Received: 28 September 2023 | Revised: 5 January 2024 | Accepted: 29 January 2024

DOI: 10.1002/edn3.522

Environmental DNA
Open Access
Dedicated to the study and use of environmental DNA for basic and applied sciences

WILEY

ORIGINAL ARTICLE

Blood meal metabarcoding of the argasid tick (*Ornithodoros turicata* Dugès) reveals extensive vector-host associations

Sujata Balasubramanian[1] | Rachel E. Busselman[1] | Nadia A. Fernandez-Santos[2,3] | Andrew P. Grunwald[4] | Nicholas Wolff[5] | Nicholas Hathaway[6] | Andrew Hillhouse[5] | Jeffrey A. Bailey[7] | Pete D. Teel[2] | Francisco C. Ferreira[2] | Sarah A. Hamer[1] | Gabriel L. Hamer[2]

[1]Department of Veterinary Integrative Biosciences, School of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, Texas, USA

[2]Department of Entomology, Texas A&M Agrilife Research, Texas A&M University, College Station, Texas, USA

[3]Instituto Politécnico Nacional, Centro de Biotecnología Genómica, Laboratorio de Biomedicina Molecular, Reynosa, Tamaulipas, Mexico

[4]Laguna Atascosa National Wildlife Refuge, U.S. Fish and Wildlife Service, Los Fresnos, Texas, USA

[5]Texas Institute for Genome Sciences and Society, Texas A&M University, College Station, Texas, USA

[6]Department of Internal Medicine, UMass Chan Medical School, Worcester, Massachusetts, USA

[7]Department of Pathology and Laboratory Science, Brown University, Providence, Rhode Island, USA

Correspondence
Gabriel L. Hamer, Department of Entomology, Texas A&M Agrilife Research, Texas A&M University, College Station, TX, USA.
Email: gabe.hamer@ag.tamu.edu

Sujata Balasubramanian, Department of Veterinary Integrative Biosciences, School of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX, USA.
Email: sbalasubramanian@tamu.edu

Present address
Andrew Hillhouse, FUJIFILM Diosynth Biotechnologies, College Station, Texas, USA

Funding information
Texas A&M AgriLife Research;
Texas Ecolab;
Coalition for Epi Response, Engagement, and Science (CERES)

Abstract

Molecular methods to understand host feeding patterns of arthropod vectors are critical to assess exposure risk to vector-borne disease and unveil complex ecological interactions. We build on our prior work discovering the utility of PCR-Sanger sequencing blood meal analysis that work well for soft ticks (Acari: Argasidae), unlike for hard ticks (Acari: Ixodidae), thanks to their unique physiology that retains prior blood meals for years. Here, we apply blood meal metabarcoding using amplicon deep sequencing to identify multiple host species in individual *Ornithodoros turicata* soft ticks collected from two natural areas in Texas, United States. Of 788 collected *O. turicata*, 394 were evaluated for blood meal source via metabarcoding, revealing 27 different vertebrate hosts (17 mammals, five birds, one reptile, and four amphibians) fed upon by 274 soft ticks. Information on multiple hosts was derived from 167 individual *O. turicata* (61%). Metabarcoding revealed mixed vertebrate blood meals in *O. turicata* while same specimens yielded only one vertebrate species using Sanger sequencing. These data reveal wide host range of *O. turicata* and demonstrate the value of blood meal metabarcoding for understanding the ecology for known and potential tick-borne pathogens circulating among humans, domestic animals, and wildlife such as relapsing fever caused by *Borrelia turicatae*. Our results also document evidence of prior feeding on wild pig from an off-host soft tick for the first time in North America; a critical observation in the context of enzootic transmission of African swine fever virus if it were introduced to the US. This research enhances our understanding of

vector-host associations and offers a promising perspective for biodiversity monitoring and disease control strategies.

KEYWORDS
African swine fever virus, *Borrelia turicatae*, soft tick trapping, tick-borne relapsing fever, wild pigs

1 | INTRODUCTION

Blood meal analysis of hematophagous arthropods can reveal local vertebrate diversity (Schnell et al., 2012) and identify host species utilized by vectors that transmit viruses, bacteria, and parasites that affect humans, domestic animals, and wildlife (Borland & Kading, 2021). Recently, blood meal analysis using a metabarcoding approach (PCR with universal primers followed by next-generation sequencing) has revealed an increase in number and diversity of hosts utilized by mosquitoes (Alonso et al., 2023; Logue et al., 2016), triatomines (Balasubramanian et al., 2022; Dumonteil et al., 2018; Murillo-Solano et al., 2021; Polonio et al., 2021; San Juan et al., 2023), and sand flies (Abbasi et al., 2019), providing insights from both the perspective of pathogen transmission and also to elucidate the community of hosts in the environment. For instance, by using this approach Kocher et al. (2017, 2023) demonstrated that locations with higher indices of anthropogenic modification were associated with lower mammal diversity, and increased relative abundance of blood meals derived from reservoir hosts for *Leishmania*, which in turn favors vectorial pathogen transmission. Therefore, blood meal metabarcoding appears to be a powerful tool to unveil vector-host interactions in areas where pathogens circulate among humans, domestic animals, and wildlife.

Soft ticks (Acari: Argasidae) are long-lived, hematophagous arthropod vectors, including approximately 200 species, known to vector a variety of pathogenic bacteria and viruses (Mans et al., 2019; Manzano-Román et al., 2012). The genus *Ornithodoros* includes important vectors of medical and veterinary importance globally. In the USA, *O. turicata* is found in Florida (recognized as *O. turicata americanus* by Beck et al. (1986)) and from the southwestern US from Texas to California including the high plains of Kansas and Colorado, extending into Mexico (Donaldson et al., 2016; Dworkin et al., 2002). Different species of *Ornithodoros* are important vectors and reservoirs for pathogenic bacteria in the spirochete genus *Borrelia* that cause tick-borne relapsing fever (TBRF) in humans and in domestic animals in the Americas (Faccini-Martínez et al., 2022). *Ornithodoros turicata* transmit *Borrelia turicatae*, an agent of TBRF, which is found in Texas and other western states associated with caves or animal burrows (Beeson et al., 2023; Campbell et al., 2019; Dworkin et al., 2008). Multiple *Ornithodoros* ticks, including *O. turicata*, are also competent vectors of African swine fever virus (ASFV). ASFV causes 100% mortality in domestic swine (Manzano-Román et al., 2012), and is currently considered the most impactful disease for the pig production industry (Jori et al., 2023). ASFV is a DNA virus (family Asfiviridae) discovered in Africa that has emerged in Europe, Asia, and the Caribbean (Gonzales et al., 2021; Jean-Pierre et al., 2022; Penrith et al., 2013). Though not currently in the US, Texas is included in the list of US states at elevated vulnerability for ASFV introduction (Herrera-Ibatá et al., 2017; Wormington et al., 2019). There is no broadly distributed vaccine for ASFV and the current method of control relies on biosecurity measures to prevent contact between the non-infected domestic pigs with virus sources (Brake, 2022; USDA, 2022), including *Ornithodoros* ticks, which can transmit the virus for at least one year after infection (Boinas et al., 2011).

Ornithodoros ticks feed on a broad diversity of vertebrates including reptiles, birds, livestock, companion animals (e.g., dogs), and humans (Busselman et al., 2021; Kim et al., 2021; Kleinerman et al., 2021; Palma et al., 2013). DNA from prior blood meals is retained for years in soft ticks (Beck et al., 1986; Francıs, 1938; Kim et al., 2021), and our recent study using *O. turicata* reared under laboratory conditions confirmed chicken (*Gallus gallus*) and domestic pig (*Sus scrofa*) blood meals via Sanger sequencing 1105 and 622 days, respectively, after feeding took place (Busselman et al., 2021). The habitat of *O. turicata* includes caves and animal burrows with nidicolous species of vertebrates (Adeyeye & Butler, 1989; Francıs, 1938; Manzano-Román et al., 2012). Because soft ticks can live for many years in environments with favorable temperature and humidity conditions and feed multiple times throughout their lifetime (Dworkin et al., 2002), analyzing their blood meals may become an efficient way to characterize local vertebrate host communities.

In this study, a metabarcoding approach was applied to identify the diversity of hosts utilized as blood meal sources by *O. turicata* collected from two natural areas in Texas, USA between 2019 and 2022. Understanding the host preferences and blood-feeding habits of *O. turicata* contributes to informing preventive and control measure. This tool could also help identify soft tick-vertebrate host networks in other regions of the world such as Africa, Europe, and Asia where ASFV is endemic, which was recently highlighted as an important knowledge gap (Jori et al., 2023).

