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ORIGINAL PAPER

Using DNA barcode to relate landscape attributes to small vertebrate roadkill

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Abstract Large vertebrates are the main focus of roadkill studies because their greater size facilitates taxonomic identification and the collection of statistical data. However, these studies fail to effectively include and identify small vertebrates and correlate roadkill events with the surrounding landscape. Here we showed the effectiveness of molecular data to identify small vertebrate roadkill, and we correlated landscape structure attributes with the location of roadkill for functional groups of varying mobility. The extraction of DNA from roadkilled individuals was followed by the amplification of two mitochondrial genes. We compared each DNA sequence to a database and used the highest similarity values for species identification. The species were classified according to their taxa and degree of mobility: birds, reptilia and amphibia with low and intermediate movement capability. After calculating the landscape attributes for each roadkill point, we used a competing model approach based on Akaike Information Criteria to determine which landscape variable best explained the occurrence of roadkills. Combining molecular and morphological characteristics, we identified 82.93% of the roadkilled animals. DNA barcoding allowed the identification of 310% more specimens than by morphological characteristics alone. Roadkilled birds with intermediate movement capability were strongly influenced by dominated areas by agriculture and sugar cane monocultures. Roadkilled reptiles with low movement capability were positively correlated with the presence of forest remnants, while those with intermediate movement capability seemed to be more

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frequent in heavily anthropized landscapes. We showed that molecular data is a powerful tool for precisely identifying small-sized roadkilled animals. Our results also highlight that different landscape structure attributes enable the prediction of roadkill occurrence along roads, which in turn allows us to identify roadkill hotspots and plan appropriate mitigation actions.

Keywords Functional groups · Species identification · Cytochrome oxidase I (COI) gene · 16S gene

Introduction

Each species adapts differently to changes in their natural habitat. During their movements between heterogeneous areas, they face composite matrix, urban areas and roads (Fahrig 2003; Gorman and Raffaelli 2008). While crossing the matrix, they are exposed to various threats such as hunting or predation (Laurance et al. 2009), disease transmission (Cleaveland et al. 2001) and a high roadkill probability when traversing roads (Forman and Alexander 1998).

Assessing roadkill effects on vertebrates

Roadkill has become an issue for the maintenance of biodiversity and gene flow, particularly for those species that need to disperse or have suffered population reduction. Jackson and Fahrig (2011) showed that although the barrier effect of roads contributes to the reduction of genetic diversity, a reduction in population by roadkills has a higher impact. Species are differentially affected by roadkill: (a) species that have intermediate movement capability and that use heterogeneous matrix have the greatest probability of being killed (Beebee 2013); (b) large-bodied mammals with low reproductive rates and high mobility species have been indicated as those most afflicted by road effects (Rytwinski and Fahrig 2012); (c) the degree of mobility of bird species was key in defining their susceptibility to traffic effects, with the more mobile species being at greater risk (Rytwinski and Fahrig 2012); (d) species that do not cross roads or altered areas present lower roadkill probability (Hawbaker et al. 2006); (e) roads and traffic negatively influenced small-bodied frogs as well as toads with lower reproductive rates during the early years of sexual maturity (Rytwinski and Fahrig 2012).

Studies on roadkill focus mainly on the number of animal hits, i.e. the rate of hits per segment of road per time. Analyses of these data have revealed the effects of road types on mortality and on the spatial distribution of roadkilled vertebrates (Clevenger et al. 2003), the ecological effect of roads (Coffin 2007), the barriers created by roads and their effects on genetic diversity (Balkenhol and Waits 2009; Sork and Smouse 2006). Some studies also evaluated the response of wildlife to crossing structures (Bager and Fontoura 2013). While a large number of small individuals/species suffer road effects (ex. Clevenger et al. 2003; Glista et al. 2008; Gomes et al. 2009; Kociolek et al. 2011; Langen et al. 2009; Ramp et al. 2005; Shepard et al. 2008), they have not received attention until recently, because the loss of their morphological characteristics due to roadkill events makes taxon identification difficult.

DNA barcoding contribution to roadkill studies

DNA barcoding become a powerful tool for bypassing the difficulty of identifying small-sized roadkilled animals quickly and accurately, which is crucial when morphological features are lost. In addition to accurate identification, the application of genetic markers using fauna samples from roadkill studies benefits the collection of information for conservation, e.g. the presence of rare and endangered species, or species-level identification when a large number of small individuals are hit (Clark et al. 2010; Klippel et al. 2015; Munshi-South 2012; Riley et al. 2006; Simmons et al. 2010; Taylor and Goldingay 2010).

Mitochondrial cytochrome c oxidase 1 (COI) gene is preferable for establishing the genetic barcode of species because its variety of phylogenetic signals has greater divergence between species than other mitochondrial genes, and it has been used to successfully identify new species (Hebert et al. 2004). Some studies have argued that there is no single gene for a universal barcode that pertains to all domains of life while also having sufficient divergence for species discrimination (Stoeckle 2003). The 16S gene is suggested as an alternative *locus* for vertebrate barcoding (Vences et al. 2005a, b) and is proposed as supplementary to the COI gene in molecular identifications (Smith et al. 2008).

