Mycobacterium leprae in Nine-Banded Armadillos (*Dasypus novemcinctus*), Ecuador

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We found *Mycobacterium leprae*, the most common etiologic agent of Hansen disease or leprosy, in tissues from 9 (18.75%) of 48 nine-banded armadillos (*Dasypus novemcinctus*) collected across continental Ecuador. Finding evidence of a wildlife reservoir is the first step to recognizing leprosy zoonotic transmission pathway in Ecuador or elsewhere.

The World Health Organization Global Leprosy Strategy targets the long-term goal of leprosy elimination through interruption of disease transmission (*1*). One factor that can impair that goal is environmental or animal reservoirs that contribute to persistence of the *Mycobacterium leprae* bacteria and potential spillover into the human population. The Strategy acknowledges that *M. leprae* zoonotic transmission exists but with a lower risk and highly localized in North America (*1*), possibly because of a lack of research on new and existing animal reservoirs in other locations.

M. leprae, the main causative agent of leprosy, has a broad range of animal hosts, including wild armadillos (*Dasypus* spp.) in the Americas, red squirrels (*Sciurus vulgaris*) in the British Isles. and nonhuman primates in the Philippines and Africa (*2*). Armadillos are a family of medium-sized mammals, belonging to the Xenarthrans, which also includes sloths and anteaters (*3*). At least 20 armadillo species have been recognized (*3*). The Cingulata order encompasses >9 *Dasypus* species, including the nine-banded armadillo (*D. novemcinctus*), considered the main *M. leprae* reservoir in the Americas (*2*).

Ecuador, located in northwestern South America, is a medium-income economy nation with ≈18 million inhabitants (*4*). Officially, Ecuador eliminated leprosy as a public health threat, which means incidence is <1 new case/10,000 inhabitants; only 41 new leprosy cases were registered in 2022 (*5*). Scientific literature on leprosy in Ecuador is scarce; nonetheless, the Ministry of Public Health suggests a higher disease incidence across the country (*6*). Armadillos are found throughout Ecuador and are valued as a protein source and a cultural item in many rural settings (*7*). In view of the uncertain epidemiologic landscape of leprosy in Ecuador and the occurrence of a possible animal reservoir in the country, we investigated *M. leprae* infection in armadillos in Ecuador.

The Study

We gathered tissue samples from 45 armadillos via local hunters who use the mammal as a protein source for their families and communities. The Instituto Nacional de Biodiversidad (Quito, Ecuador) also donated 3 additional samples stored in 70% ethanol, for a total of 48 armadillos. We performed tissue collection according to a protocol approved by the Ministerio del Ambiente, Agua y Transición Ecológica, as part

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Table. Characteristics of animals and samples tested in a study of *Mycobacterium leprae* in nine-banded armadillos (*Dasypus novemcinctus*), Ecuador*

Categories	No. (%)	*M. leprae*–positive, no. (%)
Tissue samples		
Liver	38 (45.24)	8 (21.05)
Spleen	26 (30.95)	4 (15.38)
Muscle	10 (11.90)	1 (10)
Heart	3 (3.57)	0 (0)
Kidney	3 (3.57)	0 (0)
Lung	3 (3.57)	0 (0)
Ear	1 (1.19)	0 (0)
Total	84 (100)	13 (15.48)
Armadillo species		
Dasypus novemcinctus	40 (83.33)	9 (22.50)
Dasypus spp.	6 (12.5)	0 (0)
D. pastasae	1 (2.08)	0 (0)
Cabassous centralis	1 (2.08)	0 (0)
Total	48 (100)	9 (18.75)
Provinces		
Esmeraldas	10 (20.83)	1 (10)
Sucumbíos	9 (18.75)	1 (11.11)
Guayas	7 (14.58)	0 (0)
Santo Domingo de los Tsáchilas	7 (14.58)	3 (42.86)
Morona Santiago	4 (8.3)	0 (0)
Pastaza	3 (6.25)	1 (33.33)
Imbabura	2 (4.17)	1 (50)
Manabí	2 (4.17)	1 (50)
Cañar	1 (2.08)	1 (100)
Cotopaxi	1 (2.08)	0 (0)
Los Ríos	1 (2.08)	0 (0)
No information	1 (2.08)	0 (0)
Total	48 (100)	9 (18.75)