2 | METHODS

2.1 | Locations and soft tick sampling

Soft ticks were collected from two natural areas in Texas, USA. One site was Government Canyon State Natural Area (GCSNA), San Antonio, TX (Lat: 29.549316, Lon: −98.764715; Texas Parks and Wildlife State Park Scientific Study Permit No. R3-02-19). Samples from this location

were described in a prior study (Busselman et al., 2021). Sampling took place in March 2019 from three caves (Bone Pile, Little Crevice and Mad Crow). As previously described, dry ice-baited sticky traps were placed at the opening of the three caves for two consecutive days with one check and reset (removal of ticks, replacement of dry ice and sticky material) at 24 h. The glue board material (Trapper Max Mouse & Insect, Sheffield, UK) remained sticky even with moisture or rain.

The second sampling site was Laguna Atascosa National Wildlife Refuge (LANWR), Los Fresnos, TX (Lat: 26.22901, Lon: −97.34725; US Fish and Wildlife Service Research & Monitoring Special Use Permit No. 2022-LA-001). Sampling at LANWR took place between February and June 2022. LANWR was established as a National Wildlife Refuge for migrating and wintering birds with a diverse community of vertebrates including bird, mammal, and reptile species. The landscape consists of thorn scrub, coastal prairies, salt flats, and estuaries and given the absence of caves in this landscape, traps were placed in multiple animal burrows. For this study, we screened soft ticks from burrows designated LA-1, LA-2, LA-6_14, LA-7, LA-8, LA14, LA15, LA-16 LA-23, LA-24, LA-25, LA-26. The dry-ice-baited sticky traps used at LANWR differed from those used in GCSNA and which were modeled after those described by Miles (1968). Dry ice was placed in a 1.9 L cooler with 1–2 m of clear vinyl tubing (4.8 mm inside diameter and ~ 10.1 mm outside diameter) inserted into the cooler drinking spout. At the sampling location, the tubing was secured to a natural stick about 0.5 m to 1 m long with duct tape and inserted into the burrow. At the terminal end of the tubing and stick, a 4 cm strip of glue board (Trapper Max Mouse & Insect, Sheffield, UK) was attached creating a u-shape with the adhesive side on the interior surface (Figure S1). The sticky trap was inserted into nests or burrows and the dry ice sublimation from the cooler caused CO_2 release at the end of the tube, attracting hematophagous arthropods which, when approached, became immobilized on the adhesive part of the tape. Traps were left in the burrows for approximately 24 h, after which soft ticks were removed from the traps with forceps and placed in vials with 70% ethanol.

2.2 | Camera trap data

Data for visual observations of animal activity around Little Crevice, and Mad Crow caves at GCSNA from 2105 to 2016 were procured from Kim (2017). At LANWR, camera traps (Stealth Cam 2022 G42NG 32MP No-Glow Trail Camera) were set up between March and August 2023 facing directly at the burrows as well as nearby locations within 100 m that directed towards openings well-traveled by animals based on tracks. Animal species in the pictures were identified morphologically to the lowest taxonomic unit.

2.3 | Soft tick processing, DNA extraction, molecular barcoding

Soft ticks from GCSNA were processed as described earlier (Busselman et al., 2021). Soft ticks from LANWR were measured, sexed, and identified using taxonomic keys (Guzmán-Cornejo et al., 2019; Pratt & Stojanovich, 1963) as *Ornithodoros turicata*. Specimens from each location have been deposited into Texas A&M University Insect Collection (TAMUIC) under voucher number TAMUIC764. Soft ticks were surface sterilized in 10% bleach for 15 s followed by two rinses in DNAse-free water for 15 s each. DNA was extracted from individual ticks randomly and selected to represent all sizes and life stages from all burrows without discerning by collection date. If more than 25 ticks were collected from a burrow, a subset of 25 were selected for extraction; if fewer than 25 were collected, all ticks were used. The only exception to this was LA6_14 from where a total of 42 ticks were processed. This resulted in the extraction of DNA and evaluation of 19 soft ticks from LA-1, 25 from LA-2, 42 from LA-6_14, 24 from LA-7, 25 from LA-8, 25 from LA-15, one soft tick from LA-16, three soft ticks from LA-23, 19 from LA-24, 19 from LA-25, and 24 from LA-26. For this, the MagMAX CORE Nucleic Acid Purification Kit (Applied Biosystems Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used following manufacturer instructions. Total nucleic acid was eluted into 50 μL of elution buffer. In addition to morphological identification, a subset of randomly selected soft ticks (but including specimens from all sites within GCSNA and LANWR) were subjected to a barcoding PCR targeting a ~700 bp fragment of the mitochondrial cytochrome c oxidase subunit I gene (COI) using primers LCO1490 (5′GGTCAACAAATCATAAAGATATTGG3′) and HCO2198 (5′TAAACTTCAGGGTGACCAAAAAATCA3′) (Folmer et al., 1994). For this, the FailSafe 2× premix buffer E (Biosearch Technologies, Petaluma, CA, USA) was used with 0.5 μL of Enzyme mix, 1 μL of each primer (10 pmol), and 3 μL of DNA template in a 25 μL of final volume per reaction. Cycling conditions involved an initial denaturation at 94°C for 3 min, 40 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 8 min. PCR products were visualized in a 2% agarose gel, positive samples were purified with ExoSap-IT (USB Corporation, Cleveland, OH, USA) following the manufacture's protocol, and bi-directionally sequenced by Eton Bioscience (San Diego, CA, USA). Sequences were quality-trimmed and assembled using Geneious Prime 2023.2.1 (Biomatters Inc. San Diego, CA, USA), and the consensus results were used as a query in the NCBI Basic Local Alignment Search Tool, BLASTn (Altschul et al., 1990).

2.4 | Metabarcoding PCR and sequence analysis

PCR amplification of a ~145 bp fragment from the 12S rRNA mitochondrial gene using Kapa HiFi DNA Polymerase (Roche Sequencing, Indianapolis, IN, USA) was performed. Both forward (5′CAAACTGGGATTAGATACC3′) and reverse (5′AGAACAGGCTC CTCTAG3′) primers (Humair et al., 2007; Kieran et al., 2017) had 10-base barcode tags added (Shokralla et al., 2014). Barcode tags on forward and reverse primer pairs were identical (Schnell et al., 2015). Conditions for the metabarcoding PCR consisted of an initial denaturation at 95°C for 3 min, followed by 40 cycles of 30 s at 98°C, 60 s at 63°C, and 1 min at 72°C. PCR for every sample was performed in duplicate with different barcodes for replicates (Schnell et al., 2015).

If one of the two replicates for a sample amplified and the other did not, one more attempt was made to generate a second amplicon. Replicates were successfully generated for 162 samples (55 GCSNA and 107 LANWR). PCR products were submitted to the Texas A&M Institute for Genome Sciences and Society for library preparation and sequencing. Sequencing libraries with Illumina sequencing adapters were made from the barcoded PCR product pools using xGen™ ssDNA & Low Input DNA Library Prep kit from Integrated DNA technologies (Coralville, IA, USA) following manufacturer instructions, and run on the NovaSeq6000 platform (Illumina, San Diego, CA, USA).

For analysis of sequencing reads, paired reads were matched using BBmerge (https://jgi.doe.gov/data-and-tools/software-tools/bbtools/). Merged reads were analyzed using Seekdeep (https://seekdeep.brown.edu/) (Hathaway et al., 2018). Sequencing data processing commands have been deposited into DataDryad (Balasubramanian et al., 2024). Barcodes and primers were trimmed out, sequences were filtered for a minimum length of 100 bp, and reads that had a quality score of less than 25 across 75% length were rejected. Filtered reads were binned on single base difference. Chimeras were marked and removed. Samples with fewer than 1000 total reads were removed from all analyses. When replicates were available for samples, only sequences that were present in both replicates were accepted. Resulting sequences were matched with hostnames using Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information, US National Library of Medicine) using MegaBLAST parameters against the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) (Altschul et al., 1990). Sequences showing 99% or higher identity to database sequence were identified to species level. GenBank matches with identities between 94.79% and 99% were accepted when only one species of the genus occurs in the US according to the GBIF database (https://www.gbif.org/); this included collared peccary (*Pecari tajacu*), North American porcupine (*Erethizon dorsatum*), barking frog (*Craugastor augusti*), nine-banded armadillo (*Dasypus novemcinctus*), and Virginia opossum (*Didelphis virginiana*). In other instances where multiple species occur within Texas or the county of sampling, the lowest common taxonomic unit was assigned; this included Passeriformes – order level, Sciuridae – family level, *Crotalus*, *Eleutherodactylus*, *Neotoma*, *Scaphiopus* – genus level. The possibility of presence of a vertebrate host in the study area was taken into consideration. Vertebrates whose presence could not be established, given currently available information (GBIF database https://www.gbif.org/) were removed. Since the target DNA is mitochondrial, chromosomal matches identified by NCBI BLAST search were also removed. As a final stringency measure in multiple host samples, vertebrate hosts with either fewer than 500 reads or fewer than 1% of the total reads for that sample, whichever higher, were removed. Many steps were taken to minimize contamination such as using filter pipette tips and single-use consumables, conducting all laboratory steps in disinfected biosafety cabinets working in batches to limit the number of tagged primers used at a given time, working in separate areas of the laboratory and by including negative controls in all metabarcoding PCR runs, all of which were negative.

