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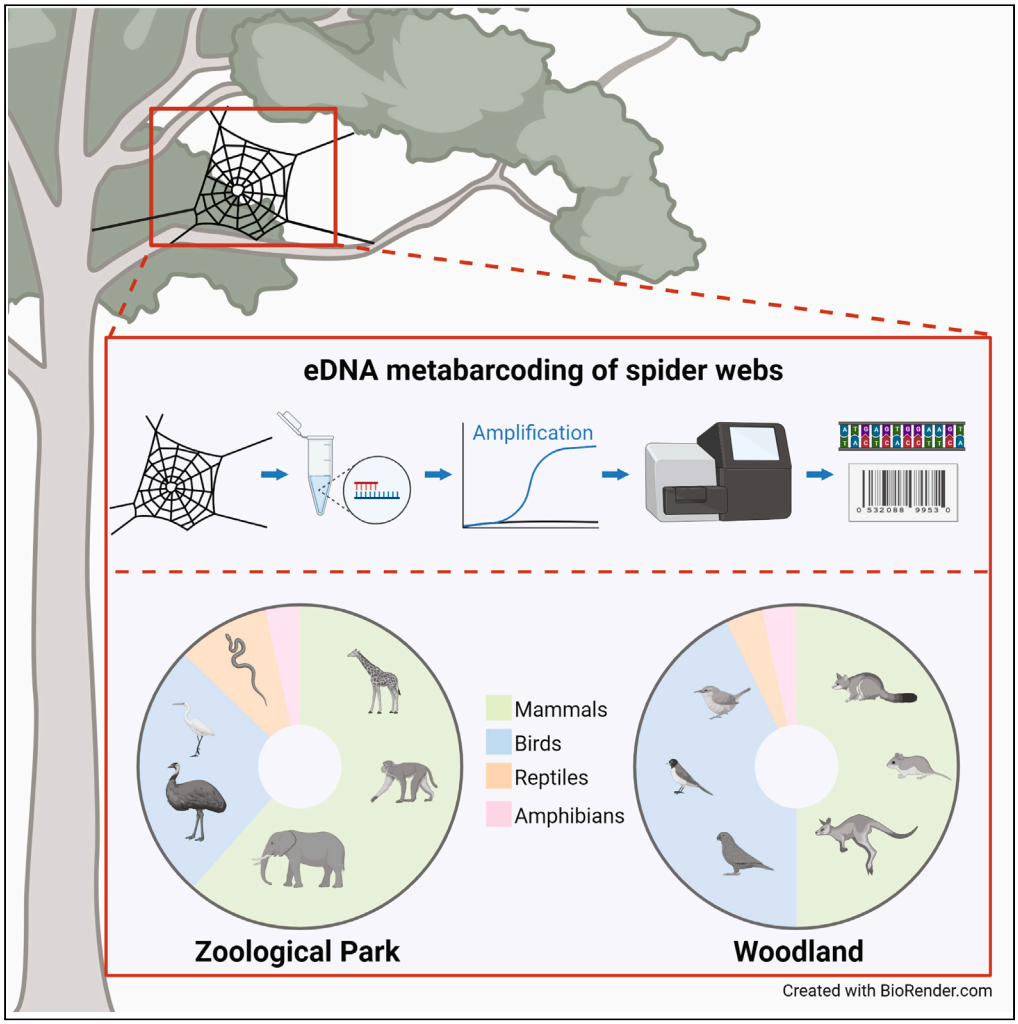

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Highlights

Environmental DNA from vertebrates was sequenced directly from spider webs

Webs from woodland and zoo show eDNA from native and non-native fauna, respectively

Biomass and distance to zoo enclosure is correlated with eDNA species detectability

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Spider webs capture environmental DNA from terrestrial vertebrates

