases have been evaluated in Curitiba, the ninth biggest city in Brazil, during a 6-year period (2010–2015). A retrospective analysis of bat complaints, bat species identification and rabies testing of bats, dogs and cats has been performed using methodologies of seasonal decomposition, spatial distribution and kernel density analysis.

Results: Overall, a total of 1003 requests for bat removal have been attended to, and 806 bats were collected in 606 city locations. Bat species were identified among 13 genera of three families, with a higher frequency of *Nyctinomops* in the central-northern region and *Molossidae* scattered throughout city limits. Out of the bats captured alive, 419/806 (52.0%) healthy bats were released due to absence of human or animal contacts. The remaining 387/806 (48.0%) bats were sent for euthanasia and rabies testing, which resulted in 9/387 (2.32%) positives. Linear regression has shown an increase on sample numbers tested over time (regression: $y = 2.02 + 0.17\times$; $p < 0.001$ and $r^2 = 0.29$), as well as significant seasonal variation, which increases in January and decreases in May, June and July. The Kernel density analysis showed the center-northern city area to be statistically important, and the southern region had no tested samples within the period. In addition, a total of 4769 random and suspicious samples were sent for rabies diagnosis including those from dogs, cats, bats and others from 2007 to 2015. While all 2676 dog brains tested negative, only 1/1136 (0.088%) cat brains tested positive for rabies.

Conclusion: Only non-hematophagous bats were collected during the study, and the highest frequency of collections occurred in the center-northern region of the city. Rabies spillover from bats to cats may be more likely due to the registered exposure associated with cats' innate hunting habits, predisposing them to even closer contact with potentially infected bats. Although associated with a very low frequency of rabies, cats should always be included in rabies surveillance and vaccination programs.

Keywords: Non-hematophagous bat, Dog, Cat, Rabies, AgV-3. Geo-referencing, Kernel, Seasonal decomposition

Background

Bats (order Chiroptera) have been considered one of the most diverse worldwide mammal groups, accounting for 20.7% of 5416 currently known mammal species, with 18 families and 1120 species [1, 2]. The presence of bats has been reported in all geographic areas of the world except the Arctic, Antarctic, extreme desert areas, and some isolated oceanic islands [3]. Brazil has been ranked as the second highest country in bat species, harboring 178 (15.9%) of the known species worldwide [4, 5].

Of the species of bats identified worldwide, only three feed exclusively on blood: *Desmodus rotundus*, *Diphylla ecaudata* and *Diaemus youngi*. *D. rotundus* is known as the common vampire bat and is the only one that feeds on mammalian blood, while the other two species feed on bird blood. Vampire bats are distributed from Mexico to South America [6]. Deforestation has drastically reduced the number of natural prey for *D. rotundus*;

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faced with this change, vampire bats have found a great source of food in cattle, which were introduced by man in South America. This has given rise to the numbers of vampire bats and their contact with cattle and man, causing a direct impact on human and animal health by the transmission of the rabies virus [7].

The rabies virus (RABV) can affect all mammals; however, the orders Carnivora and Chiroptera act as reservoirs for the virus [8]. The rabies virus (RABV) has been divided into two main variants: the first is associated with carnivores, mostly dogs, on an urban cycle, and the second is associated with bats, raccoons, and skunks on a sylvatic cycle [6–9]. The rabies cycle is divided into 4 cycles in several publications in South America: urban (domestic dog and cat), rural (livestock, cattle, horses, pigs, etc.), sylvatic (fox, raccoon, opossum, etc.) and air cycles (bats). However, in this study, this context was simplified to two major cycles, urban (dog and cat) and sylvatic (which covers all free-living animals, including bats) [10].

Although human cases in developing countries have been mostly associated with dog bites, bat species may also be infected by RABV, and human fatalities in Latin America have recently been connected to spillover from hematophagous, insectivorous and frugivorous bats [10, 11]. Not surprisingly, the highest recorded rabies outbreaks in Brazil were bat-transmitted and occurred in Brazilian northern rural (21 deaths) and remote areas of the Amazon forest (16 deaths) due to rabies virus variant 3 (AgV3), which is mainly found in *Desmodus rotundus*, a vampire bat species [10, 12, 13].