Landscape as a roadkill predictor

After species identification, statistical models can explain the relationship between their most frequent points of collision and the surrounding landscape. If we are able to predict the positions along the road where species are more prone to be killed due to the surrounding landscape structure of these positions, we can build a predictive model for estimating the roadkill probability of a species. We can then use this information to identify potential roadkill hotspots; such information is important not only for those road segments where we have roadkill information, but also for other segments without data (Clevenger et al. 2003; Laurance et al. 2009; Rytwinski and Fahrig 2012). The emphasis is on *potential hotspots*, because we are also interested in identifying areas without data, which is the case of many roads worldwide. Differences among landscapes and among species' movement capabilities warrant the use of functional groups (Fahrig and Rytwinski 2009) in models and allow a new perspective for data analysis.

In this study, we (a) used DNA barcoding to identify roadkilled species whose morphological characters were lost, and (b) correlated the location of roadkill species grouped by movement capacity, by the surrounding landscape structure. We predicted that the DNA barcoding method would contribute significantly to species-level identification of small-sized animals by distinguishing an increased number of carcasses, and that more roadkills would correlate to the surrounded landscape structure attributes, but that these attributes would vary depending on the species or their movement capacity. More specifically, we expected that: (1) DNA barcoding would significantly increase the number of identified individuals when compared to morphological identification; (2) all species would be equally identifiable by DNA barcoding, independent of the taxon, degree of mobility or gene used; (3) birds, reptilia and amphibia would be the taxa killed more frequently compared to mammalia; (4) species of intermediate mobility would have greater probability of being killed than those of low mobility, independent of the taxon; (5) the variety of landscape elements (forest, sugarcane and agriculture, urban, water body and swamps, forestry and pasture) surrounding the roadkill position would significantly contribute to the predictability of roadkills for each taxon and mobility level, but the relative contribution would vary between landscape elements, taxon and mobility; (6) roadkill frequency to be higher in areas of natural habitat (forest, water body and swamps) and lower adjacent to anthropogenic landscapes (urban, forestry, agriculture and pasture).

Methods

Study area and data collection

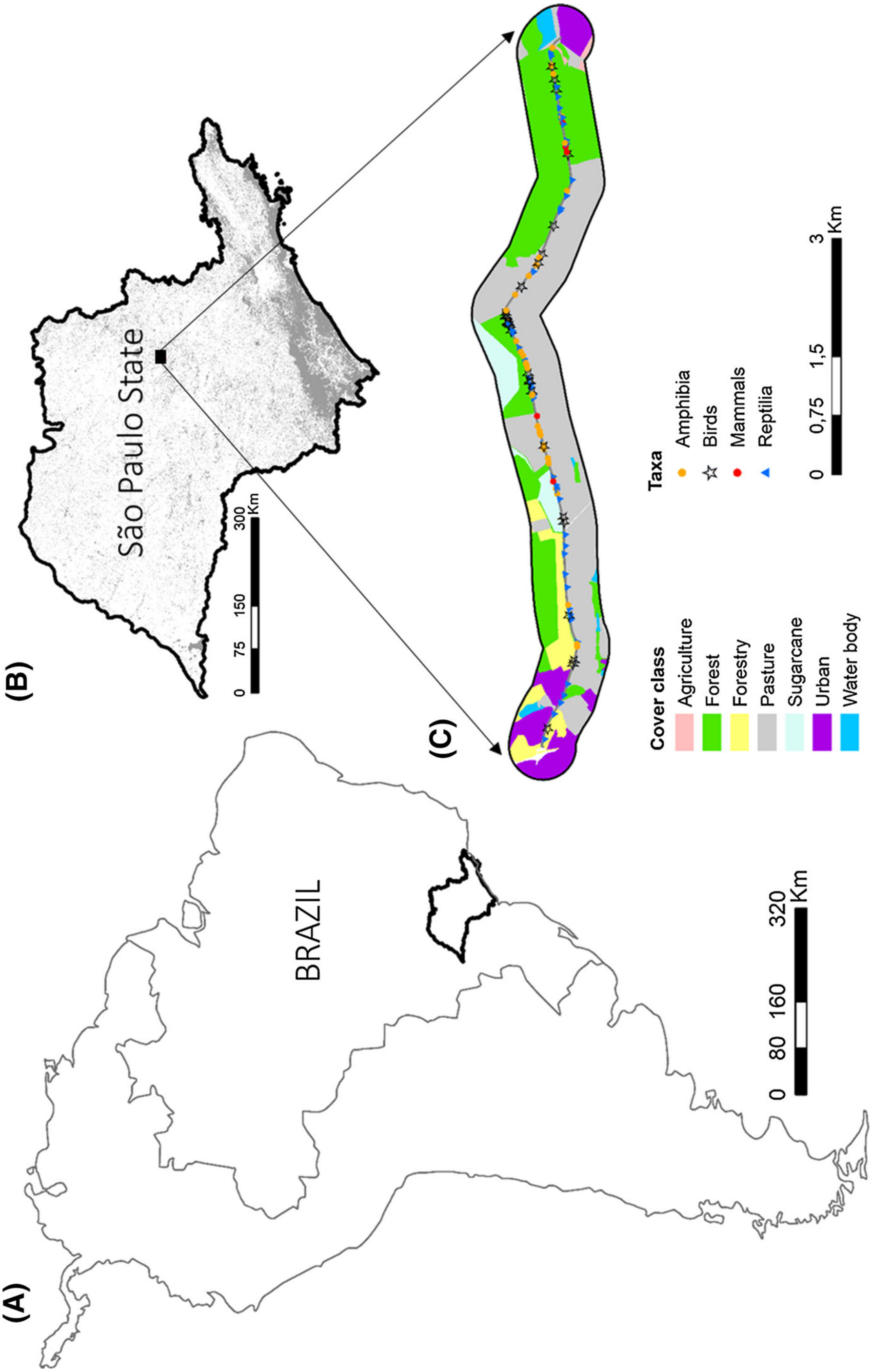
We collected roadkilled individuals on the Guilherme Scatena road (SCA-010, São Carlos-SP, Brazil), a single two-way road with a car speed limit of 60 km/h ($\sim$37 mph). The road is located between 21°59′27″S and 21°54′55″S latitude and, 47°52′57″W and 47°48′57″W longitude (Fig. 1), at an elevation of 750 m.a.s.l. Cerrado—a typical Brazilian savanna—is the predominant original vegetation in the region, which exhibits a high rate of species endemism (Klink and Machado 2005; Myers et al. 2000). Currently, however, the interior of São Paulo presents a high portion of human-modified lands and consequent habitat loss and fragmentation, with only about 7% of the vegetation remaining in the region (Ribeiro et al. 2009). The climate presents two dominant seasons throughout the year: a wet summer and a dry winter.