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SUMMARY

Environmental DNA holds significant promise as a non-invasive tool for tracking terrestrial biodiversity. However, in non-homogenous terrestrial environments, the continual exploration of new substrates is crucial. Here we test the hypothesis that spider webs can act as passive biofilters, capturing eDNA from vertebrates present in the local environment. Using a metabarcoding approach, we detected vertebrate eDNA from all analyzed spider webs (N = 49). Spider webs obtained from an Australian woodland locality yielded vertebrate eDNA from 32 different species, including native mammals and birds. In contrast, webs from Perth Zoo, less than 50 km away, yielded eDNA from 61 different vertebrates and produced a highly distinct species composition, largely reflecting exotic species hosted in the zoo. We show that higher animal biomass and proximity to animal enclosures increased eDNA detection probability in the zoo. Our results indicate a tremendous potential for using spider webs as a cost-effective means to monitor terrestrial vertebrates.

INTRODUCTION

The assessment of DNA obtained from environmental samples, or "environmental DNA" (eDNA), has shown great promise as a non-invasive method for monitoring terrestrial biodiversity. With only trace amounts of DNA needed to identify species, the data obtained have the ability to strengthen biodiversity assessments through improving the detection and monitoring of rare, cryptic, or protected species,[1,2] increasing the taxonomic resolution of biodiversity surveys,[3–5] allowing for increased sampling of inhospitable or challenging environments to survey,[6] and aiding in the early detection of invasive species.[7,8]

Within terrestrial system careful consideration of the targeted substrate/s is essential because each type will likely sample different components of the biodiversity, depending on how the species are interacting with that specific substrate.[9,10] Many terrestrial eDNA substrates rely on direct interaction with vertebrate species to "absorb" the DNA, and this interaction may be limited to a small subset of taxa that are existing in the area being monitored. Some species will have contact with certain substrates over others, with the physiology, biology, and behavior of target species playing a major role in where the DNA will be found in terrestrial systems (e.g., biomass, habitat use, shedding rate).[6,11,12] Consequently, variability in both species diversity[6,10,13,14] and detection rates[15–17] are often seen between substrate types. For example, Sales et al.,[17] detected ten additional mammalian species in water samples compared to sediment samples. Additionally, Kyle et al.,[15] testing both swabs of cover objects and soil samples for the detection of reptiles, showed swabbing to have a higher detection probability than that of soil samples.

After DNA has been deposited in the environment, it starts degrading, but the rate at which this occurs differs between sample types and environmental conditions.[2] The variability in DNA degradation rates influences the effectiveness of a sample type as a successful agent for eDNA biomonitoring. For example, Ryan et al.,[18] demonstrated that sediment samples taken from within log hollows exhibit greater species richness when compared to exposed soil samples collected at the entrance of these hollows. This difference in richness is attributed to both increased animal interaction as well as the stable micro-climate found within the hollows, which allows for greater DNA accumulation and preservation. At the extreme ends of the spectrum eDNA, in many aquatic environments seems to disappear within 24–48 h,[19,20] whereas in permafrozen soil it has been shown to preserve for up to 2 million years.[21]

For these reasons, new eDNA substrates are continually being explored, and eDNA based biomonitoring using water,[22] soil,[23] ingested or invertebrate-derived DNA (iDNA),[24,25] flowers,[26,27] and swabbed surfaces,[6,15] are now common. Recently, the detection of species through airborne DNA has also been trialled to detect vertebrate taxa. Using specially designed powered air samplers (i.e., air drawn through a filter media via a fan) these studies successfully detected vertebrates within a zoo environment[12,28] and natural environments.[29,30] Similarly, alternative collection methods such as open containers filled with water[31] and dust collectors,[32] have also highlighted not only the movement of

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airborne vertebrate DNA but also the potential for passive collection. While this shows great promise, the use of these methods for vertebrate surveys in natural habitats requires optimization and further development to be applied to future terrestrial vertebrate studies.