Meanwhile, a switch in the habits of non-hematophagous bats has also been recently observed, with migration from rural to urban areas probably due to increased food supply in urban centers and environmental impact on their natural habitats, increasing potential contact with domestic and wild animal populations and human beings [14, 15]. As a result, 20/41 (49.1%) positive bat specimens currently reported for rabies in Brazil were from non-hematophagous species, followed by 12/41 (29.0%) hematophagous and 9/41 (21.9%) unidentified species [16]. In addition, despite a decrease in human and canine rabies in Brazil, human cases have mostly (78.0%) occurred from bat variants between 2000 and 2009 [17, 18].

Cats have been considered a high-risk species for rabies transmission to humans in some European countries mainly due to their hunting habits, particularly toward flying animals including bats, which may connect rabies from the sylvatic-aerial cycle to urban settings [19]. Such scenarios may similarly occur in major cities of Brazil such as Curitiba, the ninth biggest Brazilian city, where a cat has been diagnosed with bat variant rabies after almost 30 years of no pet rabies cases [20].

Accordingly, this study aimed to analyze the bat rabies surveillance and risk factors for rabies spillover in an area without human cases in southern Brazil during a 6-year period (2010–2015). In addition, a retrospective analysis of bat complaints, bat species identification and rabies testing of bats, dogs and cats in the same area has been performed using methodologies of seasonal decomposition, spatial distribution and kernel density analysis.

Methods

Curitiba (25°25′48″ S, 49°16′15″ W), the capital of Paraná state, southern Brazil, has been currently ranked as the ninth biggest Brazilian city with approximately 1.8 million inhabitants [21]. Although categorized as a 100% urban area, Curitiba city has been considered to be environmentally friendly and the first in sustainability and quality of life in Brazil, with a high green-area ratio distributed throughout more than 40 city parks and preservation areas [22].

Since 1984, an official central telephone system has been used in Curitiba as a communication channel between the population and public managers; this system allows the population to request government services of all areas (health, urbanism, education, etc.), and among the available services are requests for the collection of dead animals (dogs and cats), removal of fallen bats inside houses and removal and/or observation of aggressive animals. Complaints of dead animals have been used as a source of brain samples from dogs and cats, most of which are sent for rabies diagnosis at the Parana State Reference Laboratory (LACEN) and used for monitoring rabies virus circulation. In addition, complaints for bats have followed another specific protocol: local inspection by professionals from the Curitiba Zoonosis Control Center (ZCC), capture or collection of bats, an epidemiological questionnaire and bat health status. If bats were healthy and had no human or pet contact, they were released using an open box at sunset of the same day at the ZCC, which was located nearby preserved areas at the time. If bats were dead, had contact with pets or human beings, or were unhealthy (no flying, neurological signs, injuries), they were euthanized, and their brains were sent for rabies testing at the LACEN.

Official city records of bat complaints, local inspections and bat destinations were obtained from January 2010 to December 2015. Additionally, records of bats, dogs and cats sent for rabies testing were obtained from the ZCC from January 2007 to December 2015. Bats were individually identified based on two standard taxonomy references [23, 24]. All rabies tests were performed by the Central Reference Laboratory of the State of Paraná (LACEN-PR) following international guidelines for laboratory and diagnostic techniques and using the fluorescent antibody test (FAT) with a panel of monoclonal antibodies as well as intracerebral inoculation in 21-day-old mice [25, 26].

A database was constructed with a commercially available statistical package (Microsoft Excel 2007, Microsoft Company, Redmond, WA, USA) and included collection location, situation in which the animal was collected or captured, number of animals, animal genus and species, procedures at ZCC, date and rabies result. Descriptive statistics were conducted with frequencies and distributions, followed by calculation of seasonal indices and a linear regression model with significance of 5% with Minitab software (Minitab 17 Statistical Software (2010). [Computer software]. State College, PA: Minitab, Inc.) [27]. A simple linear model was performed after tests were fitted to a normal distribution of data.

