UNIVERSIDADE FEDERAL DE SÃO CARLOS

Centro de Ciências Biológicas e da Saúde

Programa de Pós-Graduação em Ecologia e Recursos Naturais

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Sexagem molecular em Xenarthras, avaliação espaço-temporal de atropelamentos em tamanduás e diversidade genética em *Myrmecophaga tridactyla*: implicações para conservação

São Carlos

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“Here's to the crazy ones. The misfits. The rebels.
The troublemakers. The round pegs in the square holes.
The ones who see things differently. They're not fond of
rules. And they have no respect for the status quo. You
can quote them, disagree with them, glorify or vilify them.
About the only thing you can't do is ignore them. Because
they change things. They push the human race forward.
And while some may see them as the crazy ones, we see
genius. Because the people who are crazy enough to think
they can change the world, are the ones who do.”

Steve Jobs.

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Resumo geral

É esperado que espécies que vivem em ambientes altamente fragmentados e que possuem características biológicas como dieta especializada, tempo de geração longo e baixa fecundidade, tendam a sofrer mais pelos impactos antrópicos, modificando a sua biologia, ecologia e genética. Deste modo, espécies com essas características apresentam altas taxas de mortalidade devido aos impactos, além de mostrar baixa diversidade genética, elevados coeficientes de endogamia, sinais de redução no fluxo genético e tamanhos efetivos populacionais pequenos. Utilizando um total de 581 amostras biológicas provenientes de carcaças assim como amostras de tecido de indivíduos vivos (capturados) de *Myrmecophaga tridactyla* e *Tamandua tetradactyla*, nós realizamos: (I) a identificação do sexo dos indivíduos coletados, utilizando duas sequências nucleares (SRY e *Zinc finger*); (II) a avaliação temporal e espacial dos agrupamentos dos pontos de atropelamento para as duas espécies, utilizando abordagens de ecologia de estradas (III) a avaliação da diversidade genética e das mudanças demográficas da espécie ameaçada, *M. tridactyla*. Assim, nós encontramos que houve (I) uma taxa de sucesso de amplificação de 87 % no método utilizado, conseguindo a identificação de 151 machos atropelados para 82 fêmeas atropeladas na espécie *M. tridactyla* (2:1), e 128 machos atropelados para 51 fêmeas atropeladas na espécie *T. tetradactyla* (3:1). Adicionalmente a isso, (II) nós não encontramos variáveis temporais (climáticas) que explicassem o número de atropelamentos das espécies, mas encontramos um padrão sazonal respondido pelas fêmeas das espécies. Um total de dez pontos quentes de atropelamento foi encontrado para ambas espécies (cinco para cada uma), no entanto, a avaliação de variáveis espaciais que poderiam explicar os pontos quentes não foi explicativa. Os modelos construídos com variáveis espaciais em toda a paisagem (ex, porcentagem de vegetação) explicaram para *M. tridactyla* 27 % da variação encontrada, enquanto para o *T. tetradactyla*, 39 %. Por último, (III) nós encontramos ausência de sinais de redução de fluxo genético e uma diversidade genética moderada (Ho = 0,53) para *M. tridactyla*. Adicionalmente, nós encontramos um coeficiente endogâmico de 0,13 ($p \leq 0,05$) junto a sinais de redução do tamanho populacional efetivo da espécie. Concluindo, nós encontramos evidências do impacto das rodovias nas espécies de tamanduás da região do centro-oeste brasileiro e por tanto, recomendamos que os nossos dados sejam utilizados para a avaliação de medidas de mitigação na área.

Palavras chaves: Xenartros, sexagem molecular, ecologia de transporte, genética de populações, história demográfica.

General abstract

It is expected that species that live in highly fragmented environments and have biological characteristics such as specialized diet, long generation time and low fecundity, tend to suffer more from anthropic impacts, modifying their biology, ecology and genetics. Using a total of 581 biological samples from carcasses as well as tissue samples from living (captured) individuals of *Myrmecophaga tridactyla* and *Tamandua tetradactyla*, we performed: (I) the identification of the sex of the individuals collected, using two nuclear sequences (SRY and Zinc finger); (II) the temporal and spatial evaluation of the groupings of the run over points for the two species, using road ecology approaches (III) the evaluation of the genetic diversity and demographic changes of the threatened species, *M. tridactyla*. Thus, we found that there was (I) an 87 % amplification success rate in the method used, achieving the identification of 151 males run over for 82 females run over in the species *M. tridactyla* (2:1), and 128 males run over for 51 females run over in the species *T. tetradactyla* (3:1). Additionally, (II) we did not find temporal variables (climatic) that would explain the number of run over of the species, but we did find a seasonal pattern responded by the females of the species. A total of ten hotspots of running over were found for both species (five for each), however, the evaluation of spatial variables that could explain the hotspots was not explanatory. Models constructed with spatial variables across the landscape (e.g. percentage of vegetation) explained for *M. tridactyla* 27 % of the variation found, while for *T. tetradactyla* 39 %. Finally, (III) we found no signs of reduced gene flow and moderate genetic diversity (Ho = 0.53) for *M. tridactyla*. Additionally, we found a coefficient of inbreeding of 0.13 ($p \leq 0.05$) along with signs of reduction in effective population size of the species. In conclusion, we found evidence of the impact of roads on anteater species in the central western region of Brazil and therefore we recommend that our data be used for the evaluation of mitigation measures in the area.

Keywords: Xenarthra, molecular sexing, roads ecology, population genetics, demography changes.

LISTA DE ABREVIAÇÕES E SIGLAS

DNA	Ácido desoxirribonucleico
ABC	*Approximate Bayesian Computation*
ºC	Centigrados
$MgCl_2$	Cloreto de Magnésio
dNTPs	Desoxirribonucleotídeos Fosfatados
Zf	Gene *Zinc finger*
ha	Hectares
ICMBio	Instituto Chico Mendes de Conservação da Biodiversidade
m	Metros
µl	Microlitro (10 x E-06)
mM	Milimolar
NCBI	*National Center of Biotechnology Information*
pb	Pares de bases
RFLP	Polimorfismos de comprimento de fragmentos de DNA
Kg	Quilogramas
km^2	Quilômetros quadrados
PCR	Reação em cadeira da polimerase (*Polymerase Chain Reaction*)
CR	Região controle
SRY	*Sex-determining region Y protein*
SSR	*Short sequence repeat*
SISBIO	Sistema de Autorização e Informação em Biodiversidade
IUCN	*International Union for Conservation of Nature*

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1. APRESENTAÇÃO

Com o intuito de apresentar o conteúdo deste estudo, a tese se inicia com uma fundamentação teórica que abordará aspectos da ecologia e biologia da conservação bem como um panorama atual dos trabalhos já publicados utilizando as nossas espécies alvo.

O capítulo I, intitulado “Molecular sexing of Xenarthra: A tool for genetic and ecological studies” foi submetido na revista Conservation Genetics Resources. Nesse trabalho, nós avaliamos o potencial das ferramentas moleculares para identificação do sexo em amostras biológicas provenientes de indivíduos atropelados de sete espécies de Xenarthra. Pequenas sequencias nucleares de DNA presentes exclusivamente nos cromossomos X e Y (Gene *Zinc finger* e SRY) foram utilizadas para a padronização do método de sexagem na super ordem. Portanto, esse capítulo culmina no primeiro estudo que combina métodos moleculares com abordagens de ecologia de transporte e explora a presença de polimorfismos de única base em espécies de Xenarthra.

O capítulo II, intitulado “How do Temporal and Spatial Features Affect anteater’s Roadkill in Brazil” foi organizado e escrito sobre as normas da revista Journal of Environmental Management (candidata para publicação). Esse manuscrito teve como objetivo principal testar a influência de variáveis temporais e espaciais em quatro rodovias da região centro-oeste do Brasil nos atropelamentos de duas espécies da super ordem Xenarthra (*Myrmecophaga tridactyla e Tamandua tetradactyla*). Levando-se em consideração a relação entre a mortalidade das duas espécies com as características temporais e espaciais do entorno, como por exemplo, a proporção de vegetação. Nós coletamos informações no entorno das quatro rodovias estudadas, e construímos modelos que pudessem explicar os atropelamentos para as duas espécies.

O capítulo III, intitulado “Genetic diversity and demographic changes in the endangered giant anteater, *Myrmecophaga tridactyla*, living in an anthropized area” foi redigido sob as normas da revista Molecular Ecology (candidata para publicação). Nesse manuscrito, utilizando marcadores microssatélites, nós avaliamos como as ameaças, perda e fragmentação de habitat natural, podem estar afetando a diversidade genética e a estruturação genética populacional da espécie *Myrmecophaga tridactyla*. Essa, uma espécie tem sido classificada como vulnerável pela lista internacional de espécies ameaçadas (IUCN, 2014). Adicionalmente identificamos assinaturas genéticas de reduções no tamanho efetivo populacional da espécie ao longo dos últimos séculos. Esse estudo se configura como pioneiro no âmbito genético populacional dos xenartros, já que utiliza um número alto (dez) marcadores altamente polimórficos e o maior número de amostras biológicas para a espécie estudada.

Após a apresentação de todos os capítulos supracitados, estão as considerações finais junto às conclusões da tese, que reúne todos os resultados dos capítulos e considera as implicações para a conservação e manejo das espécies estudadas. Adicionalmente apresentamos as perspectivas futuras assim como as aplicações para a conservação das espécies estudadas. É importante ressaltar que cada capítulo possui sua própria formatação, referências bibliográficas e dois deles representam só um *proof* para uma possível submissão do manuscrito, estando, portanto, os capítulos II e III sujeitos a alterações.

2. FUNDAMENTAÇÃO TEÓRICA

2.1 Biodiversidade no Brasil: Ameaças

O território brasileiro pertence à região neotropical do globo, que concentra a maior diversidade do nosso planeta (MYERS et al., 2000). Graças a uma enorme variação de ecossistemas e ambientes dentro do território brasileiro, existe uma diversidade biológica exuberante, indo desde florestas amazônicas, maiores florestas tropicais úmidas do mundo, até caatingas, florestas semiáridas. A variedade de biomas que o Brasil tem, reflete sua riqueza de flora e fauna, e o permite ocupar uma posição de destaque, sendo considerado mega diverso por abrigar cerca de 10 a 20% do total de espécies conhecidas no planeta (LEWINOHN, 2006; MMA, 2010).

No entanto, a última avaliação realizada pelo Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) entre os anos de 2010 e 2014 incluindo todos os vertebrados trouxe um pouco de preocupação. Foram avaliados cerca de 12.256 táxons da fauna (732 mamíferos, 1980 aves, 732 répteis, 973 anfíbios e 4.507 peixes) dentre os quais 1.173 estavam ameaçados no Brasil (ICMBio, 2014). Como resultado, foi encontrada uma significativa correlação entre o número de espécies ameaçadas e o número de espécies conhecidas de cada ordem, o que indica que ordens mais conhecidas e com alta riqueza de espécies, possuem um número maior de espécies ameaçadas.

Na atualidade, já foram citados 17 tipos de ameaças como as principais causadoras do declínio das espécies. A maior parte das espécies está ameaçada pela destruição de habitat (88,4 %) e pelo desmatamento (73,9 %), fatores mais intensos no bioma Cerrado, considerado único no território da América do Sul e catalogado como *hotspot* de biodiversidade (LINO et al., 2019; SCHLAEPFER et al., 2018). Além da perda de habitat causada pelo desmatamento, a caça e perseguição entraram como ameaças que afetam 53,6 % e 23,2 % das espécies, respectivamente, sendo consideradas os segundos fatores que apresentam maior impacto a biodiversidade. Em terceiro lugar, outros fatores não menos importantes são o turismo, apontado como uma ameaça para 7,2 % (cinco espécies marinhas) das espécies e a malha viária afetando menos de 5 % da biodiversidade, mas crescendo exponencialmente com o passar dos anos, junto com a proporção de espécies impactadas.

O Cerrado brasileiro, considerado de grande importância não apenas pela sua beleza intrínseca mas também por ser o segundo maior bioma do Brasil, depois da floresta amazônica, abriga uma enorme diversidade de flora e fauna (CARVALHO; DE MARCO; FERREIRA, 2009; MYERS et al., 2000). Nos seus mais de 2 milhões de quilômetros quadrados, o Cerrado conta com três super centros de diversidade, determinados por barreiras altimétricas (Cerrados litorâneos, Cerrados meridionais e Cerrados do planalto central). Esse bioma abriga mais de 300 mil espécies (mais de 250 de mamíferos, dentre os quais 32 são endêmicos) que são afetadas pelo intenso processo de industrialização e urbanização que ocorre há décadas (COLLI; VIEIRA; DIANESE, 2020).

Junto com o avanço no desenvolvimento da agricultura houve um rápido crescimento populacional humano, assim como um grande avanço na estrutura viária que levaram ao desaparecimento da vegetação nativa em diferentes áreas deste Bioma (IBGE, acessado em junho de 2020). Da mesma maneira, o grande crescimento da malha viária no começo da década de 70 no território brasileiro afetou drasticamente biomas como o Cerrado (MYERS et al., 2000), diminuindo quase num 50% sua proporção de vegetação nativa.

2.2 Ecologia de transporte no Brasil: Impacto das estradas nos xenartros

Infraestruturas de transporte tais como as ferrovias, rodovias e canais marinhos podem ter grandes impactos no ambiente. Os efeitos ecológicos da estrada incluem a destruição do habitat das espécies assim como a fragmentação e poluição. Assim a crescente preocupação com esses efeitos conduziu ao aparecimento de uma nova disciplina dentro da ecologia chamada de ecologia de transporte ou ecologia de transporte (BALKENHOL; WAITS, 2009). O principal objetivo da ecologia de transporte é providenciar planos com base científica que mostrem como é possível evitar vários dos impactos causados pelas infraestruturas assim como minimizas ou mitigar os impactos atuais das mesmas (BALKENHOL et al., 2014).

A mortalidade registrada atualmente a causa do desenvolvimento da infraestrutura, que cada vez é maior em áreas que contém e mantêm uma alta diversidade de espécies (BENÍTEZ-LÓPEZ; ALKEMADE; VERWEIJ, 2010) vem aumentando desde a colonização do território brasileiro, com a chegada dos primeiros portugueses no ano de 1500. Isso, junto o avanço da estrutura viária que começa com a construção da primeira rodovia brasileira no ano de 1856 e que continua crescendo até hoje, os impactos registrados na biodiversidade também têm sofrido um incremento exponencial (GRILO et al., 2018).

No território brasileiro, são aproximadamente 1,6 milhões de km de rodovias, com 14,5 % asfaltadas, muitas construídas através ou no entorno de áreas do sudeste brasileiro, com um possível crescimento de 20 % nos próximos 20 anos (BAGER; BORGHI; SECCO, 2015). Assim, com o passar dos anos, o impacto das estruturas de transporte tornam-se mais fortes em algumas regiões naturais do Brasil, causando alterações que promovem a perda de habitat e fragmentação de até quase 80 % da vegetação nativa dos diferentes biomas em algumas regiões (BAGER; FONTOURA, 2012). Isso é evidente, por exemplo, só se considerarmos que a largura média de uma estrada que é de 10 m e sua área de influência que é de 50 a 100 m para cada lado. Pensando nisso, por tanto, já temos milhões de hectares diretamente afetadas do bioma e, centenas de espécies afetadas pela perda e fragmentação do seu habitat, assim como múltiplas barreiras causadas só pela estrutura da rodovia.

No Brasil foi estimado um total de 473 milhões de mortes de vertebrados por ano até o ano 2014 (BAGER, 2020; DORNAS et al., 2012), aumentando exponencialmente o número de mortes e os impactos causados a cada ano que passa. No ano de 2018 (GRILO et al., 2018) foi realizada uma compilação de dados georreferenciados sobre as mortes nas estradas, a partir de dados publicados e não publicados no Brasil. Essa compilação conseguiu um conjunto de dados de 21.512 registros que inclui 31 espécies de anfíbios, 90 espécies de répteis, 229 espécies de aves, e 99 espécies de mamíferos mortos nas diferentes rodovias do país, principalmente no sul brasileiro. Segundo a mesma compilação feita até ano 2018, as espécies com maior número de registros encontrados principalmente no sudeste de Brasil foram: *Didelphis albiventris* (n=1.549), *Volatinia jacarina* (n = 1,238), *Cerdocyon thous* (n = 1,135), *Helicops infrataeniatus* (n = 802) and *Rhinella icterica* (n = 692) (GRILO et al., 2018), sendo espécies abundantes em algumas regiões e pouco ameaçadas segundo a IUCN. No entanto, mesmo que os maiores registros sejam para espécies com essas características, espécies classificadas como ameaçadas, vulneráveis ou quase ameaçadas pela IUCN dentro do território brasileiro (nove ameaçadas e 12 quase ameaçadas) foram registradas, mesmo que em um número menor a 300, aumentando a preocupação de risco para a fauna brasileira.

Na última década, Cáceres et al. (2010) em um levantamento de fauna atropelada, realizou um top 5 das espécies mais impactadas pelas rodovias, sendo elas *Euphractus sexcinctus, Dasypus novemcinctus, Cabassous tatouay, Myrmecophaga tridactyla* e *Tamandua tetradactyla* , e sugerindo urgentemente que medidas de conservação fossem tomadas. Da mesma maneira, Ribeiro; Silveira Miranda; Rodrigues de Araújo (2017), em um estudo de levantamentos de fauna atropelada em rodovias que atravessam o Cerrado, identificaram a super ordem Xenarthra como a mais afetada pelos atropelamentos. Assim, sabendo o grande impacto da malha vária neste grupo, Ribeiro; Silveira Miranda; Rodrigues de Araújo (2017) explicaram como as características fisiológicas das espécies que constituem a super ordem (visão limitada, taxas reprodutivas baixas, movimentação reduzida) assim como estruturas morfológicas (tamanho do corpo, pelagem e até coloração) aumentam a probabilidade destes animais serem mortos pelos atropelamentos nas rodovias que atravessam ou contornam o Cerrado. Por outro lado, e não menos importante, Bertassoni (2012) em um estudo feito em duas comunidades tradicionais do centro-oeste brasileiro sobre as percepções de duas espécies de Xenarthra (*M. tridactyla* e *T. tetradactyla*) mostrou que muitas das percepções populares das espécies por parte dessas comunidades está relacionada com a sorte, indicando que muitos eventos de atropelamentos causados nas espécies poderia ser intencional por estar relacionado às crenças populares.

Do mesmo modo, a fragmentação e perda de habitat vem produzindo a redução dos tamanhos populacionais das espécies, resultando em populações com baixo tamanho efetivo, limitação no fluxo genético e alto coeficiente endogâmico (FAHRIG, 2003; REED; FRANKHAM, 2003a). A combinação de todos esses cenários provocados a raiz das atividades e matrizes antrópicas, pode por tanto minimizar a probabilidade de adaptação dos indivíduos causando (em cenários extremos) até a extinção local das espécies (RIPPLE et al., 2016; SOULÉ; WILCOX, 1980)

2.3 Biologia da conservação: Genética da conservação

O surgimento do termo biodiversidade criado por Walter G. Rosen, do National Research Council/National Academy of Sciences em 1985 foi acunhado em um momento no que as preocupações com a conservação da vida tomavam impulso. No entanto, desde 1979 o livro *The sinking Ark: A new look at the Problem of Disappearing Species*, publicado pelo ecólogo ambientalista inglês Norman Myers, já discutia a extinção das espécies e as ameaças que surgiam com a relação à destruição de habitats, devastação das florestas tropicais e o crescimento na infraestrutura social.

A partir dos anos 80's, com o surgimento do conceito de biodiversidade, a questão de conservação da vida devido às ameaças relacionadas ao crescimento populacional humano tomou força (JOSÉ LUIZ DE ANDRADE FRANCO, 2013). Muitos livros de autores formados em biologia foram importantes para o debate sobre a conservação da natureza, surgindo diversas teorias que ajudaram a explicar os impactos da fragmentação de habitats na diversidade genética das espécies. Dessa maneira, a partir de 1987, os trabalhos que utilizaram os termos de biodiversidade e conservação aumentaram consideravelmente em diversas áreas da biologia (FRANCO, 2013; SOULÉ; WILCOX, 1980).

Devido à impacto que teve o termo de conservação da biodiversidade, importância atribuída não simplesmente à riqueza e abundância de espécies, mas também à variação genética das mesmas (MCNEELY; MILLER; REID, 1990) os trabalhos que foram realizados utilizando ferramentas moleculares e informações genéticas em diferentes grupos, principalmente em grupos carismáticos como os mamíferos, aumentou consideravelmente nas últimas décadas. Sendo assim, a identificação dos impactos genéticos encontrados e causados possivelmente por fatores antrópicos, assim como as medidas de manejo utilizadas, se fortaleceu após a integração de várias áreas com objetivos comuns, conservação da fauna silvestre ameaçada.

Por exemplo, alguns como Forman (1998) descrevem a perturbação da mobilidade dos indivíduos interferindo nas migrações entre populações e consequentemente, na estrutura e viabilidade populacional das espécies graças a falta de fluxo genético entre as populações. Da mesma maneira, outros autores (FRANKHAM, 2005; REED; FRANKHAM, 2003a) descrevem a falta de fluxo genético como a causa do isolamento gerado pela fragmentação de habitat promovendo a redução na diversidade genética das populações, podendo causar maiores riscos de extinção em panoramas futuros. Por tanto, a partir dos 90’s, o isolamento reprodutivo é identificado como uma causa da perda de fluxo de indivíduos entre populações, que causa o aumento da ação de diversas forças evolutivas (deriva genética e coeficiente de endogamia principalmente) sobre as populações e que por tanto, diminui a plasticidade adaptativa das mesmas frente às possíveis alterações ambientais ao longo do tempo (FRANKHAM, 1998; REED; FRANKHAM, 2003a).

Partindo desse panorama teórico, houve um investimento para aprimorar as técnicas moleculares que permitiriam acessar a diversidade genéticas das espécies. Em ordem cronológica, os primeiros marcadores utilizados para análises populacionais foram as isoenzimas, na década dos 90. Subsequentemente veio o uso de enzimas de restrição e a Reação em Cadeia da Polimerase (PCR) associada a outros métodos de biologia molecular, como clonagem, polimorfismos de comprimento de fragmentos de DNA (RFLP) e sequenciamento, que permitiram o acesso a distintas informações moleculares. E por último, finalmente, os marcadores microssatélites, neutros, que devido a sua alta taxa mutacional se tornaram marcadores altamente eficientes na resolução de diversas questões a respeito da genética populacional das espécies (FRANKHAM; BALLOU; BRISCOE, 2010).

2.4 Xenarthra: Panorama atual

A super ordem Xenarthra possui características morfológicas únicas dentro dos placentários como o número de articulações atípicas entre as vertebras, resultando na origem do nome Xenarthra derivado do grego, *xenos* = estranho e *arthros* = articulação, em português xenartros. Da mesma maneira, outras características como a dentição reduzida e nula em tamanduás, assim como as temperaturas corporais baixas, junto as baixas taxas metabólicas(CAMILO-ALVES; MOURÃO, 2006; MCNAB, 1984) fazem dos xenartros um grupo particularmente importante.

Filogeneticamente, os xenartros constituem um dos maiores e mais basais clados dos mamíferos placentários, sendo o primeiro grande clado placentário com o genoma total mitocondrial de todas as espécies vivas descrito (GIBB et al., 2016). Aparentemente, os primeiros xenartros em aparecer no Mioceno foram os mirmecófagos, animais que deram origem a linhagens omnívoras e herbívoras dentro da super ordem, atributo importante para conhecer várias das relações evolutivas dentro dos mamíferos (GAUDIN; CROFT, 2015). As análises de diversificação feita por Gibb et al. (2016) mostraram que os xenartros são um clado antigo com uma taxa de diversificação constante ao longo do tempo e com uma rotação de espécies impulsionada por uma elevada taxa de constantes extinções. É um grupo que possui mais diversidade extinta do que existente, melhorando e ajudando a definição das espécies. Segundo Gaudin; Mcdonald (2008), existem atualmente somente 31 espécies vivas, com 21 espécies de tatus, seis espécies de preguiças e quatro espécies de tamanduás (Figura 1).

Figura 1. Representantes dos três grandes grupos da super ordem Xenarthra (esquerda para direita): ordem Cingulata (tatus) e ordem pilosa (tamanduás e preguiças, respectivamente)

Fonte: Arkive (Download 21 de abril, 2020)

Mais da metade das espécies da super ordem está distribuída na América do Sul (19 espécies), algumas espalhadas na América central e só o tatu galinha (*Dasypus novemcinctus*) no sul dos Estados Unidos. Por tanto, os xenartros ocorrem ao longo de um gradiente de ambientes secos e abertos, indo de florestas úmidas até campos abertos e secos (SUPERINA; LOUGHRY, 2015), mas também mostrando certo grau de preferência de habitats em algumas espécies (MORAES-BARROS; ARTEAGA, 2015). Dessa maneira, as espécies dentro da super ordem podem variar de espécies estritamente arbóreas, como o tamanduaí (*Cyclopes didactylus*), encontrado principalmente na zona norte do Brasil, até espécies fossoriais (adaptados para cavar e viver no subsolo), como o tatu de 15 kg ou tatu canastra (*Priodontes maximus*) (ANACLETO; MARINHO-FILHO, 2001; BERTASSONI, 2010; BERTASSONI et al., 2017; HAYSSEN, 2011; MEDRI; MOURÃO, 2005; MIRANDA JÚNIOR; BERTASSONI, 2014).