2.5 | Diversity analysis and host network

A vector-host network to show the diversity of hosts associated with each collection site was created (Figure 1). To compare the diversity of hosts identified through our metabarcoding approach within each community (GCSNA or LANWR), we calculated Simpson's index of diversity for each cave or burrow within GCSNA and LANWR using the number of different hosts found in each site's population of ticks:

$$1 - D, \text{where } D = \frac{\Sigma n(n-1)}{N(N-1)}$$

where n = number of individuals within each host species and N = total number of individuals identified across all hosts. A value closer to 1 indicates a more diverse host community and a value closer to 0 indicates a less diverse host community. This metric takes into consideration both the richness and evenness of species diversity. For each site, bootstrap resampling techniques were used to generate 95% confidence intervals of the Simpson's index of diversity (Figure S3) (Efron & Tibshirani, 1993; Tinio & Sebuala, 2021). To bootstrap, the population of hosts at each site was sampled a number of times based on how many ticks had been collected at that site, and the Simpson's index of diversity was calculated; this was repeated 10,000 times to generate confidence intervals around the originally calculated Simpson's index of diversity at each site. The indices and 95% confidence intervals were then compared across sites within each larger community, such that when the 95% confidence intervals did not overlap between locations, their diversity indices are significantly different from one another (p-value < 0.05). Only sites with more than one host identified were included. Statistical analyses and data visualization were performed in Program R (R Core Team, 2023) using R Studio (Posit, 2023) with package bipartite (Dormann et al., 2008) and boot (Canty & Ripley, 2022; Davison & Hinkley, 1997). Code used to perform these analyses has been deposited into DataDryad (Balasubramanian et al., 2024).

3 | RESULTS

3.1 | Ornithodoros turicata collections

A total of a total of 381 soft ticks were collected from three caves within GCSNA, identified as *O. turicata*; 32 were female, 55 male, the rest were unidentifiable for sex, and 285 were nymphs, as previously reported in Busselman et al. (2021). A total of 407 soft ticks were collected from 11 locations within LANWR from February to June 2022. Specimens included 35 larvae, 235 nymphs, and 137 adults, which were morphologically identified as *O. turicata*. Molecular barcoding of 25 soft ticks – eight from GCSNA (three adults, four

Environmental DNA
Open Access
Dedicated to the study and use of environmental DNA for basic and applied sciences
WILEY

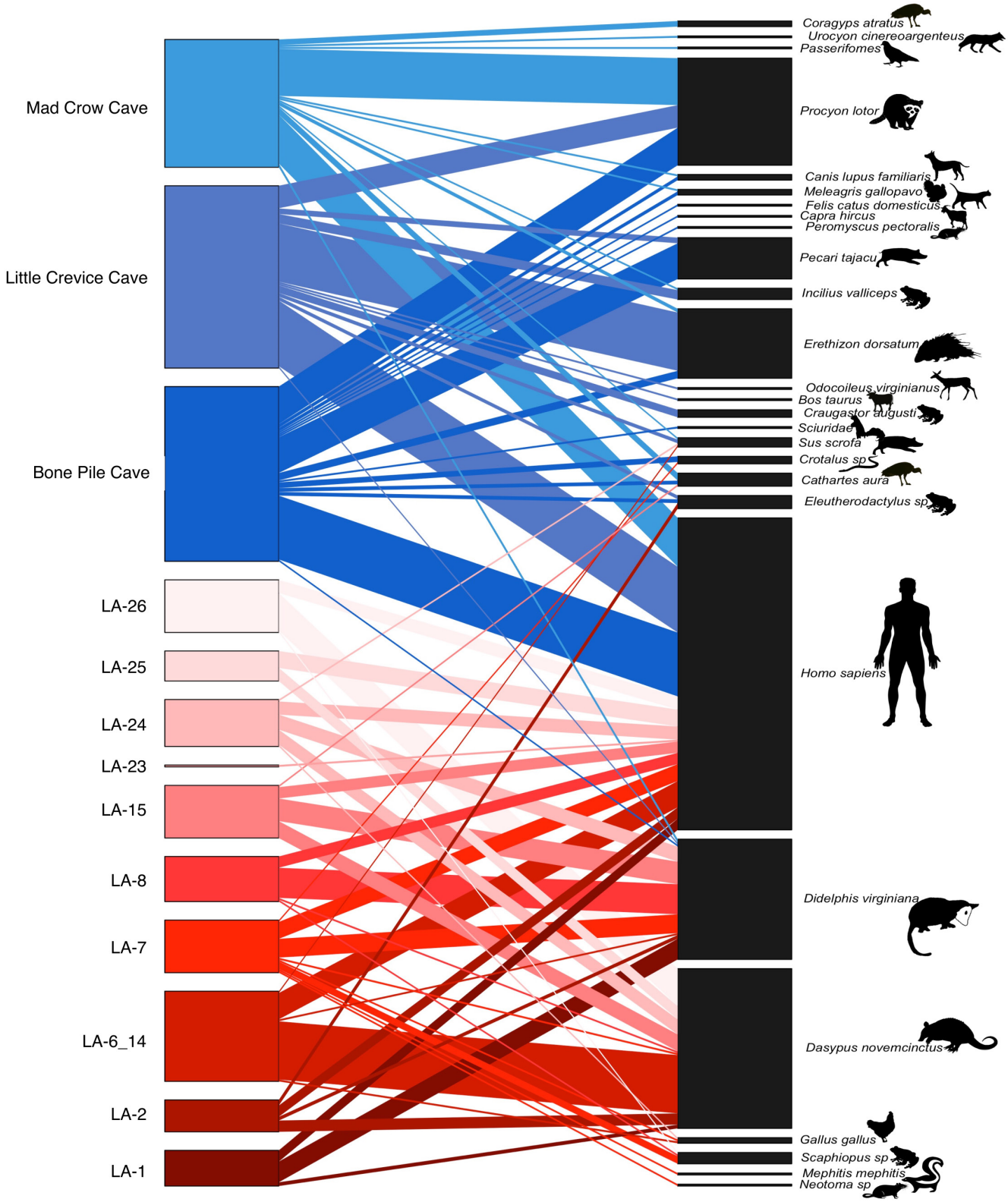

FIGURE 1 Graphic depiction of sites within Government Canyon State Natural Area (GCSNA) (left bars in blue) and Laguna Atascosa National Wildlife Refuge (LANWR) (left bars in red) and vertebrate hosts detected by blood meal metabarcoding (right column). The length of any given site's bar is proportional to the number of hosts found, and the lengths of the host bar reflect the number of times that hosts have been detected in blood meals in the study. The width of the networking lines indicates the number of times the host has been found at that location with the widest lines being the most frequent hosts for that location.

nymphs, one unknown) and 17 from LANWR (7 adults, 10 nymphs) – were all confirmed as *O. turicata*. Sequences binned into three *O. turicata* COI haplotypes that were 99.07–99.84% identical to each other (differences were 1, 5, and 6 bp). One haplotype (GenBank accession number OR980950) was present in both locations, while one (GenBank accession number OR980949) was found only once in LANWR, with a third one found twice in GCSNA (GenBank accession number OR980951). These sequences matched GenBank *O. turicata* records with 99.07–99.8% identity (GenBank accession number ON800887).

3.2 | Metabarcoding analysis

From the 381 soft ticks collected at Government Canyon State Natural Area (GCSNA), 168 (69 adults and 97 nymphs, 2 undetermined) were subjected to the metabarcoding PCR. Products were visualized for 122 samples, 121 of which yielded sequencing reads. Hosts were identified from 118 samples (47 adults, 70 nymphs, one unknown) at an average of 2.5 vertebrates identified as blood meal source per soft tick (Appendix S1). Mammals were 14 of the 22 hosts, four were avian, one reptilian, and three amphibian (Table 1, Figure 1). After humans, the most frequent hosts were raccoon (*Procyon lotor*) followed by North American porcupine and collared peccary. The maximum number of hosts detected from one soft tick was six (ST-47A) from Bone Pile cave: raccoon, collared peccary, turkey vulture (*Cathartes aura*), turkey (*Meleagris gallopavo*), human (*Homo sapiens*) and rattlesnake (*Crotalus* sp.) (Appendix S1 GCSNA), while 5%, 19%, 58%, and 16% of the soft ticks had fed on four, three, two and one host, respectively. All three caves where soft ticks were collected at GCSNA had relatively high Simpson's index of diversity values (Bone Pile Cave: $1-D=0.79$, 95% CI 0.74–0.85; Mad Crow Cave: $1-D=0.72$, 95% CI 0.66–0.80; Little Crevice Cave: $1-D=0.75$, 95% CI 00.71–0.81) with overlapping 95% confidence intervals; thus, no significant difference in diversity indices between caves was identified (Figure S3).