A geo-referencing approach was applied on the address data, using the "RDSTK" package [28] in the R software environment [29]. A map was built in commercial software [30] and contained bat points (positives/negatives), urbanization information, and neighborhood boundaries with shape files obtained from the City Geography Services (Institute of Urban Planning and Research of Curitiba, IPPUC). Finally, a kernel density analysis was performed with the "stats" package in the R environment [29]. These spatial treatments of data were performed to visualize the points (the map build) of bats collection and to test patterns of their distribution (kernel analysis). The kernel analysis is a density analysis that estimates the contribution of each point when compared to the distance to other points. The contribution extension is dependent on the bandwidth adopted (in this study, 50 m, considering the households as reference), and this analysis provides a density evaluation in which the hot areas represent the most important areas of the study when compared to the cold areas [31].

Results

Overall, a total of 4769 samples were sent for rabies diagnosis, including dogs, cats, bats and other animal species, from 2007 to 2015 (Table 1). The highest number of brain samples were collected from dogs (2676; 56.1%), followed by cats (1136; 23.8%), bats (940; 19.7%) and other animals (17; 0.35%), which included three rabbits (*Oryctolagus* sp.), three bush dogs (*Speothos venaticus*), two ferrets (*Galictis* sp.), two horses (*Equus ferus caballus*), a non-human primate (*Cebus* sp.), a squirrel (*Sciurus ingrami*), an opossum (*Didelphis albiventris*), a deer (*Cervus* sp.), a raccoon (*Procyon* sp.), a marmoset (*Callithrix* sp.), and a gerbil (*Meriones* sp.). Out of the tested samples, only 9/4769 (0.18%) bats and 1/4769 (0.02%) cats were positive for the rabies virus.

The central phone system had registered 1003 bat removal requests from 2010 to 2015 (Table 2), resulting in a total of 806 captured or collected bats. Due to environmental preservation and no evident risk of rabies transmission, 419 healthy bats that did not have contact with other animal species or human beings were systematically released within city preserved areas. The remaining 387 bats were immediately submitted for euthanasia and rabies testing, resulting in 9/387 (2.32%) positive bats.

Table 1 Animal samples sent for rabies surveillance in Curitiba, Parana, Brazil from 2007 to 2015

Year	Dogs	Cats	Bats	Other	Total
2007	93	8	52		153
2008	49	3	37	1 (ferret)	90
2009	26	1	45		72
2010	38	119	54		211
2011	21	116	64	2 (non-human primate and rabbit)	203
2012	250	173	86	2 (rabbit and horse)	511
2013	911	235	66	2 (bush dog)	1214
2014	916	230	351	5 (rabbit, horse, bush dog, squirrel and opossum)	1502
2015	372	251	185	5 (deer, raccoon, ferret, marmoset, gerbil)	813
Positives[a]	0	1	9	0	10
Total	2676	1136	940	17	4769

[a]Values not added to avoid overlapping

During the investigation, a total of 806 bats were captured or collected, and they were categorized in 13 genera from three families (*Molossidae*, *Vespertilionidae* and *Phyllostomidae*). The family *Molossidae* was the most frequent with 658/806 (81.5%) bats, followed by *Vespertilionidae* with 57/806 (7.1%) bats and *Phyllostomidae* with 45/806 (5.6%) bats; 46/806 (5.8%) bats were not identified (Table 3).