O nível de ameaça nos xenartros é alto sendo que dois dos Cingulata (tatu-canastra - *Priodontes maximus;* tatu bola - *Tolypeutes tricinctus*); e dois dos Pilosa (tamanduá-bandeira - *Myrmecophaga tridactyla*; preguiça de coleira - *Bradypus torquatus*), estão classificados como Vulneráveis (VU) nas listas vermelhas nacionais e internacionais (MARQUES et al., 2002; MIRANDA et al., 2015; TORRECILHA et al., 2017). Ou seja, um quinto (21 %) das 19 espécies que habitam o território brasileiro encontra-se em risco de extinção. Adicionalmente, Miranda et al. (2005) aponta que 83,3 % das espécies carece de estudos sobre limites de distribuição geográfica, 77,7 % dos táxons apresentam carência em dados ecológicos e 55,5 % das espécies precisam de maiores esforços de pesquisa sobre biologia e reprodução. Desse modo, o panorama exposto anteriormente apresenta os xenartros como um grupo pouco estudado e subvalorizado, além de altamente impactado, especialmente quando comparado com outros grupos taxonômicos equivalentes, tais como primatas, morcegos, roedores e ungulados (SUPERINA; LOUGHRY, 2015).

Nos xenartros, Nardelli et al. (2016) realizaram o levantamento dos estudos populacionais publicados utilizando marcadores nucleares (microssatélites). No total, encontraram nove estudos populacionais em somente cinco das 31 espécies vivas da super ordem. Todos os estudos reportados, revelam panoramas díspares devido às diferentes abordagens utilizadas; as amostragens esses estudos foram realizados entre o ano de 1996 e 2012, utilizaram entre 4 e 18 marcadores microssatélites e entre 15 e 310 espécimenes, encontrando uma diversidade genética observada (heterozigosidade observada, Ho) em xenartros que vária de 0,059 em tamanduá-bandeira até 0,71 em preguiça comum (*Bradypus variegatus*). No entanto, apenas dois estudos (COLLEVATTI et al., 2007; GARCIA et al., 2005; SARTORI, 2018) foram realizados para avaliar o panorama genético populacional de um xenartro ameaçado (tamanduá-bandeira) utilizando marcadores neutros que responderiam padrões contemporâneos, objetivando a implantação de medidas de conservação e mitigação para esta espécie.

2.4.1 Família Myrmecophagidae

Essa família é constituída por dois gêneros: *Myrmecophaga* com uma única espécie viva, o tamanduá-bandeira (*Myrmecophaga tridactyla*) e duas do gênero *Tamandua* o tamanduá-mirim (*Tamandua tetradactyla*) e o tamanduá do norte (*Tamandua mexicana*), distribuídas ao longo de Centro e Sul América (Figura 2). O nome genérico de Tamanduá, vem da língua tupi (uma língua dos povos do vale do rio Amazonas) que significa apanhador de formigas. No caso de *T. tetradactyla*, o nome é traduzido a tetra = quatro, dactyla = dedos.

Embora o número de estudos acerca da ecologia, biologia e genética dessas duas espécies seja limitado (SUPERINA; LOUGHRY, 2015), é sabido que espécies solitárias e especialistas na mirmecofagia, alimentando-se do mesmo tipo de espécies de formigas sociais e térmitas (MONTGOMERY; LUBIN, 1977).

Figura 2. Distribuição das três espécies vivas da família Myrmecophagidae. *Myrmecophaga* com uma espécie viva, o Tamanduá-bandeira (*Myrmecophaga tridactyla*) e *Tamandua*, com duas espécies vivas, o tamanduá-mirim (*Tamandua tetradactyla*) e o tamanduá do norte (*Tamandua mexicana*).

Fonte: Figura adaptada da IUCN. (Download 22 de abril de 2020).

Tamanduá-bandeira (*Myrmecophaga tridactyla*, Linnaeus, 1758)

O tamanduá-bandeira, (Figura 3) se distribui desde o Panamá até o Sul do Brasil e nordeste da Argentina (MEDRI; MOURÃO, 2005; REDFORD, 1994) (Figura 2). Atualmente o tamanduá-bandeira é considerado o mamífero mais ameaçado da América Central, já estando extinto em Belize, Guatemala e muito provavelmente na Costa Rica. Na América do Sul está extinto no Uruguai (EISENBERG; REDFORD, 1999).

Sendo a maior das quatro espécies de tamanduá existentes e podendo atingir até 39 kg (GAUDIN; MCDONALD, 2008), o tamanduá-bandeira tem gestação longa, com idade de maturidade aproximada de 4 anos (NOWAK, 1991), criando somente um filhote por ano que é alimentado e cuidado durante aproximadamente seis meses pela fêmea (Figura 3) (MONTGOMERY & LUBIN, 1977). Possui um sistema de acasalamento poligínico, no qual cada animal seleciona novos parceiros em cada época reprodutiva (DESBIEZ; BERTASSONI; TRAYLOR-HOLZER, 2020). Adicionalmente, não existem registros da evidência de características que permitam identificar indivíduos adultos e jovens morfologicamente sem contar a ausência de dimorfismo sexual aparente na espécie (MIRANDA, 2004). Os machos da espécie possuem criptorquidia[1] e externamente um saco urogenital similar ao órgão reprodutivo das fêmeas, o que torna a sexagem impossível somente através de caracteres secundários sendo unicamente possível com a manipulação dos indivíduos (DINIZ; COSTA; OLIVEIRA, 1995; POCOCK, 1924; SHAW; CARTER, 1980).

No Brasil, a espécie ocorre em todos os biomas ocupando uma grande variedade de habitats, desde florestas fechadas até campos limpos do Cerrado (BERTASSONI et al., 2017; DESBIEZ; MEDRI, 2010; DI BLANCO; PÉREZ; DI BITETTI, 2015). Os indivíduos têm hábito terrestre e a sua alimentação é constituída principalmente por formigas e cupins (MEDRI; MOURÃO, 2003; RODRIGUES et al., 2008). Possuem atividade crepuscular, evitando horários com picos muito frios ou quentes do dia, para sua movimentação (CAMILO-ALVES; MOURÃO, 2006; DESBIEZ; MEDRI, 2010), o que ajuda na termorregulação.

Figura 3. Fêmea de tamanduá-bandeira (*Myrmecophaga tridactyla*), carregando o filhote no dorso. A idade estimada do filhote é de aproximadamente seis meses.

Fonte: Lydia Möcklinghoff.

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[1] Criptorquidia: Condição médica que ocorre quando um ou os dois testículos não descem para a bolsa escrotal no momento da formação.

As principais causas da vulnerabilidade desta espécie é a deterioração, fragmentação e perda de habitat (FONSECA et al., 1999), a caça (PERES, 2000), os atropelamentos (DE FREITAS; JUSTINO; SETZ, 2014; ZANZINI et al., 2018; ZIMBRES et al., 2013) e os incêndios florestais (SILVEIRA et al., 1999). Recentemente, alguns estudos têm apontado os impactos das matrizes antrópicas na distribuição da diversidade genética da espécie, apontando a perda de diversidade genética em algumas regiões do Brasil, junto com o aumento da endogamia da espécie, assim como a estruturação populacional em algumas áreas de distribuição de tamanduá bandeira.

Garcia et al. (2005) desenvolveram os marcadores microssatélites específicos para a espécie e também analisaram 15 indivíduos de diferentes regiões, inclusive do estado de São Paulo (sete animais capturados no Parque Nacional de Emas e oito de espécimes mortos, coletados em estradas nos estados de São Paulo e Mato Grosso do Sul). Collevatti et al. (2007) estudaram populações de tamanduá-bandeira situadas no Parque Nacional das Emas, utilizando os mesmos marcadores microssatélites utilizados por Garcia et al. (2005) e concluíram que as populações exibiram níveis muito baixos de diversidade genética (média de alelos = 3; He= 0,482 e Ho= 0,059), além de um elevado grau de endogamia (Fis =0,879). Adicionalmente, um estudo realizado no sudeste brasileiro (estado de São Paulo), utilizando marcadores moleculares para identificação da diversidade e estrutura da espécie, identificou uma estrutura genética entre as populações, assim como um fluxo genético limitado entre elas. Isso, devido provavelmente as matrizes antrópicas da região (SARTORI, 2018). No entanto, a diversidade genética identificada para cada população encontrada, foi maior do que era esperado, indicando que algumas populações de espécies vulneráveis e ameaçadas de extinção são capazes de manter altos níveis de diversidade genética, mesmo em áreas intensamente fragmentadas (SARTORI, 2018).

Tamanduá-mirim (*Tamandua tetradactyla,* Linnaeus 1758)

O tamanduá-mirim (Figura 4), também conhecido como tamanduá de colete, pode alcançar até 7 kg em biomassa (NOWAK, 1991). No Brasil ocorre em todos os biomas, apresentando preferência por áreas florestais, mas sendo possível encontrá-lo forrageando em áreas abertas de Cerrado e/ou vegetação ribeirinha (FONSECA; HERRMANN; LEITE, 1999). Da mesma maneira que o tamanduá-bandeira e outras espécies de xenartros, o tamanduá-mirim não possui dimorfismo sexual aparente entre machos e fêmeas, dificultando a identificação sexual dos indivíduos em campo (RODRIGUES et al., 2008). Adicionalmente, não existem informações sobre a razão sexual de nascimentos da espécie, sistemas de acasalamento, intervalo entre nascimentos, idade de maturação ou possíveis sazonalidades reprodutivas (MIRANDA et al., 2015). Do mesmo modo que o tamanduá-bandeira, o tamanduá mirím, tem gestação longa, cerca de 190 dias, criando somente um filhote por ano, indivíduo que é carregado (Figura 4), alimentado e cuidado pela fêmea muitas vezes durante todo seu primeiro ano de vida (PINTO, 1968).

A atividade desta espécie é predominantemente em períodos noturnos, sendo os períodos que possuem as menores temperaturas do dia, passando seus momentos de descanso em ocos de árvores e tocas deixadas por outros animais (RODRIGUES; MARINHO-FILHO, 2003). Possui hábito solitário e requer grandes áreas de vida para sobreviver, tendo registros em regiões de Cerrado no Tocantins que oscilam entorno das 100 ha (TROVATI; BRITO, 2009). Essa espécie se alimenta de uma combinação variável de formigas e cupins disponíveis no chão ou nas árvores, pelo que a disponibilidade de recursos é maior as dos tamanduás bandeira.

Figura 4. Fêmea de tamanduá mirim, com filhote no dorso. Idade estimada do filhote, cinco meses.

Fonte: Onçafari.

Segundo a avaliação do risco dos xenartros brasileiros feita no ano de 2015 (ICMBIO, 2015) a espécie é considerada relativamente comum (DESBIEZ; MEDRI, 2010; HAYSSEN, 2011), dada sua ampla ocorrência no território brasileiro (MIRANDA et al., 2015). No entanto, o número de informações sob a biologia da espécie junto as poucas informações obtidas até hoje a partir de dados de cativeiro, nos levam a pensar que esta espécie é altamente subestimada e pouco estudada.

Os fogos, os atropelamentos rodoviários e a caça, por exemplo, podem ser algumas das ameaças que estejam afetando a densidade populacional da espécie, no entanto, em áreas naturais protegidas já foram descritas altas densidades desses indivíduos (AGUIAR, 2004). Atualmente a espécie é considerada pela IUCN como menos preocupante (Least Concerned – LC), porém os longos períodos de gestação descritos para a espécie, o baixo número de filhotes por ano, os longos intervalo entre partos, as baixas taxas metabólicas descritas para xenartros, a preferência de habitat dessa espécie, assim como a diversidade genética afetada pelas ameaças externas, não tem sido consideradas num panorama real para a avaliação da viabilidade populacional da espécie.

3. ÁREA DE ESTUDO

O estado de Mato Grosso do Sul (MS) possui uma área de 357.660 km^2, tendo 79 municípios e aproximadamente 2.778.998 milhões de habitantes (SCHUTZE et al., 2020). Possui uma localização geográfica privilegiada e estratégica, fazendo fronteira com a Bolívia e o Paraguai, e divisas com os estados de Paraná, São Paulo, Minas Gerais, Goiás e Mato Grosso e por tanto, sendo uma das primeiras regiões a explorar o uso do seu solo e aumentar a sua economia. O clima da região é caracterizado por ser um clima subtropical de inverno seco, com temperaturas inferiores a 18 ºC, e verão quente com temperaturas superiores a 22 ºC.

Contendo três dos cinco biomas característicos do Brasil (Cerrado, Pantanal e Mata Atlântica) o estado do MS possui maior parte de sua área coberta pelo Cerrado (“IBGE”, 2020). O Cerrado é uma savana tropical caracterizada por conter grande parte da biodiversidade do país, estações climáticas definidas e vegetação baixa e arbustiva (FERNANDES et al., 2016). O Cerrado do MS tem perdido aproximadamente 76 % da sua vegetação original. Já o Pantanal, considerado o mais protegido dos três biomas do estado, possui 86 % da sua vegetação original, compreendendo a maior zona úmida tropical do mundo. O terceiro e último bioma, Mata Atlântica, abrange quase 18 % da área do estado, sendo umas das áreas menos degradadas desde o ano de 2008 (“IBGE,” 2020)

Desde o ano de 1970, a pecuária e a agricultura são as únicas atividades econômicas do estado de MS. As grandes pastagens naturais da região e as demandas dos estados vizinhos, tem convertido quase 80 % da sua área natural total em pastagem para criação de gado (SCHUTZE et al., 2020). O incentivo ao cultivo da lavoura no estado, tem se tornado maior a começar pela soja a partir da década dos 70's, e aumentando mais recentemente, a produção de cana-de-açúcar que atravessou as fronteiras do estado de São Paulo (SP) e se apropriou de uma grande área do estado de MS, quadriplicando-se desde o ano de 2003 (SCHUTZE et al., 2020).

No estado de MS a história de implantação de medidas para conservação é bastante recente, sendo um dos últimos membros da federação a criar unidades de conservação. Atualmente, o estado possui 28 unidades de Conservação de Proteção Integral abrangendo 320.170,62 hectares que representam 0,9 % da sua superfície territorial (TORRECILHA et al., 2017). Essa criação tem ajudado no desenvolvimento de projetos de levantamento da biodiversidade dentro do estado nos seus diferentes biomas, levando a identificação de muitas espécies de plantas e animais dentro do território.

A lista de mamíferos mais recente do estado foi atualizada com base em registros primários realizados nas áreas. Cento e sessenta e seis (166) espécies foram listadas como de ocorrência no estado, sendo 47 mamíferos de médio e grande porte, 46 de pequenos mamíferos e 73 de morcegos. Segundo Myers et al. (2000) o Cerrado e a Mata Atlântica brasileira constituem dois dos 25 *hotspots* de biodiversidade do mundo, alojando 2.5 % da fauna endêmica do Brasil. A maior proporção de Cerrado e Mata Atlântica estão localizadas na região sudeste do Brasil (atualmente nos estados de São Paulo e Minas Gerais) e região centro-oeste brasileira (Mato Grosso e Mato Grosso do Sul) (IBGE, 2015). Esses estados, por tanto, são considerados foco para medidas de mitigação que ajudem a contornar os impactos e por tanto aumentem a conservação das espécies.

4. PERGUNTAS E HIPÓTESES

Com base na fundamentação teórica mostrada anteriormente, a fragmentação e a perda de habitat consequência das matrizes antrópicas pode alterar padrões genéticos e ecológicos nas populações. Em um cenário de constante alteração e aumento das atividades antrópicas, espécies com comportamentos mais plásticos e generalistas, com capacidade de deslocamento grande, mesmo em ambientes alterados, podem ter mais vantagens sobre outras com comportamentos mais especialistas e restritos. No entanto, mesmo que essas espécies possam ser menos afetadas do que outras, tais alterações podem causar viés nos padrões de dispersão e deslocamento, interferindo no fluxo genético das mesmas e aumentando o impacto das pressões habituais (endogamia, deriva genética e seleção natural). Por tanto, a necessidade de entender o impacto dos atropelamentos na diversidade das espécies, assim como levantar as possíveis variáveis espaço-temporais que explicam os padrões de atropelamento das espécies, se torna uma alternativa plausível nas medidas de conservação. O nosso estudo surgiu a partir de várias perguntas que foram organizadas em capítulos. As perguntas e hipóteses serão mostradas por capítulo.

Capítulo I

Perguntas: Os genes nucleares comumente utilizados em mamíferos para identificação do sexo possuem o mesmo desempenho de identificação a partir de carcaças de três espécies de xenartros? Existe polimorfismo de única base entre as regiões homologas do gene *Zinc finger* (*Zfx* e *Zfy*) dentro e entre as espécies de xenartros?

Capítulo II.

Perguntas: (i) Qual espécie é mais atropelada? (ii) Existe um viés de atropelamento para um sexo, em cada espécie estudada? (iii) As taxas de atropelamentos são influenciadas por variáveis climáticas e/ou (iv) variam sazonalmente? (v) Existem pontos quentes de atropelamento, ou seja, os atropelamentos não acontecem de forma aleatória? (vi) Se existir, os pontos quentes (*hotspots*) estão relacionados a características ambientais diferenciadas que eles possuem?

Hipóteses: (i) Espécies de hábitos mais generalistas quanto ao uso da paisagem e com maior capacidade de dispersão, serão mais atropeladas em todas as áreas; enquanto espécies com hábitos mais restritos e menor capacidade de dispersão serão menos atropeladas; (ii) A proporção de machos e fêmeas atropeladas será a mesma, devido a que adoptamos para ambas a espécies uma proporção de nascimentos de 1:1; (iii) Espécies com taxas metabólicas baixas nas quais os padrões de movimentação são relacionados a temperatura (iv) A mortalidade é diferente entre as estações, devido ao possível efeito da sazonalidade nas espécies, relacionado ao momento de maior movimentação no período reprodutivo ou à disponibilidade de recursos; (v) Os atropelamentos não acontecem aleatoriamente, especialmente perto de fragmentos com vegetação e agua, e longe de áreas urbanas e tráfego intenso; (vi) Os atropelamentos estão positivamente relacionados com a quantidade de vegetação e agua, assim como da intensidade do tráfego e negativamente relacionados com o grau de fragmentação e áreas urbanas

Capítulo III

Perguntas: (i) Indivíduos geograficamente mais próximos possuem diferenças genéticas menores que indivíduos geograficamente mais afastados? Existe um viés sexual de dispersão na espécie? (ii) populações de *Myrmecophaga tridactyla* possui baixa diversidade genética e redução do fluxo genético? (iii) A redução e fragmentação de habitat pode ter causa uma mudança no tamanho efetivo populacional da espécie no passado?

Hipóteses: (i) Considerando os estudos que tem relatado filopatria para as fêmeas da espécie, esperamos encontrar uma distância genética menor entre fêmeas da espécie, quando comparada com as dos machos da espécie, indicando um viés de dispersão para machos; (ii) populações de tamanduás que habitam um grande polígono na região centro-oeste do Brasil mostrariam perda de diversidade genética e sinais de redução do fluxo gênico entre as populações locais. Além disso, colocamos a hipótese de que (iii) há uma redução no tamanho efetivo da população de tamanduás devido à alta perda de habitat natural.

5. OBJETIVOS

5.1 Objetivo geral

Identificar as consequências das matrizes antropizadas nos atropelamentos das espécies *Myrmecophaga tridactyla* e *Tamandua tetradactyla*. Além disso, utilizando como modelo o *M. tridactyla*, identificar a distribuição da sua diversidade genética em uma paisagem antropizada.

5.2 Objetivos específicos

5.2.1 Identificar polimorfismo molecular entre os sexos e desenvolver um método para a identificação molecular do sexo em xenartros.

5.2.2 Avaliar o padrão temporal e espacial dos atropelamentos em *M. tridactyla* e *T. tetradactyla*.

5.2.3 Identificar quais variáveis temporais e espaciais explicam os locais de maior taxa de atropelamento (*hotspots*) para as espécies.

5.2.4 Determinar locais prioritários para planos de mitigação de *M. tridactyla* e *T. tetradactyla*.

5.2.5 Avaliar parâmetros genético populacionais para espécie ameaçada *M. tridactyla*.

5.2.6 Identificar quais processos demográficos históricos respondem a variação genética atual de *M. tridactyla*.

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Capítulo I

Molecular sexing of Xenarthra: A tool for genetic and ecological studies

(Artigo em revisão na revista Conservation Genetic Resource)

Molecular sexing of Xenarthra: A tool for genetic and ecological studies

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Abstract

Although sex assignment is essential to study biology and ecology of a given animal, in Xenarthra there is still no standardized assay for gender genetic identification. Here, we evaluate the potential of two nuclear fragments [SRY (~180 bp) and Zinc finger (~400 bp) genes] for sex identification of specimens that have lost their morphological characteristics because of road-kills. DNA samples from seven Xenarthra species were amplified and sequenced for one or both segments. Finally, we performed a case study using tissue samples from road-kill carcasses, which supported the suitability of our markers for poor-quality DNA. The method proved to be efficient for different types of samples and may be especially useful for studies using road-killed and hunted animals.

Keywords: Cingulata, Pilosa, Sex identification, Road Ecology.

Introduction

Molecular sexing has become a useful tool with the advent of noninvasive DNA analysis (CHAVES et al., 2012; PRITHIVIRAJ; MELNICK, 2001; SARANHOLI; CHÁVEZ-CONGRAINS; GALETTI, 2017; SHAW; WILSON; WHITE, 2003), either for conservation (GRIFFITHS; TIWARI, 1993) or forensic (KOBAYASHI et al., 1988) purposes. In parallel, roads have become an important threat for terrestrial fauna, especially medium to large sized mammals (FORMAN; ALEXANDER, 1998), and molecular tools are often the only way for sex identification of road-kills (MIOTTO et al., 2012; SARANHOLI; CHÁVEZ-CONGRAINS; GALETTI, 2017). Short nuclear DNA sequences exclusively present in the Y or in both X and Y chromosomes have been used for molecular sex identification in mammals (COTTS et al., 2019; MARTINELLI et al., 2010; MURATA; MASUDA, 1996; PRITHIVIRAJ; MELNICK, 2001; TAKAMI et al., 1998). Although species of Xenarthra are a quite frequent taxonomic group among road-kills (BERALDI et al., 2019; FERREGUETTI et al., 2020; ZIMBRES et al., 2013) there is still no standardized molecular assay for sex identification of the superorder.

In mammals, the SRY gene is present exclusively on the Y chromosome (DI FIORE, 2005; TAKAMI et al., 1998) and, therefore, its PCR amplification has been widely used for the identification of males (MURATA; MASUDA, 1996; STATHAM; TURNER; REILLY, 2007). It is expected that females will not show PCR product for this gene. However, the absence of amplification is not always indicative of females, as false negatives can occur (JOSHI; DE; GOYAL, 2019; PAGÈS et al., 2009). To prevent an error based on false negative, often other mitochondrial or nuclear genes are co-amplified with the SRY sequence (KAMIMURA et al., 1997; PALSBØLL et al., 1992), as for instance a portion of the Zinc finger (*Zf*) gene, existing in both X and Y chromosomes (PAGE et al., 1987). Males can be identified either by a small deletion in the Y chromosome, which includes a region encoding Zinc finger domains (Page et al. 1987), or by single nucleotide polymorphisms. This Zinc finger protein gene is thought to be present in all eutherian mammals and in most cases this region shows single nucleotide polymorphisms between the X and Y chromosomes, characterizing the *Zfx* and *Zfy* genes, respectively (WILLIAMS; BRECK; BAKER, 2004). In *Bradypus*, for instance, a polymorphism between the *Zfx* and *Zfy* sequences allowed the use of Restriction Fragment Length Polymorphism (PCR-RFLP) for sex identification (MARTINELLI et al., 2010).

In the present, the main goal was to investigate the performance of the PCR amplification of both SRY and Zinc finger genes for molecular sex identification of road-kill carcasses of three neotropical Xenarthra species. Additionally, we explored the presence of polymorphic sites between the *Zfx* and *Zfy* among seven Xenarthra species.

Material and methods

A total of 601 tissue samples of road-killed individuals belonging to three different Xenarthra species (271 giant anteaters, *Myrmecophaga tridactyla*; 220 southern anteaters, *Tamandua tetradactyla* and 110 yellow armadillos, *Euphractus sexcinctus*) were analyzed. These samples were collected during road-kill surveys in four Brazilian roads, carried out by the Anteaters and Highways project (www.giantanteater.org), from April 2013 to March 2014, and February 2017 to January 2018. The gender of some individuals was identified by animal necropsy and was used as control to evaluate the success of the molecular sexing methodology.

Tissue samples were conserved in absolute ethyl alcohol and stored in a freezer at -20°C. Total DNA was extracted using the phenol-chloroform method (SAMBROOK; FRITSCH;

MANIATIS, 1989). The DNA concentration was measured using NanoVue Plus (Biochrom) and all DNA samples were standardized to 30 ng/µl. The DNA quality was also checked by electrophoresis in 1% agarose gel stained with Gel Red ™ (Biotium, Hayward, CA, USA).