Spider webs represent another potential passive eDNA filter able to collect both bioaerosols and invertebrate-derived DNA. They are ubiquitous in many natural and anthropogenic environments, found in an array of microhabitats worldwide, and naturally selected to act as sticky traps.[33] To date, researchers have successfully identified genetic signatures of both the host spider and its prey in spider webs,[34,35] confirming the feasibility of amplifying DNA from webs. More recent DNA metabarcoding studies have demonstrated that spider webs capture genetic traces from a diverse range of invertebrates (Arthropoda, Nematoda, and Rotifera), fungal, and bacterial taxa.[36–38] To our knowledge, the ability to detect vertebrate biodiversity from spider webs has not been tested.

With no initial set up and easy to collect, spider webs can be rapidly sampled, and would thereby add a very convenient substrate to the eDNA toolbox for the detection of terrestrial vertebrates. Hence, we here seek to determine if vertebrate DNA can be detected from spider webs. As a proof-of-concept, we sampled spider webs from two locations in Western Australia and used eDNA metabarcoding to identify vertebrate species. Firstly, we tested spider webs sampled from an Australian woodland to explore the effectiveness of spider webs for the eDNA monitoring of terrestrial vertebrate communities in a natural environment. Secondly, we examined spider webs from Perth Zoo as a source of known exotic animal diversity and abundance, allowing us to track DNA sources and assess the impacts of biomass and distance on detectability.

RESULTS

In total, from the 49 spider webs collected across both sites, 2,458,990 metabarcoding reads were generated by the three assays used to detect vertebrate DNA. We excluded all ZOTUs identified as human (1,056,989 reads), which accounted for 24% of the 12S-V5 reads, 54% of the 16Smam reads, and 30% of the 16Srep reads. In addition, we conservatively excluded ZOTUs unable to be assigned to species level (*Calidris* sp., *Zanda* sp., *Thylogale* sp.), which very likely reflected gaps in the reference sequence databases, and the complexity of closely related species. The remaining ZOTUs, representing 1,314,149 assigned reads, were assigned to 85 vertebrate species known to occur at the two sampling locations. Of these, the 12S-V5 assay yielded 452,072 sequences from 43 samples (mean = 10,513, SE = 1,620) representing 64 species, including 25 species uniquely identified by this assay. The 16Smam assay yielded 705,916 sequences from 42 samples (mean = 16,808 SE = 2,304) representing 38 species, including 7 species uniquely identified by this assay. The 16Srep assay yielded 156,101 sequences from 32 samples (mean = 4,880, SD = 635) representing 45 species, including 8 species uniquely identified by this assay (Figure 1).

We detected non-human vertebrate DNA in all tested web samples; yielding a final list of taxa that included 85 non-human vertebrate species across both sample sites (Figure 1). To assess the detection of vertebrates from spider webs within a natural setting, 24 samples were taken from Karakamia Wildlife Sanctuary (Karakamia), a 268 ha feral-predator free conservation property ~50 km east of Perth WA, owned and managed by the Australian Wildlife Conservancy. Detections here included a total of 32 vertebrate species, including 16 mammals, 14 birds, 1 reptile, and 1 amphibian. We detected 26 out of the 177 species known to occur within the fenced sanctuary grounds (15%). This included 11 out of the 20 known mammals (55%) e.g., brush-tailed phascogale (*Phascogale tapoatafa*), 13 out of the 118 known birds (11%) e.g., Spotted Pardalote *(Pardalotus punctatus*), 1 out of the 9 known amphibians (11%), the motorbike frog (*Litoria moorei*) and 1 out of the 30 known reptiles (3%) the snake-eyed skink (*Cryptoblepharus australis*). Additionally, we also identified 3 invasive species the red fox (*Vulpes vulpes*), house mouse (*Mus musculus*) and black rat (*Rattus rattus*), and several domestic and livestock animals, including cow (*Bos taurus*), sheep (*Ovis aries*) and pigs (*Sus scrofa*) not known to occur within the sanctuary but farmed throughout the region (Figure 1). Of the species that are actively being excluded from Karakamia, we detected red fox DNA from two spider webs located approximately 250 and 235 m within the boundary fence. Similarly, cow DNA was detected in webs collected at a maximum distance of 200 m from the boundary fence, while sheep and pig DNA was detected at distances of 550 and 500 m from the fence, respectively. Sheep, widespread throughout the area (but not within the fence), were detected in 18 of the 24 web samples.