The case distribution map showed all the points where bats were captured or collected in Curitiba from 2010 to 2015, including the nine positive cases (Fig. 1). A seasonal decomposition was made for the same period to identify in which part of the year more captures or collections had occurred (Fig. 2). The kernel density for negative cases presented a homogeneous distribution, despite the aggregation observed in downtown Curitiba (Fig. 3a). The kernel density estimation for positive bats

Table 2 Bat complaints and proceedings for rabies surveillance in Curitiba, Parana, Brazil, 2010 to 2015

Year	Complaints	Collected	Released	Rabies test	Positive
2010	129	54	27	27	1
2011	72	64	21	43	3
2012	139	86	28	58	1
2013	140	66	22	44	0
2014	250	351	267	84	2
2015	273	185	54	131	2
Total	1003	806	419	387	9

Table 3 Family and genus of bats collected for rabies surveillance in Curitiba, Parana, Brazil from 2010 to 2015 (Additional file 1)

Family	Genus	n	tested	Positives[a]	Genus (%)	Families (%) (Positives, %)
Molossidae (Total: 658)	*Molossus*	241	136	2 (1.47%)	29.9	81.6 (7/283, 2.47%)
	Promops	61	50	2 (4.00%)	7.5	
	Tadarida	19	10	–	2.3	
	Nyctinomops	336	86	3 (3.48%)	41.6	
	Eumops	1	1	–	0.12	
Vespertilionidae (Total: 57)	*Eptesicus*	13	10	–	1.6	7.1 (1/43, 2.32%)
	Myotis	23	16	1 (6.25%)	2.8	
	Histiotus	7	5	–	0.86	
	Lasiurus	14	12	–	1.7	
Phyllostomidae (Total: 45)	*Artibeus*	27	18	–	3.3	5.6 (1/32, 3.12%)
	Sturnira	14	12	1 (8.33%)	1.7	
	Glossophaga	3	1	–	0.37	
	Pygoderma	1	1	–	0.12	
Not identified		46	29	–	5.7	5.7
Total		806	387	9 (2.32%)	100	100

[a]Values were not added to avoid overlap and show the percentage of positive test results

showed an aggregation of bat points in north Curitiba (Fig. 3a).

Discussion

Although the Brazilian National Program for Rabies Control and Prevention has historically recommended a 0.2% sampling of total estimated city dog population, consisted by dead dogs sent every year for rabies testing [32], animal sampling has increased above dog population growth, particularly between 2012 and 2014 (Table 1). Moreover, majority of samples were dogs (mostly killed by car, elderly or euthanized in shelters), which all resulted negative for rabies. Despite Curitiba has been reportedly considered a free-rabies city since 1975 [20], such "healthy" dog sampling not based on suspicious nervous clinical signs or critical bat rabies areas may have lowered the surveillance sensitivity through these years.

On the other hand, one cat tested positive for rabies virus variant 4 in 2010, compatible with isolates from insectivorous bat *Tadarida brasiliensis* [20], which may suggest that a direct contact between a bat and a cat occurred [33]. The predatory behavior of cats may include bat hunting, which can raise the risk of cat rabies infection, making cats a potential rabies source for other animal species and human beings [34]. The last human case of rabies in the nearby São Paulo state was recorded in 2001, when a woman was likely infected by a bite from her cat with variant 3, commonly found in vampire bats (*Desmodus rotundus*) [35]. In 2008, in Santander de Quilichao, Colombia, rabies transmission was recorded from a cat, leading to the death of two people; in both cases, the virus type was AgV3, which is mostly associated with hematophagous bats [36]. Colombia recorded another human case of rabies in 2013, with the owner bitten by a cat described as a bat hunter; the rabies type was identified as variant 4, which is associated with insectivorous bats [37].

A recent study has shown the importance of rabies spillover from bats to other animal species and the likelihood of rabies transmission through the bat-cat-human chain, but it did not estimate the risk of bat-dog and bat-cat transmission [38]. Recipient hosts have been exposed to virus source in a sufficient amount to establish an infection, showing susceptibility to the virus [38, 39]. The positive cat rabies case from Curitiba reported in 2010 [20], associated with the data presented herein, may emphasize the importance of the surveillance service and monitoring to suspect bats for rabies, providing substantial information to authorities to establish strategies and actions.