We identified robust primers in the literature based on their success in sexing across different mammal groups (BENTO et al., 2019; MARTINELLI et al., 2010; TAKAMI et al., 1998). We used a multiplex polymerase chain reaction (multiplex PCR) to amplify simultaneously the sex-determining region Y (SRY) using the primer pair described in Fain and LeMay (1995), and the Zinc finger gene using a universal primer pair (P1-5EZ and P2-3EZ) reported by Aasen and Medrano (1990). This latter gene has been widely used as PCR control (DECANDIA et al., 2016; FISCHBACH et al., 2008) since it shows homology between the mammalian X (*Zfx*) and Y (*Zfy*) chromosomes, providing amplification for both sexes. PCR reactions for all samples were performed using 1x *Taq* buffer, 1 mM $MgCl_2$, 0.25 mM of each dNTPs, 0.5 uM of each primer, 0.5 unit (U) *Taq* Polymerase (Invitrogen Platinum *Taq* DNA Polymerase, Carlsbad, CA, USA), 0.5 uM of BSA and 30 ng genomic DNA to a 12µl final volume. The amplification program consisted of an initial denaturation at 94° C for 3 min; 35 cycles of denaturation at 94° C for 45 s, annealing at 60° C for 45 s, and extension at 72° C for 1 min, and an additional final extension at 72° C for 10 min. Resulting products were visualized in a 3% agarose gel stained with GelRed ™ and visualized on an ultraviolet transilluminator. Product sizes were determined by using the 1-Kb Plus DNA molecular weight ladder (Invitrogen). The number of bands expected by gender was only one band corresponding to *Zfx*/*Zfx* for females, and a total of three bands, two of them when *Zfx* and *Zfy* show different sizes, and a third one corresponding to SRY for males. Males can only show two bands, corresponding to the Zinc finger and SRY genes, when *Zfx* and *Zfy* have the same size. We included negative and positive controls in all independent PCRs. For increasing confidence in the results, each sample was twice independently amplified when identified as a male and three times when sexed as female. The flowchart of PCR reactions is shown in Fig1.

Tissue samples (muscle, ear, hair, and skin) of other three Xenarthra species, nine-banded armadillo (*Dasypus novencinctus*), giant armadillo (*Priodontes maximus*), and southern naked-tailed armadillo (*Cabassous unicinctus*), previously collected by the Anteaters and Highways project, and brown-throated sloth (*Bradypus variegatus*) collected in the Rio Largo municipality (AL) were added to verify polymorphisms between *Zfx* and *Zfy*. One to four specimens (males and females) of each species were analyzed. All tissue samples (Table S1) were deposited in the biological sample collection of Laboratório de Biodiversidade Molecular e Conservação, Departamento de Genética e Evolução/UFSCar (SISGEN CED2669).

The morphological sex of all individuals analyzed was previously known to confirm the molecular sex identification obtained. Each obtained sequence was compared to the of the GenBank database using the Basic Local Alignment Search Tool (BLAST) (ALTSCHUL et al., 1990) to confirm the fidelity of the primers.

Using the "find the heterozygotes" plugin implemented in the Geneious software (KEARSE et al., 2012), we identify double peaks with 70% of peak similarity in the electropherograms of each sequence analyzed. The *Zfx* and *Zfy* sequences obtained with heterozygote sites were deposited in GenBank, and those sites are identified as degenerated bases (Table S2). The restriction sites on the X or Y fragment, and the enzymes which can only cut one of them, were selected for each species using the "find restriction sites" in the Geneious software (Kearse et al. 2012).

The biological sampling authorization was obtained through Biodiversity Authorization and Information System – SISBIO, number 53798-4. The research was approved by Ethic Committee on the Use of Animals, Universidade Federal de São Carlos (CEUA/UFSCar) according to protocol 1584280817. The genetic resource access was registered under SisGen A9F8717.

Results

Both SRY and Zinc finger genes were successfully amplified in multiplex PCR of *M. tridactyla*, *T. tetradactyla* and *E. sexcinctus*, and produced fragments from ~180 bp and ~400 bp, respectively. In addition, the amplification of the Zinc finger gene for all species (totaling seven Xenarthra species) showed no fragment length differences between *Zfx* and *Zfy* and produced a single band (~400 bp) in both sexes when observed in agarose gel.

From a total of 601 road-kill tissue samples tested, we were able to identify the gender of 522 individuals of the three focused species, with a success rate of about 87 % (Table 1). On the other hand, the PCR amplification of the Zinc finger gene was effective in all seven Xenarthra species evaluated and produced fragments between 360 bp to 400 bp. The *Zfx* and *Zfy* fragments were determined and compared within and between species (Table S2). Double peaks observed only in the electropherograms of males of most species, enable us to recognize the *Zfy* sequence by the heterozygote sites. Subsequently, we identify exclusive *Zfy* restriction sites for *Bradypus variegatus* and *Cabassous unicinctus* and exclusive *Zfx* restriction sites for *Tamandua tetradactyla* and *Dasypus novemcinctus* (Table 2). No polymorphism between the *Zfx* and *Zfy* fragments were notably observed in *M. tridactyla, P. maximus* and *E. sexcinctus*.

Discussion

Our results showed that multiplex PCR amplification with Zinc finger and SRY genes using DNA from road-kill carcasses can successfully be used for sex identification of giant anteater, southern anteater and yellow armadillo (Table 1). The success in molecular sexing using both Zinc finger and SRY genes was expected since the primers tested here was also successfully used in molecular sexing of other Xenarthra species, such as *Bradypus torquatus* and *Choloepus didactylus* (Murata et al., 1996; Martinelli et al., 2010) and other phylogenetically distant mammals, such as whales and wolves (Rutledge et al., 2010; Wade et al., 2011). We were also able to carry out the sex identification of biological samples with poor quality such as road-kill carcasses, representing a wide number of genera within Xenarthra.

Most road-kill samples collected here provided low DNA quantity due to prolonged exposure to environmental conditions, resulting in a poor quality of the tissue samples and degradation of the DNA extracted. This fact reinforces the applicability of this method which was able to identify the individual sex for most of the carcasses analyzed.

The PCR amplification of the Zinc finger gene region showed four of the seven studied species exhibiting nucleotide polymorphisms between *Zfx* and *Zfy*. Males of these four species were readily distinguishable from females, suggesting that PCR-RFLP method can be useful for molecular sex identification of these species (Table 2), as previously reported for other species (DALLAS et al., 2000; MARTINELLI et al., 2010). In contrast, the absence of polymorphisms in the amplified region of the Zinc finger gene in *M. tridactyla*, *P. maximus* and *E. sexcinctus* species indicates high intra-species conservation of these fragments, impairing the use of restriction enzymes and PCR-RFLP in these species. Thus, although the Zinc finger gene amplification could result in an easy and quick way for identification of restriction sites useful for PCR-RFLP for discriminating the *Zfx* and *Zfy* homologous fragments, this is not a common feature among Xenarthra. Only using the multiplex PCR described here we were able to carry out the sex identification in *M. tridactyla*, *P. maximus* and *E. sexcinctus*, where the SRY was used as a male marker and Zinc finger as a positive control.

In summary, the approach used here was able to accomplish the sex identification of biological samples of poor quality often resulting in degraded DNA, as road-kill carcasses, using a wide number of genera within Xenarthra. In the absence of polymorphism between *Zfx* and *Zfy*, the use of multiplex PCR was able to accomplish the sex identification in *M. tridactyla*, *P. maximus* and *E. sexcinctus*.

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Data Accessibility

DNA sequences: Genbank accessions MT298925- MT298949.

Author Contributions

- Designed research and data acquisition: CBR, EPF, KGRC, PMGJ.
- Contributed new reagents or analytical tools: CBR, EPF, KGRC, PMGJ.
- Analyzed data: CBR, EPF, KGRC, ALLJD, PMGJ.
- Wrote the paper: CBR, EPF, KGRC, ALLJD, PMGJ

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Tables and Figures

Table 1. Total number of carcasses with molecular sex identification, using SRY and Zinc finger genes in three Xenarthra species

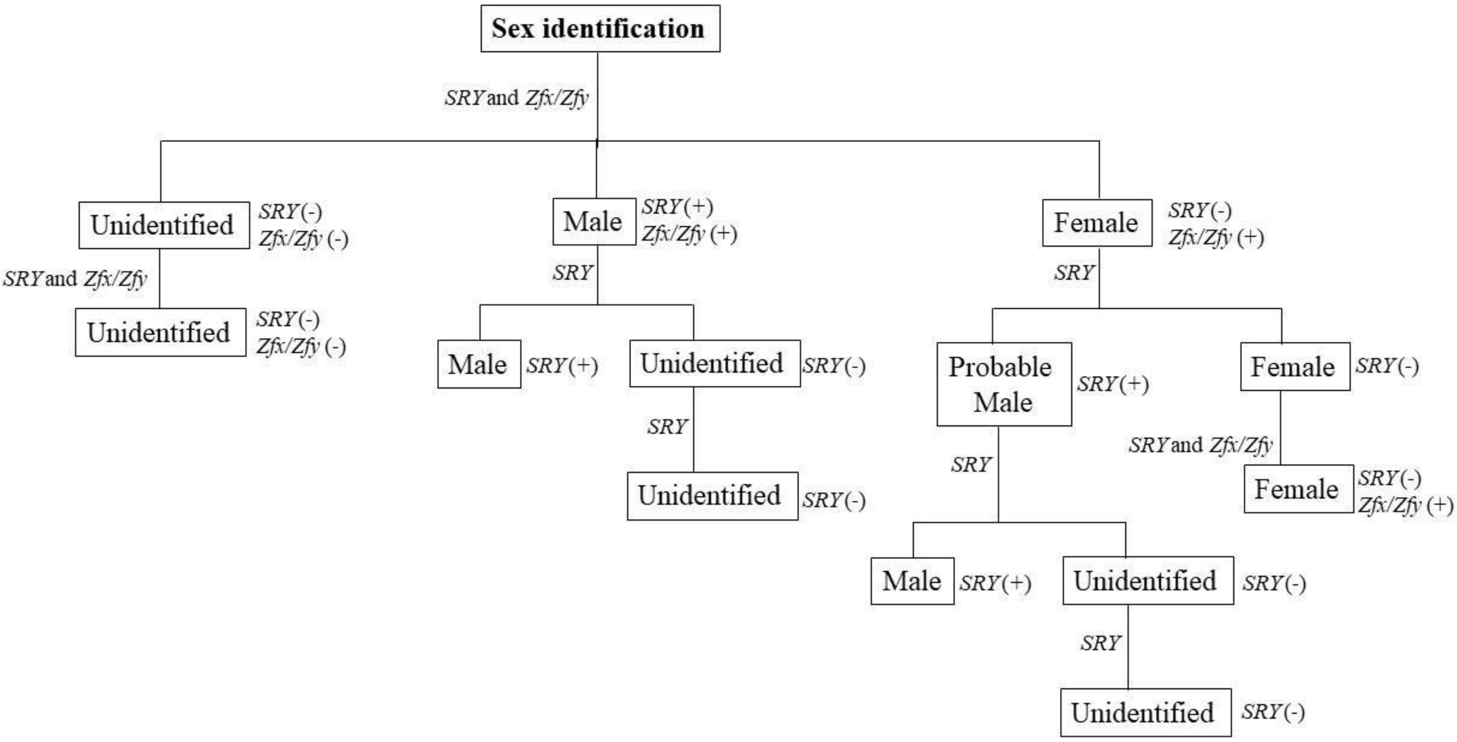
	Male	Female	Unsuccess (%)	Total
M. tridactyla	151	82	38 (14%)	271
T. tetradactyla	128	51	41(18%)	220
E. sexcinctus	62	36	12 (11%)	110

Table 2. Restriction enzymes selected for digestion of Zinc finger region which can only cut one of alleles (*Zfx* or *Zfy*) for molecular sex identification. Restriction positions are numbered with reference to the 1st base of the sequenced fragment.

	Restriction enzyme	Position of restriction site
Bradypus variegatus	BccI	210
*Tamandua tetradactyla**	Hpall,Mspl	237
*Dasypus novemcinctus**	Eco57I, Acul	182
Cabassous unicinctus	Psti	283

*Restriction enzymes for restriction digestion of *Zfx*

Figure 1. Flowchart showing the PCR reactions for molecular sex identification from road-kill carcasses using two nuclear gene markers (Zinc finger (*Zfx* and *Zfy*) and SRY).



Supplementary Material

Table S1. GenBank accession numbers and information of Xenarthra species used for amplification of Zinc finger gene. The gender of each specimen was identified in field.

Accession	ID	Organism	Gender	Country	Lat	Long
MT298925	Seq1	*Bradypus variegatus*	Female	Brazil	-35.914455	-9.5165920
MT298926	Seq2	*Bradypus variegatus*	Male	Brazil	-35.984002	-9.5517561
MT298927	Seq3	*Cabassous unicinctus*	Female	Brazil	-20.324254	-56.208493
MT298928	Seq4	*Cabassous unicinctus*	Female	Brazil	-19.282618	-55.787776
MT298929	Seq5	*Cabassous unicinctus*	Male	Brazil	-20.463976	-54.050209
MT298930	Seq6	*Cabassous unicinctus*	Male	Brazil	-20.456210	-53.008825
MT298931	Seq7	*Dasypus novencinctus*	Female	Brazil	-20.562642	-54.657353
MT298932	Seq8	*Dasypus novencinctus*	Male	Brazil	-20.447393	-55.147727
MT298933	Seq9	*Dasypus novencinctus*	Male	Brazil	-21.650470	-53.602570
MT298934	Seq10	*Euphractus sexcinctus*	Female	Brazil	-20.471961	-54.091716
MT298935	Seq11	*Euphractus sexcinctus*	Female	Brazil	-20.828762	-54.155526
MT298936	Seq12	*Euphractus sexcinctus*	Male	Brazil	-20.405610	-53.930900
MT298937	Seq13	*Euphractus sexcinctus*	Male	Brazil	-19.253568	-55.832900
MT298938	Seq14	*Myrmecophaga tridactyla*	Female	Brazil	-20.460920	-54.032650
MT298939	Seq15	*Myrmecophaga tridactyla*	Female	Brazil	-20.503091	-55.870670
MT298940	Seq16	*Myrmecophaga tridactyla*	Male	Brazil	-20.573045	-52.478154
MT298941	Seq17	*Myrmecophaga tridactyla*	Male	Brazil	-20.954378	-54.109211
MT298942	Seq18	*Priodontes maximus*	Female	Brazil	-20.047146	-56.784851
MT298943	Seq19	*Priodontes maximus*	Female	Brazil	-19.205192	-55.777188
MT298944	Seq20	*Priodontes maximus*	Male	Brazil	-21.386502	-52.764282
MT298945	Seq21	*Priodontes maximus*	Male	Brazil	-20.425416	-54.613937
MT298946	Seq22	*Tamandua tetradactyla*	Female	Brazil	-21.011316	-54.011687
MT298947	Seq23	*Tamandua tetradactyla*	Female	Brazil	-20.518406	-52.613682
MT298948	Seq24	*Tamandua tetradactyla*	Male	Brazil	-20.878590	-54.140460
MT298949	Seq25	*Tamandua tetradactyla*	Male	Brazil	-20.502575	-55.871285