Using a motorcycle, a 12-km stretch of the road was searched for roadkill twice daily for 20 weeks during the summer (10) and winter (10) of 2010–2011. For each roadkilled individual discovered, we recorded its geographic coordinate using a GPS device and collected a tissue sample, preserving it in absolute ethyl alcohol. Every roadkill was photographed at the locale and properly transported to the laboratory. A specialist morphologically identified each sample to the most detailed taxonomic level possible.

Molecular identification and species classification

Total DNA of each sample was extracted according to the methodology described by Sambrook et al. (1989). We did PCR analysis in 25 µl for molecular identification of the samples (50 ng of DNA; buffer tris–KCl 1× [Tris–HCl 20 mM pH 8.4 and KCl 50 mM]; 2.0 mM of $MgCl_2$ [50 mM]; 0.3 µM of each primer; 0.25 mM of dNTPs; and 0.5 unit of *Taq* DNA Polymerase Platinum [Invitrogen]). We initially amplified the pair of universal primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al. 1994) in order to amplify a fragment of mitochondrial cytochrome c oxidase subunit I (COI) gene (670 base pairs, bp). We used 16SarL (5′-CGCCTGTTTACCAAAAACAT-3′) and 16SbrH (5′-CCGGTCTGA ACTCAGATCACGT-3′) primer pair (Palumbi et al. 1991) to amplify the mitochondrial 16S gene (550 bp). Both mitochondrial genes were used to amplify all samples and compare results.

PCR conditions for COI included 4 min denaturation at 94 °C; 5 initial cycles of 4 min at 94 °C; 90 s at 47 °C; 60 s at 72 °C; 40 additional cycles of 60 s at 94 °C; 90 s at 50 °C; 60 s at 72 °C; and elongation for 5 min at 72 °C. We conducted 16S PCR for 5 min denaturation at 94 °C; 30 cycles for 30 s at 94 °C; 45 s at 57 °C; 45 s at 72 °C; and a final extension for 10 min at 72 °C. All amplifications were checked on 2% agarose gel, including the negative controls and molecular weight marker. We purified PCR products with polyethylene glycol, following the protocol proposed by Sambrook and Russell (2001). For the obtained fragments, we used direct sequencing by the chain-termination


(A)
BRAZIL
0 80 160 320 Km
(B)
São Paulo State
0 75 150 300 Km
(C)
Cover class
Agriculture
Forest
Forestry
Pasture
Sugarcane
Urban
Water body
Taxa
Amphibia
Birds
Mammals
Reptilia
0 0,75 1,5 3 Km

◀**Fig. 1** Roadkill points distribution of birds, reptilia, amphibia and mammals on a road segment and land cover types within highly fragmented and heterogeneous landscape, Guillerme Scatena Municipal Road, São Carlos-SP, Brazil. **a** Location of São Paulo State within Brazil and South America; **b** remaining vegetation of São Paulo State and the location of study area; **c** land use and land cover map within a buffer of 500 m around the studied road, where the roadkills for taxa area presented

reaction technique (Sanger et al. 1977) in the automatic sequencer ABI 3730xl DNA Analyzer. When necessary, the samples were repeated at least twice to ensure sequence accuracy and clear electropherograms.

