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ARTICLE

Using noninvasive techniques to monitor game species targeted by poaching in Misiones, Argentina

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ABSTRACT

Misiones, Argentina contains the largest remnant of Upper Paraná Atlantic forest; however, half of it is unprotected. The long-term survival of its biodiversity is threatened by poaching and habitat loss, which eliminate animal populations and decrease genetic variability in species. Noninvasive techniques were used to evaluate the presence of four mammals [white-lipped peccary (*Tayassu pecari*), collared peccary (*Pecari tajacu*), tapir (*Tapirus terrestris*), and paca (*Cuniculus paca*)] often targeted by poachers. With detection dogs, 179 scats were collected across intact and modified habitats in the northern-central zones of Misiones. Of the samples collected, 76.5% (n = 137) could be genetically confirmed as one of the three targeted prey: 98 white-lipped peccaries, 13 collared peccaries, and 26 tapirs. A greater proportion of white-lipped peccary and collared peccary samples were associated with heterogeneous landscapes (74.5% and 76.9%, respectively), which contrasts with tapirs that had a higher proportion (76.9%) in native forest. While collared peccaries and tapirs had close association with protected areas (84.6% and 96.2%, respectively), over half (57.1%) of the white-lipped peccary samples were located outside of protected areas. Despite a higher proportion of survey coverage in the central zone (64.0%), the majority (81.8%) of prey samples were in the northern zone. While samples were found across habitats that varied in integrity and degree of protection, the restrictions seen among prey species distributions indicate concern for their long-term survival if the threats imposed by poaching, habitat loss, and human expansion are not controlled, especially in the central zone of Misiones.

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Detection dogs; genetics; poaching; scat

Introduction

Misiones, Argentina contains the largest remnant of Upper Paraná Atlantic forest (471,204 km^2 [1]), which is part of the Green Corridor (Provincial Law Nº 3.136), a multiuse conservation area of more than 1,000,000 ha [2–4]. However, connectivity across the region is threatened by ongoing habitat conversion, growing human populations/activities, and an expanding network of roads [5–7]. This landscape includes existing protected areas that are becoming increasingly isolated, situated in a matrix of altered habitat with forest patches that vary in size and connectivity. The threats that species face as they navigate this heterogeneous landscape include poaching, which can reduce genetic variability and could lead to the extinction of the isolated populations [8,9]. This phenomenon known as defaunation, not only directly impacts the biodiversity of mammalian communities but it can also trigger trophic cascades whose top-down effect has the potential to shift overall organization in an ecosystem [10–13]. Consequences can include impoverished native forests that suffer from "empty forest syndrome" [14,15].

While the hunting of wild animals is one of the most practiced activities in the forests of South America [16–18], it is prohibited by law in Misiones, Argentina. However, between hunting being a culturally, deeply rooted activity [19] and the high demand for bushmeat locally and transboundary, the threats to wildlife are real. In this sense, the aim of the present work was to evaluate the presence and distribution of four mammal species that are often poached in the northern-central zones of Misiones: white-lipped peccary (*Tayassu pecari*), collared peccary (*Pecari tajacu*), tapir (*Tapirus terrestris*) and paca (*Cuniculus paca*). Data was collected using noninvasive techniques (detection dogs and genetic analyses of scats), which allowed searches to be carried out independent of habitat type, degree of protection afforded to the area, and presence of humans [5,6,20–26]. Using DNA extracted from the collected scats, species identity was confirmed and used to evaluate species presence and distribution relative to sample location (e.g. habitat, protective status).

Materials and methods

Study area. – Misiones, Argentina, which is bordered by Brazil and Paraguay, contains the largest remnant of Upper Paraná Atlantic forest, with 67.0% of this biodiversity hotspot located in the northern-central zones of Misiones. However, only 46.1% of this native forest is

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found in a series of protected areas that vary in size, isolation, and degree of protection (Figure 1). The remaining portion is in a mosaic of monoculture plantations (*Pinus* sp., *Eucalyptus sp*., native *Araucaria angustifolia*), small-scale agriculture (perennial crops of *Camellia sinensis* and *Ilex paraguariensis*), areas of subsistence agriculture, pastures, bare ground, and urban areas (Figure 1 [6,7,27]).