The identification of bat species can be important to understanding rabies transmission. The behavior of some species can expose them more or less to the virus [17]. *Tadarida brasiliensis*, *Molossus rufus*, and *Molossus molossus* (species identified in this study) form maternal colonies, which may push males to competition and segregation and make females have more body contact [40, 41]. Spatially, the *Molossidae* family (insectivorous family in general) may be attracted to insects near urban artificial lights and may find artificial shelters in roofs, ceilings, attics, etc. [42, 43]. This is reflected in the study at hand, where the highest bat capture was at the central-northern region, the high human population density of the city, providing artificial shelters and food supply [6, 44].

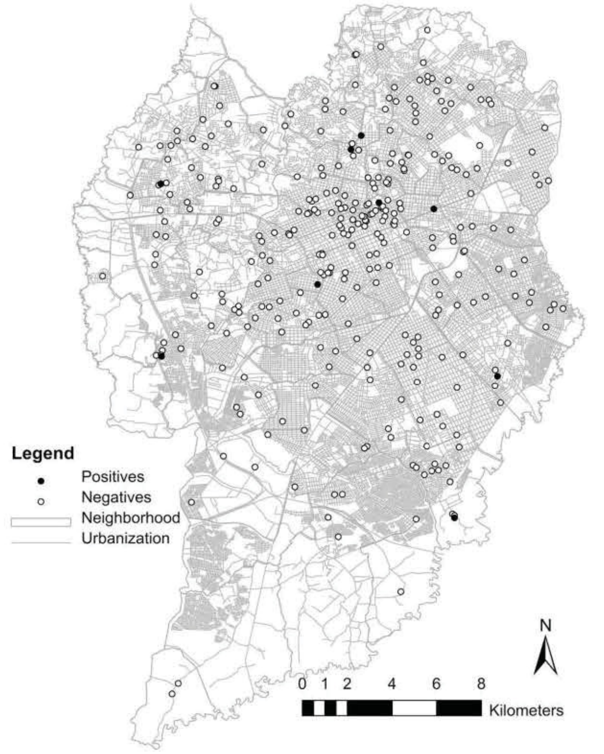

Fig. 1 Urbanization map of the city of Curitiba showing the site where bats were collected during the period 2010–2015. The dark circles indicate bats collected that were positive for the rabies virus (9 bats); the white circles indicate bats collected that were not positive for the rabies virus

Requests to remove the bats were higher than the number of animals collected since requests have occasionally involved bat colonies, which were not considered an imminent risk for rabies transmission by the Curitiba ZCC (Table 2). However, identification of bat colony genus and geo-referencing has been prioritized by the ZCC for rabies sanitary blocking, preventive informative and pet vaccination programs [20, 43].

The analysis of seasonal distribution has shown a close relationship between the warmer tropical months with the number of requests made by citizens for bat removal. Studies of *Tadarida brasiliensis* bats made in Argentina showed that weather conditions directly influence the bats' behavior; on very hot days (temperatures > 27 °C), they were more active [40]. Higher temperatures were recorded from December to March in the study area, which may have led to increasing food supply for non-hematophagous bats, mainly insectivorous bats.

Insectivorous bats were collected throughout the study area but more frequently in the central region, and the same was observed for fruit bats, probably due to the abundance of food and shelter for bats in the region. Food source may be a key factor influencing bat activity during periods of high temperatures, which may increase the activity of flying insects and consequently attract bats to areas of high concentration of insects due to food availability [6, 44]. Another important point regarding higher temperature periods has been people's habit of leaving windows opened, which may facilitate bat entry overnight, bat sightings the next day and phone system complaints, accounting for most of the requests for bat removal by the surveillance service. The area with the highest concentration observed in Fig. 3 corresponds to the Matriz sanitary district, which houses the most populous region of the city. In this sector, the ZCC technical staff identified several artificial shelters, such