The spider webs sampled within the Perth Zoo yielded a total of 61 vertebrate species, comprising of 33 mammals, 21 birds, 5 reptiles, and 2 amphibians. Detections included 34 out of the 98 species housed outdoors at the zoo (37%). This included 23 of the 47 mammals (49%) e.g., pygmy marmoset (*Cebuella pygmaea*), meerkat (*Suricata suricatta*) and Sumatran orangutan (*Pongo abelii*) and 11 of the 34 birds (32%) e.g., Emu (*Dromaius novaehollandiae*) and Royal Spoonbill (*Platalea regia*). No reptiles or amphibians housed outdoors were detected at Perth Zoo. A further 22 species that represent either feed items e.g., cow and sheep, pest species e.g., black rat, house mouse, or other non-zoo species known to occur in and around Perth Zoo including common brushtail possum (*Trichosurus vulpecula*) and Gould's wattled bat (*Chalinolobus gouldii*; Figure 1). All reptiles detected within the Perth Zoo (n = 5) were from spider web samples (n = 3) taken from within the dedicated indoor reptile room. Of these species, four are known occupants of the reptile room, shingleback (*Tiliqua rugosa*) and blue-tongued skinks (*Tiliqua occipitalis*) from open air enclosures and two snakes the Burmese python (*Python bivittatus*) and dugite (*Pseudonaja affinis*) housed within individual closed terrariums.

All the species detected are well-documented within the study regions (excluding feed species). Of these, a total of 10 species were detected at both sampling sites. Notably, 84% of species detected at the zoo site were found only in samples from the zoo, whereas at Karakamia 66% of the species detected were unique to that location. A Wilcoxon rank-sum test ($\alpha = 0.05$) found a significant difference in the ZOTU richness between Karakamia and zoo web samples ($W_1 = 100$, $p < 0.001$), with a greater number of species per spider web detected from zoo samples (Figure S3). Moreover, from spider webs collected at Perth Zoo, we found a significant relationship between animal biomass and likelihood of being detected (Deviance explained = 11.40%; df = 1,95 $p < 0.001$), with higher animal biomass increasing detection probability (Figure 2A).