The province is characterized by a humid, subtropical climate with no distinct dry season [28]. Average monthly rainfall typically exceeds 100 mm; however, in October and November rainfall is >200 mm. The hot season (September to mid-April) is characterized by warm days (28–33°C) and moderate nights (14–20°C). In contrast, the cool season has moderate days (23–27°C) and cool nights (9–12°C).

Scats were collected from June to August 2016, when lower daily temperatures were optimal for the detection dogs. A total of 68 unique routes that covered 512.6 km were surveyed (Figure 1). While an effort was made to have equal coverage across the northern and central zones, a higher proportion of the routes (64.0%) were in the central zone. While sampling occurred outside and inside protected areas, the majority (60.8%) were outside of protected areas. Protected areas included those with high levels of protection (30%) and areas of mixed used with varying levels of protection (70%) [5,6,27]. Surveys outside protected areas included private properties, areas in colonies and farms, and properties managed by large forestry companies.

Detection dog and handler training. – Two field-experienced detection dogs were used in the surveys:

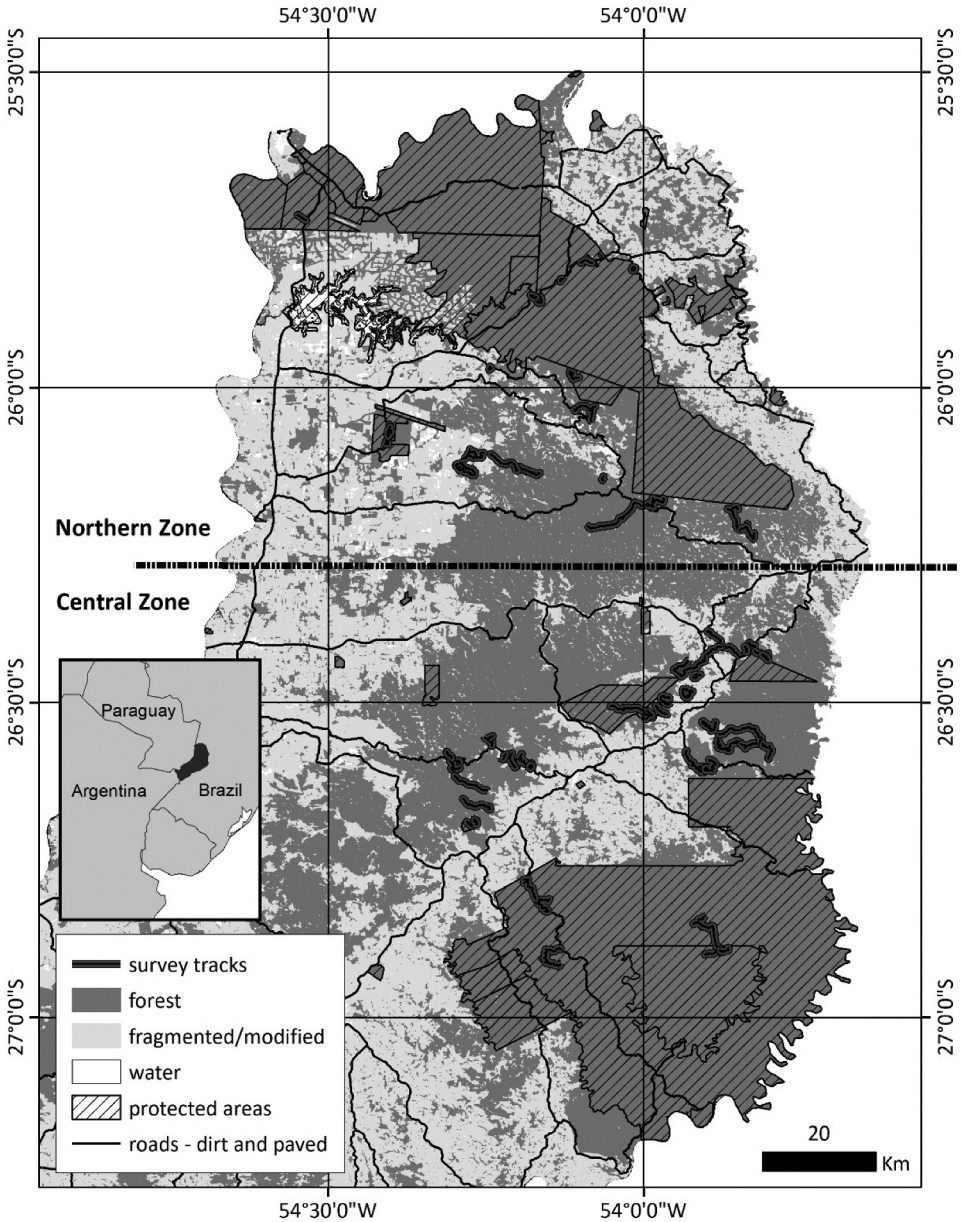

Figure 1. Location of Misiones, Argentina, in South America (inset). Map of Misiones province showing protected areas across the northern and central zones. These areas are shown in relation to the land-use pattern existing in Misiones in 2009: forest, fragmented or altered areas, and large bodies of water [27].