The sequences were edited using the electropherograms and were aligned according to the Clustal method (Thompson et al. 1994) using the BioEdit software (Hall 2004). We compared each sequence to the databases of the National Center of Biotechnology Information (NCBI), using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) and, for the COI gene, also using the Barcode of Life Data Systems database (BOLD). To prevent false identification comparisons, each gene was independently compared to each database. To establish the most likely species of each individual, we searched for sequences showing the highest similarity value, considering only values greater than 98%. The most likely species was then compared to the photographic record made during data collection. We analyzed the species occurrence in the study area using the lists of species recorded in Brazil (Dos Reis et al. 2011; Sawaya et al. 2008; SBH 2010; Souza 1998). When a conflict emerged between COI or 16S species identification, or when similarity values on databases were lower than 98%, we identified to a level higher than species (genus, family, order). To identify the statistical difference between the number of individuals identified by morphological characters or by DNA barcode, we used a Chi square test, expecting that the DNA barcode and morphological characters would have the same resolution to identify species.

After species identification, we classified them according their taxa (birds, amphibia, reptilia or mammalia) and then according to their functional group, which considers the mobility of species. To determine each species' functional group based on their daily movement capability, we referred to information from species specialists and from the literature (Forester et al. 2006; Indermaur et al. 2009; Perry and Garland 2002), or to their taxonomic classification when species identification was not possible. Depending on the movement capability, we define two groups: low movement capability, for radius <100 m of landscape influences around roadkills; and intermediate movement capability, for radius >200 m of landscape influence (Table 1). The spatial extent, or buffer size, that should be considered as a scale of influence in a landscape ecology study has not been firmly established and often depends on the specialists' opinion. The species' natural history, the context in which observations are made and, in some cases, the ecological process or response variable of interest are important considerations when setting the buffer size. For example, when studying the variability of spatial patterns of urban-based landscape structure to predict urban bird diversity in the city of Belo Horizonte, Brazil, J. Pena (personal comm.) ascertained 160 m as the best spatial extent. (Boscolo and Metzger 2011) evaluated the influence of different spatial extents on forest-dependent occurrence within Brazilian Atlantic Forest and observed that the buffer sized varied from 600 to 1000 m, depending on the species of interest. However, no multiple-scale analysis was done here, because it is beyond the scope of present study. Regarding the functional groups, many studies have used this approach for landscape-based research, though the method of species classification varies: birds classified according to their sensitivity to fragmentation [low, medium and high] (Martensen et al. 2012); mammals classified based on their

Table 1 Taxonomic classification and functional groups according to movement capability of the road-killed individuals surveyed at Guilherme Scatena Municipal Road, São Carlos-SP, Brazil

Class	Species		No. of samples	Movement capability
Birds	*Synallaxis frontalis*[c]		1	Intermediate
	Falco sparverius		1	
	Coryphospingus cucullatus		5	
	Turdus amaurochalinus		1	
	Aratinga leucophthalmus[b]		1	
	Basileuterus culicivorus[c]		1	
	Volantinia jacarina[c]		3	
	Guira guira		1	
	Columbina talpacoti		2	
	Synallaxis albescens[c]		1	
	Turdus sp.		1	
	Furnarius rufus[c]		1	
	Caprimulgus parvulus[c]		2	
	Zonotrichia capensis[c]		2	
	Phaethornis pretrei[c]		1	
	Sporophila caerulescens		1	
	Crypturellus parvirostris[b]		1	Low
	Gallus gallus[c]		1	
	Cariama cristata[b]		2	
	Nothura maculosa[c]		1	
Reptilia[a]	Amphisbaenia	Amphisbaenidae[c]	9	Low
	Lacertilia	*Ameiva ameiva*[c]	8	Low
		Lizards	5	
		Mabuya dorsivittata[c]	1	
		Tupinambis merianae	3	Intermediate
	Squamata	Snake	4	Intermediate
		Oxyrhopus guibei[b]	9	
		Bothrops neuwiedi[c]	1	
		Micrurus altirostris[b]	1	
		Crotalus durissus	4	
		Sibynomorphus mikanii[c]	2	
		Boa constrictor	1	
		Philodryas patagoniensis[c]	1	
Amphibia	*Bufo schneideri*[c]		2	Intermediate
	Hyla albopunctata[c]		2	Low
	Scinax fuscovarius[c]		20	
	Aplastodiscus cochranae[c]		1	
	Leptodactylus fuscus[c]		6	
	Physalaemus nattereri[c]		2	
	Physalaemus cuvieri[c]		2	
	Physalaemus centralis[c]		1	
	Hyla faber[c]		1	
	Leptodactylus sp.		1	

Table 1 continued

Class	Species	No. of samples	Movement capability
Mammalia	Carnivora	1	High
	Oligoryzomys flavescens[c]	3	Low
	Mus musculus[c]	1	
	Cabassous unicinctus	1	
Total		123	

[a] Additional classification within Reptilia, following the taxonomic classification in suborder (Lacertilia, Squamata and Amphisbaenia)

[b] Identified species using only morphological characters

[c] Identified species (or family, only in the case of Amphisbanidae) using only molecular characters

fragmentation sensitivity and mobility for roadkill analysis [low mobility sensitive, high mobility generalist, high mobility sensitive] (Ciocheti 2014; Ciocheti et al. in review). We excluded from the analysis mammals and amphibians with intermediate movement capability and birds with low movement capability, because the sample sizes (6, 2 and 5, respectively) were insufficient to perform statistical analyses.