*A total of 84 tissue samples corresponding to 48 individual armadillos from 4 species were tested for *Mycobacterium leprae*. Percentage of *M. leprae* positives is calculated with the total sampling of each row. No samples were positive for *M. lepromatosis*.

of the Genetic Resources Access Framework contract (contract no. MAATE-DBI-CM-2021-0172). We established definitive armadillo species identification by morphological features, known geographic distributions, and molecular diagnosis (Appendix 1, https//wwwnc.cdc.gov/EID/article/30/12/23-1143-App1.pdf). We processed ≥2 tissues from 36 armadillos and only 1 tissue for the other 12; we examined each tissue ≥2 times (Appendix 1). We performed DNA extraction and pathogen identification via real-time quantitative PCR (qPCR) using previously well-established primers and protocols (*8*,*9*) (Appendix 1). We considered a sample positive for *M. leprae* or *M. lepromatosis* only if 2 independent qPCR runs yielded a cycle threshold (Ct) <35 (*9*,*10*).

We processed a total of 84 armadillo tissue samples (Appendix 2, https//wwwnc.cdc.gov/EID/article/30/12/23-1143-App2.xlsx), including 38 (45.24%) liver, 26 (30.95%) spleen, and 10 (11.9%) muscle samples (Table). We identified *M. leprae* DNA in 13 (15.48%) samples, mostly from liver (n = 8/38 [21.05%]) and spleen (n = 4/26 [15.38%]) (Table). For 3 armadillos with varying results between tissues, the liver was the source of positivity (Appendix 2). All 84 tissue samples were negative for *M. lepromatosis* according to our protocols.

The 48 individual armadillos belonged to 4 different species: 40 (83.33%) were *D. novemcinctus*, 6 (12.5%) *Dasypus* spp. (not identified to species), 1 (2.08%) *D. pastasae*, and 1 (2.08%) *Cabassous centralis* (Table; Figure). We detected *M. leprae* in 9 *D. novemcinctus* armadillos, for an overall prevalence of 18.75%. Ct values were 26.01–33.66 (Table, Figure; Appendix 2). Most (20.83%, 10/48) armadillos were collected in the Esmeraldas province along the coast, among which only 1 (10%) *D. novemcinctus* armadillo was *M. leprae*–positive (Figure, Table). We observed the highest prevalence (42.86%) of infected armadillos in Santo Domingo de los Tsáchilas, in the northwest, where 3 of 7 animals were *M. leprae*–positive (Figure).

To characterize potential clusters of infected *D. novemcinctus* armadillos in Ecuador, we used the localities of the 9 *M. leprae*–positive armadillos to develop a species distribution model based on 1-class support vector machine hypervolumes (*11*) and 20 environmental predictors (Figure, panel C; Appendix 1 Figure 1). The subtropical region of Ecuador, west of the Andes mountains, had the highest concentration of environments like those with *M. leprae*–positive detections (Figure, panel D). Specifically, Esmeraldas, Los Ríos, Santo Domingo de los Tsáchilas, Santa Elena, northern Bolívar and Guayas, and southern Manabí are regions with environmental similarities to locales where infected *D. novemcinctus* armadillo were found (Figure).

Conclusions

The canon of leprosy transmission has been actively rewritten in the past 2 decades (*2*). Confirmation of zoonotic *M. leprae* transmission in the United States (*7*) prompted a series of studies to evaluate the spread of leprosy bacilli in the *D. novemcinctus* armadillo across its range in the Americas (Appendix 1 Figure 2). Our research demonstrated that nine-banded armadillos from the 3 continental regions of Ecuador host *M. leprae* with an 18.75% prevalence (Table; Figure). Detection of bacilli in wild armadillos is the first step in evaluating leprosy as a zoonotic pathogen in Ecuador. All 84 tissues examined were negative for *M. lepromatosis*, in agreement with previous results for other mammals in Europe and Mexico (*12*,*13*) and for armadillo specimens from across the Americas (*8*).