Table S2. *Zfx* and *Zfy* sequences (*Zfx/Zfx* for female and *Zfx/Zfy* for male) based on the Zinc finger gene of the studied species. The degenerated bases highlight the polymorphic sites where there are differences between the *Zfx* and the *Zfy* fragment.

```
         10        20        30        40
     ....|....|....|....|....|....|....|....|
MT298925  TCTCTCATGCGGGGGCTTTGTTTACTCACAAAATGGTGCA
MT298926  ............................M...........
MT298927  ..........T.................T...........
MT298928  ..........T.................T...........
MT298929  ..........T.................Y...........
MT298930  ..........T.................Y...........
MT298931  ..........T.............................
```

```
MT298932  ..........T.............................
MT298933  ..........T.............................
MT298934  ..........T.............................
MT298935  ..........T.............................
MT298936  ..........T.............................
MT298937  ..........T.............................
MT298938  ..........T..............C..............
MT298939  ..........T..............C..............
MT298940  ..........T..............Y..............
MT298941  ..........T..............C..............
MT298942  ..........T.............................
MT298943  ..........T.............................
MT298944  ..........T.............................
MT298945  ..........T.............................
MT298946  ..........T..............C..............
MT298947  ..........T..............C..............
MT298948  .......C.TA........A....................
MT298949  .......C.TA........A....................

         50        60        70        80
     ....|....|....|....|....|....|....|....|
MT298925  TAAGGAAAAAGGAGCCAACAAAATGCACAAGTGTAAATTC
MT298926  Y.......................................
MT298927  .........G..............................
MT298928  .........G..............................
MT298929  .........G..............................
MT298930  .........G..............................
MT298931  .........G..............................
MT298932  .........G........Y......Y..............
MT298933  .........G........Y......Y..............
MT298934  .........G..............................
MT298935  .........G..............................
MT298936  .........G..............................
MT298937  .........G..............................
MT298938  ........................................
MT298939  ........................................
MT298940  ........................................
MT298941  ........................................
MT298942  .........G..............................
MT298943  .........G..............................
MT298944  .........G..............................
MT298945  .........G..............................
MT298946  ........................................
MT298947  ........................................
MT298948  ............CA..........................
MT298949  ............CA..........................

         90       100       110       120
     ....|....|....|....|....|....|....|....|
MT298925  TGTGAATATGAGACTGCTGAACAAGGGTTACTGAATCGTC
```

```
MT298926  ..............Y...............T.........
MT298927  ..............C...........T..GT.......C.
MT298928  ..............C...........T..GT.......C.
MT298929  ..............C...........T..GT.......C.
MT298930  ..............C..Y........T..GT.......C.
MT298931  ..............C...........A..GT.......C.
MT298932  ..............C...........AY.RT.......C.
MT298933  ..............C...........AY.RT.......C.
MT298934  ..........................A..GT.......C.
MT298935  ..........................A..GT.......C.
MT298936  ..........................A..GT.......C.
MT298937  ..............C...........A..GT.......C.
MT298938  ........C.....................T.........
MT298939  ........C.....................T.........
MT298940  ........C.................R...T.......Y.
MT298941  ........C.....................T.......Y.
MT298942  ..............C...........A..GT.......C.
MT298943  ..............C...........A..GT.......C.
MT298944  ..............C...........A..GT.......C.
MT298945  ..............C...........A..GT.......C.
MT298946  ........C.....................T.........
MT298947  ........C.....................T.........
MT298948  ........C.....A..C........A..GT.......C.
MT298949  ........C.....A..C........A..GT.......C.

        130       140       150       160
     ....|....|....|....|....|....|....|....|
MT298925  ACCTTTTGGCAGTCCACAGCAAGAACTTTCCTCATATTTG
MT298926  ........................................
MT298927  .......A..G.............................
MT298928  .......A..G.............................
MT298929  .......A..G..Y..........................
MT298930  .......A..G..Y..........................
MT298931  ..........G.............................
MT298932  ..........T.............................
MT298933  ..........T.............................
MT298934  ..........G.............................
MT298935  ..........G.............................
MT298936  ..........G.............................
MT298937  ..........G.............................
MT298938  ........................................
MT298939  ........................................
MT298940  ........................................
MT298941  ........................................
MT298942  .......A..G.............................
MT298943  .......A..G.............................
MT298944  .......M..G.............................
MT298945  .......M..G.............................
MT298946  ........................................
MT298947  ........................................
```

```
MT298948  .T................................C.....
MT298949  .T................................C.....

         170       180       190       200
     ....|....|....|....|....|....|....|....|
MT298925  TGTGGAGTGCGGTAAAGGTTTTCGTCATCCGTCAGAGCTC
MT298926  ....................G.........A.........
MT298927  ..............................A..G......
MT298928  ..............................A..G......
MT298929  ..............................A..G......
MT298930  ..............................A..G......
MT298931  ..............................A.........
MT298932  ..............................A.........
MT298933  ..............................A.........
MT298934  ..............................C.........
MT298935  ..............................C.........
MT298936  ..............................C.........
MT298937  .........S....................C.........
MT298938  ......A..T..C.................T.....A...
MT298939  ......A..T..C.................T.....A...
MT298940  ......R..T..C.................T.....A...
MT298941  ......A..T..C.................T.....A...
MT298942  ..............................A..G......
MT298943  ..............................A..G......
MT298944  ..............................A..G......
MT298945  ..............................A..G......
MT298946  ......A..T..C.................T.....A...
MT298947  ......A..T..C.................T.....A...
MT298948  .........T....................C........T
MT298949  .........T....................C........T

         210       220       230       240
     ....|....|....|....|....|....|....|....|
MT298925  AAAAAGCACATGCGAATCCACACTGGGGAGAAGCCGTACC
MT298926  .......................Y................
MT298927  ....................T...................
MT298928  ....................T...................
MT298929  ....................T..Y........K..R....
MT298930  ....................T..Y........K..R....
MT298931  ....................T...................
MT298932  ....................T...................
MT298933  ....................T...................
MT298934  ....................T...................
MT298935  ....................T...................
MT298936  ....................T...................
MT298937  ....................T...................
MT298938  .......................C.....A..........
MT298939  .......................C.....A..........
MT298940  .......................C.....A..........
MT298941  .......................C.....A..........
```

```
MT298942  ..............T.....T..C................
MT298943  ..............T.....T..C................
MT298944  ..............W.....T..C................
MT298945  ..............W.....T..C................
MT298946  .......................C.....A..........
MT298947  .......................C.....A..........
MT298948  ........................................
MT298949  ........................................

             250       260       270       280
     ....|....|....|....|....|....|....|....|
MT298925  AGTGCCAGTACTGCGAATATAGATCTGCAGACTCCTCTAA
MT298926  .........................C..............
MT298927  .A....................G..C..............
MT298928  .A....................G.................
MT298929  .A....................G..Y..............
MT298930  .A....................G..Y..............
MT298931  ......................G.................
MT298932  .R....................G..C..............
MT298933  .R....................G..C..............
MT298934  .A....................G..C..............
MT298935  .A....................G..C..............
MT298936  .A....................G..C..............
MT298937  .A....................G..C..............
MT298938  .A.......................C..............
MT298939  .A.......................C..............
MT298940  .A.......................C..............
MT298941  .A.......................C..............
MT298942  .A....................G..C..............
MT298943  .A....................G..C..............
MT298944  .A....................G..C..............
MT298945  .A....................G..C..............
MT298946  .A.......................C..............
MT298947  .A.......................C..............
MT298948  .........................C.....T........
MT298949  .........................C.....T........

             290       300       310       320
     ....|....|....|....|....|....|....|....|
MT298925  CTTGAAAACTCATGTAAAAACTAAGCATAGTAAAGAGATG
MT298926  ........................................
MT298927  .........C..............................
MT298928  .........Y..............................
MT298929  .........Y..............................
MT298930  .........Y..............R...............
MT298931  .........C..............................
MT298932  .........C..............S...............
MT298933  .........C..............................
MT298934  .........C....................C.........
MT298935  .........C....................C.........
```

```
MT298936  .........C....................C.........
MT298937  .........C....................C.........
MT298938  ............C...........................
MT298939  ............C...........................
MT298940  ............Y...........................
MT298941  ............C...........................
MT298942  .........C..............................
MT298943  .........C..............................
MT298944  .........C..............................
MT298945  .........C..............................
MT298946  ............C.......................A...
MT298947  ............C.......................A...
MT298948  ...A.....G...A.........................A
MT298949  ...A.....G...A.........................A

             330       340       350
       ....|....|....|....|....|....|....|
   MT298925  CCATTCAAGTGTGACATTTGTCTTCTGACTTTC
  MT298926  .................................CT
  MT298927  ........A........................CT
  MT298928  Y.......R........................CT
  MT298929  K.......R........................CT
  MT298930  Y.......R........................CT
  MT298931  ........A........................CT
  MT298932  T................................CT
  MT298933  Y.......A........................CT
  MT298934  .................................CT
  MT298935  .................................CT
  MT298936  .................................CT
  MT298937  .................................CT
  MT298938  .................................CT
  MT298939  .................................CT
  MT298940  .................................CT
  MT298941  .................................CT
  MT298942  ........A........................CT
  MT298943  ........A........................CT
  MT298944  ........A........................CT
  MT298945  ........A........................CT
  MT298946  .................................CT
  MT298947  .................................CT
  MT298948  .................................CT
  MT298949  .................................CT
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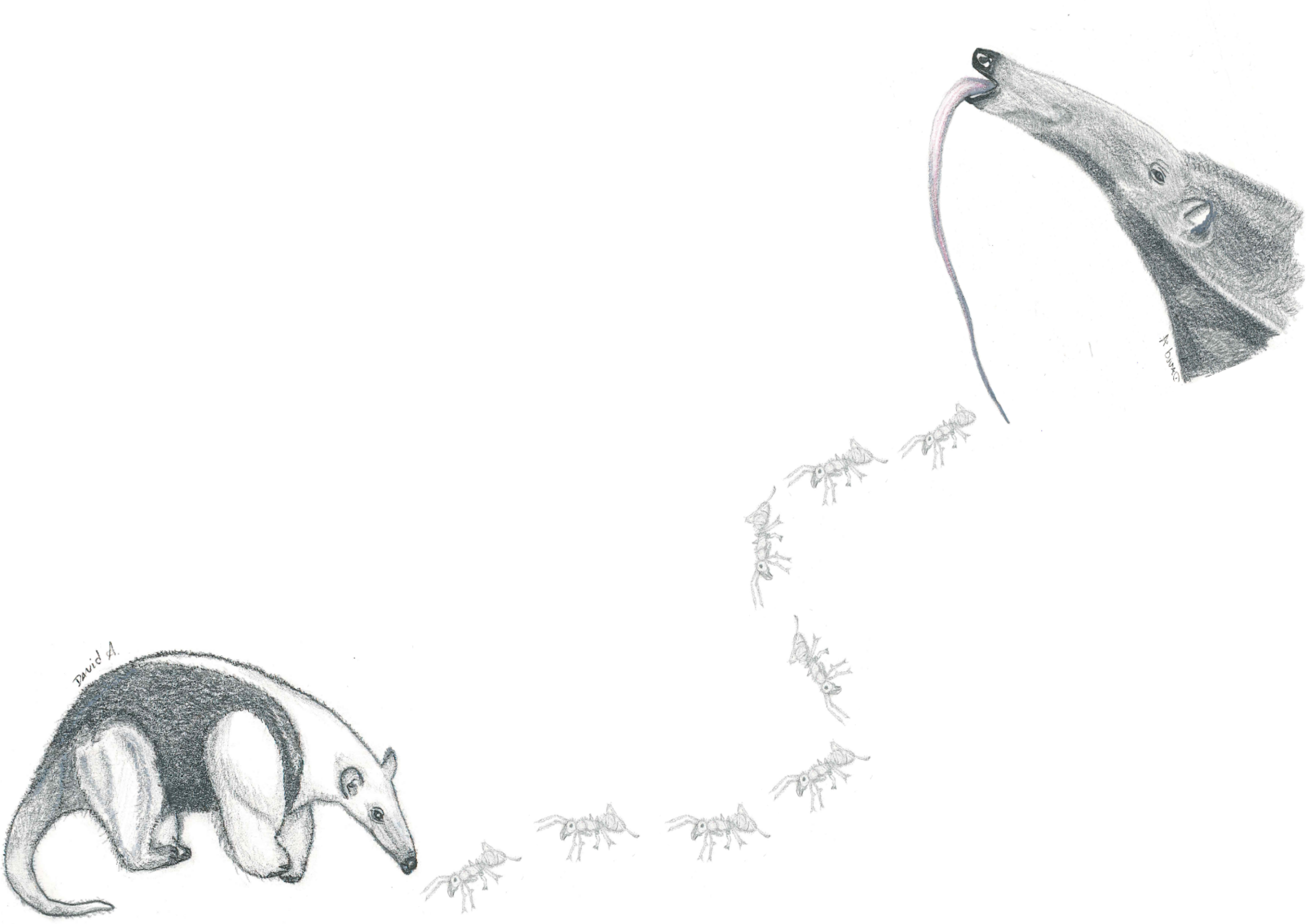
Capítulo II

How do Temporal and Spatial Features Affect Anteater Roadkill in Brazil?

(Artigo a ser submetido para o Journal of Environmental Management)



How do Temporal and Spatial Features Affect Anteater Roadkill in Brazil?

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Abstract

Movement, dispersion, ecology and animal behavior are possible determining factors for the mortality rate of species on roads. Understanding the magnitude of roadkill and the possible features that affect the specie mortality via vehicular collision are essential to propose conservation measures. Medium-large mammals are highly vulnerable to collision given their general high mobility, low reproductive rates, specialist diets and low population densities. Among them, the superorder Xenarthra are in the top 10 of species most affected by the roadkill collision in Brazil. However, there is no research containing information about the influence of temporal and spatial features on Xenartha’s mortality rate. Therefore, we evaluated the patterns of roadkill of two species, the giant anteater (*Myrmecophaga* *tridactyla*) and the southern tamandua (*Tamandua tetradactyla*) across a temporal and spatial gradient in the Brazilian Center-Western region. The surveys were conducted along four different road transects (1,259 km) between April 2017 and March 2018. A total of 303 individuals (1.2 individuals/100 km/year) were recorded, including 174 giant anteater and 129 southern tamanduas. We tested whether roadkills vary seasonally, and additionally, we identified the hotspots for each species across each highway using the modified 2D-Ripley K test and the 2D-Hotspot identification analysis. We used regression analyses and generalized linear models to test the influence of temporal (e.g. temperature and humidity), and spatial (e.g. forest coverage %) features on roadkill rates, respectively. Males of the southern tamandua were killed at a proportion of 3:1, while the roadkill rate for male giant anteaters was 1.5:1, revealing an equal tendency in the roadkills' genders, if we consider the natural sexual ratio is 1:1 for both species. No influence of temperature and humidity were registered in any evaluated roadkill pattern. However, the female roadkill of both species peaked in the rainy season which can reveal the influences of seasonal factors on female's movement, contributing to an increased collision rate. Males were killed on roads at similar rates throughout the year. We found in total ten roadkill hotspots for both species on all roads. In general, the numbers of giant anteater roadkill were negatively related to traffic and the proportion of vegetation and positively related to density of fragment, with these variables contributing together to the roadkill patterns. Thus, the spatial aggregation of roadkills is explained by both proportion of vegetation and traffic characteristics of roads, which may influence the anteaters' behavior of crossing this barrier, and landscape structures around roads, which seems to affect their movement patterns. This study reinforces the value of using specific traits to analyze roadkill rates and the need for the integration of areas to provide efficient mitigation measures.

Keywords: Mammals, Xenarthra, Seasonality, Conservation, Road Ecology, traffic, landscape structure

Introduction

Mortality of wild animals due to roads is one of the most important threats to biodiversity in the world (ASSIS; GIACOMINI; RIBEIRO, 2019; BERALDI et al., 2019; GRILO; BISSONETTE; SANTOS-REIS, 2009). Although considered connected to the development of human action, roads cause landscape fragmentation and habitat loss, which, summed with the roadkill of animals, may result in a loss of biodiversity (JACKSON; FAHRIG, 2011; JOCHIMSEN, 2006). Billions of roadkill records in the world are registered every year (BISHOP; BROGAN, 2013; ERICKSON; JOHNSON; JR, 2005; GRILO et al., 2020; SEILER; HELLDIN; SEILER, 2004), affecting not only individuals that survive, but also population plasticity and persistence.

Mortality due to roads can affect the dynamic of populations by the reduction of the size of natural populations (DUFFETT et al., 2020; FORMAN; ALEXANDER, 1998; PINTO et al., 2018). Additionally, the populations can be divided by this anthropogenic barrier. The combined effect of reductions in population size and changes of individuals' flux dynamics may lead to the loss of genetic diversity and local extirpation (Reed and Frankham, 2003). Therefore, local extinctions may be possible when the loss of individuals affects the immigration and/or exceeds the number of individuals born in the population (FORMAN; ALEXANDER, 1998).

Generally, roadkill does not happen randomly, but at certain points on roads and during some periods of the year, with certain seasonality in some species (ASCENSÃO et al., 2017; CLEVENGER; CHRUSZCZ; GUNSON, 2003; FERREGUETTI et al., 2020). Extrinsic factors such as temporal and spatial features (e.g. density of vegetation, proportion of water and traffic) has proven to have a direct relationship with roadkill rates, explaining the patterns of roadkill in some taxa (CACERES, 2011; CLEVENGER; CHRUSZCZ; GUNSON, 2003; COELHO; KINDEL; COELHO, 2008b; FERREGUETTI et al., 2020; GARRIGA et al., 2017). For example, the temporal variation in roadkill may be related to the phenology of biological events, such as mate searching, dispersal and migration periods, and these activities can lead to a gender- and life stage-specific differential mortality (FERREGUETTI et al., 2020; GRILO; BISSONETTE; CRAMER, 2013). On the other hand, variations in: vehicle traffic between road stretches, highway design (which influences vehicle speed and the driver's visibility of road), and surrounding landscape composition and arrangements (which influence fauna abundance and mobility) are some factors than may be responsible for spatial aggregations of roadkills (CLEVENGER; CHRUSZCZ; GUNSON, 2003; DUFFETT et al., 2020; SEILER; HELLDIN; SEILER, 2004).

Most studies about road mortality only provide check lists of killed taxa, with no attached information on the patterns and consequences of mortality. Particularly, few studies have focused on the relationships between spatiotemporal variables and sex ratio roadkill patterns in Xenarthras, one of the top groups in mortality by collisions (ASCENSÃO et al., 2017; CÁCERES et al., 2010; ZIMBRES et al., 2013). Here, we aimed to evaluate the impact of roads on the mortality of two species of anteater along a large extension of a heterogeneous urban-rural landscape. We evaluated the spatial and temporal roadkill pattern of the southern tamandua (*Tamandua tetradactyla*) and the giant anteater (*Myrmecophaga tridactyla*) for one year along 1,259 km of pavemented one-way and two-way roads.

Xenarthra are one of the ancient placental group (Gibb et al., 2016) with two species of anteaters belonging to the Myrmecophagidae family inhabiting almost the whole territory of

Brazilian (90 %) and are considered particularly vulnerable due to habitat loss and fragmentation, due to wildfires and roadkill in some Brazilian states (BERTASSONI et al., 2019; DINIZ; BRITO, 2013; MIRANDA; BERTASSONI; ABBA, 2014; SILVEIRA et al., 1999; SUPERINA; LOUGHRY, 2015). The anteaters are in the top 10 of species with high mortality by roadkill in Brazil (ASCENSÃO et al., 2019; CÁCERES et al., 2010; DINIZ; BRITO, 2013; GARRIGA et al., 2017) and thus, they are especially vulnerable to roadkill because they have large spatial requirements, small populations, tend to live at low densities, and occupy small geographic ranges or exhibit migratory behaviors (CACERES, 2011; FERREGUETTI et al., 2020; GRILO et al., 2020).

Both anteaters, the giant anteater and the southern tamandua, are also largely found in different landscape, from open grassland savanna in the Cerrado, wetlands in the Pantanal, up to transitional forests and mountain tropical regions (ASCENSÃO et al., 2017; CLOZATO et al., 2015; DESBIEZ; MEDRI, 2010). Nevertheless, although southern tamandua have a preference for forested areas for feeding and resting (DESBIEZ; MEDRI, 2010), giant anteaters are habitat generalist with preferences for heterogeneous habitats (QUIROGA et al., 2016; VYNNE et al., 2011). Both species are myrmecophagous and termitophagous with lower body temperatures and lower basal metabolic rates than others mammals (MCNAB, 1984). Their daily activities are diurnal, nocturnal to diurnal (crepuscular) or nocturnal, with habitat use widely related to ambient temperature (CAMILO-ALVES; MOURÃO, 2006; RODRIGUES et al., 2008). Therefore, all the above features and difference of biological characteristics contribute to the increase of impacts by roads and possibly to differences in spatial and temporal patterns of mortality.

Considering the background, we expected that: (i) the majority of roadkill data would be represented by species with larger body masses, because of their high dispersion capacity and larger home range; (ii) we expected that males and females would have the same number of deaths, since we adopted that the proportion of births for the two species is 1:1; (iii) the anteater's mortality rates caused by roadkill would be influenced by climatic variables with increased number of events in the drier and hotter months, as temperatures encourage animals to reduce their activity patterns due to thermoregulation costs; (iv) mortality would be different between seasons, due to the possible effect of seasonality on species movement, related to the time of greatest movement in the reproductive period or the availability of resources. Spatially, we expected that (v) the roadkill events would aggregate at different specific points along the roads, especially near vegetation fragments and water, and far from urban areas and intense traffic. Additionally, we expected that (vi) landscape features at larger scales would influence roadkill patterns due to their influence on anteater movement dynamics. We expected that roadkills would be positively related to the amount of habitat and water, as well as traffic, and negatively related to density of habitat patches, and urban areas. This expected pattern was based on the behavior of each species and gender, with more roadkill events in landscapes where the anteater has to move more intensely to supply their resources requirements. We tested and discussed each environmental variable and the possible implication for conservation and mitigation measures of each species.