Table 1. The three species-specific mitochondrial (mtDNA) sequences (110 bp) generated from the scats in this study. Sequences were compared with entries in GenBank to confirm species identity. n= number of species identified.

	n	1																			20
white-lipped peccary	98	T	G	G	C	A	A	T	A	C	G	T	A	T	C	C	C	A	T	G	A
collared peccary	13	_	_	_	T	_	G	G	_	_	A	_	_	_	_	_	T	_	_	A	_
tapir	26	C	_	_	_	_	_	A	_	_	_	_	_	G	_	_	_	_	_	_	_
		21																			40
white-lipped peccary		A	G	G	C	T	G	T	A	G	C	T	A	T	A	G	T	C	A	T	T
collared peccary		_	_	_	_	G	_	_	T	_	_	_	_	_	T	A	C	T	G	_	_
tapir		_	_	_	_	_	_	_	_	_	_	C	_	_	_	A	_	_	G	_	G
		41																			60
white-lipped peccary		A	G	T	A	G	A	A	T	A	A	T	A	A	T	T	C	C	G	G	T
collared peccary		_	_	_	_	_	G	_	G	_	_	_	_	_	C	_	_	_	_	A	_
tapir		_	A	_	_	_	T	_	G	_	_	_	_	_	_	_	_	_	_	A	_
		61																			80
white-lipped peccary		G	T	T	T	C	A	T	G	T	T	T	T	T	A	G	A	A	A	T	A
collared peccary		_	_	_	_	_	_	_	_	_	_	_	C	_	_	_	_	_	_	_	_
tapir		_	_	_	_	_	_	_	_	_	_	_	C	_	_	_	G	_	_	_	G
		81																			100
white-lipped peccary		G	G	T	A	G	G	A	T	C	C	G	T	A	A	T	A	T	A	G	T
collared peccary		_	_	_	_	_	_	_	C	_	_	A	_	_	G	_	_	_	_	_	_
tapir		T	_	_	_	_	_	_	_	_	_	A	_	_	_	_	_	C	_	A	A
		101									110										
white-lipped peccary		C	C	T	C	G	G	C	C	T	A										
collared peccary		_	_	_	_	_	_	_	_	_	_										
tapir		_	_	_	_	_	T	_	_	_	_										

a nine-year adult male rescued Chesapeake Bay Retriever and a five-year adult female rescued Border Collie. The male, who had seven years of field experience in Argentina and USA working with five carnivores, was handled by K. DeMatteo [5–7,25]. The female, which had three-years of field experience with three primates and one carnivore, was handled by P. Delgado. Both dogs were adopted at a young age and selected due to their strong ball drive and high energy level, two key factors that make a successful conservation detection dog [20,29].

While these two handlers varied in their years of experience, both were certified in the handling and training of conservation detection dogs. This training allows the handler to interpret nonverbal cues from their dog and maximize the ability of their dog to find samples in varying field conditions [29]. Since her initial training in 2007 [5], K. DeMatteo has expanded her skills through field surveys in various locations with multiple dogs, hands-on training opportunities, and applied instruction to new handlers. Included in the latter, was P. Delgado, who completed an intensive three-week course followed by field instruction at the start of this study.

Both detection dogs were trained using scat samples from captive and wild animals and to detect four target species (white-lipped peccary, collared peccary, tapir, and paca) and to ignore several nontarget species [deer (*Mazama* sp.), agouti (*Dasyprocta azarae*), and various poisonous snakes (e.g. *Porthidium* sp. and *Bothriechies* sp.)]. The importance of incorporating scats from nontarget species has been demonstrated as being essential in fine-tuning the dog's search image [6,29].