On the other hand, from the 407 *O. turicata* collected at Laguna Atascosa National Wildlife Refuge (LANWR), 226 (78 adults, 133 nymphs, and 15 larvae) were tested via metabarcoding PCR and products were obtained from 159 samples. Information on blood meal hosts was obtained from 156 soft ticks (65 adults, 90 nymphs, and one larva; 69% of the total ticks tested), revealing 11 host taxa across four vertebrate classes (Table 2, Appendix S1). An average of

TABLE 1 Table of caves and hosts for Government Canyon State Natural Area (GCSNA), San Antonio, Texas, indicating the number of times each host was identified in *Ornithodoros turicata* using metabarcoding at the 12S rRNA mitochondrial locus. Visual confirmation is based on records from September 2015 to October 2016 (Kim, 2017).

Vertebrate host	Overall	Bone pile	Little crevice	Mad crow
Craugastor augusti/Barking frog	4	0	4	0
Coragyps atratus/Black vulture	3	0	0	3
Felis catus domesticus/Cat	1	1	0	0
Bos taurus/Cattle	1	0	1	0
Eleutherodactylus marnockii/Cliff chirping frog	4	2	2	0
Canis lupus familiaris/Dog	3	2	0	1
Sus scrofa/Wild pig	3	0	2	1
Capra hircus/Goat	1	1	0	0
Urocyon cinereoargenteus/Gray Fox	1	0	0	1
Incilius valliceps/Gulf Coast toad	6	0	5	1
Homo sapiens/Human	95	34	35	26
Erethizon dorsatum/North American Porcupine	36	4	30	2
Passeriformes	1	0	0	1
Pecari tajacu/Collared peccary	22	19	3	0
Procyon lotor/Raccoon	56	19	12	25
Crotalus sp/Rattlesnake	3	3	0	0
Sciuridae	1	1	0	0
Meleagris gallopavo/Turkey	3	2	0	1
Cathartes aura/Turkey vulture	6	2	0	4
Didelphis virginiana/Virginia opossum	4	1	1	2
Peromyscus pectoralis/White-ankled mouse	1	1	0	0
Odocoileus virginianus/White-tailed deer	1	0	1	0

1.5 hosts per individual tick was identified. The nine-banded armadillo was the most frequently identified host, followed by human and Virginia opossum. A maximum of four different host species were identified in one soft tick from LANWR (LA-24R – nine-banded armadillo, human, chicken, Virginia opossum). Based on the estimated 95% confidence intervals of the Simpson's index of diversity for each site, three LANWR sites had significantly lower diversity metrics than other LANWR sites ($p < 0.05$, Figure S3). LA-6_14 (1-D = 0.51, 95% CI 0.40–0.63), LA-25 (1-D = 0.53, 95% CI 0.45–0.66), and LA-26 (1-D = 0.44, 95% CI 0.29–0.63) all had significantly lower diversity indices than three other sites (LA-2: 1-D = 0.75, 95% CI 0.68–0.91; LA-7: 1-D = 0.77, 95% CI 0.70–0.90; and LA-24: 1-D = 0.74, 95% CI 0.70–0.85). No other significant differences in Simpson's index of diversity were noted.

3.3 | Integration with camera trap data

Camera trap images at GCSNA showed 15 vertebrates in the vicinity of two of the three caves (Little Crevice Cave and Mad Crow cave) (Kim, 2017). At LANWR, 25 vertebrates were observed at and around the 11 burrows sampled for soft ticks. An overlap of 5 vertebrates was noted among the two locations (coyote (*Canis latrans*), bobcat (*Lynx rufus*) turkey vulture, Virginia opossum, wild pig) (Table S1). At GCSNA, counting both metabarcoding and camera trap vertebrates, 26 vertebrates were identified, with 22 of the 26 (85%) detected by metabarcoding. These 22 were evenly split with 11 vertebrates detected only through metabarcoding and 11 hosts detected both by metabarcoding and camera trap. Ringtail (*Bassariscus astutus*), coyote, bobcat, and black-tailed jackrabbit (*Lepus californicus*) were observed on trail cameras but were not detected in blood meals (Table 1, Table S1). At LANWR, 30 vertebrates were identified by both methods combined – five by metabarcoding alone, six by both methods, and 19 on camera alone so metabarcoding detected 11 of the 30 (37%) of all vertebrates combined (Tables S1 and S2).

4 | DISCUSSION

This study is the first utilizing a metabarcoding approach to characterize the diversity of hosts fed upon by soft ticks, revealing a total of 27 different vertebrate species (22 in GCSNA, 11 in LANWR, and six common to both locations) among blood meals of individual *O. turicata* sampled in Texas. Adult *O. turicata* have been documented to live over 7 years (Francıs, 1938), while those of *Argas brumpti* have been observed to live over 26 years (Shepherd, 2022). Such long-lived arthropods have opportunities to take multiple blood meals throughout their lifetime. Host DNA from blood meals remains viable in their abdomens for long periods and we have already documented the ability to detect blood meals of *O. turicata* over 3 years of age (Busselman et al., 2021), making them ideal arthropods to characterize vertebrate host communities by metabarcoding.

TABLE 2 Trap site and hosts identified for Laguna Atascosa National Wildlife Refuge (LANWR), Los Fresnos, Texas, indicating the number of times each host was identified as a host by metabarcoding at the 12S rRNA mitochondrial locus. Visual confirmation is based on camera records from 2023 (see Section 2). Parentheses indicate percent of the total number of meals for the given burrow.

Vertebrate host	Overall	LA-6_14	LA-15	LA-1	LA-24	LA-25	LA-26	LA-2	LA-23	LA-7	LA-8	Visual confirmation
Cathartes aura/Turkey vulture	1 (0.4%)	0	1 (4%)	0	0	0	0	0	0	0	0	Y
Crotalus spp/Rattlesnake	1 (0.4%)	0	0	0	0	0	0	0	0	1 (4%)	0	N
Dasypus novemcinctus/Nine-banded armadillo	85 (36%)	31 (64%)	9 (32%)	2 (11%)	8 (32%)	7 (44%)	20 (71%)	6 (35%)	0	1 (4%)	1 (4%)	Y
Didelphis virginiana/Virginia opossum	60 (26%)	1 (2%)	12 (43%)	11 (58%)	8 (32%)	0	0	2 (12%)	0	10 (36%)	16 (67%)	Y
Eleutherodactylus sp/ Rain frog	3 (1%)	0	0	0	0	0	0	3 (18%)	0	0	0	N
Gallus gallus/Chicken	3 (1%)	1 (2%)	0	0	1 (4%)	0	0	0	0	1 (4%)	0	N
Homo sapiens/Human	71 (30%)	14 (29%)	6 (21%)	6 (32%)	7 (28%)	9 (56%)	7 (25%)	6 (35%)	1 (100%)	9 (32%)	6 (25%)	N
Mephitis mephitis/Striped skunk	1 (0.4%)	0	0	0	0	0	0	0	0	1 (4%)	0	Y
Neotoma spp/Woodrat	1 (0.4%)	0	0	0	0	0	0	0	0	1 (4%)	0	Y
Scaphiopus sp/ North American spadefoot toad	6 (3%)	0	0	0	0	0	1 (4%)	0	0	4 (14%)	1 (4%)	N
Sus scrofa/Wild pig	2 (0.9%)	1 (2%)	0	0	1 (4%)	0	0	0	0	0	0	Y
Total of vertebrate meals	234	48	28	19	25	16	28	17	1	28	24	

This study also allowed side-by-side comparisons between Sanger sequencing (see Busselman et al., 2021) and metabarcoding approaches in their efficiency to detect vertebrate hosts in blood meal analyses of *O. turicata*. In samples from GCSNA, metabarcoding showed an improvement in the rates of vertebrate detection in soft ticks from 11.3% (19) to 72% (118) of individuals tested and compared to Sanger sequencing. This method revealed multiple host blood meals in 99 of 118 (84%) of the samples, adding 18 host species to the four different host species detected by Sanger sequencing. In the subset of 19 *O. turicata* that revealed hosts by Sanger sequencing, metabarcoding revealed that 11 had fed on multiple hosts, two of those feeding on four hosts each, and three soft ticks did not yield amplicons in the metabarcoding assay. While the Sanger sequencing approach yields only one host for all tested samples, only five of the same 19 soft ticks showed a single blood meal host by metabarcoding. Hosts identified by Sanger sequencing were also identified by metabarcoding in all but one instance (ST-78I) (Table 3). Overall, more than one host was detected in 167 of the 274 (61%) *O. turicata* with metabarcoding blood meals (128 with two hosts, 31 with three hosts, seven with four hosts, and one *O. turicata* with six hosts). Sanger sequencing reveals single-host blood meals only (Busselman et al., 2021; Kim et al., 2021; Palma et al., 2013) or requires multiple PCR and sequencing runs to detect multiple hosts (Kleinerman et al., 2021) underscoring the advantages of the metabarcoding method to reveal cryptic vector-hosts interactions.