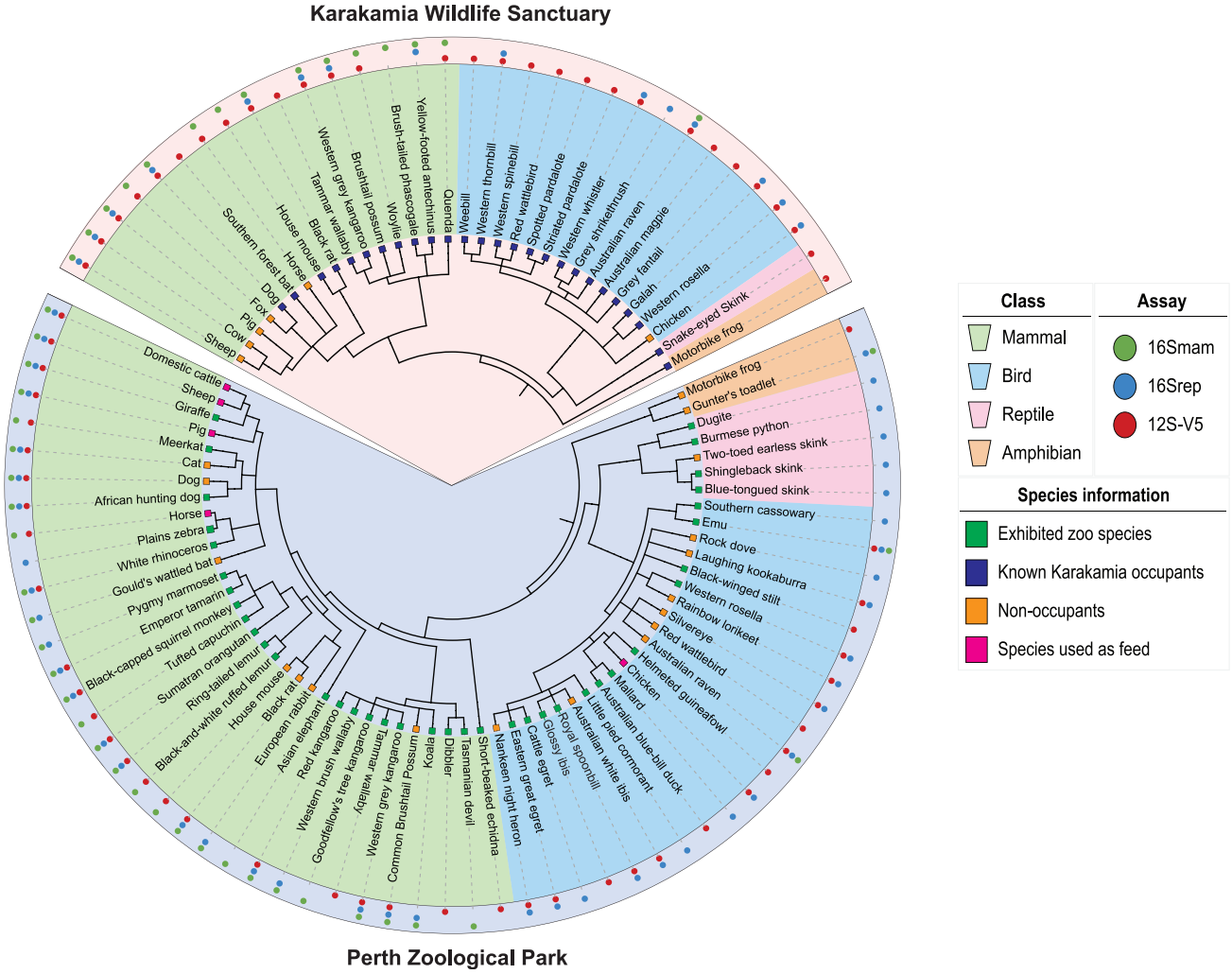

Figure 1. Vertebrate taxa identified from 49 spider web samples collected from both Perth Zoo (top) and Karakamia Wildlife Sanctuary (bottom), using three metabarcoding assays

Classes are identified by leaf color. The outside circles represent the assays a species was detected in. Inside squares provide information a species relationship to the sample sites.

From spider webs collected outdoors at the Perth Zoo, 50 individual detections of 32 zoo animals were made. Of these, we found a significant relationship between the distance a web was located from the DNA source and the likelihood of detecting that DNA (Deviance explained = 17.31%; df = 1, 757 $p < 0.001$), with species more likely to be detected in webs collected closer to their enclosures (Figure 2B). The maximum distance observed from a web containing DNA from a certain species and to the nearest point of that species' enclosure was 195 m. These were Asian elephant (*Elephas maximus*) and giraffe (*Giraffa camelopardalis*) - two of the largest species at the zoo. Apart from these exceptions, 94% of the species detection came from spider webs sampled within the same named "zone" that housed the detected zoo animal for example, white rhinoceros (*Ceratotherium simum*) was detected within the African Savannah zone and koala (*Phascolarctos cinereus*) was detected within the Australian Bushland zone. In fact, our results show relatively modest movement of eDNA from the source with a mean distance detected of 22 m (+/− 5.6 m). Furthermore, 53% of detections occurred within just 5 m of an animal's enclosure (Figure 2D; Table S6). Total read counts were also highest closer to source locations with 50% of reads found within 5 m of an animal's enclosure (Figure 2C; Table S6).