One limitation of our study was that our sampling scheme depended on local hunters who collect armadillos; thus, systematic sampling representing specific

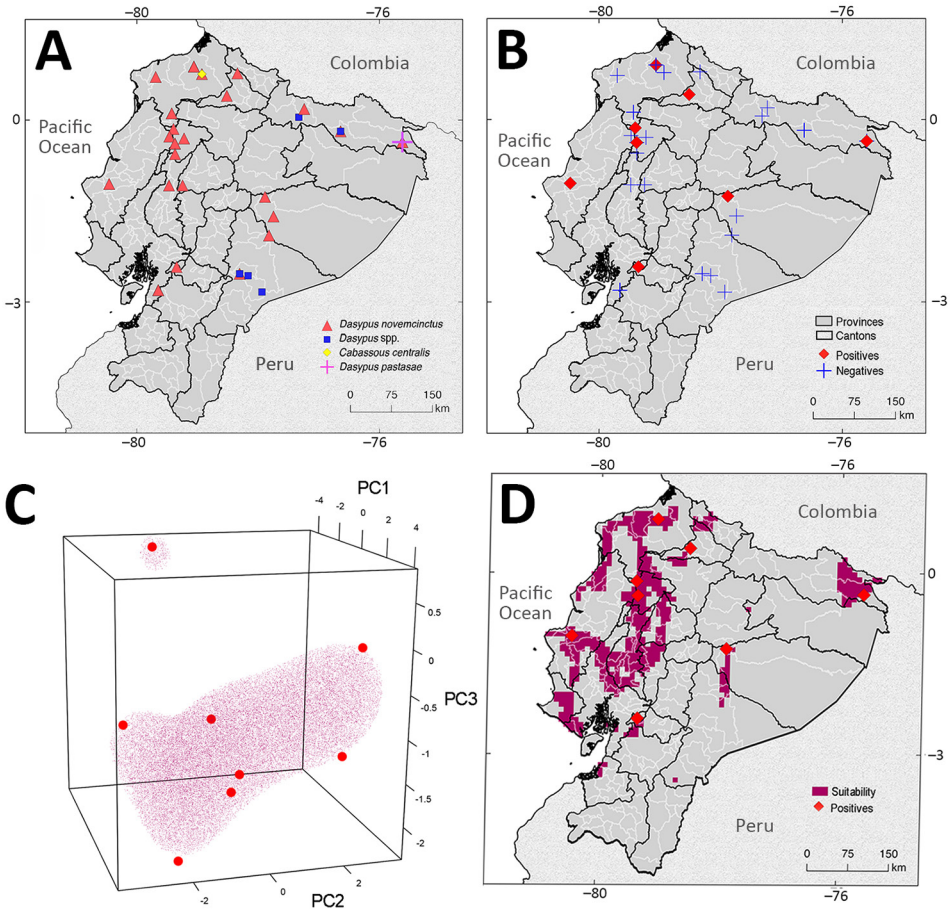

Figure. Locations of and geographic range *Mycobacterium leprae* detections in a study of *M. leprae* in nine-banded armadillos (*Dasypus novemcinctus*), Ecuador. A) Locations of armadillo collections and species identified. B) Locations from which *M. leprae*–positive armadillos samples collected. In southern Santo Domingo de los Tsáchilas, >1 armadillo was collected (Appendix 2, https://wwwnc.cdc.gov/EID/articles/30/12/23-1143-App2.xlsx). No samples were positive for *M. lepromatosis*. C, D) Vector machine hypervolume and its projected geography. C) One-class support vector machine hypervolume with enclosed regions of environmental similarity to areas with *M. leprae* detections (red points); D) map with projected geography for *M. leprae* detections. Latitude and longitude are shown at edges. Mapping developed with the information available in Appendix 2. PC, principal component (see Appendix 1, https://wwwnc.cdc.gov/EID/articles/30/12/23-1143-App1.pdf).

ecologic regions was unfeasible. Moreover, sampling scope was initially limited to tissues in ethanol, preventing serology and histopathology investigations. Nevertheless, we were able to collect tissues from across the country and observed consistency in the molecular detection of *M. leprae* with multiple rounds of qPCR in DNA extracted from various tissues from the same armadillo (Table; Appendix 2). Of note, a Ct value <35 in 2 independent qPCR rounds per tissue as criteria for *M. leprae* positivity is conservative, yet informative of the pathogen in nine-banded armadillos in the country.