All GCSNA sites had similar and relatively high Simpson's diversity indices, indicating few differences in the high richness and evenness of hosts used by soft ticks at GCSNA across sites (Figure 1, Figure S3). Sites across LANWR had greater variation in Simpson's diversity indices, with some sites having significantly lower diversity indices based on the host communities than others (Figure S3). This indicates some populations at LANWR had lower richness and/or evenness of hosts represented in their identified blood meals. When visually comparing soft tick hosts at GCSNA and LANWR as a whole, only five overlapping hosts (turkey vulture, rattlesnake, Virginia opossum, cliff chirping frog/rain frog (*Eleutherodactylus marnockii*/*Eleutherodactylus* sp.) and wild pig) other than human hosts were detected in soft ticks in both locations, while host sharing within sites appears to be common (Figure 1). This may indicate differences in host communities between community-level locations based on blood meal metabarcoding, which differed in the habitat where traps were deployed (caves and burrows), showing that host use varies at small spatial scales. Blood meal hosts identified from GCSNA showed differences in host feeding from cave to cave, with collared peccary being completely absent from Mad Crow cave and North American porcupine being more frequent in Little Crevice cave than the other two. Similarly, barking frog and most of the Gulf Coast toad (*Incilius valliceps*) blood meals are identified from Little Crevice cave (Table 1, Figure 1). In LANWR, while the most frequent blood meals were nine-banded armadillo or Virginia opossum, there are differences in the number of hosts between sites, with some sites having significantly lower Simpson's diversity indices – representing blood meal host species evenness and richness – than others. This may indicate and identify populations of soft ticks that are more limited with respect to the type of blood meal hosts available than others. The diversity of hosts covering four vertebrate classes and limited

TABLE 3 Comparison of results between Sanger sequencing (Busselman et al., 2021) and metabarcoding of blood meals from *Ornithodoros turicata* collected at Government Canyon State Natural Area (GCSNA), San Antonio, Texas.

		Vertebrate blood meal host	
Cave/site name	Tick ID	Sanger sequencing	Metabarcoding
Bone Pile Cave	ST-18E	*Procyon lotor*	*Procyon lotor* *Homo sapiens* *Felis catus domesticus* *Eleutherodactylus marnockii*
Bone Pile Cave	ST-19B	*Procyon lotor*	*Homo sapiens*
Bone Pile Cave	ST-19C	*Procyon lotor*	*Procyon lotor* *Homo sapiens*
Bone Pile Cave	ST-20B	*Procyon lotor*	*Procyon lotor* *Homo sapiens* *Canis lupus familiaris*
Bone Pile Cave	ST-21E	*Procyon lotor*	*Procyon lotor*
Bone Pile Cave	ST-22B	*Crotalus molossus*	*Crotalus* spp *Homo sapiens*
Bone Pile Cave	ST-40B	*Procyon lotor*	*Procyon lotor* *Homo sapiens* *Meleagris gallopavo* *Cathartes aura*
Bone Pile Cave	ST-46B	*Procyon lotor*	*Homo sapiens* *Procyon lotor*
Little Crevice Cave	ST-65E	*Homo sapiens*	*Procyon lotor* *Homo sapiens*
Little Crevice Cave	ST-68A	*Procyon lotor*	*Procyon lotor* *Homo sapiens*
Mad Crow Cave	ST-02A	*Procyon lotor*	*Procyon lotor* *Homo sapiens*
Mad Crow Cave	ST-02B	Procyon lotor	No amplicon
Mad Crow Cave	ST-05B	*Coragyps atratus*	No amplicon
Mad Crow Cave	ST-72H	*Coragyps atratus*	*Coragyps atratus*
Mad Crow Cave	ST-74E	*Procyon lotor*	No amplicon
Mad Crow Cave	ST-78C	*Procyon lotor*	*Procyon lotor*
Mad Crow Cave	ST-78I	*Procyon lotor*	*Homo sapiens*
Mad Crow Cave	ST-80B	*Procyon lotor*	*Procyon lotor* *Homo sapiens*
Mad Crow Cave	ST-80H	*Procyon lotor*	*Procyon lotor* *Homo sapiens*

overlap in species detected between the two broad sampling locations, confirms that *O. turicata* are opportunistic feeders with host choice likely varying with local availability of vertebrates.

At GCSNA, blood meal metabarcoding revealed host feeding on 22 vertebrate species out of the 26 species detected by camera traps and metabarcoding combined. At LANWR, only 11 species of hosts were detected in blood meals out of the 30 vertebrate species detected by camera traps and metabarcoding combined. Nine-banded armadillos were reported as blood meal hosts in every LANWR burrow (except LA-23 where only one of three soft ticks yielded host information) while camera traps only observed armadillos to be present in four of the burrows, and instead, camera traps captured many additional vertebrates utilizing the burrows. This corroborates the recent camera trap observations of DeGregorio et al. (2022) that observed the importance of armadillos as ecosystem engineers providing shelter to diverse vertebrate communities. This past study in Arkansas used camera traps to observe animals associated with armadillo burrows and found many vertebrate species entering and exiting the active nine-banded armadillo burrows; Virginia opossums co-inhabited the burrows with nine-banded armadillos. In our study, camera traps on burrow LA-6_14 documented concurrent use of the burrow by nine-banded armadillo and Virginia opossums (Figure S2), while nine-banded armadillos and humans were the only vertebrates documented as *O. turicata* blood meal hosts from that burrow. A nine-banded armadillo was observed entering the LA8 burrow when soft tick traps were being set up at the same location. One year later, the camera trap on that burrow documented several additional vertebrates, including Virginia opossum; 67% of the tick blood meals from that burrow were from opossum with only 4% from armadillo. While much of the *O. turicata* host feeding appears opportunistic, we also observed frequent vertebrates associated with the burrows and fewer blood meals than we would expect. For example, eastern cottontails were observed at 6 of the LANWR camera trap locations, but no soft tick blood meals were observed from this species. Additionally, multiple species of rodents were frequently observed entering and exiting the burrows but only 1 out of 235 identified blood meals came from a rodent. Our use of camera trapping was not able to identify unique individuals of each species or estimate relative densities which precluded our ability to measure *O. turicata* host selection or the over- and under-utilization of different vertebrates relative to abundance (Fikrig & Harrington, 2021). While *O. turicata* appears overall to be opportunistic, this study indicates that some vertebrates may be over and under-utilized relative to availability, which could be pursued in future controlled studies.

Ornithodoros turicata is a vector of *Borrelia turicatae* in Texas and in Mexico, which causes tick-borne relapsing fever in humans and dogs (Beeson et al., 2023; Bissett et al., 2018). This study's results identify the diverse community of potential reservoir hosts that could contribute to the enzootic maintenance of *B. turicatae* in nature. From the four mammal species detected as potential hosts of *B. turicatae* (Armstrong et al., 2018), two (raccoons and gray fox) were found in *O. turicata* blood meals. All these cases happened in caves where human and dog DNA was also detected. While acknowledging the potential for human blood meal results to originate from environmental DNA, the current study presents a potential mechanism of bridge transmission of *B. turicatae* from the enzootic cycle to humans and domestic animals. The evidence of blood meal derived from dogs in *O. turicata* sampled in caves provides clues about the locations where *B. turicatae* may be transmitted. Clinical cases of canine tick-borne relapsing fever have been reported in dogs in Texas (Piccione et al., 2016; Whitney et al., 2007). Human exposure to *O. turicata* bites leading to exposure to *B. turicatae* has been known to occur when cavers and campers enter and stay in caves (Davis et al., 2002; Dworkin et al., 2008; Forrester et al., 2015) and even from attending a conference in a rustic setting (Bissett et al., 2018).

Evidence of soft tick feeding on wild pigs was not uncommon from these locations – 3 individual *O. turicata* at GCSNA and 2 at LANWR. *Ornithodoros turicata* have been identified as competent for the transmission of ASFV (Manzano-Román et al., 2012), and prior observations have documented the rare on-host observations of *O. turicata* on wild pigs (Sames & Teel, 2022). Texas is included in the region of the US with elevated risk for transmission of ASFV given the presence of competent vectors and wild pigs, domestic swine, and free-range common warthog (*Phacochoerus africanus*) documented in 4 counties (Mayer et al., 2020). The results of this study provide evidence of soft ticks and swine contact which could be occurring more frequently than previously known (Brown & Bevins, 2018; Herrera-Ibatá et al., 2017; Wormington et al., 2019), aided in part by molecular methodological advancements. In addition, the simple CO_2-baited sticky trap deployed into the burrows in LANWR represents a technique that is not widely used for collecting soft ticks in endemic or non-endemic regions for ASFV (Jori et al., 2023). This technique can utilize dry ice, as in the current study, but also CO_2 cylinders or sugar-yeast fermentation to increase the flexibility in diverse regions when resources are often limited.