Material and methods

Ethics Statements

The biological sampling authorization was obtained through the SISBIO-ICMBio (Authorization System and Biodiversity Information, Chico Mendes Institute for Biodiversity Conservation, Ministry of Environment, Brazil), under the number 53798-4. The research was approved by the Ethics Committee on the Animal Experimentation (CEUA/UFSCar) protocol number 1584280817, and the genetic resource access was registered under SisGen A9F8717.

Study Area and Data Collection

The study was carried out in the Brazilian Center-Western region across four main roads in the state of Mato Grosso do Sul (MS). The Center-Western vegetation is predominantly Savanna, but also includes the Pantanal and flooded areas, and the region's economy is based on livestock, soybeans, and tourism, with a large highway system present mostly in the central region. The study area presents subtropical climate (Aw in the Köppen classification) characterized by a dry winter, with temperatures below 18 ºC, and a wet summer with temperatures above 22 ºC. During the study period the mean temperatures and humidity were 23.94 ± 2.19 °C and 56.77 ± 10.67 % in the dry (from April to September) and 25.33 ± 2.14 °C and 63.17 ± 10.35 % in the rainy (from October to March) seasons (t-test =-5.48, df = 29.09, $p < 0.001$).

We conducted the surveys along four different one- and two-way roads, defined as our transects and referred hereafter as 'T1', 'T2', 'T3' and 'T4' (Figure 1). The municipality of Campo Grande (20°28'15" S - 54º37'15" W) was the starting point of each transect: T1, along BR-262 to the bridge over the Paraguay River (total extension: 397 km); T2, along BR-262 to Três Lagoas (305 km); T3, along BR-163 and BR-267 to Nova Andrandina (275 km) and T4, along MS-040 to Bataguassu (300 km; see Figure 1). All transects covered 1,259 km of paved road subjected to different land cover and climatic variability along their extensions. The transects were surrounded by portions of Pantanal and Savanna.

We monitored the four roads fortnightly, maintaining regular intervals, from April 2017 to March 2018, resulting in 25 surveys. We performed the surveys by car maintaining a regular speed of maximum 60 km h^{-1} in the daytime period from 6:00 am until the daytime necessary to cover the entire stretch. We surveyed a total of 5,036 km every month, considering the four sampled transects. Each road was surveyed in two-directions on the same day, and the positions of all anteater carcasses were annotated with a GPS information receiver. Since the methodology used and the effort was the same for all transects, the results of the surveys are comparable. We removed the animal carcasses from the road once they had been recorded and so we collected tissue samples for further molecular sex identification (Chapter I).

Sampling of Environmental Variables

For all transects we recorded two temporal variables i) temperature (ºC), ii) relative humidity (%). The climate variables data were obtained from the National Institute of Meteorology (INMET, www.inmet.gov.br). The highways were sectioned in 20 km-long segments, based on the home range registered for species (BERTASSONI et al., 2017; DESBIEZ; MEDRI, 2010; MEDRI; MOURÃO, 2005; SHAW; MACHADO-NETO; CARTER, 1987). To obtain more accuracy in the variable values for each segment, we measured the Euclidean distance (m) between the central point of each segment and all nearest climatological stations and chose the station with the shortest distance to obtain climatic data. In total, we collected information from ten climatological stations located in ten municipalities from the state of Mato Grosso do Sul (Agua Clara, Aquiduana, Bataguassu, Campo Grande, Corumbá, Ivinhema, Miranda, Rio Brilhante, Sindrolândia, Três Lagoas).

Land cover and sampling of spatial variables

We obtained the land use and cover maps from the 2018 MapBiomas Collection 4.1 of Brazil, with 30 m of spatial resolution. First, using Quantum GIS v. 3.4.10-Madeira software (QGIS Development Team, 2020), we summed up the categories of land cover to four classes: native vegetation (Forest formation, Savanna formation, Wetland and Grassland formation), urban (Urban infrastructure, other non-vegetated and mining areas), water (Rivers and Lakes) and agriculture (Forest plantations, pastures, annual and perennial crops, semi perennial crops). Then, around each segment, within a 10 km-radius buffer (the largest possible until overlap between contiguous segments), we recorded five landscape variables: 1) proportion of vegetation, 2) proportion of urban area, 3) proportion of agriculture, 4) proportion of water, and 5) density of vegetation fragments (Table 1). For extracting the proportional values of each category for each segment, we used the LecoS-Landscape Ecology Statistics plug-in implemented in QGIS (QGIS Development Team, 2020). Additionally, for each segment we collected data on traffic intensity (daily traffic volumes in 2018; http://servicos.dnit.gov.br/) for each transect. No recent traffic counts were available for T4. For this reason, the T4 was not included in the traffic analyses.

Data Analysis

Roadkill Description

We evaluated the number of roadkill for both species (giant anteater and southern tamandua) separately and compared roadkill rate results between sexes, according to sex identification information obtained previously (Chapter I). To characterize the roadkill patterns, we evaluated whether the number of road-killed individuals were different between sex per species than the expected value, using Chi-square tests. We compared our sexual proportion of roadkill with sex ratio values previously obtained for giant anteaters: 1:1 (DESBIEZ; BERTASSONI; TRAYLOR-HOLZER, 2020), 2:1 (CAMILO-ALVES, 2003), and 3:1 (MOURÃO; MEDRI, 2002). Since no data on sex ratios for southern tamandua are available, we considered the expected ratio of roadkills between species 1:1, but we felt it necessary to conduct our analysis with the same sexual ratio described for the giant anteater.

Temporal and climatic patterns on roadkills

We grouped months by season to test whether roadkills vary seasonally, performing a two-tail unpaired t-tested analysis. When the data did not fit the requirements of the parametric tests, even after log transformation, we performed the non-parametric Mann-Withney test. We performed all analyses in R software v. 3.2.5 (R Development Core Team, 2019). Additionally, to test the influence of temperature and humidity on roadkill rates (individuals/ 100 km) we used multiple regression analysis with the months as the replicas.

Spatial pattern of roadkills

To investigate the spatial aggregation patterns of roadkills, initially we used the function "mortality rate estimate" in SIRIEMA v. 2.0 (COELHO et al., 2011) to estimate the road mortality rate for specie and transect. More details about this approach can be found in Teixeira et al. (2013). Additionally, we multiplied the roadkill rate (roadkill/km/day) per 100 to standardize all results. To evaluate the non-randomness of the spatial distribution of events over multiple scales (Coelho et al., 2008), we analyzed the roadkill events in each transect separately using SIRIEMA v. 2.0 program (COELHO et al., 2011). We analyzed by specie: giant anteater and southern tamandua and by sex per species: males and females. We used the modified 2D-Ripley K test (Coelho et al., 2008; 2012) and the 2D-Hotspot identification analysis for identification of the highest roadkill aggregation points (hotspots) in each transect. The first test was done with an initial radius of 500 meters, radius increments of 1000 meters, 1,000 simulations, and a confidence limit of 95 %. The initial radius choice was the used for mitigation measures, such as speed reducers (TEIXEIRA et al., 2013). The second test was used to identify the segment with highest roadkill aggregations (hotspots). This test was done by dividing each transect into segments with the same length (100 meters). The radius used was 500 meters, 1,000 simulations, a confidence limit of 95 %. More details of analysis can be search in Coelho et al. (2011).

After identifying the roadkill hotspots per species and sex, we investigated the characteristics of such points. To generate a comparison dataset, we used QGIS v. 3.4.10-Madeira to generate random points in the same number of detected hotspot points along each transect. To analyze the land cover around each hotspot, we designed a buffer with a radius of 500 m around the hotspots and the random points to identify what features surround each heat point. Then we extracted the following variables from the landcover map: 1) proportion of vegetation, 2) distance to urban area, 3) proportion of agriculture, and 4) traffic. These analyses were processed using QGIS v. 3.4.10-Madeira software and the size and percentage of the classes for each buffer were calculated using LecoS-Landscape Ecology Statistics plug-in (JUNG, 2016). Finally, to test whether the evaluated variables differed between random points and hotspots, we performed Chi-square tests to the proportion data and two-tailed unpaired t-tests to traffic data.

At a landscape level, to understand which spatial features influence roadkill rates, we used generalized linear models (GLMs) in a multi-model averaging approach to test if the predictor variables (proportion of urban area, density of vegetation, proportion of water, proportion of vegetation and traffic) explained the number of roadkills of each species or the presence of roadkill for each sex of giant anteater. The low presence of southern tamandua females in our roadkill data did not permit the gender analysis to be carried out for this species. We considered the negative binomial distribution of number of roadkills analysis since it best fits the data, correcting the overdispersion issue; and binomial distribution to presence/absence of roadkill data. We previously checked for the absence of multicollinearity among predictors by calculating the variance inflation factor (VIF), using package “car” in R software v. 3.2.5 (R Development Core Team, 2019). We excluded the agriculture proportion variable, since they were inflating the variance, due to its strong correlation with proportion of native vegetation cover ($r = -0.96$). After this exclusion, all VIF values were lower than 2, suggesting independence among predictors (NETER et al., 1996).

The number of roadkills of each species, and the presence or absence of giant anteater females and males were obtained for each segment. For each model, we calculated Akaike Information Criterion (AIC) and the difference of each model and the best model (ΔAIC). Models with $\Delta AIC \leq 2$ were considered as equally plausible (ZUUR; IENO; ELPHICK, 2010), and the Akaike's weight of evidence (w_i, ranging from 0 to 1, with larger numbers indicating greater support) values for all possible combinations. We ranked the models based on the w_i from the higher ranked model until the total of sum be > 0.95, which represents the set of models that best explain the numbers or gender of the roadkill. Then, we evaluated the relative importance of each predictor variable by the $\sum w_i$ of each candidate model in which this variable appeared and of all equally plausible models. Furthermore, we calculated for each predictor their unconditional variance from 95 % confidence set of models to assess the association between each predictor and the response variable. Thus, we considered important the predictor variables that had: a high sum of w_i and the model-averaged unconditional variance was lower than the model-averaged parameter estimates. We performed all analyses with the "glmulti" package in R software v. 3.2.5 (R Development Core Team, 2020). The same methods were used to select the best models to explain the factors for species and sex.

Results

We recorded 320 killed individuals (1.28 individuals/100 km/day), including 191 giant anteaters (60 females, 85 males, and 46 without gender) and 129 (17 females, 75 males, and 37 without gender) southern tamandua for one year. The roadkill number was the same for both species ($X^2 = 3.073$, df = 1, p=value = 0.080). Transect T1 had the largest number of killed individuals (1.68 individuals/100 km/day), followed by T3 (1.27 individuals/100km/year), T2 (1.03 individuals/100 km/day), and T4 (0.96 individuals/100 km/day) with considered all roadkill events. The roadkill number for both evaluated species were higher in the T1 than the others transects ($F_{3,44}$ = 0.864, R^2 = 0.019, p = 0.025); Figure 2a). The giant anteater roadkills were equal among the transects ($F_{3,44}$ = 0.036, R^2 = 0.510, p = 0.829; Figure 2b). The southern roadkills were higher in the T1 than the other transects ($F_{3,44}$ = 0.456, R^2 = 0.190, $p < 0.001$; Figure 2c), which is the same pattern found for all roadkills.

For giant anteaters, the roadkill of females and males happened every month, while no female roadkill was reported for the southern tamandua in five months (May, July, August, September and January; Figure 3). The highest roadkill rate was in February, likely due to the highest number of male southern tamandua roadkills. The roadkill rate found by male giant anteaters was significantly different than the expected sexual ratio of 3:1 (males and females, respectively), while the mortality of southern tamandua males was significantly higher than expected in a sexual ratio of 1:1 and 2:1 (Table 2).

We did not find a relation between the variation of temperature and humidity per month that would explain the temporal pattern of run over for both species (Table 3, Figure 3). Estimated total roadkill rates did not vary significantly between seasons, but the test of female roadkill rates from both species were significantly higher during colder months (roadkill rates from giant female anteaters, w = 185, p = 0.031 and roadkill rate from southern tamandua of females, w = 216, p = 0.037, Table 4).

Ripley's statistical analyses showed that clustering of roadkills occurred at scales up to 200 meters, indicating that roadkills are not randomly distributed along the roads. The 2D-Hotspot analysis showed several places along the road where there are noticeable higher frequency of roadkills and that may be the focus for further efforts to determine priorities in conservation management. For both species we identified ten sections that were classified as hotspots and only one section coincided for the two anteaters (Figure 4). For giant anteater hotspot analyses we found five hotspots for all transects. Three for T1 and one for T2 and one for T3, while for southern tamandua hotspots we found two for T1, and one for T2, T3 and T4 (Figure 4). All hotspots occurred in the agricultural areas for giant anteaters and southern tamanduas, with a mean proportion of 0.19 and 0.35, respectively. However, the comparison of composition spatial features between hotspots and random points (with absence of roadkill aggregations) do not identify significant differences (Table 5). The distance of each hotspots from urban areas never had any effect on roadkills.

Giant anteaters and southern tamandua roadkills had a reasonable association with certain landscape attributes (explained deviance = 27 % and 39 % for giant anteaters and southern tamanduas respectively). At landscape level, we found support for three spatial variables (e.g. proportion of vegetation, proportion of water, and proportion of traffic) that can explain roadkill occurrences for giant anteaters and southern tamanduas. Anteater roadkills were positively related to density of fragment and negatively related to the proportion of vegetation and traffic (Table 6, Figure 5). Overall, the models could explain a small fraction of the spatial patterns for roadkills of each gender (Table 7) but had a reasonable power for each species. The importance of models was lower when we try explaining the gender differences in roadkills.

Discussion

Roadkills show a relevant impact on anteater populations, because of the intense mortality rate registered for both southern tamanduas and giant anteaters and the influence of roads on anteaters’ population dynamics revealed by the spatiotemporal pattern of roadkills. Roadkill numbers are similar for both species, contradicting our first prediction (i) that the tendency for mortality should be higher for species with larger home range, dispersion capacity, and higher body mass. However, the roadkills by sex ratios are different between species (prediction ii).

The roadkills seem to affect mainly the males of southern tamanduas and may vary from equal proportion by sex ratio to mainly effecting females according to some scenarios of natural sex ratio. Additionally, there is no relation between roadkill rate and climate variables, contradicting our prediction (iii) on thermoregulatory influences on movement dynamics. But we show that the patterns of female roadkills are related to seasonality, revealing some influence of seasonal factors on species' movement dynamics, as expected (prediction iv) (ASCENSÃO et al., 2017). Spatially, as we expected (prediction v) the roadkill patterns are nonrandom, but the evaluated local environmental characteristics of hotspots do not explain the aggregation of roadkills in such points. However, we highlight the importance of both local (traffic) and landscape features (fragmentation degree and habitat amount) in roadkill mortality, although not all variables had the expected relationship (prediction vi).

The larger number of anteater roadkills may be considered high when we compared our results with other studies with similar analyses effort, as found by Ascensão et al. (2017) who found 124 giant anteaters and 116 southern tamanduas in the same region of our study, in a period of 13 months in an analysis effort of 23,000 km. Anteaters are usually found in all list of taxa killed on roads in Brazil (ARAUJO et al., 2020; ASCENSÃO et al., 2017, 2019; GARRIGA et al., 2017; RIBEIRO; SILVEIRA MIRANDA; RODRIGUES DE ARAÚJO, 2017) probably reflecting an overall high abundance of anteaters in some Brazilian regions (CÁCERES et al., 2010). Additionally, several biological features of Xenarthras may contribute to the high incidence of roadkills (e.g. poor vision) compared with other groups (FREITAS; JUSTINO; SETZ, 2014; MERI MEDRI; MOURÃO; RODRIGUES, 2010; REDFORD, 1985). In contrast with others studies, where species with larger body mass require large areas for their survival (ANACLETO; MARINHO-FILHO, 2001; MEDRI; MOURÃO, 2003), which increases the number of casualties in some species, and despite the fact that the giant anteater can exceed 35 kg in weight, about seven times the body mass of the southern tamandua (approximately 5 kg; Rodrigues and Marinho-Filho, 2003), we found that the total of number of roadkills in both species was similar. All surveys were done following similar protocols as other studies, considering the persistence time of the southern tamandua carcasses (SANTOS; CARVALHO; MIRA, 2011) and our results can be explained basically by the biology of the species. For this reason we raise the possibility that species may deal with the roads in their habitat by altering their activity patterns and other behaviors (DE JONG, 1995; JEPSEN; TOPPING, 2004; KOMERS, 1997).

The roadkill mortality by sex was the same in males and females of giant anteaters. As expected, there was no difference between expected and observed roadkill sex ratios in giant anteaters, in the scenario for natural populations with 1:1 sex ratio. The tolerance for overlap of territory between both sexes is high for giant anteaters (Bertassoni et al., 2017; Miranda, 2004; Anteaters & Highways *pers. comm)* resulting in the same area probably having the same number of males and females. Thus, this could also partially explain the absence of sex bias mortality in giant anteaters. However, our results indicate a different overview from that found by Mourão and Medri (2002).Nevertheless, whether the sex ratio indicated by these is the current giant anteater population, this would represent a high impact on females of the species and would therefore have a greater impact for populations located near highways.

Yet, males of southern tamanduas were killed three times more than females, which may represent an intense removal of males from the population or populations naturally biased to males in the sex ratio. Currently there is no data on sex rate or the mating systems of southern tamanduas, so it is difficult to advance in the explanation the impact of roadkills on population balance. In ideal natural populations of mammals, it would be expected that there would be the same proportion of males and females in the population (1:1) (BULMER;

TAYLOR, 1980), however, in some cases it is possible to find two males for one female (2:1) which happens in some vertebrates (ARESCO, 2005; SILLERO-ZUBIRI; GOTTELLI; MACDONALD, 1996). Therefore, whether we consider these characteristics in natural populations of southern tamanduas, the biases of which animals suffered roadkills would become more evident. Thus, whether considering the same mating system of two species with an absence of differential dispersion between sexes, in any panorama previously shown, the loss of individuals in populations represents a loss in the genetic diversity of the southern tamandua species and even possible changes in their effective population size in a given time (Forman and Alexander, 1998; Reed and Frankham, 2003). In the same line, population viability analyses in giant anteaters suggest that mortality due to road kill may not necessarily lead to the extinction of local populations, yet point to a possible reduction in the population's resilience and ability to withstand or recover from other anthropogenic threats on species (DESBIEZ; BERTASSONI; TRAYLOR-HOLZER, 2020)