Sample collection. – Scat samples indicated by either detector dog were collected as long as mold was not observed on their surface, which meant the condition of selected scat ranged from fresh with a moist mucus layer to hard and dry. Scats where mold was evident were not collected, as DNA extraction success from these types of samples was predicted to be lower due to DNA degradation [25,26]. Even though environmental factors, such as rain, sun, and insects, can cause an inaccurate assessment of scat condition, a best guess was made when classifying each scat as fresh (≤24 h), moderately old (between 24 h and 3 days), or old (>3 days) [5–7]. Each scat location was georeferenced using a GPS unit (Garmin 62S) and condition/approximate age [26], composition/contents, location relative to trail or road, position in/out of a protected area, and habitat heterogeneity were recorded. With the latter, type of environment was classified as heterogeneous (matrix of native forest, pastures, monoculture plantations, and fruit trees) or native forest. The latter includes various plant communities [30,31], such as gallery forests, bamboo forests, palm groves (*Euterpe edulis*), araucaria (*Araucaria angustifolia*), guatambú (*Balfourodentrum riedelianum*), and palo rosa (*Aspidosperma polyneuron*).

The surface of the scats was swabbed with a cotton-tipped applicator, soaked in 1x phosphate bufferedsaline solution to collect cells sloughed from the digestive tract of the animal [32,33]. Each scat was swabbed in triplicate (two for DNA extraction and one to be kept in the collection of the Ministerio de Ecología y Recursos Naturales Renovables) and each swab stored in a 2.0 mL polypropylene tube, labeled, and secured with parafilm. Each swab was taken from a different area on the scat's surface to maximize the quantity of DNA obtained. In addition, each scat was collected and stored in a labeled 18-oz Whirlpak® bag (Nasco) as backup for DNA extraction and future analyses (e.g. diet, parasites). At the end of each field day, samples were placed in a −20°C freezer.

Genetic Analyses. – DNA was extracted from two independent swabs using two extraction protocols. Initially, all samples

Table 2. A summary of how the number of scats (% of total) of the three targeted species (white-lipped peccary, collared peccary, and tapir), plus a total of them, are distributed across Misiones, Argentina. They are shown specified by zone (northern *vs* central), and combining both, with the total of all samples noted (n) in every case. For each zone, the data are summarized in two distinct ways: (1) relative to the type of habitat; specifically, whether the scats were in intact (native forest) or fragmented (heterogeneous landscape) environments and (2) relative to protected areas; specifically, whether samples were located inside or outside of protected areas.

		Targeted Species			
Zone (n)	Location	White-lipped peccary	Collared peccary	Tapir	Total
North (112)	Habitat type				
	native forest	9 (9.2%)	3 (23.1%)	18 (69.2%)	30 (21.9%)
	heterogeneous landscape	67 (68.4%)	9 (69.2%)	6 (23.1%)	82 (59.9%)
	Protected area				
	inside	26 (26.6%)	11 (84.6%)	23 (88.5%)	60 (43.8%)
	outside	50 (51.0%)	1 (7.7%)	1 (3.8%)	52 (38.0%)
Central (25)	Habitat type				
	native forest	16 (16.3%)	0 (0%)	2 (7.7%)	18 (13.1%)
	heterogeneous landscape	6 (6.1%)	1 (7.7%)	0 (0%)	7 (5.1%)
	Protected area				
	inside	16 (16.3%)	0 (0%)	2 (7.7%)	18 (13.1%)
	outside	6 (6.1%)	1 (7.7%)	0 (0%)	7 (5.1%)
Combined (137)	Habitat type				
	native forest	25 (25.5%)	3 (23.1%)	20 (76.9%)	48 (35.0%)
	heterogeneous landscape	73 (74.5%)	10 (76.9%)	6 (23.1%)	89 (65.0%)
	Protected Area				
	inside	42 (42.9%)	11 (84.6%)	25 (96.2%)	78 (56.9%)
	outside	56 (57.1%)	2 (15.4%)	1 (3.8%)	59 (43.1%)

were extracted using a CTAB protocol (Cetyl Trimethylammonium Bromide [34]); however, those samples that had low success were extracted using a Qiagen DNeasy™ Blood & Tissue Kit following a modified protocol suggested by Vynne [35]. Extractions were carried out in a room separated from the one in which polymerase chain reaction (PCR) amplifications were done to prevent cross-contamination of samples and PCRs. Negative controls (no scat material added to the extraction) accompanied each set of extractions and were used in species identification PCRs to test for contamination.