DISCUSSION

Here, we have shown that spider webs can act as passive filters and effectively capture vertebrate eDNA. Using three metabarcoding assays we identified vertebrate eDNA from all 49 spider web samples collected at both Karakamia and Perth Zoo, resulting in totals of 32 and 61 vertebrate species detected, respectively. This demonstrates that vertebrate eDNA is readily available in spider webs found in both natural and artificial environments. At both study sites, DNA metabarcoding of spider webs identified a diverse range of animals, including mammals (e.g., meerkats, white rhinoceroses), birds (e.g., Emu, Royal Spoonbill), reptiles (e.g., dugite, Burmese python), and amphibians (e.g.,

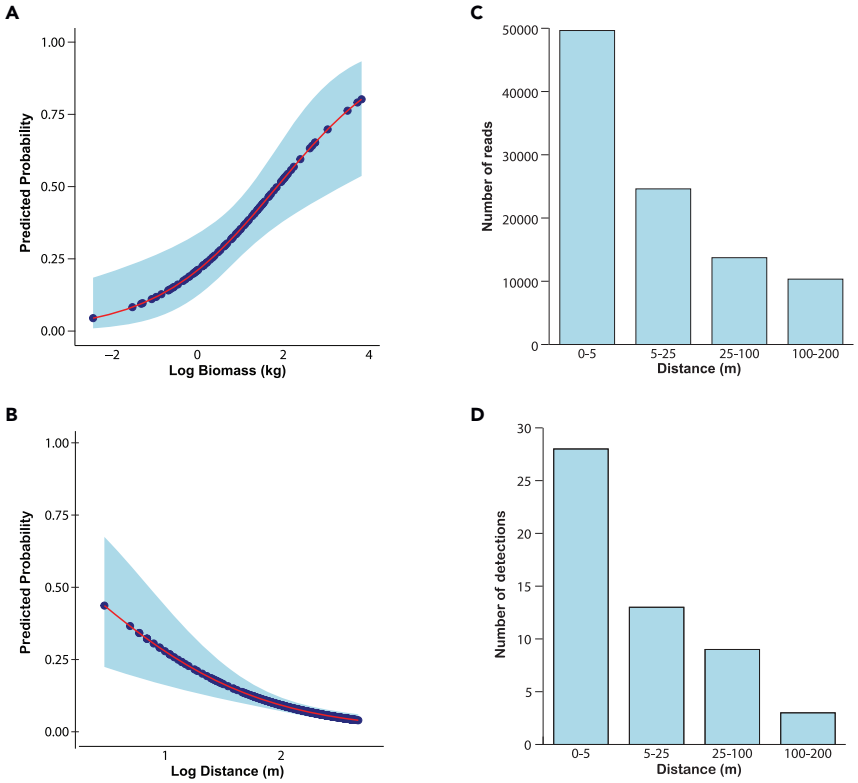

Figure 2. Number of eDNA terrestrial vertebrate detections, read counts and detection probability from spider webs collected at Perth Zoo

(A) showing relationship between eDNA detection probability and animal biomass based on models using all zoo species (both detected and undetected). Upper and lower 95% confidence intervals are presented in the blue band.

(B) showing relationship between eDNA detection probability and distance spider web samples are taken from animal enclosures, based on models generated using only vertebrate zoo species detected from spider webs. Upper and lower 95% confidence intervals are presented in the blue band.

(C) Summary of read counts from spider webs relative to source location (Distance from spider web to detected species enclosure).

(D) Number of detections from spider webs relative to source location.

motorbike frog). This study provides compelling evidence that the eDNA metabarcoding of spider webs can detect vertebrate species living in the immediate vicinity of the webs.

At the zoo, species detected ranged in sizes from the pygmy marmoset (average biomass of 0.476 kg) to the Asian elephant (average biomass of 4060 kg). However, similar to the air filtering study of Lynggaard et al.,[12] we found that greater animal biomass increased detection probability at the zoo. At both study sites animals of varying behavior and lifestyle were detected including, arboreal (e.g., common brushtail possum), ground dwelling (e.g., giraffe), nocturnal (e.g., brush-tailed phascogale) and diurnal species (e.g., Sumatran orangutan) as well as animals with fur, feathers, scales, and naked skin. In agreement with other studies, we detected more vertebrate species when using multiple primer sets in combination.[12,37,39] Overall, our results show that the eDNA metabarcoding of spider webs can be harnessed to monitor a wide range of terrestrial vertebrates with minimal sampling effort.