Our results found no evidence for the relationship between roadkill and temperature but suggest the relation between female anteater roadkills and the rainy season (from October to March). Some studies in mammals have shown that during the dry season mortality levels are higher because the demand for food forces individuals to move across heterogeneous landscapes and thus crossing roads several times (Bueno and Almeida, 2010; Grilo et al., 2009). Although some studies did not find seasonal influences on the roadkill of mammals (FERREIRA et al., 2014; ORLANDIN et al., 2015). To others, the number of road accidents in the dry season is significantly lower than in the rainy season (CACERES, 2011; FERREGUETTI et al., 2020). This variation in the results found probably depends on biological and ecological characteristics of the species studied, such as dispersion, mating systems, foraging, dispersion, and the food habits of species. In our case, the increase of female mortality in the rainy season may be related to the reproductive period of females or the greater availability of resources during this season, however, there is no information on dispersal or reproductive periods for anteaters.

Although we found roadkill hotspots for both species, the characteristics described and tested for them were no different from the rest of the road. This means that other factors may be responsible for the high aggregation found at specific points on the road. For example, variations in vehicle traffic between road stretches, highway design which influences vehicle speed, and the driver's visibility of the road, are all factors that can and should be included in future analyses.

Despite our efforts to collect the largest number of variables to explain the pattern of anteater roadkills, using the most common variables in landscape studies, the power of explanation was low. We suggest that, more than proportion of vegetation for that species, it is the traffic that is causing the pattern of mortality found, a discovery that needs more attention from mitigation measures. Although some studies show a higher number of roadkills in areas with a higher volume of traffic (COELHO; KINDEL; COELHO, 2008a; JACKSON; FAHRIG, 2011; ROW; BLOUIN-DEMERS; WEATHERHEAD, 2007), our results indicated the opposite. This result may be partially explained by the fear of the animals to the landscape with the most traffic volume (MENDES et al., 2020). For example, some studies showed the least occurrence of roadkill for birds in fragments near highways with high noise pollution and low habitat quality (BROTONS; HERRANDO, 2001; PERIS; PESCADOR, 2004). On the other hand, a study on giant anteaters highlighted the negative effect of habitat fragmentation by the road as well as the proportion of vegetation surrounding the road, indicating that many of the areas used in this study are below the minimum needed to sustain a viable population (PINTO et al., 2018). Therefore, we suggest that additional, finer resolution, field-derived habitat and road variables (road width, presence of curves, floor type, and topography) should be included in future road ecology analyses.

Conclusion

In this study, we have shown the importance of using information such as sex in the mortality analysis to better understand the patterns of roadkill by species. Understanding the seasonality and spatial variations in species roadkill patterns are directly proportional to the impact of the conservation measures. In conclusion, according to our results, road surveys and mitigation measures for anteaters in the Brazilian Center-Western region should be prioritized in the rainy season and monitoring should be enhanced for landscapes with low density and low proportion of vegetation, i.e. the most degraded areas, near the roads with low volume of vehicles.

Finally, we suggest that more studies using more specific traits be used for future road ecology analyses and mitigation measures, especially for large body mass and charismatic species. This may be a more effective measure with greater social impact.

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Tables and Figures

Table 1. Predictor variables tested for explaining the roadkill events of *Myrmecophaga tridactyla* and *Tamandua tetradactyla* in Brazilian Center-Western

	Variables name, unit (type)	**Symbol**	**Definition**
	Environmental variables		
i	Mean Temperature (ºC)	T ºC	Bioclimatic variables derived from the monthly temperature and humidity values. Data were generated from INMET (www.inmet.gov.br).
ii	Humidity (%)	RH	
	Landscape variables		
1	Proportion of vegetation	Pv	Proportion of primary or secondary succession stages of remnants of forest and savanna in the sampled area
2	Density of vegetation	Dv	Number of patches of native vegetation per sampled area (N/ km²)
3	Proportion of urban area	Pu	Proportion of urban areas and human buildings in the sampled area
4	Proportion of agriculture	Pa	Proportion of crops and pasture mosaics
5	Proportion of water	Pw	Proportion of rivers, lake and pounds
6	Distance to urban area	Du	Euclidean distance (m) between the nearest urban center and hotspot point
7	Traffic	T	Daily traffic volumes in 2018. Data were generated to Plano Nacional de Contagem de Tráfego – PNCT (http://servicos.dnit.gov.br/)

Table 2. Results of the Chi-square test (X^2) for mortality of *Myrmecophaga tridactyla* and *Tamandua tetradactyla* according to three expected scenarios of local population sexual ratio (see the main text for references about expected sexual ratio). Significant values are indicated in bold ($p \leq 0.05$).

Specie	Expected Sexual Ratio								
	1:1			2:1			3:1		
	X^2	df	p	X^2	df	p	X^2	df	p
Myrmecophaga tridactyla	2.54	1	0.111	1.03	1	0.309	**6.45**	**1**	**0.011**
Tamandua tetradactyla	**21.53**	**1**	**3.47 E 10-6**	**4.67**	**1**	**0.035**	1.48	1	0.223

Table 3. Climatic variables relationship with mortality rate of giant anteater (*Myrmecophaga tridactyla*) and southern tamandua (*Tamandua tetradactyla*). T ºC: monthly mean temperature; RH: monthly mean relative humidity.

	β	SE	t	p
All				
T ºC	0.089	0.098	0.906	0.370
RH	0.038	0.044	0.869	0.390
T ºC: RH	-0.001	0.002	-0.759	0.452
Giant anteater				
T ºC	0.224	0.128	1.753	0.087
RH	0.111	0.058	1.919	0.062
T ºC: RH	-0.004	0.002	-1.855	0.070
Males giant anteater				
T ºC	0.104	0.072	1.447	0.155
RH	0.065	0.032	2.003	0.051
T ºC: RH	-0.002	0.001	-1.918	0.062
Females giant anteater				
T ºC	0.028	0.056	0.500	0.619
RH	0.007	0.025	0.260	0.796
T ºC: RH	0.000	0.001	-0.196	0.846
Southern tamandua				
T ºC	-0.015	0.066	-0.229	0.820

RH	-0.012	0.030	-0.391	0.698
T ºC: RH	0.001	0.001	0.458	0.649
Males Southern tamandua				
T ºC	0.031	0.063	0.486	0.629
RH	0.018	0.029	0.644	0.523
T ºC: RH	-0.001	0.001	-0.508	0.614
Females Southern tamandua				
T ºC	-0.021	0.035	-0.593	0.556
RH	-0.012	0.016	-0.778	0.441
T ºC: RH	0.001	0.001	0.870	0.389

Table 4. Difference between rainy and dry season on mortality rates of giant anteater (*Myrmecophaga tridactyla*) and southern tamandua (*Tamandua tetradactyla*). In bold the significant relationships ($p \leq 0.05$).

	Test	**df**	**p**
Giant anteater	t = 0.901	42	0.373
Males	W = 369.5	46	0.093
Females	**W = 185**	**44**	**0.032**
Southern tamandua	t = 1.664	43	0.103
Males	t = 0.947	43	0.349
Females	**W = 216**	**38**	**0.038**
All	t = -0.097	42	0.923

Table 5. Analysis of spatial variables obtained in a buffer with a radius of 500 m around hotspots and random points of giant anteater (*Myrmecophaga tridactyla*) and southern tamandua (*Tamandua tetradactyla*) across all roads of Mato Grosso do Sul.

	X^2	df	p
Giant anteater			
Proportion of vegetation	10	9.0	0.351
Proportion of agriculture	10	9.0	0.351
Proportion of Urban area	4.6	4.0	0.323
Distance to urban area	t = -0.639	4.4	0.554
Traffic	t = 1.131	7.7	0.292

Male of giant anteater			
Proportion of vegetation	26.7	26.0	0.427
Proportion of agriculture	32	30.0	0.368
Proportion of Urban area	16.3	16.0	0.436
Distance to urban area	t = -0.895	21.3	0.380
Traffic	t = -0.851	14.3	0.408
Female of giant anteater			
Proportion of vegetation	10	7.0	0.189
Proportion of agriculture	10	8.0	0.265
Proportion of Urban area	5.2	5.0	0.392
Distance to urban area	t = -0.488	7.1	0.640
Traffic	t = -1.659	6.7	0.142
Southern tamandua			
Proportion of vegetation	10	9.0	0.351
Proportion of agriculture	10	9.0	0.351
Proportion of Urban area	5.2	5.0	0.392
Distance to urban area	t = 0.981	4.0	0.381
Traffic	t = 0.258	5.9	0.804

Table 6. Results of generalized linear models (GLM) that best explained giant anteater (*Myrmecophaga tridactyla*) and southern tamandua (*Tamandua tetradactyla*) roadkill events. We ranked, according to AIC, the set of models for which Akaike weights (*w*i) summed > 0.95. The values of model-averaged parameter estimate (β) and unconditional variance (UV) are also indicated. The equally parsimonious models are indicated in bold. The variables included in each model are indicated with X.

Model	Proportion of vegetation	Proportion of urban	Proportion of water	Density of vegetation	Traffic	AIC	ΔAIC	w_i
Giant anteater								
Model 1	**X**		**X**		**X**	**231.27**	**0.00**	**0.317**
Model 2	**X**		**X**	**X**	**X**	**232.99**	**1.72**	**0.134**
Model 3	**X**	**X**	**X**		**X**	**233.05**	**1.78**	**0.130**
Model 4	X				X	233.28	2.01	0.116
Model 5	X	X	X	X	X	234.90	3.63	0.052
Model 6	X			X	X	234.97	3.70	0.050
Model 7			X		X	234.99	3.72	0.049
Model 8	X	X			X	235.24	3.97	0.044
Model 9		X	X		X	236.24	4.97	0.026
Model 10			X	X	X	236.46	5.19	0.024
Model 11	X	X		X	X	236.96	5.69	0.018
β	-1.953*	0.376	-44.332	0.013*	-2.1 E-04*			Σ w_i = 0.96
UV	1.256	1.086	1.256 E +03	3.0 E-03	2.21 E-08			
Southern tamandua								
Model 1		**X**	**X**		**X**	**222.10**	**0.00**	**0.213**
Model 2		**X**	**X**	**X**	**X**	**223.21**	**1.11**	**0.122**
Model 3		**X**			**X**	**223.69**	**1.59**	**0.096**
Model 4			**X**		**X**	**223.85**	**1.75**	**0.089**

Model 5	**X**	**X**	**X**		**X**	**223.90**	**1.80**	**0.087**
Model 6	**X**	**X**	**X**	**X**	**X**	**224.07**	**1.97**	**0.080**
Model 7	X	X		X	X	224.47	2.37	0.065
Model 8		X		X	X	224.52	2.42	0.064
Model 9	X	X			X	225.18	3.08	0.046
Model 10			X	X	X	225.21	3.11	0.045
Model 11	X		X		X	225.84	3.74	0.032
Model 12	X		X	X	X	226.98	4.88	0.018
β	-0.2498*	-81.767	-0.02743	0.0567*	-1.749 E-04*			Σ w_i = 0.95
UV	0.1359	51.458	1.673 E +03	1.018 E-02	2.066 E-08			

* Values with an asterisk indicate cases in which the unconditional variance was smaller than the model-averaged parameter estimates, suggesting safe interpretation of β.

Table 7. Results of generalized linear models (GLM) that best explained gender's roadkill events in *Myrmecophaga tridactyla*. We ranked, according to AIC, the set of models for which Akaike weights (*w*i) summed ≥ 0.95. The values of model-averaged parameter estimate (β) and unconditional variance (UV) are also indicated. The equally parsimonious models are indicated in bold. The variables included in each model are indicated with X.

Model	Proportion of vegetation	Proportion of urban	Proportion of water	Density of vegetation	Traffic	AIC	ΔAIC	w_i
Males								
Model 1	**X**		**X**		**X**	**73.71**	**0.00**	**0.212**
Model 2	**X**				**X**	**73.72**	**0.01**	**0.211**
Model 3	**X**	**X**	**X**		**X**	**74.17**	**0.46**	**0.169**
Model 4	**X**	**X**			**X**	**75.35**	**1.64**	**0.093**
Model 5	**X**			**X**	**X**	**75.49**	**1.78**	**0.087**
Model 6	**X**		**X**	**X**	**X**	**75.59**	**1.88**	**0.083**
Model 7	X	X	X	X	X	76.17	2.46	0.062
Model 8	X	X		X	X	77.23	3.52	0.037
β	-4.692*	5.170	-0.289	-0.018*	-5.643 E-04			Σ w_i = 0.95
UV	4.318	1.19 E +02	1.730 E +03	8.84 E-03	8.63 E-08			
Females								
Model 1		**X**	**X**		**X**	**80.09**	**0.00**	**0.144**
Model 2	**X**	**X**	**X**		**X**	**80.39**	**0.30**	**0.124**
Model 3	**X**				**X**	**80.61**	**0.52**	**0.111**
Model 4	**X**	**X**			**X**	**80.96**	**0.87**	**0.093**
Model 5	**X**			**X**	**X**	**81.42**	**1.33**	**0.074**

Model 6	**X**		**X**		**X**	**81.63**	**1.54**	**0.067**
Model 7		**X**			**X**	**81.66**	**1.57**	**0.066**
Model 8		**X**	**X**	**X**	**X**	**82.07**	**1.98**	**0.054**
Model 9	X	X	X	X	X	82.11	2.02	0.052
Model 10	X	X		X	X	82.29	2.20	0.048
Model 11					X	82.57	2.48	0.042
Model 12	X		X	X	X	82.62	2.53	0.041
Model 13			X		X	83.00	2.91	0.034
β	-1.697*	1.209	-2.553	0.046*	-2,94E-01*		$\Sigma w_i = 0.95$	
UV	3.645	3.292 E+ 02	1.380 E+ 03	1.487 E-02	7.650 E-08			

* Values with an asterisk indicate cases in which the unconditional variance was smaller than the model-averaged parameter estimates, suggesting safe interpretation of β.

Figure 1: Map of the study area, Mato Grosso do Sul (MS) state, with the surveyed roads, hereafter, referred as ‘T1’, ‘T2’, ‘T3’ and ‘T4’. The city of Campo Grande was the central point for the begin of each transect: T1, along the BR-262 to the bridge over the Paraguay River (397km); T2, along the BR-262 to the Três Lagos (305km); T3, along the BR163 and BR-267 to Nova Andrandina (275km) and T4, along the MS040 to Bataguassu (300km). The flag symbol indicates the location of meteorological stations of MS.

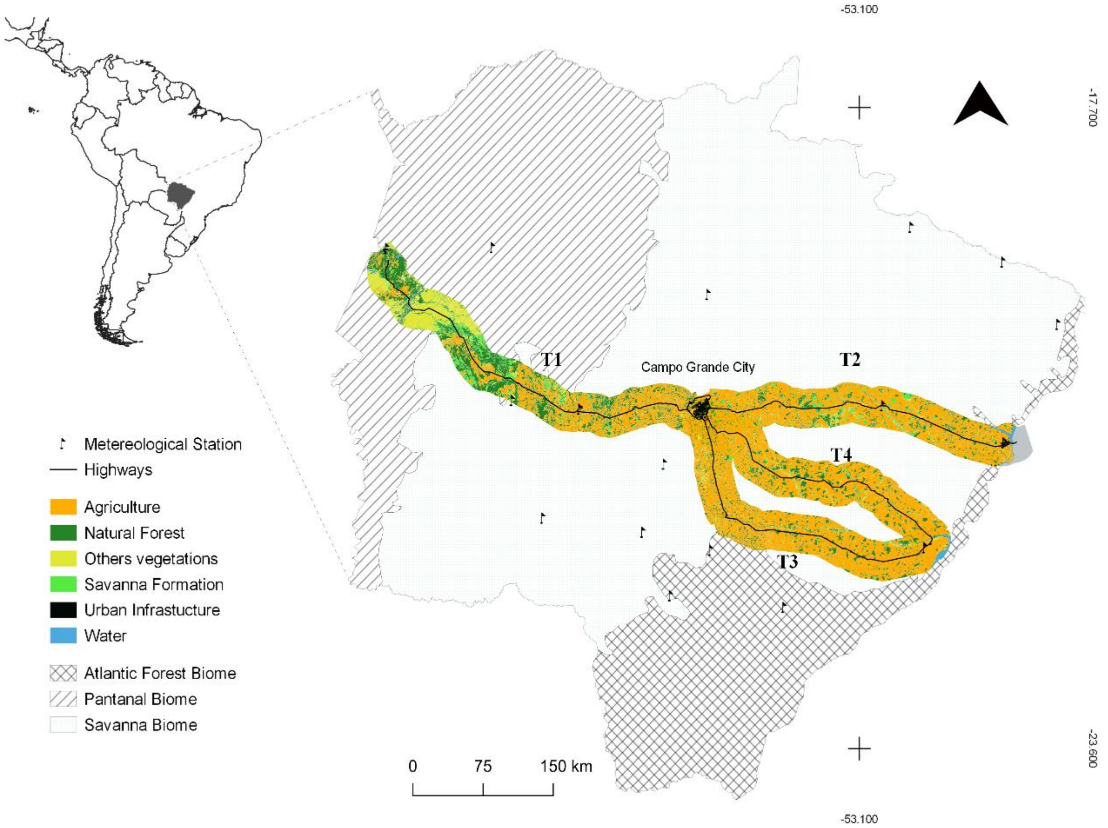


Figure 2. Number of roadkill events per evaluated transects. Numbers of roadkill (N) for summed species (A), *Myrmecophaga tridactyla* (B) and *Tamandua tetradactyla* (C). In each boxplot, the box encompasses the range of number of roadkill, the line is the median, and outliers are shown outside the white dots. The letters A and B above each bar are indicating the significant differences on roadkill between transects.

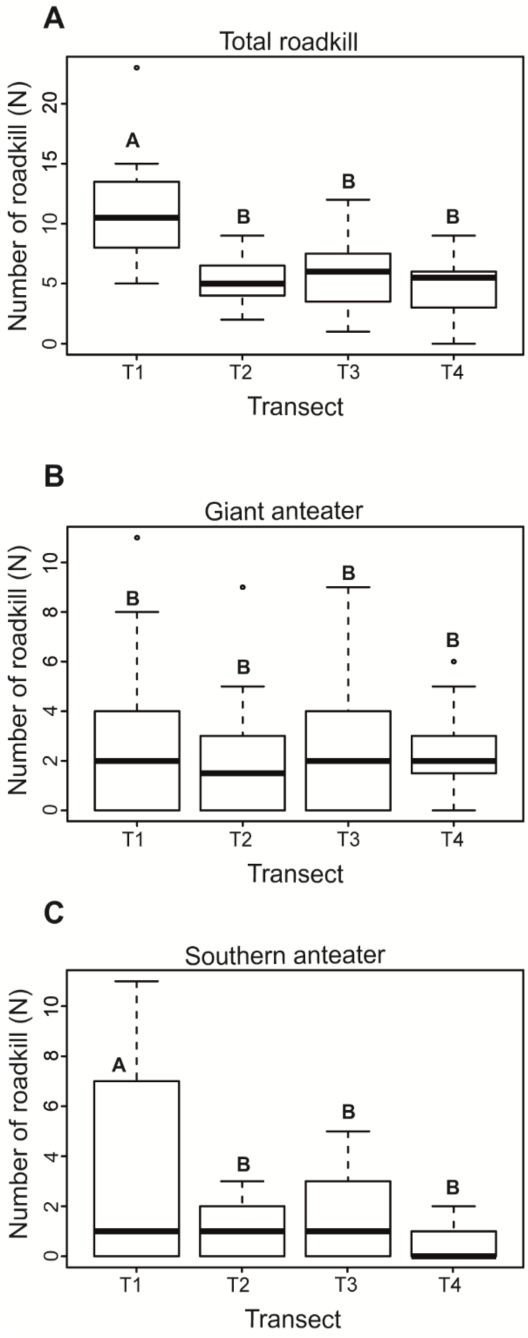


Figure 3. Monthly climatic (A) and roadkill rate for *Myrmecophaga tridactyla* (B) and *Tamandua tetradactyla* (C).

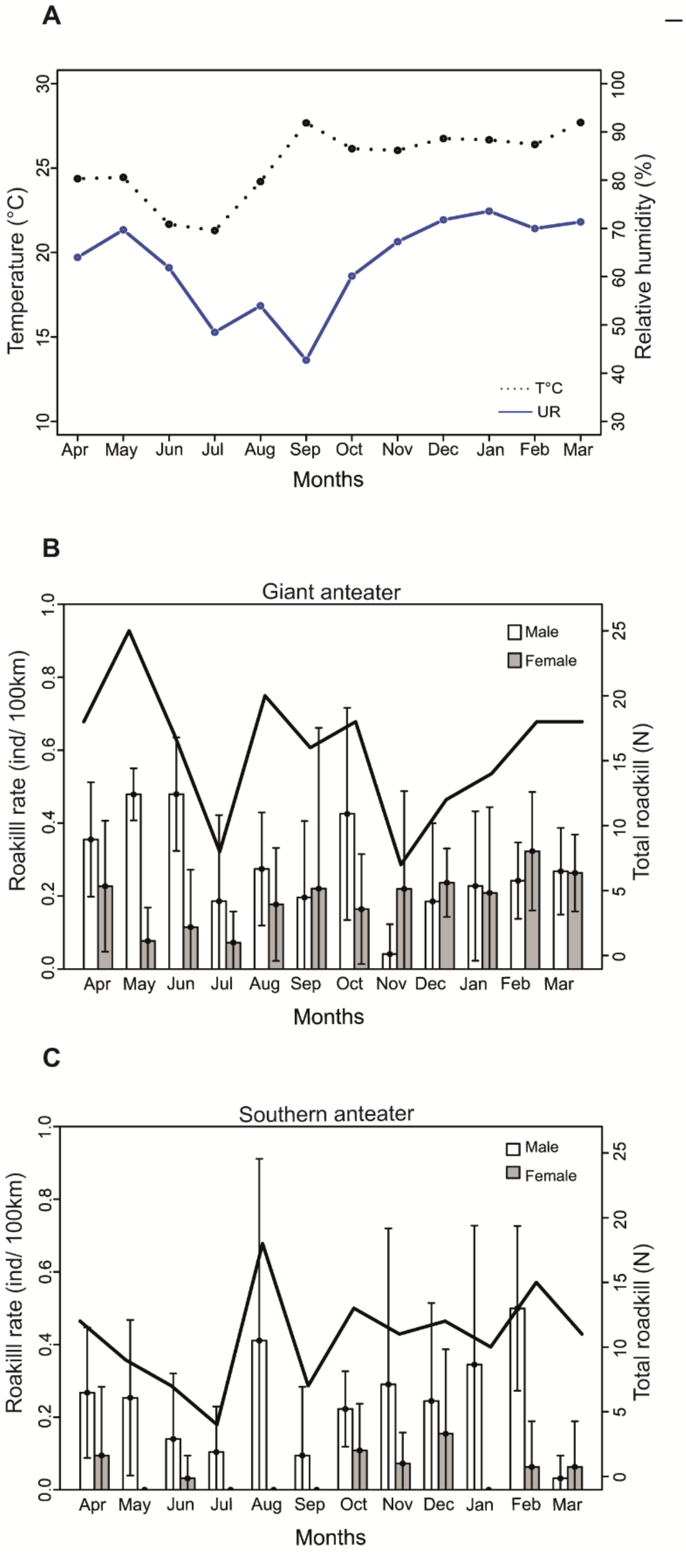

A
Temperature (°C)
10
15
20
25
30
Relative humidity (%)
30
40
50
60
70
80
90
100
T°C
UR
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan
Feb
Mar
Months
B
Giant anteater
Roakilll rate (ind/ 100km)
0.0
0.2
0.4
0.6
0.8
1.0
Total roadkill (N)
0
5
10
15
20
25
Male
Female
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan
Feb
Mar
Months
C
Southern anteater
Roakilll rate (ind/ 100km)
0.0
0.2
0.4
0.6
0.8
1.0
Total roadkill (N)
0
5
10
15
20
25
Male
Female
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan
Feb
Mar
Months

Figure 4. Roadkill hotspots along the roads studied in Mato Grosso do Sul state for giant (black) and southern (grey) anteaters.

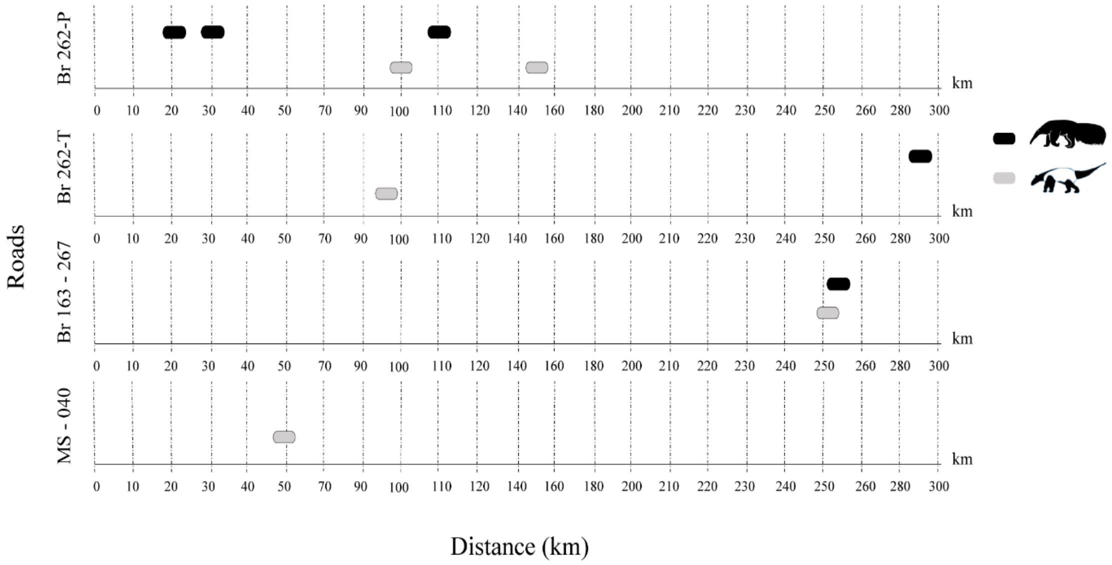
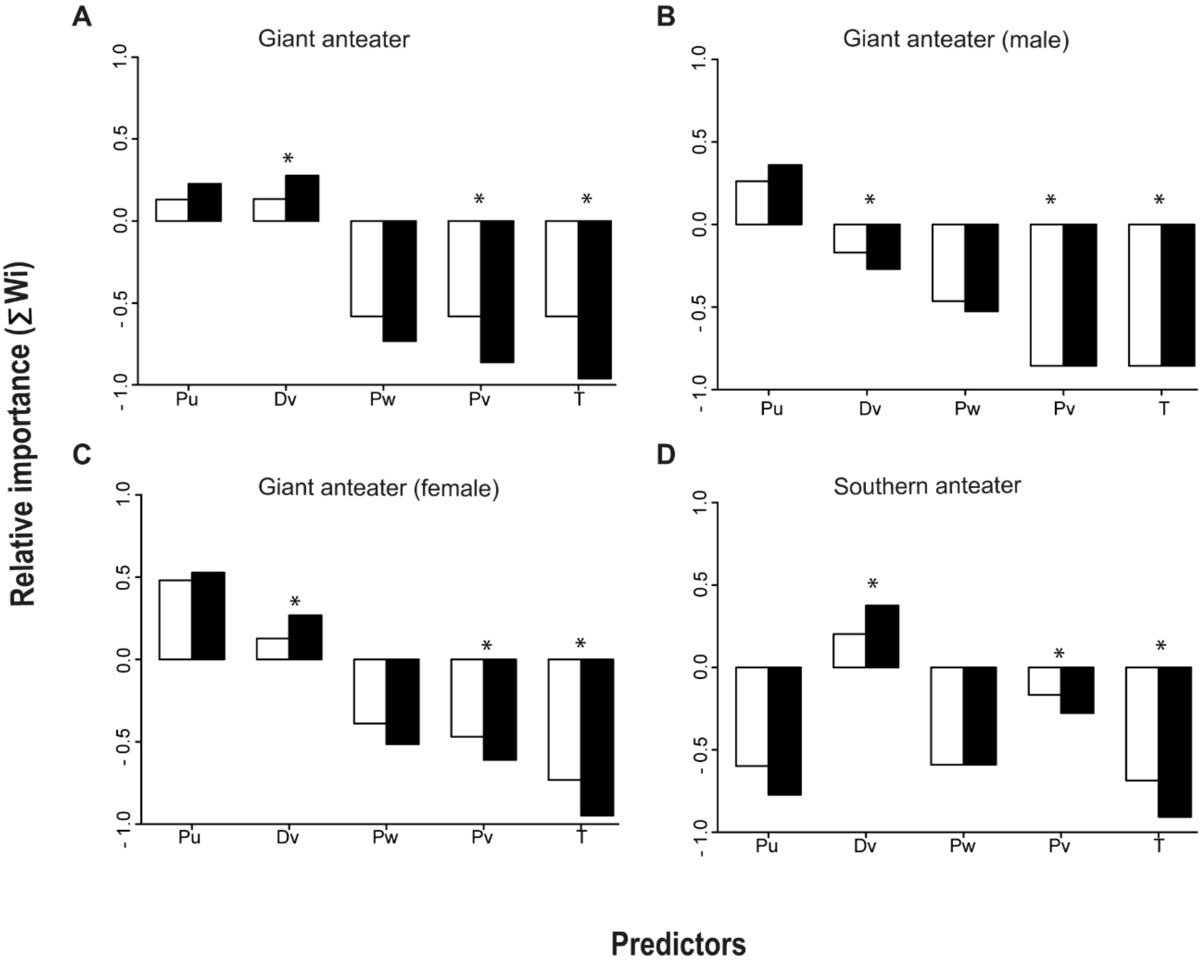


Figure 5. Relative importance of each predictor variable on the roadkill of *Myrmecophaga tridactyla* and *Tamandua tetradactyla* in Brazilian Center-Western. The predictor variables included the ΔAIC < 2 set of models (black bars) and in 95 % set of models (white bars). The importance of each variable is shown by the sum of Akaike weights (Σw_i). The sign (+/-) of Akaike weights (*w*AIC) represents the effect (positive or negative) of each predictor based on the model averaged parameters (β). Pu: Urban proportion; Dv: Density of vegetation; Pw: Proportion of water; Pv: Proportion of vegetation; T: Traffic. Bars with asterisk indicate cases in which the unconditional variance was smaller than the model-average parameter estimates.



Capítulo III

Genetic diversity and demographic changes in the endangered giant anteater, *Myrmecophaga tridactyla*, living in an anthropized area.

(Artigo a ser submetido para a Molecular Ecology)

Genetic diversity and demographic changes in the endangered giant anteater, *Myrmecophaga tridactyla,* living in an anthropized area.

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Abstract

It is expected that a species living in a highly fragmented habitat and having biological characteristics such as a specialized feeding behavior , long generation time, and low fecundity would present low genetic diversity, positive inbreeding coefficients and signs of genetic structure. However, despite the above is expected, we showed that a threatened Xenarthra, *Myrmecophaga tridactyla*, which suffers great anthropogenic impacts from hunting, human-induced fire in its habitat and roadkill has moderate levels of genetic diversity (Ho = 0.54) and no genetic structure, maintaining connectivity within the individuals in the Central-Western region of Brazil. Nevertheless, we find a significant inbreeding coefficient (Fis, 0.13 $p \leq 0.05$), accompanied by a recent bottleneck that is evidence of a population size reduction in this population. In addition, our results provide evidence for the effective population size of approximately Ne = 375.5. Therefore, although the moderated genetic diversity found, it is essential to clarify that the current scenario described here for *M. tridactyla* population does not guarantee the maintenance of the genetic diversity over time, especially considering the high anthropic pressures. The combination of the recent bottleneck and inbreeding in a scenario constant human impacts, which can promote a population decline can result in a decrease and loss of genetic diversity, reducing long term population. Monitoring *M. tridactyla* living in this anthropized area is essential to avoid genetic diversity declining and genetic structure and ensure its population viability.

Keywords: microsatellites, demography, roadkill, Myrmecophagidae

Introduction

It is quite unlikely that a large mammal once having a wide geographic distribution has not been suffering any negative consequence from the anthropic activities. During the last few decades, anthropogenic impacts have been promoting habitat loss and fragmentation by extensive agriculture, urbanization and highways, resulting in threats to biodiversity worldwide (Storfer et al., 2010) and promoting several consequences to the wild populations. Among these consequences, increasingly isolated populations have been decreasing the populations´ size (Reed and Frankham, 2003), reducing gene flow among them (Haag et al., 2010; Oliveira, Hannibal, Oliveira, and Hannibal, 2017), and becoming them more sensitive to genetic drift effects (Reed and Frankham, 2003). Consequently, local genetic variation can be reduced and genetic differentiation among populations increased, negatively impacting the long-term maintenance of wild populations (Reed and Frankham, 2003). Although the importance of these impacts on the species conservation, little is known about these genetic consequences on large mammals mainly in neotropical regions (Miotto et al., 2012; Saranholi et al., 2017), particularly for some groups little studied, as the Xenarthra.

Extant in several major biomes across Central and South America, the giant anteater, *Myrmecophaga tridactyla*, is a charismatic and large Xenarthra that has been suffering from human activities in several regions of its distribution area. Currently categorized as 'Vulnerable' with decreasing populations (IUCN, 2020), the giant anteater has disappeared in several areas of its original range distribution (IUCN, 2020), mainly due to habitat reduction (Miranda et al., 2015, 2014) and habitat fragmentation caused by anthropic activities. Its solitary habits, low fecundity, long gestation time and relatively high generation time (Eisenberg and Redford, 1999), added to a specialist diet (McNab, 1984), make this species more vulnerable and threatened in anthropic scenarios (Desbiez et al., 2020). However, little is known about genetic consequences to tamandua living in highly anthropized regions. A previous genetic study reported the genetic structure, relatedness and mating structure in giant anteater suggesting that its populations exhibit relatively low levels of genetic diversity accompanied by inbreeding due to recurrent fire events in the area (Collevatti et al., 2007). Other studies suggest a population differentiation accompanied by high levels of genetic diversity (Sartori, 2018). Of note, all these previous studies focused on small local populations inhabiting protected areas or surrounding protected areas, and there is no genetic populational analysis evaluating this genetic information in large-scale anthropized area.

The Brazilian Center-Western landscape has been undergoing an intense urbanization process, with outstanding development of agriculture (IBGE, 2020), and increase in of roads and highways construction (Grilo et al., 2019). However, such landscape modifications are relatively recent in this area, mostly occurring during the last five decades (IBGE, 2020). We predicted that the giant anteaters living in this increasingly human-modified landscape would be losing genetic diversity and showing fragmented populations with reduced gene flow. We tested the hypothesis that anteater populations inhabiting a large polygon into the Center-Western Brazil region will show loss of genetic diversity and signals of gene flow reduction among local populations. In addition, we hypothesize that would be a reduction in the effective population size of anteaters due to the high loss of natural habitat. This current study represents the first large genetic survey investigating the genetic diversity and population genetic structuring for *M. tridactyla*.

Material and Methods

Ethics Statements

The biological sampling authorization was obtained through the SISBIO-ICMBio (Authorization System and Biodiversity Information, Chico Mendes Institute for Biodiversity Conservation, Ministry of Environment, Brazil), under the number 53798-4. The research was approved by the Ethics Committee on the Animal Experimentation (CEUA/UFSCar) protocol number 1584280817, and the genetic resource access was registered under SisGen A9F8717.

Study area and sampling

The study area partially comprises three distinct biomes, the Cerrado Brazilian savanna, Pantanal wetlands, and inland Atlantic Forest in the Brazilian Center-Western region. Here, agriculture has transformed the landscape into a mosaic of monocultures, mainly soy and sugar-cane crops with pasture, and different degradation levels of natural vegetation (IBGE, 2020), besides urbanization, roads and highways.

We collected a total of 107 tissues samples, of which 66 samples were from roadkilled animals, and 41 obtained from captured wild animals. All samples were collected by the research project “Anteaters and Highways” (www.giantanteater.org). The sample collection was conducted from April 2013 to February 2017. This sampling represents the largest range for *M. tridactyla* population genetically evaluated thus far. Detailed information related to each specimen sampling is available in the Table S1.