In addition to *O. turicata* feeding on wild pig, we also observed 22 soft ticks from GCSNA to have fed on collared peccary. Although peccaries are in the new-world pig family (Tayassuidae) they share the same suborder of Suina with old-world pigs which are known to be highly susceptible to ASFV (e.g., *Sus scrofa* and *Phacochoerus* spp.). While peccaries are considered resistant to ASFV (Brown & Bevins, 2018), there are few experimental studies confirming this status. Consequently, the current observation of frequent feeding on peccaries by competent ASFV vectors warrants revisiting the ASFV vertebrate competence of new-world pigs.

Human hosts were found to be the most frequently identified meal (95 out of 256) in GCSNA and the second most frequent (71 of 234) in LANWR and were present in every sampling location. Humans are known to visit both the caves at GCSNA (camera trap evidence in Kim, 2017) and are known to recreate in the areas around burrows at LANWR. While it is plausible that some *O. turicata* human blood meal results are real, it is also possible that some may reflect environmental contamination. Human DNA is ubiquitous and is a component of all aspects of this study the placing of traps, tick collection, identification, extraction, and molecular steps. Despite

surface sterilization of the soft ticks to remove exogenous DNA and measures taken during processing to avoid human contamination, the presence of human DNA contaminating soft tick samples cannot be ruled out. There is also potential for some of these meals to be very old (Beck et al., 1986; Francıs, 1938; Kim et al., 2021). Future steps to parse sequences into human haplotypes to discern between different human encounters, contamination, and meals would be useful. Steps have been taken within this study, also, to reduce the incidence of tag jumping like using primers with identical barcode tags on both forward and reverse primers, using separate barcode tags for replicates, handling tagged primer PCRs in smaller batches which are among the recommended methods to reduce tag jumping (Schnell et al., 2015). Future work could adopt additional methods to eliminate tag jumping (Carøe & Bohmann, 2020).

We have collected unique information elucidating the feeding habits of soft ticks substantiating the diversity of hosts utilized by *O. turicata* in natural areas. The detection of multiple meals within single ticks allows better deciphering of feeding over time, possibly across years, during which pathogens could remain viable. The metabarcoding protocol developed in this study is a valuable tool to detect vertebrate blood meal from soft ticks in areas currently affected by ASFV, identifying contact between susceptible hosts and local *Ornithodoros* vectors. Not only could blood meals be used to determine the transmission chain and inform control method for *B. turicatae* and ASFV but also could inform the habits and presence of hosts.

AUTHOR CONTRIBUTIONS

Designed the research: SB, REB, PDT, SAH, GLH. Performed research: SB, REB, NF-S, FCF, AH, NW. Contributed new reagents or analytical tools NH, JAB, SAH, GLH. Analyzed data SB, REB, NF-S, APG. Wrote the paper—SB, REB, FCF, GLH. Reviewed and approved submission—All authors.

ACKNOWLEDGMENTS

This work was partially funded by a seed grant from the Coalition for Epi Response, Engagement, and Science (CERES), Texas A&M AgriLife Research, and Texas Ecolab. We also appreciate assistance sampling ticks and operating camera traps at Laguna Atascosa National Wildlife Refuge from Ester Carbajal, Danya Garza, Taylor Donaldson, Brian Rich, Odaliz Sauceda, Javier Elizondo, Salvador Solis, Daniel Obregon, and Sarah Maestas.

CONFLICT OF INTEREST STATEMENT

No conflicts of interest.

DATA AVAILABILITY STATEMENT

O. turicata barcoding sequences have been deposited into GenBank (accession numbers: OR980949-OR980951).

Sequencing raw data are deposited in DataDryad (https://datadryad.org/stash). https://doi.org/10.5061/dryad.pg4f4qrx0.

R script used to run statistical analyses has been archived along with sequencing raw data in DataDryad (https://datadryad.org/stash). https://doi.org/10.5061/dryad.pg4f4qrx0.

ORCID

Sujata Balasubramanian https://orcid.org/0009-0003-8843-5163
Andrew P. Grunwald https://orcid.org/0000-0003-2627-0087
Francisco C. Ferreira https://orcid.org/0000-0002-2034-4121

REFERENCES

Abbasi, I., Nasereddin, A., & Warburg, A. (2019). Development of a next generation DNA sequencing-based multi detection assay for detecting and identifying leishmania parasites, blood sources, plant meals and intestinal microbiome in phlebotomine sand flies. *Acta Tropica*, *199*, 105101. https://doi.org/10.1016/j.actatropica.2019.105101

Adeyeye, O. A., & Butler, J. F. (1989). Population structure and seasonal intra-burrow movement of *Ornithodoros turicata* (Acari: Argasidae) in gopher tortoise burrows. *Journal of Medical Entomology*, *26*(4), 279–283. https://doi.org/10.1093/jmedent/26.4.279

Alonso, D. P., Amorim, J. A., De Oliveira, T. M. P., De Sá, I. L. R., Possebon, F. S., De Carvalho, D. P., Ribeiro, K. A. N., Ribolla, P. E. M., & Sallum, M. A. M. (2023). Host feeding patterns of Mansonia (Diptera, Culicidae) in rural settlements near Porto Velho, State of Rondonia, Brazil. *Biomolecules*, *13*(3), 553. https://doi.org/10.3390/biom13030553

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Armstrong, B. A., Kneubehl, A., Krishnavajhala, A., Wilder, H. K., Boyle, W., Wozniak, E., Phillips, C., Hollywood, K., Murray, K. O., Donaldson, T. G., Teel, P. D., Waldrup, K., & Lopez, J. E. (2018). Seroprevalence for the tick-borne relapsing fever spirochete *Borrelia turicatae* among small and medium sized mammals of Texas. *PLoS Neglected Tropical Diseases*, *12*(10), e0006877. https://doi.org/10.1371/journal.pntd.0006877

Balasubramanian, S., Busselman, R. E., Fernandez-Santos, N., Grunwald, A., Wolff, N., Hathaway, N., Hillhouse, A., Bailey, J. A., Teel, P. D., Ferreira, F. C., Hamer, S. A., & Hamer, G. L. (2024). Bloodmeal metabarcoding of the argasid tick (*Ornithodoros turicata* Dugès) reveals extensive vector-host associations [Dataset]. *Dryad*. https://doi.org/10.5061/dryad.pg4f4qrx0

Balasubramanian, S., Curtis-Robles, R., Chirra, B., Auckland, L. D., Mai, A., Bocanegra-Garcia, V., Clark, P., Clark, W., Cottingham, M., Fleurie, G., Johnson, C. D., Metz, R. P., Wang, S., Hathaway, N. J., Bailey, J. A., Hamer, G. L., & Hamer, S. A. (2022). Characterization of triatomine bloodmeal sources using direct sanger sequencing and amplicon deep sequencing methods. *Scientific Reports*, *12*(1), 10234. https://doi.org/10.1038/s41598-022-14208-8

Beck, A. F., Holscher, K. H., & Butler, J. F. (1986). Life cycle of *Ornithodoros Turicata americanus* (Acari: Argasidae) in the Laboratory1. *Journal of Medical Entomology*, *23*(3), 313–319. https://doi.org/10.1093/jmedent/23.3.313

Beeson, A. M., Kjemtrup, A., Oltean, H., Schnitzler, H., Venkat, H., Ruberto, I., Marzec, N., Cozart, D., Tengelsen, L., Ladd-Wilson, S., Rettler, H., Mayes, B., Broussard, K., Garcia, A., Drake, L. L., Dietrich, E. A., Petersen, J., Hinckley, A. F., Kugeler, K. J., & Marx, G. E. (2023). Soft tick relapsing fever—United States, 2012–2021. *MMWR. Morbidity and Mortality Weekly Report*, *72*(29), 777–781. https://doi.org/10.15585/mmwr.mm7229a1

Bissett, J. D., Ledet, S., Krishnavajhala, A., Armstrong, B. A., Kliouneva, A., Sexton, C., Replogle, A., Schriefer, M. E., & Lopez, J. E. (2018). Detection of tickborne relapsing fever spirochete, Austin, Texas, USA. *Emerging Infectious Diseases*, *24*(11), 2003–2009. https://doi.org/10.3201/eid2411.172033

Boinas, F. S., Wilson, A. J., Hutchings, G. H., Martins, C., & Dixon, L. J. (2011). The persistence of African swine fever virus in field-infected

Ornithodoros erraticus during the ASF endemic period in Portugal. *PLoS One*, *6*(5), e20383. https://doi.org/10.1371/journal.pone.0020383

Borland, E. M., & Kading, R. C. (2021). Modernizing the toolkit for arthropod bloodmeal identification. *Insects*, *12*(1), 37. https://doi.org/10.3390/insects12010037

Brake, D. A. (2022). African swine fever modified live vaccine candidates: Transitioning from discovery to product development through harmonized standards and guidelines. *Viruses*, *14*(12), 2619. https://doi.org/10.3390/v14122619

Brown, V. R., & Bevins, S. N. (2018). A review of African swine fever and the potential for introduction into the United States and the possibility of subsequent establishment in feral swine and native ticks. *Frontiers in Veterinary Science*, *5*, 11. https://doi.org/10.3389/fvets.2018.00011

Busselman, R. E., Olson, M. F., Martinez, V., Davila, E., Briggs, C., Eldridge, D. S., Higgins, B., Bass, B., Cropper, T. L., Casey, T. M., Edwards, T., Teel, P. D., Hamer, S. A., & Hamer, G. L. (2021). Host bloodmeal identification in cave-dwelling *Ornithodoros turicata* Dugès (Ixodida: Argasidae), Texas, USA. *Frontiers in Veterinary Science*, *8*, 639400. https://doi.org/10.3389/fvets.2021.639400

Campbell, S. B., Kliioueva, A., Taylor, J., Nelson, C., Tomasi, S., Replogle, A., Kwit, N., Sexton, C., Schwartz, A., & Hinckley, A. (2019). Evaluating the risk of tick-borne relapsing fever among occupational cavers—Austin, TX, 2017. *Zoonoses and Public Health*, *66*(6), 579–586. https://doi.org/10.1111/zph.12588

Canty, A., & Ripley, B. D. (2022). *Boot: Bootstrap R (S-plus) functions*. R Package Version 1.3-28.