Across the 46 outdoor spider web samples collected, only a single reptile species was detected (snake-eyed skink at Karakamia) despite 30 reptile species documented within Karakamia, and ten species housed outdoors throughout Perth Zoo. Similarly, while an additional five reptiles were detected in webs sampled within the indoor reptile enclosure at the Perth Zoo this represents only 10% of the total number of reptiles present here. This observation lends support to the "shedding hypothesis", which suggests that organisms with a hard or keratinized exterior may shed eDNA at lower rates, reducing the detectability of reptiles in eDNA studies.[40,41]

From within the Perth Zoo, distance from the source was shown to be an important variable for the detection of vertebrates from spider web. As the distance from the animal enclosures increased, there was a significant decline in the likelihood of detecting that species. Vertebrate detections were found to be generally localized around the species enclosures, with a rapid reduction in read counts and detections past five meters from the source (Figure 2C). However, occasionally more substantial movement of DNA was observed, up to 195 m (Asian elephant and giraffe), with this study design. These findings agree with recent studies utilizing both airborne eDNA,[12,28] and invertebrate-derived DNA (iDNA) from carrion flies[24] for the detection of vertebrates, suggesting detections from both sample types to be highly localized. This reflection of the local vertebrate diversity in spider webs, is likely due to a lack of eDNA movement and/or a "dilution" of the local eDNA pool when moving away from the source. This effect may be further magnified in a zoo setting due to the built-up environment reducing direct

movement of bioaerosols or insects,[12] a presumed higher abundance of human DNA, and many animals living in close proximity. At Karakamia a slightly different pattern transpires. While most species detected within this natural system are known occupants (81%), emphasizing the increased detection probability of localized eDNA, we also detected multiple species that only exist in the bushland and farmland outside the study area (Figure 1). This highlights the potential of eDNA from spider webs to have traveled over larger distances in natural ecosystems. Other studies sampling airborne eDNA have also detected vertebrates from outside designated study areas.[28,32] In this case we do not know the exact source of the vertebrate DNA in the spider webs making it difficult to predict how far it may travel.

Further research is needed to understand the sources and the dynamics of airborne eDNA transfer. eDNA collected on spider webs likely originates in part from bioaerosols similar to that collected through air filters.[42] The movement of airborne eDNA in the atmosphere involves several factors. Initially, DNA must be shed from organisms, e.g., skin cells, hair, tissue, feces, and become airborne,[42] either still embedded in cells or as free floating (extracellular) DNA. The quantity and quality of such DNA will almost certainly vary among tissue types and taxa. Also, investigating how eDNA interacts with wind and air currents, both as bioaerosols or bound to particles, is central to understanding the movement of airborne DNA and how it ends up in spider webs. However, webs are often highly efficient in capturing insect prey and vertebrate DNA could also derive from such insects. For example, carrion and dung-feeding flies, are already well established as efficient sample types for the collection of vertebrate eDNA.[24,39,43,44] If carrion and dung-feeding flies are vectors for vertebrate DNA in spider webs, then the DNA may travel much farther from the source: upwards of 3 km.[45] The degree of mobility is critical to clarify if eDNA in spider webs is to be used to inform decision making, such as management or action plans. For example, we detected red fox DNA in Karakamia, an invasive species in Australia known to have devastating impacts on native wildlife.[46] If present inside Karakamia, essential management actions are required. However, if eDNA can travel over substantial distances, we cannot conclude that this species is present within the sanctuary grounds. In fact, intense monitoring suggests that red foxes do not occur on the property, and the detection is highly likely a result of eDNA carried in from outside the fenced area. Therefore, while it is likely that most vertebrate detections from spider webs result from the highly localized presence of the species some component of the captured eDNA is likely to have a non-local source.