DNA extraction and genotyping genetic analysis

Tissue samples from all specimens were conserved in 95 % ethyl alcohol and stored in freezer at -20 °C. Total genomic DNA was extracted using the conventional phenol-chloroform protocol (Sambrook et al., 1989). The DNA quality was checked by electrophoresis on 1 % agarose gel stained with Gel Red ™ (Biotium, Hayward, CA, USA).

A total of ten microsatellite loci were used for genotyping all the individuals. Five microsatellites (04, 07, 11, 13, and 20) were described for *M. tridactyla* (Garcia et al., 2005) and five heterologous loci (A9, B2, E3, G3, and H5) were developed for *Tamandua tetradactyla* (Clozato et al., 2014). We used a universal M13 primer fluorescent-labelled and an M13 complementary tail to the 5' position of each microsatellite forward primers (Schuelke, 2000) for genotyping each locus (Table S2). The PCR reaction was performed in a final volume of 10 µL containing 1U GoTaq DNA polymerase (Promega), 1X Buffer, 1.5 mM $MgCl_2$, 0.20 mM dNTPs, 0.8 mg/ml BSA, 2 pmol forward and 8 pmol of reverse primers, 8 pmol M13 primers and ~30 ng of DNA. PCRs were conducted in two steps. Firstly, PCRs were run with an initial denaturing step of 94 ºC at 1 min, followed by 20 cycles of 94 ºC at 1 min, 45 sec at locus-specific annealing temperature (Table S2) and 1 min at 72 ºC. In a second step, which we named M13 cycle, eight cycles of the 30 sec at 94 ºC, 45 sec at 53 ºC and 45 sec at 72 ºC were added, and a final extension for 20 min at 72 °C. PCR products were checked on 2 % agarose gel. Fragments were genotyped using ABI3730XL Applied Biosystems automatic sequencer. Allele sizes were analyzed using internal standard Rox 550 and manually determined using Geneious R7 (Biomatters Ltd) (Kearse et al., 2012). The samples consistently producing ambiguous or negative genotypes after three repetitions were treated as missing data at that locus.

Genetic population structuring and genetic diversity

The presence of null alleles and scoring errors due to allelic dropout and stutter peaks were checked using MICROCHECKER v. 2.2.3 (Van Oosterhout et al., 2004). Genetic population structuring was investigated using different methods. First, we used the Bayesian assignment analysis implemented in the STRUCTURE v. 2.3.3 software (Pritchard et al., 2000). The most likely number of clusters (K) was tested using the admixture model with sampling location as prior (LOCPRIOR) information, with 1,000,000 Markov Chain Monte Carlo (MCMC) iterations, and each value of K (1 to 5) were tested with 10 replicates and burn-in at 1000. The optimal K value was determined following Evanno (2005), using the algorithm implemented in STRUCTURE HARVESTER (Earl and vonHoldt, 2012). We also used the GENELAND package (Guillot et al., 2005), implemented in R (Core Team, 2017) to conduct a spatial clustering model. In this analysis we used the correlated frequency model, 1,000,000 MCMC iterations, thinning and burn-in parameters set at 1000 and 200, respectively. The tested group number was K = 1 - 4. The choice of K was based on the histogram of estimated K for each run and the highest mean posterior density across replicates.

Population structuring was also evaluated by a multivariate approach using Discriminant Analysis of Principal Components (DAPC; Jombart et al., 2010) from the Adegenet package (Jombart et al., 2009), implemented in the R software (R Development Core Team, 2017). Four 200 Km diameter sampling areas were artificially created to representing particular landscape where samples were obtained, named hereafter sampling site 1, 2, 3 and 4 to represent most wetland, center savanna, partial forest and eastern savanna, respectively (Figure 1).

To test the correlations between the genetic and geographic distances in the pairwise individuals and check a possible sexual dispersion bias reported by Collevatti et al. (2007), we evaluated the presence of Isolation-by-distance (IBD) using Mantel test (Mantel, 1967). For that all individuals had the sex previously assigned by molecular identification (Chapter I). To assess the genetic similarity between pair of individuals at several distance classes, we also conducted a spatial autocorrelation analysis. The distance class in kilometers for all tests was 20 Km, and the number of distance classes was 50. The significance values were assessed using 9999 permutations and 95 % confidence intervals. A significant positive autocorrelation means that individuals at a given distance class are genetically more similar than aleatory expected. The Mantel test and the spatial autocorrelation analysis was done in the GENALEX v. 6.4 software (Peakall and Smouse, 2012).

After the genetic clustering analysis, the population was redefined according to the results obtained ($K = 1$), and the microsatellite loci were tested for linkage disequilibrium (LD) and Hardy-Weinberg equilibrium (HWE), using the exact test of Guol , and Thompson (1992) for heterozygote deficit in GENEPOP v. 1.2 (Raymond and Rousset, 1994). For both tests, we estimated p-values using the Markov chain methods with 10000 dememorization steps, 1000 batches, and 10000 iterations per batch. Sequential Bonferroni correction (Rice, 1989) was applied to correct multiple simultaneous comparisons at the significance level of $p \leq 0.05$.

To measure the current genetic diversity, we estimated the number of alleles (Na), effective number of alleles (Ae), observed (Ho) and expected heterozygosity (He) in GenAlex v. 6.5.0 (Peakall and Smouse, 2012). Allelic richness (AR, Leberg 2002), inbreeding coefficient (Fis, Weir and Cockerham, 1984) with the p-value for heterozygote excess (pL) and deficit (pS), were calculated for each locus using FSTAT v. 2.9.3.2 (Goudet, 1995). We calculated the polymorphic information content (PIC) using the Cervus 3.0.3 software (Slate et al., 2000).

Demographic changes

We measured the contemporary effective population size (Ne) using the linkage disequilibrium (LD) method (Waples and Do, 2010) and the jackknife resampling method to determine the effective population size with 95 % confidence intervals. We calculated this parameter using the NeEstimator 2.0 software (Do et al., 2013).

To assess recent signature of population size reduction, we used both the Wilcoxon test (Luikart and Cornuet, 1999) and M-ratio (Garza and Williamson, 2001). Wilcoxon test was done using the infinite alleles (IAM), stepwise mutation (SMM), and two-phase (TPM) mutation models in BOTTLENECK v. 1.2.02 (Cornuet and Luikart, 1996; Luikart and Cornuet, 1999). Wilcoxon test provides relatively high power to identity significative population size reduction signatures and can be applied to data sets with few polymorphic loci. For the TPM model, a variance of 30, probability of 90 %, and 1000 interactions were assumed. Genetic bottlenecks can also leave a signature in the ratio of number alleles to the allele size range (the M-ratio), where a bottleneck depletes the number of alleles faster than reducing allele size range of the microsatellite (Garza and Williamson, 2001). We calculated the M-ratio using $M = k/r$ formula, where k is the number of alleles and $r = S_{max} – S_{min} + 1$ (S_{max} is the size of the largest allele, and S_{min} is the size of the smallest allele in the sample). Following Garza and Williamson (2001), $M < 0.68$ indicates a bottleneck, while $M > 0.80$ indicates no reduction of effective population size.

Scenarios test and Demographic history

We investigated historical changes in the effective population size using Approximate Bayesian Computation (ABC) implemented in DIYABC (Cornuet, Ravigné, & Estoup, 2010). We assessed the population size changes on *M. tridactyla* population through the time, testing three different scenarios (Figure 3): (1) the *M. tridactyla* population size has been stable during the time (null hypothesis, Na = Nr; where Na is the ancestral effective population size, and Nr is the recent effective population size); (2) the *M. tridactyla* population experiencing a reducing in the population size at coalescent time t (bottleneck event, Nr < Na); and (3) there was an expansion that led to an increase in the effective population size of *M. tridactyla* (Na > Nr). In ABC, competing population scenarios are simulated, and statistical tests are then used to assess which scenario better fit the observed data. We performed one million simulations per scenario. The prior settings for all parameters (effective population size, time, and mutation rate) are in Table S3. The summary statistics employed were the mean alleles number, mean expected and observed heterozygosity and mean allele size variance. In the final, we had 30 summary statistics once which each microsatellite was considered a distinct group no run the analysis. Reliability of scenarios was visualized through Principal Component Analysis.

To obtain the best fit scenario, the posterior probability (PP) for each scenario was estimated by logistic regression on 1 % of the simulated dataset closest to the empirical data. For the scenario with high PP, we evaluated the confidence in choice scenario estimating the posterior predictive global error using 1000 pseudo-observed dataset for the logistic regression approach. To assess the precision for each estimated parameter, we calculated the relative median of the absolute error (RMAE) (Cornuet et al., 2010). At the end, the best model was tested by comparing the summary statistics (mean allele size variance and mean Garza-Williamson's M index) between the observed and simulated datasets.

Results

Population genetic structuring and genetic diversity

The PIC values were higher than 0.5 (07, 13, 11, 20, and A9 loci) for most loci, with a mean value of 0.53 (Table 1), indicating our multi loci panel are highly informative and adequate for performance population genetic analyses carried out here in *M. tridactyla*.

All Bayesian clustering approaches were agreeing to define a single genetic population for the individuals analyzed (Figure 2). Although K= 2 was obtained under the Evanno criterion (LnP (K) = -2,251.07 and ΔK= 2.69; Figure 2 A), the assignment individuals graphical showed similar probability of a given individual belong to one or another population (Figure 2B). One population also was inferred by the spatial analysis in GENELAND (K = 1, Figure 2 C and D) and the multivariate analysis (DAPC) displayed a pattern of no genetic differentiation between sampling sites (Figure 2 E).

The spatial autocorrelation analyses showed no significant autocorrelation between individuals in all measured distances ($p \leq 0.05$), even when females and males were separately analyzed (Figure S1,1a, 2a and, 3a). Mantel test showed no association of genetic and geographic distance, neither considering total individuals or each gender in separate (Figure S1, 1b, 2b and 3b). Therefore, all results were concordant, indicating that the genetic distance is not associated to geographic distance among the analyzed individuals.

The subsequent genetic analyses considering all individuals belonging to a single large population revealed no significant linkage disequilibrium, although deviation from HWE ($p \leq 0.05$ after Bonferroni correction) occurred in two loci, G3 showing heterozygote deficit, and H5 with heterozygote excess (Table 1). The data revealed evidence of allelic stutters or large allele dropouts (genotyping errors) in three loci (04, B2, and H5). The final analyses were made with the set of all microsatellites once no difference in the results was observed when these three latter loci were withdrawn.

The total number of alleles was 55 for the ten microsatellite loci in the 107 samples. The number of alleles/locus ranged from three (B2 and G3) to eight (11 and 20) with a mean of 5.5 and the mean of effective allele (Ae) was 3.10 (Table 1). Mean observed heterozygosity (Ho) was 0.54 (ranging from 0.05 to 0.84) and mean expected heterozygosity (He) 0.61 (ranging from 0.38 to 0.83). The Fis values ranged from -0.28 to 0.47, with a statistically significant mean value of 0.13 ($p \leq 0.05$).

Effective Population Size variation

The effective population size (Ne) estimate was 375.5 (CI = 80.2 - ∞; $p < 0.05$). Signs of population reduction were significant for the TPM model ($p = 0.0048$) in the Bottleneck analysis, and the *M*-ratio also showed a signal of population reduction ($M = 0.39$).

The scenario that best explained our data was scenario 2: indicating that the *M. tridactyla* experienced a reduction in the effective population size in the past. This hypothetical scenario showed a posterior probability of 0.8339 with the posterior error rate 0.304 (Table S4). All our RMAE values was RMAE < 2 (Nr = 0.191, Na = 0.316 and t = 0.293) indicating that all parameters estimated were reliable values, suggesting a high confidence for scenario 2 (Figure 3). At this scenario, the effective population size Nr and Na had average values of 1119 (95 % CI = 660-2040) and 6370 (95 % CI = 2130 - 9830), respectively. When we applied the model checking (Figure S2), we observed that our best scenario has a good fit because the observe data set appears under the posterior predictive distribution. Thus, the possible past reduction occurred around 680 years ago using the 4 years of generation time (Desbiez et al., 2020; Nowak, 1991).

Discussion

Our different analyses concordantly showed absence of population structuring in *M. tridactyla* across the large area studied, contrary to our hypothesis that fragmented populations would be expected in the landscape studied. These findings are indicating the giant anteater has been able to move throughout the studied area. Considering that our sampling sites encompass wetlands, savanna, and forest, our results suggest that gene flow among the local populations is not restricted by these biomes, at least in the studied area. This large and single population of giant anteater in this portion of its distribution area has been already suggested by a previous mitochondrial study, in which the animals of this region were recovered in a single lineage, but different from the Amazon lineage (Clozato et al., 2017). Moreover, our results also suggest that the recent human-promoted landscape modifications are not limiting the gene flow, perhaps because these human actions have yet no time to leave any signature of population isolation and differentiation among these animals.

It is well known the giant anteater demonstrates different ranges of movement throughout the Pantanal landscape from 1 km/day (Medri and Mourão, 2005) to 8 km/day in Cerrado Biome (Bertassoni, 2010). It is likely that the absence of population structuring observed can be explained by this life-trait and biology of the species. Giant anteater has been observed from highly conserved areas to anthropogenic areas, such as agricultural fields and wood plantations of *Pinus* sp., *Acacia* sp., and *Eucalyptus* sp (Miranda 2004, Braga 2010, Vynne et al., 2011) and therefore, being considered a species associated with several environments. In general, species associated with non-forested habitats may more easily cross the matrix and move between fragments, thereby reducing the negative effects of fragmentation-like genetic differentiation (Schlaepfer et al., 2018). Both Mantel test and spatial autocorrelation analyses showed no isolation by distance among the studied individuals, even when the gender was analyzed separately. This suggests both sexes present similar patterns of dispersion, that individuals have been able to move across the modified landscape, and that they are likely reproducing throughout the area as reported by Miranda (2004) and Alves (2003).

The results of our investigation into this large and single population of *M. tridactyla* showed moderate levels of genetic diversity (Ho = 0.54; He = 0.61), similar to all previous studies that were carried out in other local populations in, or surrounding, conservation areas (Ho = 0.68, He = 0.72, Sartori, 2018; Ho = 0.60, He = 0.63, Garcia et al., 2005). Although we found a Na = 5.5, the effective number of alleles (Ae = 3.10) suggests that fewer alleles are contributing to maintain the found genetic diversity. This deviance between Na and Ae can also be a consequence of an Ne that is not large enough to retain all alleles in high frequency, since large or infinite Ne is necessary to retain more genetic diversity (Kimura and Crow, 1964). Genetic diversity has important ecological consequences in natural populations, including the maintenance of evolutionary potential and an individual's ability to survive in response to threats such as environmental change and disease (Hughes et al., 2008). The combination of increased random genetic drift, inbreeding, and reduced gene flow may substantially reduce the genetic variation of populations (Lino et al., 2019; Schlaepfer et al., 2018). Lower genetic diversity has already been described in an anteater population inhabiting a protected area (Ho = 0.059, He = 0.482) and it was associated with intense population reduction after recurrent fires, resulting in inbreeding within the remaining individuals (Collevatti et al., 2007).

The moderate genetic diversity found here suggests a certain level of genetic health of this population, but this needs to be carefully analyzed because the population showed a significant inbreeding coefficient value (F_{is} = 0.13). An increased degree of homozygosity may cause the expression of deleterious recessive alleles, which can decrease individual fitness (Reed and Frankham, 2003). Additionally, the effective population size is an important factor that contributed to genetic variability maintenance because the heterozygosity and number of alleles are less impacted in populations with large size effective population (Kimura and Crow, 1964; Reed and Frankham, 2003). It is known that effective population size vary with the generation time (Frankham, 1997; Reed and Frankham, 2003). A long generation time and lifespan can act as an intrinsic buffer against loss of genetic diversity (Hailer et al., 2006), resulting in a late detection of genetic diversity loss. In this case, the giant anteater lives from 20 to 30 years in captivity and has long generation time (Nowak, 1991), affecting the genetic diversity more slowly. In species showing one-year generation time, it is believed that N*e* = 50 is enough to avoid the negative effects of inbreeding in the short term and N*e* = 500 to prevent loss of variability by genetic drift in long term (Franklin, 1980; Soulé and Wilcox, 1980). An effective population size N*e* ≥ 1000 was indicated for retaining the evolutionary potential for fitness in perpetuity (Frankham, 2015). Our results indicated about 375 individuals present in the survey area, a relatively high effective population size potentially extant in the Center-Western region, highlighting the importance of this population for long term conservation of *M. tridactyla*. However, our demographic analyses suggested that the current *M. tridactyla* population has already suffered a recent bottleneck. The decreases in effective population size and the evidence of inbreeding indicated that part of the giant anteater genetic diversity that existed in the past has been lost.

For the first time, the demographic history of *M. tridactyla* population was addressed, and it was found that a past reduction of population size probably happened due to human expansion and land-use change in this part of the South American continent (Chaïr et al., 2011; Ripple et al., 2016). Our inference from ABC analysis showed a good correspondence between the early colonization of this territory (around five centuries ago) and the predict past population size reduction (680 years). The time estimated for this reduction response to the expansion of human civilization in the area and different anthropogenic pressures such as hunting and agriculture (Fahrig, 2003; Klink and Machado, 2005). In addition, the intensification of these human activities in the recent decades explains the more recent bottleneck and the potential ongoing inbreeding observed. For hundreds of years, the continuous impact of humans on nature has been noted in decrease of the abundance and richness of plants and animal resources (Galetti and Dirzo, 2013), and genetic consequences of these actions can be already threatening the long-term viability of *M. tridactyla*.

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Tables and Figures

Table 1. Summary information on the ten microsatellite loci used in *Myrmecophaga tridactyla*. Loci name, number of individuals (N), number of alleles per locus (Na), effective number of alleles (Ae), allelic richness (AR), observed and expected heterozygosity (Ho and He, respectively), global estimate of F_{is}, results for the Null Allele test, polymorphic information content (PIC), and the Garza-Williamson index, the number of alleles ratio to range in allele size (M-ratio). Bold values $p \leq 0.05$ after Bonferroni correction to Hardy-Weinberg equilibrium (HWE).

Loci	N	Na	Ae	AR	Ho	He	p-values	F_{is}	Null Alleles	PIC	M-ratio
4	107	4	2.06	3.99	0.27	0.51	0.0162	**0.47**	0.3243	0.42	0.44
7	74	9	6.03	9.00	0.84	0.83	0.4102	0.00	0.0028	0.81	0.52
13	98	6	3.56	6.00	0.82	0.72	0.9815	**-0.13**	0.0148	0.68	0.46
11	96	8	3.02	7.49	0.64	0.67	0.0087	0.05	0.0673	0.63	0.22
20	97	8	5.56	7.76	0.80	0.82	0.4610	0.02	0.0116	0.8	0.47
A9	82	5	3.25	4.99	0.56	0.69	0.0052	**0.19**	0.0944	0.64	0.55
B2	98	3	2.08	3.00	0.61	0.52	0.9745	-0.18	0.1049	0.46	0.6
E3	99	4	1.61	3.75	0.37	0.38	0.1298	0.01	0.0173	0.35	0.26
G3	83	3	1.80	3.00	0.46	0.44	**0.0001**	-0.03	0.0262	0.37	0.08
H5	79	5	2.04	5.00	0.05	0.51	**0.0000**	**0.90**	0.8209	0.47	0.45
Mean	91	5.5	3.10	5.40	0.54	0.61	----	**0.13**	----	0.563	----

Figure 1. Geographic location of *Myrmecophaga tridactyla* samples. Red lines represent the main Mato Grosso do Sul roads and black dots are each individual.

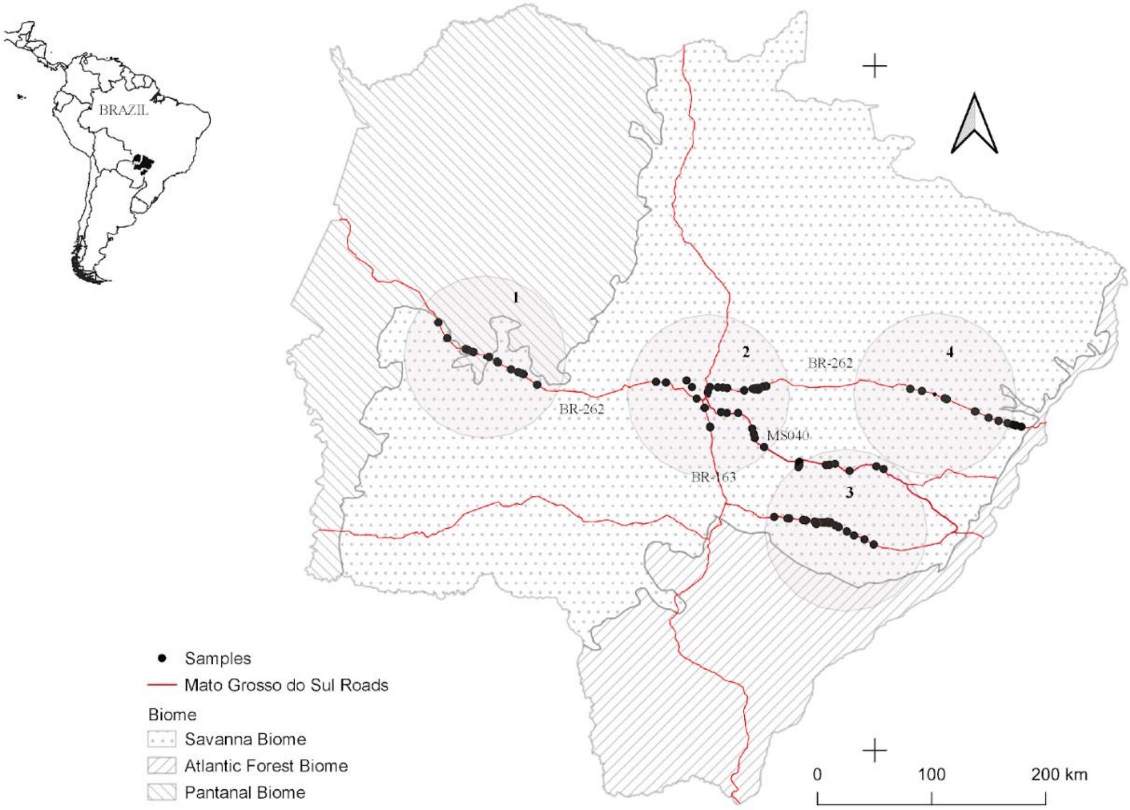


Figure 2. Genetic structure of 107 *Myrmecophaga tridactyla* specimens as assessed by different approaches based on ten microsatellite loci. **A.** Population structure results (K = 2) based on the mean likelihood Ln (K) and the ΔK statistic (Evanno et al., 2005). **B.** Graphical representation of K = 2. Each vertical bar represents an individual and each color (light grey and dark grey) represents the posterior probability of the individuals belonging to that cluster. **C.** Plot of the number of populations simulated (K=1) from the posterior distribution with GENELAND. **D.** the number of clustering among the chain. **E.** Results of the of discriminant analysis of principal components (DAPC) showing the scatterplot of the first two principal components.