Ecuador hosts at least 5 armadillo species: *Cabassous centralis*, *C. unicinctus*, *Priodontes maximus*, *D. pastasae*, and *D. novemcintus* (*14*). *D. novemcintus* was the most common (83.33%) armadillo species in our sampling and the only *M. leprae*–positive species (Table). We identified 3 other armadillo species, but all were *M. leprae*–negative (Figure, Table). Apart from *D. novemcinctus*, armadillo species in which *M. leprae* has been identified beyond Ecuador include *Euphractus sexcinctus, Dasypus* spp. nov., and *D. sabanicola*. Moreover, *D. septemcinctus* armadillos have been shown to be susceptible to *M. leprae* laboratory infections (*15*).

Species distribution models have seldom been used to characterize leprosy geographic range. Given the epidemiologic tenets of *M. leprae*, including long incubation period and human-to-human transmission, data for those models is difficult to obtain. Moreover, information on *M. leprae* prevalence in armadillos is either overrepresented as in the southern United States, or scant and dispersed as in the rest of the Americas (*2*) (Appendix 1 Figure 4). Thus, by leveraging our *M. leprae*–positive armadillo detections across the landscape of Ecuador, our model depicted clusters of environmental similarity. Considering the inherent challenges to collecting and studying armadillos (*3*), our model could be used to optimize future expedition sampling.

In conclusion, presence of a nonhuman *M. leprae* host carrier, the nine-banded armadillo, is likely to contribute directly or indirectly to the human leprosy incidence in Ecuador and other countries and will likely impair long-term goals of disease elimination. However, the detection of *M. leprae* in armadillos from Ecuador should exemplify how continued sampling and surveillance in wildlife can avert future zoonotic infections.

About the Author

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Appendix 1

Supplementary Methods

Detailed Methodology for DNA Extraction and Real-Time PCR

Detection of *Mycobacterium leprae* and *M. lepromatosis*

Two armadillo tissues were processed for 36 individuals and only one for 12 armadillos. For each tissue, at least two rounds of molecular diagnosis were applied. DNA extraction was performed using the QIAMP UCP Pathogen Minikit (Qiagen, Germany) following manufacturer instructions with minimal modifications. Briefly, ≈50–200mg of tissue were collected and rehydrated in phosphate buffer saline. After overnight incubation, the tissue was transferred to bead-beating tubes CKMix (Bertin Corp., Rockville, U.S.) and submitted to mechanical lysis for 30sec at 7200rpm in a Precellys® 24 instrument. Chemical lysis using 40µL of proteinase K (Qiagen, Germany) in ATL buffer (volume of 500µL final) was followed by incubation at 56°C until complete digestion of the tissue or overnight at 37°C. After a brief spin-down, the content of the tube was transferred to a new bead-beating tube containing 0.1mm zirconia bead. Mechanical lysis was then performed with three rounds of bead beating at 6800rpm for 30sec with five minutes on ice between rounds. The tube was then centrifuged at 10,000 g for one minute, and the supernatant was transferred to a new 2mL Eppendorf tube containing 200µL of APL2 buffer (Qiagen, Germany). After an incubation of 10min at 70°C the DNA was precipitated using 200 µL of absolute ethanol and purified on QIAmp UCP Pathogen Mini silica column as per manufacturer instructions.

All extracted samples were processed via quantitative real-time PCR (qPCR) targeting the repetitive element RLEP for *M. leprae* and the repetitive element RLPM for *M. lepromatosis* as previously described (*1*,*2*). We used 2uL of DNA to a PCR volume of 20uL containing 10uL SsoAdvanced Universal Probes Supermix (Biorad, CA, U.S.), 900 nM of each forward and reverse primers, and 250 nM of the corresponding hydrolysis probe. Initial denaturation for amplification state was done with 95°C for 10 min and 60°C for 1 minute for 40 cycles. We analyzed data using the CFX Maestro Software (BioRad, CA, U.S.). A sample was considered positive for *M. leprae* or *M. lepromatosis* only if two independent qPCR runs per tissue yielded readable cycle thresholds (Cts) below a value of 35 (i.e., Ct<35).