To identify species, a 110-bp (171-bp including primers) specific region of mitochondrial cytochrome *b* gene was amplified using Farrell et al. [36] primers (5´-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3´; 5´-TATTCTTTATCTGCCTATACATRCACG-3´) and a modified version of protocols and reagents published by Farrell et al. [36] and Miotto et al. [37] was used. Amplifications of all samples (regardless of which extraction protocol was used) were carried out on a PerkinElmer GeneAmp System 9600 (Applied Biosystems) in 25-µL final volume containing 2-µL DNA extracted, 1x PCR Green GoTaq© Flexi Buffer (Promega), 0.3-µM of each forward and reverse primer, 200 µM of dNTP, 5-mM $MgCl_2$, 150 µg/mL BSA, and 0.5-U GoTaq G2 Hot Start Polymerase (Promega). To minimize the potential for contamination in all reactions, PCR set up was prepared in a UV chamber (Ivema C9). Negative controls (no DNA added) were included in each PCR run, to test for contamination. The PCR profile consisted of 10-min denaturation at 95°C, followed by 40 cycles at 95°C for 30 s, 49°C for 45 s, 72°C for 45 s, and a final 30 min extension at 72°C. Purified PCR products were sequenced on an ABI3730XL at Macrogen Inc. (Korea). Sequences were edited (Table 1) and aligned using PROcessor of SEQuences v.2.91 and compared with reference entries in GenBank using a Local Alignment Search Tool (BLAST [38]) to identify the donor species of the sample.

Results

A total of 179 scats were collected. Of these, 137 (76.5%) could be confirmed as one of three target species: 98 white-lipped peccaries, 13 collared peccaries, and 26 tapirs. No pacas were genetically identified (Table 1). Four scats were identified as nontarget species [red brocket deer (*Mazama americana*; n = 3) and horse (*Equus caballus*; n = 1)] and likely represent urine contamination of the nontarget species on a target scat [5]. The remaining 42 scats either failed to amplify (n = 3) or had poor quality DNA (n = 35). Per the Ministerio de Ecología y Recursos Naturales Renovables de Misiones, the exact location of these samples is not provided, as a precaution and protection of these species protected by law and persecuted by poachers.

A greater proportion of white-lipped peccaries and collared peccaries samples were associated with heterogeneous landscapes (74.5% and 76.9%, respectively), which contrasts with tapirs' samples which had a higher proportion (76.9%) in native forest (Table 2). While collared peccaries and tapirs had close association with protected areas (84.6% and 96.2%, respectively), over half (57.1%) of white-lipped peccary samples were located outside of protected areas (Table 2). All three species had a higher proportion of samples collected in the northern zone, with collared peccaries and tapirs at 92.3% and white-lipped peccary at 77.6% (Table 2).

Discussion

This study demonstrated that the use of two noninvasive techniques (detection dogs and the genetic analyses of scats) were effective in collecting data on selected species targeted by poaching, regardless of behavior (solitary or group living) and habitat type, including the ability to survey independent of human presence and outside of protected areas. The latter is typically restricted or impossible with standard survey techniques like camera traps, as the risk of theft increases. It also means that species data can be collected independent of their abundance across the landscape [6,23–25]. Unlike invasive methods that involve capture or manipulation of individuals [5,39,40], these noninvasive techniques depend on samples left behind on an animal's daily movements, which can avoid stress and possible injury of the organism and/or researchers.

The application of wildlife forensic genetics can remove error associated with methods that identify scats through macroscopic parameters, including size, appearance, diet, and smell [36,41]. While this study focused on using genetic analyses to confirm donor species, the application of these techniques can be expanded to include identification of samples to the individual- and sex-level plus evaluations at the population level, such as estimating genetic diversity and population size [39,42–46]. The fact that forensic DNA recovered from scats is generally degraded, in low concentrations, and could be a mix of more than one contributor, transforms it into a challenge; however, markers do exist [36,47–49]. For species identification in vertebrates, *Cytochrome b* of mitochondrial DNA is often chosen because its sequences are conserved at the interspecific level, showing few variations at intraspecific level, and are sequences available in the GenBank database allowing for taxonomic comparison and identification [36,39,50–53].