Land cover mapping and landscape metrics

We plotted data points obtained by GPS on a QuickBird image (DigitalGlobe 2007) and Google Earth images (www.earth.google.com) using the Quantum GIS software (www.qgis.org). Through manual classification of Google Earth maps (1:10,000 scale), we classified the study area according to land use and vegetation cover—Agriculture and Sugarcane, Forest, Forestry (commercial eucalypt plantations), Pasture (for livestock), Urban Areas and Water Body (Fig. 1)—with a final resolution of 5 m. First, polygons were manually digitalized at a scale of 1:5000 to ensure optimal definition of the boundaries of each landscape element (cover classes). The polygons were then manually associated with the land use and vegetation cover described above, by visual inspection. For small extent mapping, which is the case in our study, manual classification is superior to digital image processing and automated classification techniques (Da Silveira et al. 2016; Mendes et al. 2015; Muylaert et al. 2016).

Landscape metrics calculation

Using the land use and vegetation cover map and Arc Map 9.1 (ESRI 2007), we calculated the percentage of each cover class around each pixel. These percentage values were calculated based on the two spatial extents (100 and 200 m) that were used in the analysis of functional group mobility (low or intermediate movement capability). The input map for the calculation cited above used a 5-m spatial resolution map as input. We extracted the percentage values of each land use and vegetation class for the points of roadkilled fauna. We also extracted the percentage values of cover classes for random points along the road; these random points were used as the null model. We were then able to test whether the percentage distribution of each cover class associated with roadkill points was similar to the distribution of random points along the road. The number of random points corresponded to the sample size for all roadkill points and for each functional group. Although roadkill can present nested patterns due to the influence of context where roadkill events occur, the studies that analyze this type of data do not assess spatial autocorrelation, because these nested patterns provide valuable information. For example, Dekker and Bekker (2010) evaluated the badger (*Meles meles*) road mortality in the Netherlands, without includes autocorrelation on their analysis across the country level assessment. Secco et al. (2014) analyzed the intentional snakés roadkill in Brazil also not used spatial correlation components on their study.

Density of roadkills for the functional groups

We calculated the density of roadkills per functional group for each pixel of road, using the Kernel Density function of Arc Map 9.1 (ESRI 2007). Kernel Density Function gives the relative proportion of roadkill points inside a search radius, divided by the area of the buffer around the pixel. We also used the two spatial extents of interest (100 and 200 m) to calculated roadkill density per functional group.

Data analysis

Roadkill spatial uniformity distribution along the road

As the study road lacked significant curves (almost straight), we assumed that the traffic volume and the speed limit were constant; therefore, those variables were not considered in the analyses. Using the roadkill points as reference, we evaluated whether the spatial distribution of fauna hits along road differed significantly from a uniform spatial distribution ($\chi^2 = 1.92$, $P = 0.572$; $df = 57$); see Dale and Fortin (2014) by randomly selected pixels of road (5-m spatial resolution), the sample size being equal to the number of roadkills. We then used the Chi square test to compare the number of roadkills per segment of 200 m (observed) against the number of random samples for the same segment (expected). However, when we consider the species' functional groups, the degree of uniformity of the roadkill distribution varied—intermediate mobility birds: $\chi^2 = 1.33$, $P = 0.423$, $df = 57$; low mobility birds: $\chi^2 = 7.27$, $P = 0.032$, $df = 57$; low mobility reptiles: $\chi^2 = 4.19$, $P = 0.053$, $df = 57$; intermediate mobility reptiles: $\chi^2 = 3.40$, $P = 0.075$, $df = 57$; low mobility amphibians: $\chi^2 = 5.02$, $P = 0.042$, $df = 57$. Thus, we performed analyses to determine whether this non-uniform distribution was related to the landscape; our findings are presented in the results.

Distribution of percentage of cover classes for roadkills

We tested the correlation between landscape variables, using the Spearman coefficient in the BioEstat 5.0 software (Ayres et al. 2007), to identify variable redundancy and to reduce multicollinearity. We then analyzed the distribution of each landscape variable for the observed roadkill points to test whether it differed from the null model, where uniform distribution is assumed for the same variable on the road. This analysis followed six steps: (a) select a functional group of interest; (b) extract the landscape variables (e.g. the amount of forest around every roadkill); (c) divide the range of that variable into five bins and count the number of roadkills per bin (i.e. observed frequencies); (d) select random pixels of the road (5-m spatial resolution) using the same sampling size as the functional group; (e) count the number of random points for the same bins (i.e. expected frequencies) generated in the step (c) above; (f) compare the observed and expected frequencies using Chi square tests for each functional group and landscape variable. When Chi square P-values were very low (i.e. $P < 0.05$), the landscape variables related to the roadkills differed from a random distribution in the same environmental space as the variable. We tested six landscape variables—amount of forest (Forest); agriculture and sugar cane (Agriculture); water bodies and swamps (Water body); urban and rural buildings (Urban); eucalyptus plantation (Forestry); and pasture or bare soil (Pasture)—for each functional group in each taxon.