Positive controls included 2µL of a plasmid containing the RLEP region of *M. leprae* (*2*) and 2µL of *M. lepromatosis* (DNA diluted 1:100 from the stock received) donated by Dr. Ramanuj Lahiri from the National Hansen's Disease Program. Molecular grade water served as a negative control in each PCR run.

Molecular Identification of Armadillo Species

Extractions of DNA armadillo samples were used for standard PCR amplification of the cytochrome oxidase subunit I (COI) (*3*), amplifying a universal mitochondrial marker obtaining a 710bp fragment. Amplification was performed using 100ng of DNA and 25µL of DreamTaq mastermix (Thermo Fisher, MA, U.S.), 2.5 µL of forward primer at 5 µM (LCO1490–3′-GGTCAACAAATCATAAAGATATTGG-5′) and 2.5µL of reverse primer at 5 µM (HCO2198–3′-TAAACTTCAGGGTGACCAAAAAATCA-5′).

PCR products were subjected to 1 min at 95°C followed by 40 cycles of 30 sec at 95°C, 30 sec at 52°C, and 1 min at 68°C, coupled with a final extension of 10 min at 68°C. Positive control for this step included 2µL of DNA from an armadillo specimen collected in a previous study (U.S.-am-109) (*4*); molecular-grade water served as a negative control in each PCR run. Amplified DNA was visualized using the Bio-Rad Gel Doc EZ Electrophoresis Unit in a 1% agarose gel at 120V and SybrSafe (Thermo Fisher, MA, U.S.). Amplicons were purified using ExoSap IT PCR product cleanup reagent and sent for Sanger sequencing via Genewiz. Sequences were compared with published armadillo sequences available in NCBI using Geneious Prime® 2023.2.1. Sequences have been uploaded to GenBank with accession number 13769795.

Detailed Methodology for Development Species Distribution Models

Species distribution models are relevant tools for infectious disease mapping in ecologic and biogeographic contexts with applications across different pathogen systems (*5*). These models relate geographic localities at which a particular pathogen has been detected with environmental information to infer geographies with similar conditions (*6*). Pathogens with the capacity to endure in the environment depend on abiotic conditions for survival, these conditions can be mapped as demonstrated for *Bacillus cereus* biovar *anthracis*, causing anthrax in wildlife in Africa (*7*), or *Burkholderia pseudomallei*, causing melioidosis (*8*). Classical experiments on *M. leprae* environmental tolerance have suggested that the pathogen is able to survive in wet soil for up to 46 days, which may be related to its capacity to persist inside amoeba cysts (*9*,*10*). Moreover, studies demonstrating *M. leprae* viability in soil and water samples (*11*) suggest that armadillos might be acquiring the infection directly from environmental sources

Modeling Scheme

Species distribution models were developed using one-class support vector machine hypervolumes (OC-SVM (*12*);). OC-SVM builds a hyperellipse in the environmental space around occurrences, and trims it based on established parameters to suggest environmental similarities across the study area (*12*). Outputs from OC-SVM were binarized automatically by the algorithm and interpreted as clusters of environmental similarity across Ecuadorian landscape to depict areas of suitability for *Dasypus novemcinctus* infected with *M. leprae* (Figure main text).

Environmental Predictors

Considering known details of *M. leprae* requirements for survival (*9*,*13*), we constrained our environmental predictors to those representing temperature, humidity, soil chemical properties, and vegetation. For temperature and humidity, we used the open-access repository of bioclimatic variables MERRAclim, which uses satellite imagery to derive summaries of worldwide climatic conditions (*14*). The four bioclimatic variables combining temperature and humidity (e.g., BIO8 = mean temperature of most humid quarter) are known for creating spatial artifacts and were excluded from model development (*15*). MERRAclim variables were downloaded at 5′, ≈8 km at the Equator; we used the database from 2000–2010 for temporal resolution. To represent soil chemistry, we used variables from the SoilGrids repository, at a