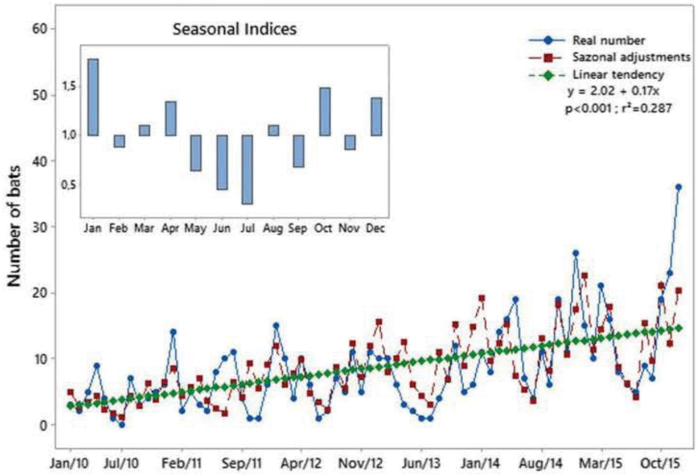

Fig. 2 Seasonal analysis of captured bats with seasonal indices and linear regression model results, showing bat capture patterns with random variation but with a tendency to occur in the warmer periods of the year

as the ceiling, attic, expansion joints, air conditioning, shutters boxes, and chimneys, among others (internal information not published).

The kernel density estimation has shown that the city's center-northern area may be characterized as a particular area for bats affected by rabies virus. Such a finding may note the importance of a monitoring service and local capturing of bats, since this service may prevent an accidental contact between an infected bat with a pet or human being. The geo-referencing may be an important tool used to identify the places where the bats were collected, providing a bat distribution overview throughout a region or city, which may be crossed with the geo-referencing of either rabies positive or negative pets, allowing health authorities to spatially combat the spread of the disease, particularly to other animal species and human beings.

Conclusion

This study showed that insectivorous bats (especially the *Molossidae* family) were important for rabies surveillance

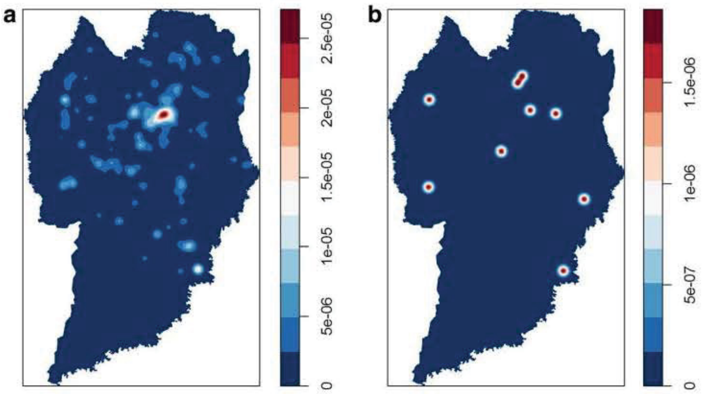

Fig. 3 Kernel maps showing the frequency of collected and positive bats for rabies surveillance in Curitiba city during 2010–2015. **a** Bats collected showing highest densities in the center-northern area of the city. **b** Bats positive for rabies virus (9 bats)

and transmission (positive bats and a cat spillover) during the period studied. There were zero positive dogs and only one positive cat, suggesting an increase in cat importance, and we recommend that public health authorities pay attention to mass vaccinations of cats in large urban centers. In addition, it is important that health services maintain and improve the monitoring of non-vampire bats in large urban centers, too.

Additional file

Additional file 1: Raw data of Table 3. (XLSX 95 kb)

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Availability of data and materials

The dataset(s) supporting the conclusions of this article are available.

Authors' contributions

JR participated in the study design, analysis and manuscript preparation. CS participated in bat collection, identification, discussion and analysis of the data about bats. CMM and FF participated with discussion and analysis of data and manuscript preparation. JP and LSU participated in the study design and manuscript preparation. AWB participated in the study design, coordination and supervision. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

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