Carøe, C., & Bohmann, K. (2020). Tagsteady: A metabarcoding library preparation protocol to avoid false assignment of sequences to samples. *Molecular Ecology Resources*, *20*(6), 1620–1631. https://doi.org/10.1111/1755-0998.13227

Davis, H., Vincent, J. M., & Lynch, J. (2002). Tick-borne relapsing fever caused by *Borrelia turicatae*. *The Pediatric Infectious Disease Journal*, *21*(7), 703–705.

Davison, A. C., & Hinkley, D. V. (1997). *Bootstrap methods and their applications*. Cambridge University Press. http://statwww.epfl.ch/davison/BMA/

DeGregorio, B. A., Veon, J. T., & Massey, A. (2022). Wildlife associates of nine-banded armadillo (*Dasypus novemcinctus*) burrows in Arkansas. *Ecology and Evolution*, *12*(5), e8858. https://doi.org/10.1002/ece3.8858

Donaldson, T. G., Pèrez de León, A. A., Li, A. Y., Castro-Arellano, I., Wozniak, E., Boyle, W. K., Hargrove, R., Wilder, H. K., Kim, H. J., Teel, P. D., & Lopez, J. E. (2016). Assessment of the geographic distribution of *Ornithodoros turicata* (Argasidae): Climate variation and host diversity. *PLoS Neglected Tropical Diseases*, *10*(2), e0004383. https://doi.org/10.1371/journal.pntd.0004383

Dormann, C. F., Gruber, B., & Fründ, J. (2008). Introducing the bipartite Package: Analysing ecological networks. *R News*, *8*, 8–11.

Dumonteil, E., Ramirez-Sierra, M.-J., Pérez-Carrillo, S., Teh-Poot, C., Herrera, C., Gourbière, S., & Waleckx, E. (2018). Detailed ecological associations of triatomines revealed by metabarcoding and next-generation sequencing: Implications for triatomine behavior and *Trypanosoma cruzi* transmission cycles. *Scientific Reports*, *8*(1), 4140. https://doi.org/10.1038/s41598-018-22455-x

Dworkin, M. S., Schwan, T. G., & Anderson, D. E. (2002). Tick-borne relapsing fever in North America. *Medical Clinics of North America*, *86*(2), 417–433. https://doi.org/10.1016/S0025-7125(03)00095-6

Dworkin, M. S., Schwan, T. G., Anderson, D. E., & Borchardt, S. M. (2008). Tick-borne relapsing fever. *Infectious Disease Clinics of North America*, *22*(3), 449–468. https://doi.org/10.1016/j.idc.2008.03.006

Efron, B., & Tibshirani, R. (1993). *An introduction to the bootstrap (Nachdr.)*. Chapman & Hall.

Faccini-Martínez, Á. A., Silva-Ramos, C. R., Santodomingo, A. M., Ramírez-Hernández, A., Costa, F. B., Labruna, M. B., & Muñoz-Leal, S. (2022). Historical overview and update on relapsing fever group *Borrelia* in Latin America. *Parasites & Vectors*, *15*(1), 196. https://doi.org/10.1186/s13071-022-05289-5

Fikrig, K., & Harrington, L. C. (2021). Understanding and interpreting mosquito blood feeding studies: The case of Aedes albopictus. *Trends in Parasitology*, *37*(11), 959–975. https://doi.org/10.1016/j.pt.2021.07.013

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, *3*(5), 294–299.

Forrester, J. D., Kjemtrup, A. M., Fritz, C. L., Marsden-Haug, N., Nichols, J. B., Tengelsen, L. A., Sowadsky, R., DeBess, E., Cieslak, P. R., Weiss, J., Evert, N., Ettestad, P., Smelser, C., Iralu, J., Nett, R. J., Mosher, E., & Baker, J. S. (2015). Tickborne relapsing fever—United States, 1990–2011. *Morbidity and Mortality Weekly Report*, *64*(3), 58–60.

Francıs, E. (1938). Longevity of the tick *Ornithodoros turicata* and of *Spirochaeta recurrentis* within this tick. *Public Health Reports (1896–1970)*, *53*(51), 2220–2241. https://doi.org/10.2307/4582740

Gonzales, W., Moreno, C., Duran, U., Henao, N., Bencosme, M., Lora, P., Reyes, R., Núñez, R., De Gracia, A., & Perez, A. M. (2021). African swine fever in The Dominican Republic. *Transboundary and Emerging Diseases*, *68*(6), 3018–3019. https://doi.org/10.1111/tbed.14341

Guzmán-Cornejo, C., Herrera-Mares, A., Robbins, R. G., & Rebollo-Hernández, A. (2019). The soft ticks (Parasitiformes: Ixodida: Argasidae) of Mexico: Species, hosts, and geographical distribution. *Zootaxa*, *4623*(3), 485–525. https://doi.org/10.11646/zootaxa.4623.3.3

Hathaway, N. J., Parobek, C. M., Juliano, J. J., & Bailey, J. A. (2018). SeekDeep: Single-base resolution de novo clustering for amplicon deep sequencing. *Nucleic Acids Research*, *46*(4), e21. https://doi.org/10.1093/nar/gkx1201

Herrera-Ibatá, D. M., Martínez-López, B., Quijada, D., Burton, K., & Mur, L. (2017). Quantitative approach for the risk assessment of African swine fever and classical swine fever introduction into the United States through legal imports of pigs and swine products. *PLoS One*, *12*(8), e0182850. https://doi.org/10.1371/journal.pone.0182850

Humair, P.-F. O., Douet, V. R., Cadenas, F. M. N., & Schouls, L. M. (2007). Molecular identification of bloodmeal source in *Ixodes ricinus* ticks using 12S rDNA as a genetic marker. *Journal of Medical Entomology*, *44*(5), 12–880.

Jean-Pierre, R. P., Hagerman, A. D., & Rich, K. M. (2022). An analysis of African swine fever consequences on rural economies and smallholder swine producers in Haiti. *Frontiers in Veterinary Science*, *9*, 960344. https://doi.org/10.3389/fvets.2022.960344

Jori, F., Bastos, A., Boinas, F., Van Heerden, J. V., Heath, L., Jourdan-Pineau, H., Martinez-Lopez, B., Pereira De Oliveira, R. P. D., Pollet, T., Quembo, C., Rea, K., Simulundu, E., Taraveau, F., & Penrith, M.-L. (2023). An updated review of Ornithodoros ticks as reservoirs of African swine fever in sub-Saharan Africa and Madagascar. *Pathogens*, *12*(3), 469. https://doi.org/10.3390/pathogens12030469

Kieran, T. J., Gottdenker, N. L., Varian, C. P., Saldaña, A., Means, N., Owens, D., Calzada, J. E., & Glenn, T. C. (2017). Blood meal source characterization using Illumina sequencing in the chagas disease vector *Rhodnius pallescens* (Hemiptera: Reduviidae) in Panamá. *Journal of Medical Entomology*, *54*(6), 1786–1789. https://doi.org/10.1093/jme/tjx170

Kim, H. J. (2017). *Habitat-Host-Vector Interactions of Ornithodoros turicata Dugès (Ixodida: Argasidae) in Texas, USA*. https://oaktrust.library.tamu.edu/bitstream/handle/1969.1/165811/KIM-DISSERTATION-2017.pdf?sequence=1&isAllowed=y

Kim, H. J., Hamer, G. L., Hamer, S. A., Lopez, J. E., & Teel, P. D. (2021). Identification of host bloodmeal source in *Ornithodoros turicata* Dugès (Ixodida: Argasidae) using DNA-based and stable isotope-based techniques. *Frontiers in Veterinary Science*, *8*, 620441. https://doi.org/10.3389/fvets.2021.620441

Kleinerman, G., Eshed, T., Nachum-Biala, Y., King, R., & Baneth, G. (2021). Transmission of the human relapsing fever spirochete *borrelia persica* by the Argasid tick *Ornithodoros tholozani* involves blood meals from wildlife animal reservoirs and mainly transstadial transfer. *Applied and Environmental Microbiology*, *87*(11), e03117-20. https://doi.org/10.1128/AEM.03117-20