resolution of 250m using a combination of field and satellite data (*16*). Specifically, we included summaries of cation exchange capacity (CECSOL), organic soil carbon (ORCDRC), water pH (PHIHOX), and nitrogen at one depth (0–5 cm). Finally, we used the Enhanced Vegetation Index (EVI) as a proxy of vegetation availability in the study area (*17*). We collected satellite images from 2010–2021 (version 6) from the Moderate Resolution Imaging Spectroradiometer (MODIS) instrument from the TERRA satellite (i.e., MOD13Q1, 16 day composites, 250m resolution (*18*);), and averaged them to obtain a summary of vegetation characteristics via Google Earth Engine (*19*). All variables were resampled to match the resolution of MERRAclim (i.e., 5′). To avoid overfitted model outputs owing to multicollinearity (*20*), we applied a singular value decomposition principal component analysis (PCA) and used the PCs summarizing >85% of the variance as final predictors for model building (i.e., PCs 1–3). Raster data collection and manipulation were performed using the 'raster' and 'kuenm' packages in R (*21*,*22*).

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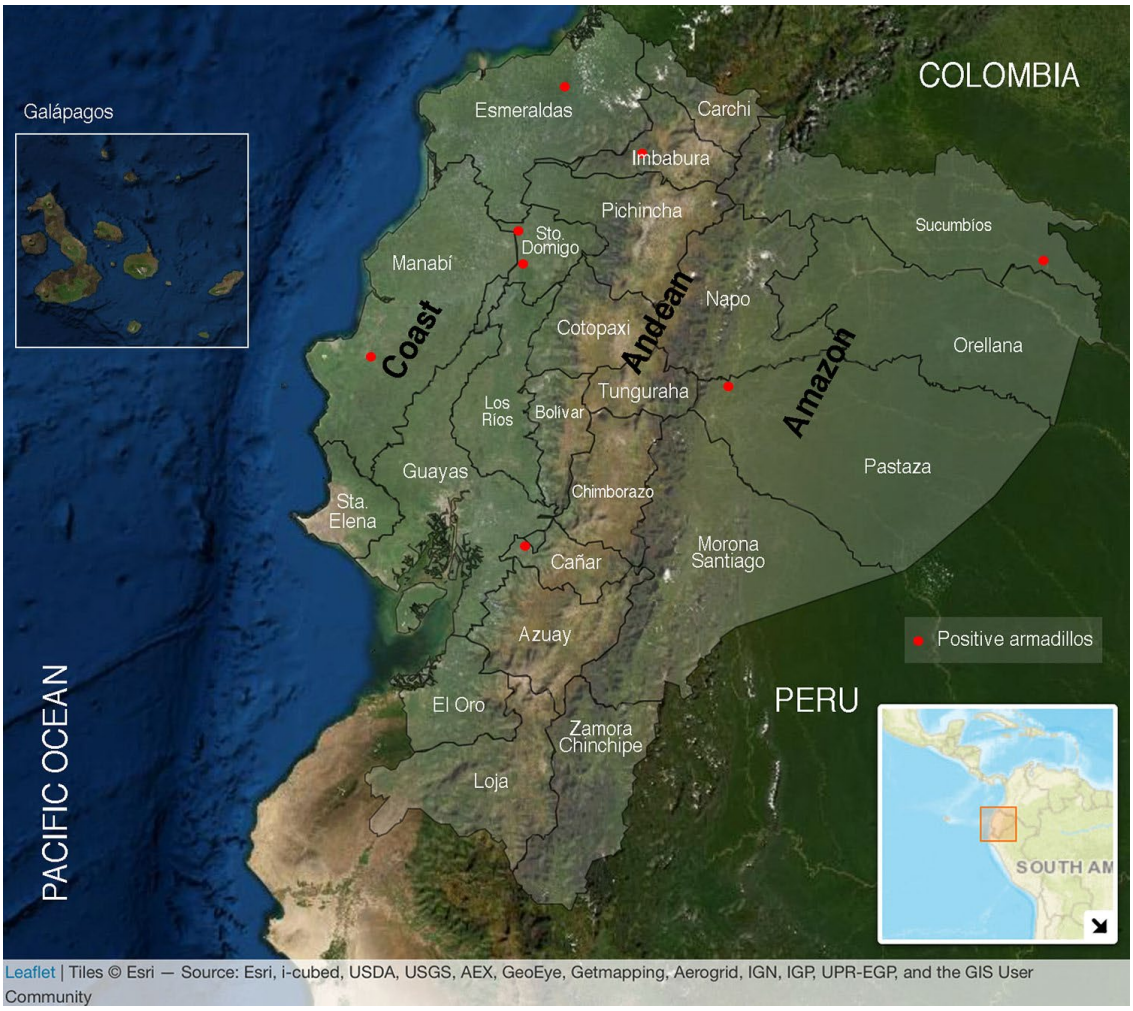
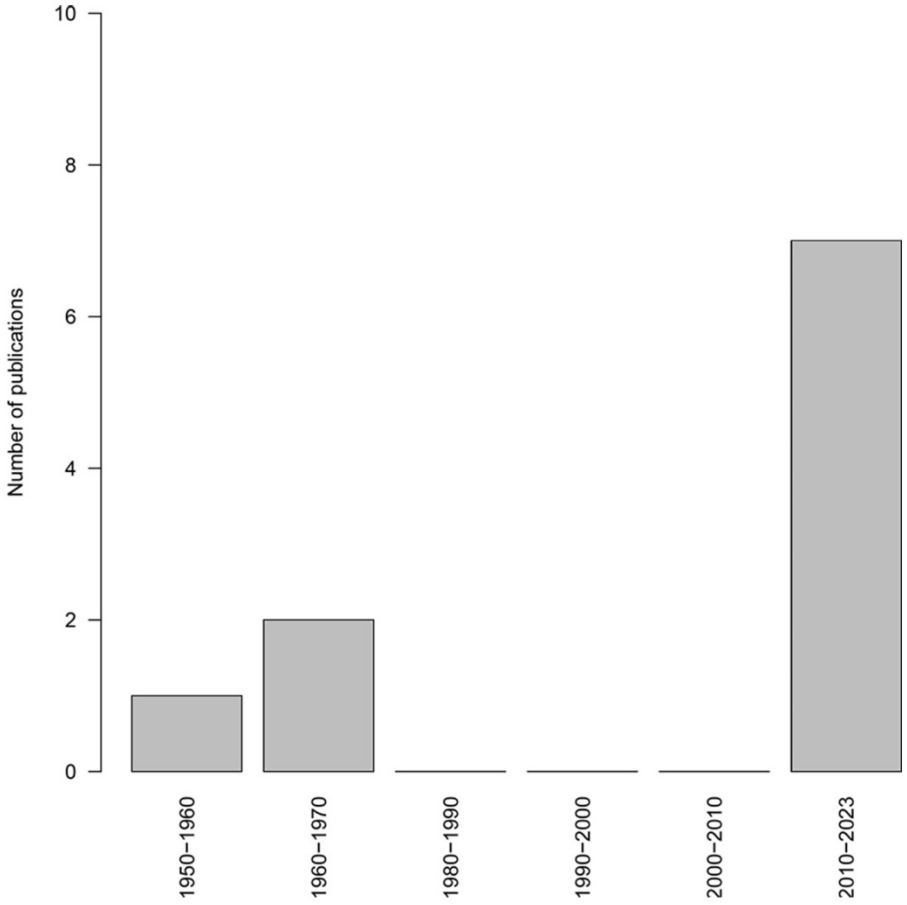
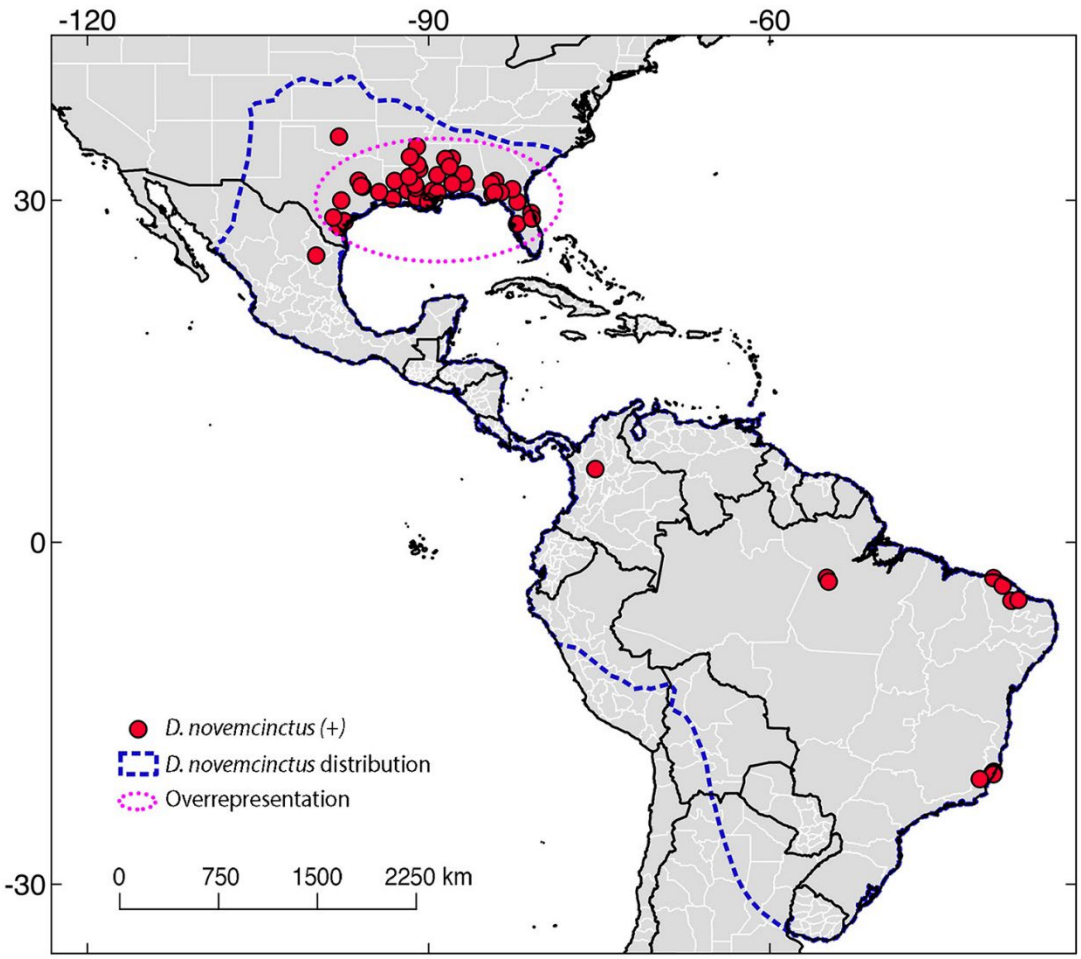