Roadkills density and landscape structure

We used a competing model approach (Burnham and Anderson 2002) to estimate the relative contribution of the percentage of each cover class to explain the roadkill densities as response variables. In this step, the sampling unit was each roadkill point for each functional group. The following explanatory variables (in percentages) were used to compose univariate models for explaining roadkill densities: (a) forest; (b) water bodies; (c) agriculture; (d) urban; (e) forestry; and (f) pasture (Table 2). We calculated the W statistic using Shapiro.test function of R to test for a normal distribution of the data, and finding that was the case. We then fitted the roadkill densities as a function of each explanatory variable using a generalized linear model GLM in R, finding that our data followed a normal distribution of residuals similar to Martensen et al. (2012). We included a null model among the competing models to simulate the absence of influence of the landscape on roadkill density. Akaike Information Criterion (AIC; Akaike 1974), and the most parsimonious model were selected (Burnham and Anderson 2002). AIC values were corrected for small samples (AICc). We used AIC to estimate the competing models' relative ability to explain the response variables, using the hypothesis that one model is better than the other, and calculated the difference between the AIC of each model and the best model with lowest AIC (ΔAIC). We also estimated the weight of evidence (wAICc) that any of the models was the best.

We used the AICc to compare all models in each functional group and determine which models best explained the distribution of roadkills in each functional group. The best models had a ΔAIC <2 and presented the highest wAICc (Burnham and Anderson 2002; Johnson and Omland 2004).

Table 2 Variables related to land use and cover used to explain roadkill density (amount of roadkill divided by the number of pixels of road within search radius) within Guilherm Scatena Municipal Road, São Carlos-SP, Brazil

Model	Variable
Forest	Percentage of forest
Agriculture	Percentage of sugarcane and other types of crops
Water body	Percentage of water body and swamps
Urban	Percentage of rural buildings and urban areas
Forestry	Percentage of commercial eucalyptus plantations
Pasture	Percentage pasture for cattle raising and bare soil

The variables of percentages were calculated within the buffers of 100 and 200 m for species with low and intermediate mobility, respectively

Results

DNA barcode and functional groups

During the 20-week sampling period, we found 123 roadkilled vertebrates (Table 1). Mitochondrial genes fragments were successfully amplified with the COI gene for 96 individuals and with 16S gene for 103 individuals. We deposited the obtained sequences in the NCBI database under the accession numbers: JQ627200–JQ627300 for the 16S gene and JQ6273301–JQ6273384 for the COI gene. Despite the success in gene amplification and molecular identification for both genes, DNA amplification was not possible with either gene for 12 individuals, due to material degradation by prolonged exposure to environmental conditions, especially for Serpentes and Lacertilia suborders, with four and five samples, respectively.

Although the 16S gene had a higher number of amplifications than the COI gene, the COI gene identified more samples to species level: 54 individuals from 19 species (46.34% of all species found) identified using the 16S gene, and 66 individuals from 28 species (68.29%) identified using the COI gene. Species identification for birds and mammals was more successful using the COI than the 16S, with 43.9 and 7.3%, respectively. However, the opposite was observed for amphibians and reptiles (24.4% for COI and 39.0% for 16S), due to both the success in amplifications and the different quantity of data available for each group in the database records.

Overall, of the 123 roadkilled individuals found on the Guilherme Scatena Municipal Road, we were able to morphologically identify 28 individuals to species level, corresponding to 14 different species (34.15% of all species found). Using the two genes (COI and 16S), we identified 87 individuals to species level, finding 37 species (90.24% of all species found). Combining both morphological and molecular characteristics, we were able to identify 102 individuals from 41 species. Compared to the number of individuals identified by morphological characteristics, DNA barcoding identified 310% more individuals, which was highly significant ($\chi^2 = 30.27$; $P > 0.0001$; $df = 1$). Morphological characteristics alone (28 identified individuals) contributed to the identification of only 17% of the total identified individuals (102), but this increase was not significant ($\chi^2 = 1.19$; $P = 0.275$; $df = 1$). We were unable to identify 21 individuals to species level; instead, two of these individuals were identified to genus (*Leptodactylus* sp. and *Turdus* sp.), nine to family (Amphisbaenidae), and ten to order (five Lacertilia and four Squamata and one Carnivora) levels.

Landscape influences analysis

Spearman correlation coefficient showed no significant correlations between the explanatory variables, since the values were less than 0.5; thus, all variables were retained for subsequent analyses. The Chi square comparison with the uniform distribution (Table S1) showed significant differences for birds with low movement capability in the forest and on bare soil or pasture. Low mobility birds experienced higher aggregation of roadkill on the road positions near forests and lower road mortality on areas with predominance of bare soil, agriculture or pasture.

The linear model revealed that, for birds and amphibians with low movement capability, the models did not explain the location of roadkills (Table 3). However, birds with an intermediate movement capability were strongly influenced by agriculture and

Table 3 Best-supported models for explaining influence of landscape structure on density of roadkill of functional groups within Guilherme Scatena Municipal Road, São Carlos-SP, Brazil

Model	Birds		Reptilia		Amphibia
	Low	Intermediate	Low	Intermediate	Low
Forest	<0.001	0.093	**0.177**	0.090	<0.001
Agriculture	–	**0.245**	0.116	0.073	<0.001
Water body	<0.001	0.094	0.143	0.145	<0.001
Urban	–	0.164	–	**0.255**	<0.001
Forestry	<0.001	0.086	0.138	0.095	<0.001
Pasture	<0.001	0.096	0.151	0.237	<0.001
Null	**1**	0.074	0.141	<0.001	**0.898**

The values are the weight of evidence (wAICc) for each model. Low and Intermediate mobility for the taxa are indicated. All the models were fit using GLM models. Comparisons must be done within each column

sugar cane monoculture, with most of the roadkill points located in proximity to this land cover type. A high number of roadkilled reptiles with low movement capability were found in forest remnants, and roadkilled reptiles with intermediate movement capability were more frequent in heavily anthropized landscapes or landscapes with man-made constructions.