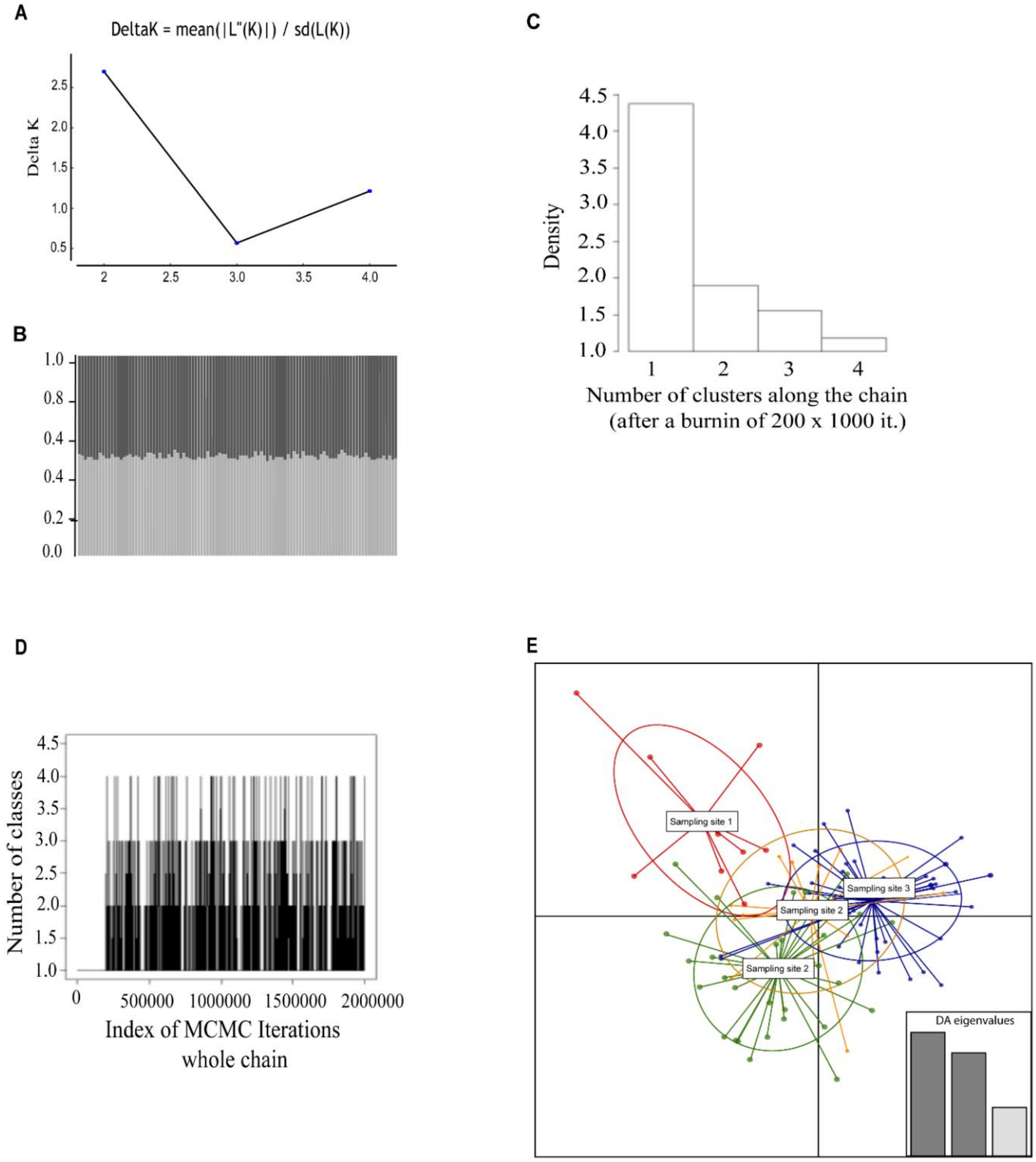
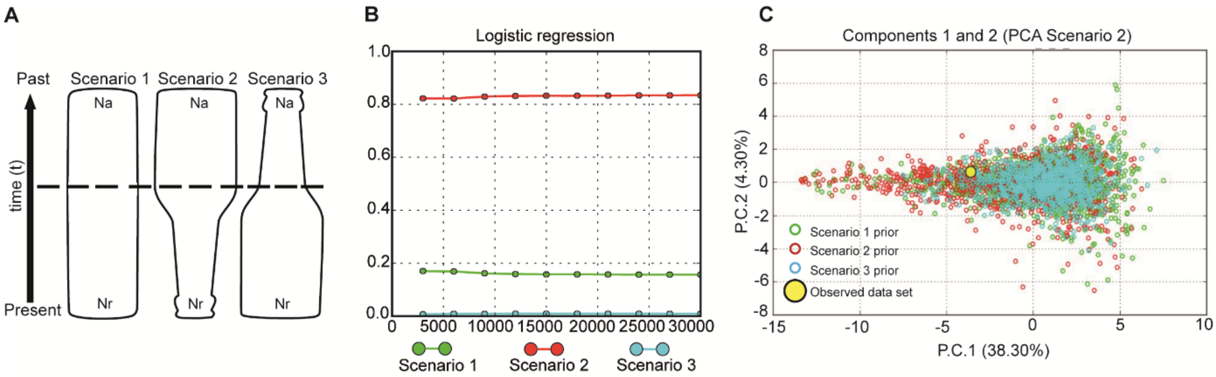


Figure 3. Possible demographic history scenarios for the *Myrmecophaga tridactyla* population. **A.** Representation of three demographic scenarios evaluated by DIYABC. Legend: the areas of the figures represent changes in population size through time. Effective population size (Ne) is represented by Na (ancestral effective population size) and Nr (recent effective population size). The time, t, in number of generations. Scenario 1 without a change in an ancestral population experiencing (null hypothesis); Scenario 2 with a change in Na at t time, representing a bottleneck event, Nr < Nr; and Scenario 3 with a change in Ne at t time, representing an expansion event, Nr > Na. **B.** Posterior probabilities of the three scenarios obtained by logistic regression of 1 % of the closest simulated datasets. the most probable demographic scenario for *M. tridactyla* population was a historical bottleneck. Posterior probability of each scenario in the y-axis and the x-axis indicates the number of simulated data closest to observed data. **C.** Graphic of Principal components analysis (PCA) generated in DYABC displaying the fit between scenarios simulated and our dataset.



Supplementary Material

Table S1: Information about 107 *Myrmecophaga tridactyla* specimens analyzed in this study. For each an individual, (ID - sample codes) we provided the following information: Sex (M-male, F- female), CD (collection date) and localities (geographic coordinates).

ID	**Sex**		**CD**	**Latitude**	**Longitude**
360	M	Roadkill	April, 2013	-20.8099701410031	-54.5183188857456
554	M	Roadkill	April, 2017	-20.6836404823444	-54.4231651812123
574	M	Roadkill	April, 2017	-21.6177577529032	-53.6918530629973
581	M	Roadkill	April, 2017	-20.2490981059027	-56.3553225935550
583	M	Roadkill	April, 2017	-21.9400850000000	-53.3132610000000
404	M	Roadkill	Dez, 2013	-20.1673224597728	-56.5573091854478
407	M	Roadkill	Dez, 2013	-20.3506323304339	-56.1424640582395
414	M	Roadkill	Fev, 2014	-21.9497602292131	-53.3161079518503
415	M	Roadkill	Fev, 2014	-20.7951707843327	-51.8946594167791
417	M	Roadkill	Fev, 2014	-20.3577399265777	-56.1271914299720
478	M	Roadkill	Fev, 2017	-21.6148799027978	-53.7161476611256
490	M	Roadkill	Fev, 2017	-20.4726345526258	-54.4095853414892
409	M	Roadkill	Jan, 2014	-21.6005080507981	-53.8509697659370
476	M	Roadkill	Jan, 2017	-20.6836404823444	-54.4231651812123
653	M	Roadkill	July, 2017	-20.4978644467484	-54.2240606560014
622	M	Roadkill	June, 2017	-21.1379850000000	-53.4910890000000
638	M	Roadkill	June, 2017	-20.6778548545702	-52.2359984793259
363	M	Roadkill	May, 2013	-19.9122775452033	-56.8609825659532
585	M	Roadkill	May, 2017	-20.7595639375226	-52.0301198747727
587	M	Roadkill	May, 2017	-20.8736330000000	-54.1415840000000
588	M	Roadkill	May, 2017	-21.1371003772023	-53.4843089013385
510	M	Roadkill	Mar, 2017	-21.6017569245764	-53.8360039244728
859	M	Roadkill	Mar, 2018	-20.5730450000000	-52.4781540000000
401	M	Roadkill	Nov, 2013	-20.4887906125484	-54.1362237957641
760	M	Roadkill	Nov, 2017	-20.4766930000000	-54.3704190000000
387	M	Roadkill	Oct, 2013	-21.6276007575350	-53.6170907192403
390	M	Roadkill	Oct, 2013	-20.1662898908932	-56.5599437446914
393	M	Roadkill	Oct, 2013	-20.7329769183708	-52.1166038355525
394	M	Roadkill	Oct, 2013	-20.7590069985773	-52.0325943103135
704	M	Roadkill	Oct, 2017	-21.7815100000000	-53.1876000000000
384	M	Roadkill	Sep, 2013	-20.1404902567460	-56.6260453767553
386	M	Roadkill	Sep, 2013	-20.6797022696362	-52.2315974568632
418	M	Roadkill	Sep, 2013	-21.6017569245764	-53.8360039244728
757	M	Roadkill	Sep, 2017	-20.9074288693110	-54.1335056613154
366	F	Roadkill	May, 2013	-20.7794692938385	-51.9547930832546

375	F	Roadkill	July, 2013	-21.7476849755710	-53.2776027716776
398	F	Roadkill	Nov, 2013	-21.6422679481749	-53.4911107346586
485	F	Roadkill	Fev, 2017	-21.5881271911959	-53.9653735773085
503	F	Roadkill	Mar, 2017	-20.1468345900431	-56.6097447035794
509	F	Roadkill	Mar, 2017	-21.7133556457266	-53.3396968290985
512	F	Roadkill	Mar, 2017	-20.4840520246359	-52.7928715391113
556	F	Roadkill	April, 2017	-21.6148799027978	-53.7161476611256
560	F	Roadkill	April, 2017	-20.3397304970204	-56.1733133081723
572	F	Roadkill	April, 2017	-20.6915878177540	-54.3742695738243
642	F	Roadkill	June, 2017	-21.1396145170700	-53.5206722356191
767	F	Roadkill	Nov, 2017	-21.8256340000000	-53.1078500000000
778	F	Roadkill	Nov, 2017	-21.1523698703842	-53.0850531255658
851	F	Roadkill	Fev, 2018	-20.7908450000000	-51.9107120000000
858	F	Roadkill	Mar, 2018	-20.5027500000000	-52.6922870000000
364	NA	Roadkill	May, 2013	-20.8161302303806	-54.5172096264141
471	NA	Roadkill	Jan, 2017	-20.4840520246359	-52.7928715391113
553	NA	Roadkill	Mar, 2017	-20.4657879506312	-54.0589371950263
595	NA	Roadkill	May, 2017	-20.4234444444000	-54.9844722222000
472	M	Roadkill	Jan, 2017	-20.7969113980321	-51.8883090795178
504	M	Roadkill	Mar, 2017	-20.4301739928254	-54.8974364019231
552	M	Roadkill	Mar, 2017	-20.5683058875048	-54.6339596969699
648	M	Roadkill	July, 2017	-20.4115856051861	-54.7214056951203
659	M	Roadkill	Aug, 2017	-20.4686937000000	-54.6741233000000
699	M	Roadkill	Oct, 2017	-20.8266095000000	-54.1552321000000
843	M	Roadkill	Fev, 2018	-20.6839750000000	-54.4252210000000
846	M	Roadkill	Fev, 2018	-20.5112800000000	-54.5382180000000
507	F	Roadkill	Mar, 2017	-21.1746596917394	-53.0225098801902
640	F	Roadkill	June, 2017	-20.6916498850332	-54.2771147181092
646	F	Roadkill	July, 2017	-20.1440669643715	-56.6168417030044
690	F	Roadkill	Sep, 2017	-20.1781830000000	-54.9319920000000
664	NA	Roadkill	Aug, 2017	-21.6751157809633	-53.4103505987422
539	M	Capture	May, 2017	-21.1590166667000	-53.7572000000000
540	M	Capture	May, 2017	-21.1397833333000	-53.7491666667000
543	M	Capture	May, 2017	-20.8106868867092	-51.8349440158646
546	M	Capture	June, 2017	-20.4266418590310	-55.0502387084270
549	M	Capture	June, 2017	-21.1377440951418	-53.4890450590052
747	M	Capture	July, 2017	-21.1401355442286	-53.7526143723369
903	M	Capture	July, 2018	-21.1132703349788	-53.7497514584710
904	M	Capture	May, 2018	-21.6365541896063	-53.5084142349243
906	M	Capture	May, 2018	-21.6410170519682	-53.5923028620391
908	M	Capture	May, 2018	-21.6323548120860	-53.4820372770272
911	M	Capture	May, 2018	-21.6303646641536	-53.5039523060799
912	M	Capture	May, 2018	-21.6291261754413	-53.5052538642057

916	M	Capture	June, 2018	-20.4830423408509	-54.1511800383002
917	M	Capture	June, 2018	-20.4815936343173	-54.0948089718569
918	M	Capture	June,2018	-20.4049617945771	-53.8866298366285
919	M	Capture	July, 2018	-21.6321500000000	-53.5021000000000
928	M	Capture	Aug, 2018	-20.4056100000000	-53.9309000000000
929	M	Capture	Aug, 2018	-20.4258352000000	-53.9661497000000
537	F	Capture	May, 2017	-21.0524000000000	-53.9241333333000
538	F	Capture	May, 2017	-21.1396333333000	-53.7513000000000
541	F	Capture	May, 2017	-20.4978644467484	-54.2240606560014
547	F	Capture	June, 2017	-20.4613393195543	-54.0361968586558
551	F	Capture	June, 2017	-21.6751157809633	-53.4103505987422
745	F	Capture	July, 2017	-21.0463685492498	-53.9214667134571
905	F	Capture	May, 2018	-21.6409638462326	-53.5951312879058
907	F	Capture	May, 2018	-21.6608511915339	-53.4374575509752
909	F	Capture	May, 2018	-21.6405560000000	-53.4805560000000
915	F	Capture	June, 2018	-20.4912279034537	-54.1024929654041
920	F	Capture	July, 2018	-21.6283600000000	-53.5040800000000
921	F	Capture	July, 2018	-21.6342900000000	-53.5185700000000
922	F	Capture	July, 2018	-21.6316100000000	-53.5964300000000
930	F	Capture	Aug, 2018	-20.4609200000000	-54.0326500000000
931	F	Capture	Aug, 2018	-20.4264700000000	-53.9695900000000
932	F	Capture	Aug, 2018	-20.4742231000000	-54.1059025000000
933	F	Capture	Jan, 2019	-21.6423170000000	-53.5968370000000
934	F	Capture	April, 2019	-21.6444700000000	-53.5989100000000
935	F	Capture	April, 2019	-21.6504700000000	-53.6025700000000
TB28	F	Capture	May,2018	-21.6255900000000	-53.4877400000000
TB29	F	Capture	Oct,2019	-21.6315640000000	-53.4957860000000
F2	NA	Capture	2019	-21.6315640000000	-53.4957860000000
F3	NA	Capture	2019	-21.6315640000000	-53.4957860000000

Table S2. Microsatellite loci used in this study for *Myrmecophaga tridactyla*. Locus name, primer sequence R (reverse) and F (forward), the repeat motif, annealing temperature (T_a), size of alleles and references. All forward sequences were labeled with M13.

Locus name	Primer sequences 5'-3'	Repeat motif	T_a	Size range (bp)	Null Alleles	Reference
04	R-TGTCTTCTTTACTCAGTGCTCC F-TGTAAAACGACGGCCAGTGGGTCAGATATCCTAATGGG	$(GT)_9$	62	172-180	0.3243	Garcia et al., 2005
07	R-TGTGTCCTGTGAAGTAATGG F-TGTAAAACGACGGCCAGTAGGAGGATAAGATTAGGCAG	$(GT)_{42}$	60	285-301	0.0028	Garcia et al., 2005
11	R-TCACCTTCATTGGAGCTTC F-TGTAAAACGACGGCCAGTTGTCTCTGTGTTAGGGTTCTTC	$(GT)_{15}$	62	171-205	0.0148	Garcia et al., 2005
13	R-TGGTAAAGAATGAGGTC F-TGTAAAACGACGGCCAGTCTGCTCAGGTAACATTCC	$(GT)_{14}$	58	238-248	0.0673	Garcia et al., 2005
20	R-CTATATGCTTGCCTTTGG F-TGTAAAACGACGGCCAGTCTTTCCTCATATCTCCCTG	$(GT)_{14}$	60	168-184	0.0116	Garcia et al., 2005
A9	R-TGTGAGCCACTGATCGTGTT F-TGTAAAACGACGGCCAGTTCCAAGTCTCAGGTCCCAT	$(AC)_8$	60	157-194	0.0944	Clozato et al., 2014
B2	R-AATGGTGGGGCACTAAGATG F-TGTAAAACGACGGCCAGTCCTTTGGGTCCTGATTGAGA	$(TG)_9$	60	191-233	0.1049	Clozato et al., 2014
E3	R-TGCTTACGCGTGGACAAAT F-TGTAAAACGACGGCCAGTCACCACGACACCACACTACC	$(GT)_{23}$	60	104-134	0.0173	Clozato et al., 2014
G3	R-TGGACTAACTGGGCTTCTGC F-TGTAAAACGACGGCCAGTTGGACCCGCCATATAAACAT	$(GT)_{18}$	60	174-222	0.0262	Clozato et al., 2014
H5	R-CCACGTCACAATCACCT F-TGTAAAACGACGGCCAGTCCCGCAGTATAGAAGCAG	$(TC)_6$	59	214-216	0.8209	Clozato et al., 2014

Table S3. Descriptions of prior setting for all parameters used in DIYABC. Ancestral effective population size (Na), recent population effective size (Nr), and coalescent time (t; in generations: 4 years for *M. tridactyla*; Nowak, 1991).

Parameter	Min	Max	Parameter distribution
Effective population size			
Na	10	10000	Uniform
Nr	10	10000	Uniform
Coalescent time scale in generations			
t	10	10000	Uniform
Mutation model			
Mean mutation rate	$1x10^{-4}$	$1x10^{-3}$	-

Table S4. Summary of posterior probabilities of three demographic history scenarios evaluated in DIYABC analysis using microsatellite data.

Scenarios	1	2	3
Posterior probabilities	0.1568	0.8339	0.0092
Confidence intervals	0.1293-0.1844	0.8059-0.8620	0.0038-0.0146

Figure S1. Mantel test and spatial autocorrelation analysis results. In 1 (all individuals combined), 2 (40 females), and 3 (55 males). The correlograms (1a, 2a, and 3a) indicate the hierarchical spatial autocorrelation analysis showing the correlation coefficient *r* (solid blue line) as a function of geographic distance across defined spatial distance classes. Dashed red lines represent upper (U) and lower (L) bounds of the null hypothesis of no spatial structure based on 10000 random permutations. Error bars represent 95 % confidence intervals about *r* based on 1000 bootstraps. In 1b, 2b, and 3b, plotting pairwise codominant genotypic distance versus pairwise Euclidean distances (km) across all individuals, females, and males, respectively. Each point represents a pairwise comparison among individual giant anteater.

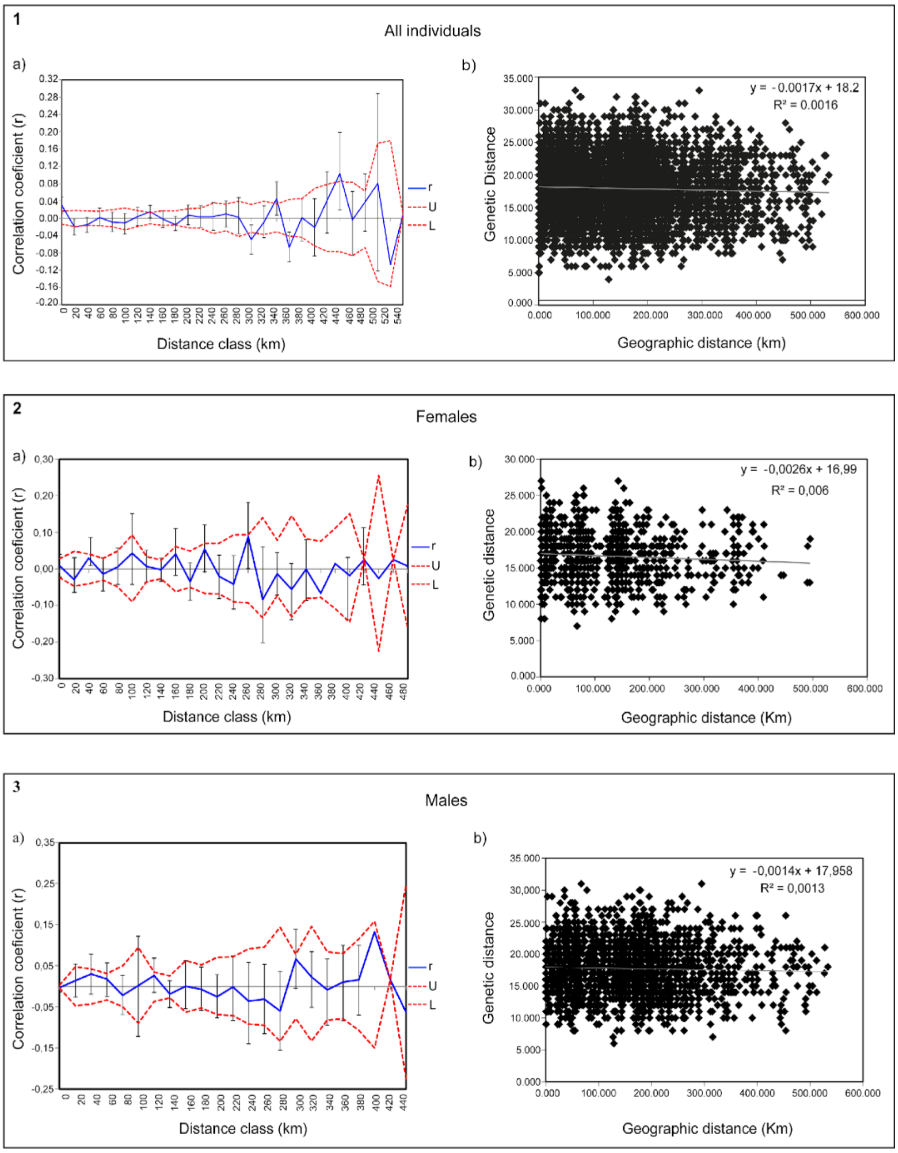
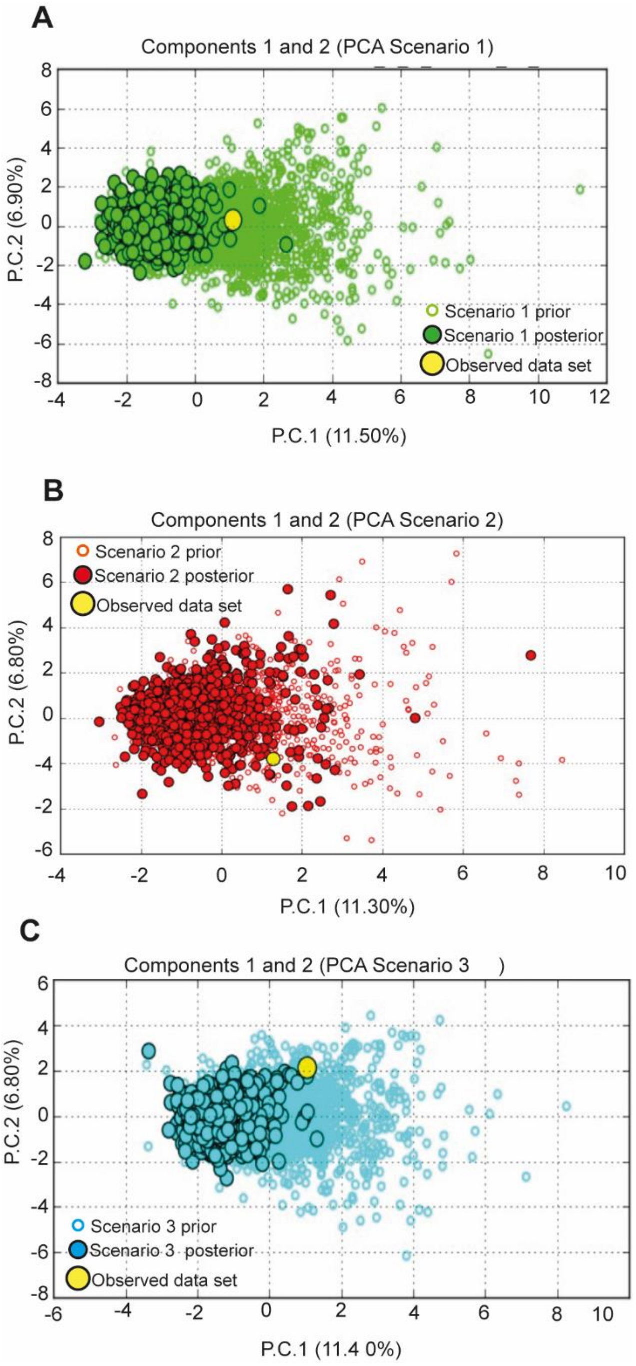


Figure S2. Model checking by applying a PCA on the best-supported scenario (scenario 2) in DIYABC analysis. **A.** Graphic of Principal components analysis (PCA) generated in DYABC displaying the process checking scenario 1 demographic scenario for *Myrmecophaga tridactyla*. **B.** Graphic of PCA to check the best scenario 2. **C.** Graphic of PCA to check the scenario 3.



7. CONSIDERAÇÕES FINAIS E CONCLUSÕES

Neste trabalho nós mostramos como diferentes áreas dentro da biologia (ecologia de transporte e genética) podem integrar-se para a avaliação populacional das espécies. Da mesma maneira, concluímos que o surgimento de pesquisas e estudos que avaliem e integrem métodos e conceitos para a conservação da biodiversidade são necessários para entender a dinâmica das populações e os impactos das alterações em espécies com algum risco de ameaça ou pouco conhecidas.

Também é importante ressaltar que a avalição feita aqui, mostra como as altas taxas de perda de habitat e fragmentação da paisagem, assim como elevado número de atropelamentos nos xenartros, junto aos poucos trabalhos reportados, torna-os modelos de estudo ideais para avalição de impactos. Ainda com relação a super ordem, é preocupante saber que dentre das 31 espécies de xenartros, somente cinco possuem análises genético populacionais e dessas somente duas foram feitas em uma espécie vulnerável. Portanto, os resultados apresentados neste trabalho através dos três capítulos suportam a ideia de que existem muitas informações a serem investigadas para as espécies e confirmam que os xenartros são altamente subestimados (ICMBIO, 2015; SUPERINA; LOUGHRY, 2015).

Primeiramente, o nosso protocolo de identificação molecular do sexo, a partir de amostras de indivíduos atropelados de várias espécies de xenartros, traz um nova perspectiva para os levantamentos de fauna silvestre atropelada. A nossa metodologia proporciona uma alternativa de identificação do sexo para espécies sem dimorfismo sexual aparente, muito comum dentro da super ordem dos xenartros. A sugestão de enzimas de restrição feita no trabalho, levanta informações uteis para identificação do sexo de várias espécies dentro da ordem e facilita a implementação da técnica em futuras pesquisas nas que se pretenda a identificação desta informação mesmo que com outro tipo de amostras biológicas.

A análise de ecologia de transporte feita no trabalho, utilizando a identificação do sexo dos indivíduos, só reforça as premissas levantadas por Grilo et al. (2020) fazendo-se mais evidente que o grau de impacto da mesma ameaça pode mudar entre espécies e até mesmo entre sexos da mesma espécie. Portanto, se observarmos o viés sexual de atropelamentos encontrado para ambas as espécies, assim como se considerarmos a avalição da influência da sazonalidade no número de atropelamentos de fêmeas das duas espécies, resultados não identificados em estudos feitos para xenartros anteriormente (ASCENSÃO et al., 2017, 2019), entendemos a importância dessas informações nas análises.

O viés da razão sexual de atropelamentos para machos de ambas as espécies, e significativamente maior para tamanduá mirim, em duas das três proporções de razão sexual testada, nos mostra o grande impacto das rodovias. Se pensarmos numa população ideal, com uma alta densidade populacional e com uma proporção sexual que se mantem ao longo do tempo equilibrada (um macho para uma fêmea; 1:1), e avaliarmos o impacto das proporções sexuais de atropelamento encontradas neste trabalho, encontramos um quadro populacional futuro pouco viável. Isso, dado que o viés de atropelamentos para machos nesse panorama, claramente estaria afetando o tamanho efetivo populacional futuro, assim como a densidade populacional da espécie, morrendo mais indivíduos do que nascem normalmente.

Por outro lado, diferente do que esperávamos, o levantamento das variáveis do entorno dos *hotspots* encontrados para ambas espécies não foram significativamente diferentes como esperado. Isso, poderia estar indicando que os atropelamentos estão sendo influenciados por outras variáveis que não foram testadas no trabalho, causando uma maior agregação de pontos de atropelamento em certos locais. No entanto, como era de se esperar devido à distribuição das espécies e o comportamento delas, o maior número de atropelamentos aconteceu em áreas abertas como Cerrado, aonde a proporção de vegetação foi inversamente proporcional a proporção de agricultura. Adicionalmente, nós esperávamos que os atropelamentos acontecessem principalmente em meses mais frios, conforme já foi descrito por Camilo-Alves e Mourão (2006), nos quais os indivíduos possuem maior atividade em dias com temperaturas mais baixas, descansando na floresta quando as temperaturas excediam os 30 ºC no Pantanal do Mato Grosso do Sul. No entanto, os nossos resultados indicaram pouca influência das variáveis climáticas nos eventos de atropelamentos registrados, e uma influência sazonal na mortalidade de fêmeas de tamanduás. Assim, esses resultados podem ser explicados por comportamentos de dispersão, movimentação entre fragmentos em procura de recursos alimentares e até processos de reprodução que ainda não foram descritos para as espécies (ASCENSÃO et al., 2019; FERREGUETTI et al., 2020; GARRIGA et al., 2017).

Apesar de termos avaliado como alto o impacto das matrizes antrópicas para os tamanduás, a avaliação genético populacional do tamanduá-bandeira, espécie ameaçada dentro dos tamanduás brasileiros, nos apresenta padrões genéticos populacionais já mostrados para outras espécies de habitat generalista, com plasticidade de movimentação entre ambientes heterogêneos, densidade populacional relativamente alta e tamanho efetivo alto (ANDRÉN; ANDREN, 1994; HAAG et al., 2010; SCHLAEPFER et al., 2018). A conectividade ainda mantida entre os pontos extremos da área estudada distantes uma da outra em aproximadamente 500 km, é evidência de um fluxo de indivíduos entre as áreas. Além do mais, a ausência de associação entre distância genética e geográfica, indica padrões semelhantes de dispersão para machos e fêmeas, contrário ao que foi encontrado para tamanduás bandeiras no Parque Nacional das Emas (COLLEVATTI et al., 2007) no qual houve um viés de dispersão para machos, sendo um dado também corroborado pela razão sexual de atropelamentos encontrados no capítulo II (igual ao esperado) para a espécie.

Em contraste a este panorama viável para tamanduá-bandeira, as análises demográficas mostraram que houve uma redução no tamanho efetivo populacional da espécie há aproximadamente 1000 anos. Adicionalmente, as análises mais contemporâneas (100-50 anos atrás) que avaliam a proporção de heterozigotos na população, indicaram do mesmo modo uma redução no tamanho efetivo da população. Por tanto, a redução populacional encontrada no tamanduá-bandeira em escalas de tempo diferentes, junto as pressões evolutivas constantes (deriva, seleção e coeficiente de endogamia) e impactos das matrizes antropogênicas, estão interferindo na viabilidade populacional da espécie. Apesar disso, uma análise de viabilidade populacional feita recentemente para a espécie (DESBIEZ; BERTASSONI; TRAYLOR-HOLZER, 2020), sugere que a mortalidade causada pelos atropelamentos não necessariamente leva a extinção das populações locais da espécie mas sim à redução na metade da taxa de crescimento na população. No entanto, é necessário levar em consideração que as análises feitas na avaliação da viabilidade populacional de tamanduá-bandeira, foram feitas sem dados genético populacionais; podendo causar um viés nos resultados encontrados.

Essas conclusões sugerem, por tanto, que o monitoramento de fauna atropelada depende não só de análises descritivas globais para os grupos mais atropelados, senão também requer de informações mais específicas acerca da dinâmica das populações desses grupos, que permitam a realização de medidas de conservação e mitigação de maior impacto. Um bom entendimento sobre a razão sexual de atropelamentos de uma população, assim como o conhecimento da influência de fatores ambientais para cada sexo, otimiza e melhora os resultados dos programas de monitoramento de fauna atropelada. Adicionalmente, contar com informações genético populacionais que permitam identificar panoramas passados, atuais e futuros acerca das populações afetadas por este impacto tornaria ainda mais eficiente os planos de conservação das espécies.

8. PERSPECTIVAS FUTURAS

Apesar de que nosso estudo ter levantado várias informações importantes para a família Myrmecophagidae, ainda são necessários outros estudos ecológicos dentro da super ordem Xenarthra. Surpreendentemente são poucos os estudos de ecologia que investigaram essas características, por exemplo, no tamanduá mirim. Até o momento não existem informações referentes a razão sexual, idade de maturidade, sistemas de acasalamento e incluso, densidade da espécie na maioria das áreas de ocorrência.

Embora a ecologia de transporte seja uma área relativamente nova dentro da ecologia e ainda esteja em crescimento, é importante que pesquisadores que trabalham com a área entendam a relevância das abordagens moleculares. Abordagens genéticas são altamente valiosas para os planos de conservação e têm sido pouco utilizadas na ecologia de transporte. Como foi evidenciado pelo nosso trabalho, é possível entender os impactos das rodovias desde diferentes ângulos (ecológicos, genéticos ou na interação das áreas). No entanto, o mais interessante e eficaz seria olhar para a combinação dos resultados obtidos a partir das análises nas duas áreas e responder de maneira mais precisa e funcional os efeitos. Da mesma maneira, embora as análises de ecologia de estrada tenham focado mais em responder padrões gerais dos atropelamentos para mamíferos e todas as espécies, é importante entender como mesmo dentro das espécies os padrões mudam. Isso, por causa de comportamentos que não se repetem as vezes nem entre indivíduos. O fato de termos realizado as análises utilizando o sexo dos indivíduos, realça essa limitação nas análises gerais, mas fortalece a necessidade de compreender mais a biologia das espécies. No entanto, somente com mais pesquisas abordando aspectos semelhantes poderemos corroborar se esses padrões se mantem para outras espécies.

No que diz respeito a genética populacional para tamanduás, é imprescindível caracterizar a diversidade genética das espécies para estudos de conservação. Inicialmente, devem ser realizados estudos de taxonomia integrada que esclareçam a filogenia da super ordem, para assim futuramente trabalhar com genética populacional. No entanto, é importante atualmente a obtenção de amostras biológicas que permitam a elaboração de pesquisas que utilizem ferramentas moleculares para responder padrões ecológicos. Assim, são necessárias as parcerias entre instituições que realizem monitoramentos de fauna atropelada com laboratórios de biologia molecular para a realização de estudos populacionais.

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