Appendix 1 Figure 1. Physical map of Ecuador depicting provinces as political divisions of the country. Localities with positive *Mycobacterium leprae* detections in nine-banded armadillos are shown as red dots (n = 9); the southern locality at Santo Domingo de los Tsáchilas had 2 armadillos from the same locale). Armadillo species listed in the main text. Map developed with the package 'leaflet' in R (The R Project for Statistical Computing, https://www.r-project.org) and modified with Adobe Photoshop Elements (https://www.adobe.com).



Appendix 1 Figure 2. Frequency of academic publications related to leprosy in Ecuador according to an advanced search in PubMed database performed on June 20, 2023. Search query used: (((((LEPRA[Title/Abstract]) OR (LEPROSY[Title/Abstract])) OR (ENFERMEDAD DE HANSEN[Title/Abstract])) OR (HANSEN'S DISEASE[Title/Abstract])) AND (ECUADOR[Title/Abstract])). We found a total of 10 research papers, the majority published between 2010–2023.



Appendix 1 Figure 3. We collected occurrences of *Dasypus novemcinctus* infected with *Mycobacterium leprae* across the Americas since the 2000s and suggested an updated distribution of *D. novemcinctus* (blue line). Given sampling overrepresentation in the southern United States (pink dotted line) and scant data on *M. leprae* in *D. novemcinctus* elsewhere (red dots), we developed a species distribution model aiming to identify clusters of environmental similarity across the Ecuadorian landscape using the positive *M. leprae* detections of our study (See main text Figure).