Discussion

DNA barcoding increases roadkill species identification

The number of roadkilled small animals in the study area may suggest that the road severely impacts the local fauna. A wide variety of animals around the world are roadkill victims (Coffin 2007); however, the majority of roadkill records represent large vertebrates, for which taxonomic identification and the collection of statistical data are easier than for small animals. If we also account for small animals, the roadkill rate in Brazil could reach 8.65 animals/km/year (Dornas et al. 2012). The high rate of roadkills demands that the impact on species be studied, particularly in biomes with a high rate of endemism. Such a study requires species identification and information on the characteristics of affected groups. Our results show that molecular identification at species level allowed the identification of 70.7% of the roadkilled animals collected for this study; the method's success depends on the quality of the sample, the amount of information in the molecular databases, and the genes chosen for amplification.

In studies analyzing different taxonomic groups, such as an assessment of roadkills, the use of universal primers is the best choice for DNA amplification. However, some genes perform better than others, depending on the taxonomic group. For amphibians, Vences et al. (2005b) showed that amplifications with universal primers for the 16S gene were more reliable than those for the COI gene. However, the data we obtained for amphibians indicated high reliability for both genes. Although we identified an almost equal number of individuals through the use of either gene, most individuals identified by the COI gene belonged to the same species. The 16S gene identified a wider variety of amphibian species; however, the absence of a specific database for the 16S gene impaired the use of 16S information for all major taxonomic groups.

Identification by functional groups

Bird individuals usually die on impacts with a car windshield; they then fall on the shoulders of the road, where their bodies are protected from being run over repeatedly. This fact not only helped their morphological identification, but also aided the molecular identification of these samples, as the well-preserved biological material allowed the extraction of high quality DNA and efficient DNA amplification. The ease of molecular identification for this group was also due to the amount of information stored in the COI database.

On the other hand, amphibians—one of the taxonomic groups most affected by roadkill (Beebee 2013)—lose most of their diagnostic features, making morphological identification almost impossible. We were unable to morphologically identify any of the species in this class, thus demonstrating that the use of the DNA barcode was decisive for amphibian roadkill identification. Moreover, the use of two mitochondrial genes and two public databases allowed us to identify 90% of the roadkilled amphibians (37 individuals), compared to 66.7% of species identified by Klippel et al. (2015) through only the COI gene and the BOLD database.

For all taxonomic groups, the combined use of both morphological and molecular characteristics was favorable, promoting fast and reliable species identification. However, we also obtained some false molecular identifications (three species) of related species that do not occur in the study area—for example, *Cariama cristata* was misidentified as *Ciconia boyciana* (88% identity)—indicating a lack of previous information in the molecular database for a few of the studied species. It is important to highlight that the similarity values were lower than 97% in these cases. Clearly, a database bias exists, whereby a vast source of molecular information is available for some regions of the world, while for other regions, such as the Neotropics, the local fauna is under-represented.

Movement capacity influences roadkill rates

According to Fahrig (2007), species' movement throughout a landscape differs depending on their sensitivity and response to environmental changes. Species with an intermediate movement capability may be more vulnerable to mortality on roads than species with lower movement capability (Carr and Fahrig 2001), or they may show different relationships with the landscape. Additionally, Rytwinski and Fahrig (2012) stated that large-sized mammal species that present low reproductive rates and greater mobility were more negatively affected by roads. Miotto et al. (in review) observed the same effect on pumas: although the road network is not yet a barrier to their movement, the elimination of breeding males is catastrophic for the genetic diversity of the population, particularly in species with low reproductive rates, because the population cannot compensate for mortality through higher reproduction. Rytwinski and Fahrig (2012) also asserted that highly mobile birds were more negatively affected by roads than less mobile birds; furthermore, amphibians, reptiles in general, small-sized frogs and toads in the early years of sexual maturity suffered greater negative influence of roads and traffic. In our study, we found no uniformity in the roadkill distribution for the functional groups, and there were differences between the analyzed groups, which in some cases were related to landscape context. For example, birds exhibiting intermediate movement capability—or more mobile birds according to Rytwinski and Fahrig (2012)—were strongly influenced by the percentage of agriculture