Kocher, A., Cornuault, J., Gantier, J.-C., Manzi, S., Chavy, A., Girod, R., Dusfour, I., Forget, P.-M., Ginouves, M., Prévot, G., Guégan, J.-F., Bañuls, A.-L., de Thoisy, B., & Murienne, J. (2023). Biodiversity and vector-borne diseases: Host dilution and vector amplification occur simultaneously for Amazonian leishmaniases. *Molecular Ecology*, *32*(8), 1817–1831. https://doi.org/10.1111/mec.16341

Kocher, A., de Thoisy, B., Catzeflis, F., Valière, S., Bañuls, A.-L., & Murienne, J. (2017). iDNA screening: Disease vectors as vertebrate samplers. *Molecular Ecology*, *26*(22), 6478–6486. https://doi.org/10.1111/mec.14362

Logue, K., Keven, J. B., Cannon, M. V., Reimer, L., Siba, P., Walker, E. D., Zimmerman, P. A., & Serre, D. (2016). Unbiased characterization of *anopheles* mosquito blood meals by targeted high-throughput sequencing. *PLoS Neglected Tropical Diseases*, *10*(3), e0004512. https://doi.org/10.1371/journal.pntd.0004512

Mans, B. J., Featherston, J., Kvas, M., Pillay, K.-A., De Klerk, D. G., Pienaar, R., De Castro, M. H., Schwan, T. G., Lopez, J. E., Teel, P., Pérez De León, A. A., Sonenshine, D. E., Egekwu, N. I., Bakkes, D. K., Heyne, H., Kanduma, E. G., Nyangiwe, N., Bouattour, A., & Latif, A. A. (2019). Argasid and ixodid systematics: Implications for soft tick evolution and systematics, with a new argasid species list. *Ticks and Tick-borne Diseases*, *10*(1), 219–240. https://doi.org/10.1016/j.ttbdis.2018.09.010

Manzano-Román, R., Díaz-Martín, V., la Fuente, J. d., & Pérez-Sánchez, R. (2012). Soft ticks as pathogen vectors: Distribution, surveillance and control. In M. M. Shah (Ed.), *Parasitology* (pp. 125–162). INTECH.

Mayer, J., Smyser, T., Piaggio, A., & Zervanos, S. (2020). Wild pig taxonomy, morphology, genetics, and physiology. In B. K. Strickland, G. J. Roloff, J. C. Beasley, J. J. Mayer, K. C. VerCauteren, & S. S. Ditchkoff (Eds.), *Invasive wild pigs in North America: Ecology, impacts, and management* (pp. 7–32). CRC Press.

Miles, V. I. (1968). A carbon dioxide bait trap for collecting ticks and fleas from animal Burrows1. *Journal of Medical Entomology*, *5*(4), 491–495. https://doi.org/10.1093/jmedent/5.4.491

Murillo-Solano, C., López-Domínguez, J., Gongora, R., Rojas-Gulloso, A., Usme-Ciro, J., Perdomo-Balaguera, E., Herrera, C., Parra-Henao, G., & Dumonteil, E. (2021). Diversity and interactions among triatomine bugs, their blood feeding sources, gut microbiota and *Trypanosoma cruzi* in the Sierra Nevada de Santa Marta in Colombia. *Scientific Reports*, *11*(1), 12306. https://doi.org/10.1038/s41598-021-91783-2

Palma, M., Lopes de Carvalho, I., Osório, H., Zé-Zé, L., Cutler, S. J., & Núncio, M. S. (2013). Portuguese hosts for *Ornithodoros erraticus* ticks. *Vector-Borne and Zoonotic Diseases*, *13*(10), 775–777. https://doi.org/10.1089/vbz.2012.1070

Penrith, M.-L., Vosloo, W., Jori, F., & Bastos, A. D. S. (2013). African swine fever virus eradication in Africa. *Virus Research*, *173*(1), 228–246. https://doi.org/10.1016/j.virusres.2012.10.011

Piccione, J., Levine, G. J., Duff, C. A., Kuhlman, G. M., Scott, K. D., & Esteve-Gassent, M. D. (2016). Tick-borne relapsing fever in dogs. *Journal of Veterinary Internal Medicine*, *30*(4), 1222–1228. https://doi.org/10.1111/jvim.14363

Polonio, R., López-Domínguez, J., Herrera, C., & Dumonteil, E. (2021). Molecular ecology of *Triatoma dimidiata* in southern Belize reveals risk for human infection and the local differentiation of *Trypanosoma cruzi* parasites. *International Journal of Infectious Diseases*, *108*, 320–329. https://doi.org/10.1016/j.ijid.2021.05.083

Posit. (2023). *Posit*. https://www.posit.co/

Pratt, H. D., & Stojanovich, C. (1963). Acarina: Illustrated key to some common adult female mites and adult ticks. In H. D. Pratt (Ed.), *Mites of public health importance and their control* (pp. 26–37). U.S. Communicable Disease Center.

R Core Team. (2023). *R: A language and environment for statistical Computing_*. R Foundation for Statistical Computing. https://www.r-project.org/

Sames, W. J., & Teel, P. D. (2022). Ornithodoros turicata (Dugès) on a feral hog in Real County, Texas, USA. *Journal of Wildlife Diseases*, *58*(1), 238–240. https://doi.org/10.7589/JWD-D-21-00042

San Juan, E., Araya-Donoso, R., Sierra-Rosales, C., Correa, J. P., Quiroga, N., Campos-Soto, R., Solari, A., Llewellyn, M., Bacigalupo, A., & Botto-Mahan, C. (2023). Humans as blood-feeding sources in sylvatic triatomines of Chile unveiled by next-generation sequencing. *Parasites & Vectors*, *16*(1), 225. https://doi.org/10.1186/s13071-023-05841-x

Schnell, I. B., Bohmann, K., & Gilbert, M. T. (2015). Tag jumps illuminated–reducing sequence-to-sample misidentifications in metabarcoding studies. *Molecular Ecology Resources*, *15*(6), 1289–1303. https://doi.org/10.1111/1755-0998.12402

Schnell, I. B., Thomsen, P. F., Wilkinson, N., Rasmussen, M., Jensen, L. R. D., Willerslev, E., Bertelsen, M. F., & Gilbert, M. T. P. (2012). Screening mammal biodiversity using DNA from leeches. *Current Biology*, *22*(8), R262–R263. https://doi.org/10.1016/j.cub.2012.02.058

Shepherd, J. G. (2022). Record longevity and reproduction of an African tick, *Argas brumpti* (Ixodida: Argasidae). *Journal of Medical Entomology*, *59*(2), 777–778. https://doi.org/10.1093/jme/tjab205

Shokralla, S., Gibson, J. F., Nikbakht, H., Janzen, D. H., Hallwachs, W., & Hajibabaei, M. (2014). Next-generation DNA barcoding: Using next-generation sequencing to enhance and accelerate DNA barcode capture from single specimens. *Molecular Ecology Resources*, *14*(5), 892–901. https://doi.org/10.1111/1755-0998.12236

Tinio, J. N. G., & Sebuala, C. J. (2021). On the efficiency of some alpha diversity indices: A simulation study using bootstrap resampling. *Journal of Wildlife and Biodiversity*, *5*(1), 32–39. https://doi.org/10.22120/jwb.2020.127654.1142

USDA, A. (2022, April 25). *African swine fever virus vaccine passes tests required for regulatory approval*. USDA ARS. https://www.ars.usda.gov/news-events/news/research-news/2022/african-swine-fever-virus-vaccine-passes-tests-required-for-regulatory-approval/

Whitney, M. S., Schwan, T. G., Sultemeier, K. B., McDonald, P. S., & Brillhart, M. N. (2007). Spirochetemia caused by *borrelia turicatae* infection in 3 dogs in Texas. *Veterinary Clinical Pathology*, *36*(2), 212–216. https://doi.org/10.1111/j.1939-165X.2007.tb00213.x

Wormington, J. D., Golnar, A., Poh, K. C., Kading, R. C., Martin, E., Hamer, S. A., & Hamer, G. L. (2019). Risk of African swine fever virus sylvatic establishment and spillover to domestic swine in the United States. *Vector-Borne and Zoonotic Diseases*, *19*(7), 506–511. https://doi.org/10.1089/vbz.2018.2386

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Balasubramanian, S., Busselman, R. E., Fernandez-Santos, N. A., Grunwald, A. P., Wolff, N., Hathaway, N., Hillhouse, A., Bailey, J. A., Teel, P. D., Ferreira, F. C., Hamer, S. A., & Hamer, G. L. (2024). Blood meal metabarcoding of the argasid tick (*Ornithodoros turicata* Dugès) reveals extensive vector-host associations. *Environmental DNA*, *6*, e522. https://doi.org/10.1002/edn3.522