Of the three species, white-lipped peccary had the highest proportion of collected samples (71.5%) with most of these samples located in heterogeneous landscapes (74.5%) and outside of protected areas (57.1%; Table 2). The latter fact is unique among the three species. Previous publications on white-lipped peccaries provide conflicting evidence on their abundance and distribution in Misiones, including a high abundance in the northern zone but only in selected protected areas [54] to dramatic shifts in species abundance in various locations [28,55–58]. It is thought that these changes in abundance could be due to the species nomadic nature and broad movement patterns, negative effects of poaching, or death from disease or epidemics. It is also possible that the populations of white-lipped peccaries have a natural population cycle with gradual increases followed by rapid decreases, as occurs in other mammals [58–60]. While it appears that the white-lipped peccaries are flexible in their ability to use fragmented habitat outside of protected areas [61], this means that the long-term survival of this species will be at risk through increased proximity to

humans, potential for poaching, and exposure to diseases (e.g. domestic pigs) [62].

In contrast to the white-lipped peccaries, only 9.5% of samples were confirmed as collared peccary. While these samples were primarily associated with heterogeneous habitats (76.9%), the majority were in protected areas (84.6%); specifically, mixed-use areas with a lower level of protection (Table 2). While this close association with protected areas aligns with Paviolo et al. [54], the need for protection is unclear because there is evidence that the species is tolerant to habitat fragmentation and hunting pressures, with healthy populations in highly degraded areas [18,57]. One question is whether there are specific behaviors of the collared peccaries that make them an easier poaching target outside of protected areas. That is, are the lower numbers of collared peccaries in general and outside of protected areas associated with a lower overall abundance of the species? In either case, caution is seen in that even if collared peccaries are found to be able to use heterogeneous landscapes outside of protected areas, they will face the same risks and concerns of white-lipped peccaries, putting their long-term survival in question.

Concern extends to the tapirs. While 19% of the samples were confirmed as tapir, this species was found to be strongly associated with native forest (76.9%) and almost exclusively located in protected areas (96.2%; Table 2). Previous studies have found this species to have a link to native forest [54] and very sensitive to hunting pressure [17,18,63], with the latter related to the species' low reproductive rate and late sexual maturation [17,64]. While select protected areas in northern Misiones are reported to have high abundance of the species, increasing isolation of protected areas, road kills, and disease have negatively affected this species [65,66]. In fact, the species has lost at least 30% of its distribution in the last 40 years [67] and appears to have little tolerance for fragmentation.

While multiple collected scats (n = 7) were located by the detection dogs and visually identified in the field as belonging to paca, the DNA obtained was insufficient for a genetic confirmation. Whether this reflects a reality for the species is unknown. It is possible that the areas surveyed failed to sufficiently cover those areas that are reportedly preferred by this species, including densely forest areas near water courses [68,69]. In addition, it is known that paca is one of the most coveted prey by hunters in Misiones; however, Giraudo and Abramson [70] reported that this species can endure relatively high levels of exploitation. Additional surveys and work with this species are needed to understand the effects of hunting and fragmentation on this important rodent.

While the white-lipped peccary, collared peccary, and tapir were successfully found across habitats that varied in integrity and degree of protection, the restrictions and limitations seen among the individual species distributions indicate concern for their long-term survival. The concern is especially high in the central zone of Misiones, with most of all samples located in the northern zone (81.8%; Table 2) despite a higher proportion of coverage in the central zone (64.0%). This lower proportion in the central zone is likely causally linked to a higher hunting pressure in this region [54]. Indirect evidence of illegal poaching combined with lower relative abundance of prey and carnivores [71] suggests that the central zone is at risk of defaunation or "empty forest syndrome". Even though some species such as the white-lipped peccary appear to be able to tolerate fragmentation, it inevitably puts its long-term survival at risk. Expanding these data into individual identification and overlapping with carnivore data can possibly identify areas that fall along a spectrum of stability and risk. These efforts combined with those that are trying to make a multispecies corridor a reality in the region [7] along with supports of anti-poaching endeavors can help ensure the long-term survival of these targeted by poaching species in Misiones and their ecosystem.

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Authors Contributions

Pryscilha M. Delgado: Conceptualization, Methodology, Investigation, Formal Analysis, Investigation, Writing Original-Draft, Visualization. Carina F. Argüelles: Conceptualization, Methodology, Validation Verification, Formal Analysis, Resources, Writing-Review & Editing, Visualization, Supervision, Project Administration. Karen E. DeMatteo: Conceptualization, Methodology, Validation Verification, Formal Analysis, Investigation, Resources, Writing-Review & Editing, Visualization, Supervision, Project Administration, Funding Acquisition.

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