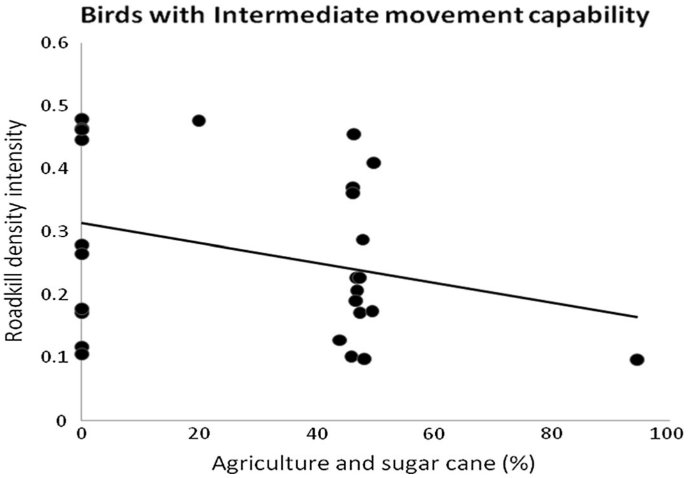


Fig. 2 Influence of percentage of agriculture and sugar cane on roadkill density for birds with intermediate movement capability within the Guilherme Scatena Municipal Road, São Carlos, SP, Brazil

and sugar cane monoculture (Fig. 2). Roadkilled reptiles with low movement capability were associated with forest remnants, while those exhibiting intermediate movement capabilities were more frequent in heavily anthropized landscapes. In the meta-analysis of Rytwinski and Fahrig (2012), the authors present information about the negative effects of roads on herpetofauna in general; however, while their discussion focuses on body size and reproductive phase, we are more interested in understanding how landscape structure influences the likelihood of species being killed on roads.

We found no relationship between amphibian roadkills and the presence of water bodies, contrary to the finding of Glista et al. (2008). Although we used high-resolution mapping, we were unable to map all watercourses and water bodies, possibly because many of them are hidden within dense vegetation. We did not identify any Cerrado endemic species, and most of the identified species that were associated with the major land cover classes are typically able to exist in human-modified habitat.

Combining molecular and morphology-based identifications

We confirmed the effectiveness of animal identification by means of molecular characters when there is a loss of morphological characteristics, as is the case for roadkilled individuals. The locations with the highest roadkill incidence were successfully identified using our molecular and morphology-based approaches. We related these locations to the position of animal carcasses and their surrounding landscape structure, testing the hypothesis that the frequency of roadkills differs significantly among functional groups, depending on the structure and composition of the surrounding landscape. Our study shows that most of the roadkilled animals are small. The presence of generalist species and the absence of Cerrado endemic fauna among roadkills may indicate the poor quality of this environment, which is heavily modified by human activities in the region (Ribeiro et al. 2016). Despite the negative effects of roads on fauna, roadkills can be an important source of (1) local wildlife information for records of species occurrence and (2) tissue samples from roadkilled animals for DNA studies; such data may benefit local fauna conservation.

When planning roads and buildings, we emphasize the need to adopt a variety of approaches to mitigate roads impacts on the fauna, with particular consider of the different responses of the key functional groups of interest. For instance, birds with intermediate movement capability require the preservation of some sort of vegetation that enables them to adapt in an altered environment, while for lower movement capability reptiles, the priority should be the maintenance of as much native forests as possible, ideally more than 30%, as suggested by Martensen et al. (2012) and Muylaert et al. (2016).

Conclusions

Wildlife loss due to roadkills is a global problem; however, roadkill analysis can contribute to the conservation of wildlife. Incorporating DNA analysis into roadkill studies means that much more data becomes available for ecological interpretation. Landscape analysis, related to the functional group of the identified species, also supports conservation through discovering the places with the highest incidence of roadkills. This information can contribute to wildlife conservation by informing roadkill mitigation strategies so that they can target the needs of specific taxonomic and functional groups. Below we present the top five mitigation strategies that we consider important for wildlife maintenance within a road-influenced context:

- Underpasses—e.g. (Administration 2002), open culverts (Goosem 2001)—for adapted fauna in order to reduce the number of small vertebrate roadkills.
- Pole barriers (Zuberogoitia et al. 2015) would be an appropriate strategy for birds of intermediate movement capability that are strongly influenced by agriculture and sugar cane monoculture; this measure would reduce the number of birds than die from impact with a windshield, because pole barriers, most of time, impede birds crossing the roads our stimualte them fly at higher height.
- Measures to control speed, such as speed bumps (Anon 1980) and increasing driver awareness with signs and pamphlets (Jones 2000), would reduce the number of low mobility roadkilled reptiles, which are related to forest remnants.
- Use of DNA barcode technique in roadkill studies to increase the number of identified species in roadkill analysis, which would aid in the selection of more appropriate mitigation measures.
- Further studies focused on functional groups and their relationship with the landscape, because there is a strong relationship between functional group and the type and location of mitigation strategy. Without this knowledge, road ecology with the purpose of the roadkill mitigation will be less effective for wildlife conservation.

Finally, we conclude that: (1) DNA barcoding increases the quality and amount of data that can be obtained in addition to those derived from morphological characteristics of fauna carcasses; (2) Landscape structure plays a crucial role for species most affected by roads, and we must combined roadkill data, DNA barcoding and landscape-based approaches to increase our understanding of how roads influence local and regional fauna maintenance; (3) Although roadkills are not desirable, the data generated is an important source of information about the biodiversity within any region, and these data this information is essential to the determination of mitigation strategies; (4) Despite the focus of many roadkill studies being a species-specific definition of mitigation strategies with a bias for medium and large-sized vertebrates, we advocate the adoption of functional group-based strategies for small-sized fauna (birds, reptiles, amphibians), because many species may have generally similar responses to roads within different landscape contexts.

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