Significantly higher species richness was found in the zoo samples, which reflects the higher diversity of vertebrates here than in the natural environment. A greater proportion of the vertebrate taxa known to occur at the site were also detected within Perth Zoo samples (37% Perth Zoo, 13% Karakamia). Similarly, comparisons of airborne eDNA studies undertaken in Denmark within both mixed forest[30] and zoo environments,[12] allude to reduced detections within natural environments. This likely reflects the contrast between natural systems where animals are less densely distributed and able to freely move throughout the environment, in comparison to the density and confined nature of artificial zoo environments. However, we note that differences in spider web types collected between sites may also have some influence on this. Distinct spider species tend to inhabit specific ecological niches, and the webs opportunistically gathered in Karakamia were primarily from the Araneidae (n = 9) and Phonognathidae (n = 10) families, both with typical two-dimensional orb webs, consisting of non-sticky as well as sticky threads, well suited to the capture of flying insects.[33] In contrast, the majority of webs at Perth Zoo were from the Desidae (n = 13) and Theridiidae (n = 9) families, both with tangled, irregular web arrangements not only designed for the capture of insects, but also long lasting likely increasing the possible collection of dirt and bioaerosols. Such disparities in web types may affect the types and quantity of DNA collected. For example, Gregorič et al.,[38] showed that sheet webs which are non-sticky and long-lasting, accumulate higher alpha diversity of both bacterial and fungal taxa compared to orb webs, which are sticky and rebuilt daily. Moreover, significant differences in invertebrate, bacterial, and fungal community composition were detected between web types. It is plausible then that different silk types and web architectures, customized for different prey capture strategies, may lead to web-dependent variations in the eDNA diversity and quantity accumulated.

Spider webs emerge from this study as a powerful natural biofilter for the eDNA detection of vertebrate species. Importantly, they are widespread within both natural and anthropogenic environments, spanning numerous ecosystems and microhabitats, and they require minimal sampling effort and no direct interaction with the target taxa. These are all features that promote spider webs as a cheap, easy-to- use, and non-invasive method for monitoring vertebrate species diversity.

Limitations of the study

We show that spider webs provide a sensitive and easy method to characterize vertebrate occurrences, but we note that the ability to capture vertebrate eDNA might be context dependent. Spiders spin webs and other silken structures for varying purposes such as capturing prey, constructing shelters, and reproducing, resulting in a range of unique characteristics that will likely affect the ability to capture and preserve eDNA. Factors such as web locality, web architecture and suspension time, silk type and adhesive substances, composition of the trapped material, enzymes secreted by the spider for the pre-digestion of prey, and various environmental conditions may all play a role in determining a webs ability to act as eDNA substrate. Some of these variations may be advantageous for biomonitoring, for example short-lived webs such as those rebuilt daily by some orb web spiders (e.g., *Zygiella x-notata*),[47] would in theory allow for high temporal precision in species detection. However, further research is needed to fully understand the potential effects of web type and their implications for genetic material trapped in spider webs.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.108904.

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AUTHOR CONTRIBUTIONS

Conceptualization, JPN, MEA, PWB, and PN; methodology, JPN, MEA, MAC, PWB, and PN; investigation, JPN and MAC; formal analysis, JN; writing – original draft, JPN; writing – review and editing, JPN, MEA, MAC, PWB, and PN; supervision, MEA, PWB, and PN; funding acquisition, PN.

DECLARATION OF INTERESTS

The authors declare that they have no conflict of interest.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
23 spider web samples	Karakamia Wildlife Sanctuary, Western Australia	N/A
26 spider web samples	Perth Zoological Park, Western Australia	N/A
Chemicals, peptides, and recombinant proteins		
MgCl2	Thermofisher	Cat# 4311820
1× PCR Gold buffer	Thermofisher	Cat# 4311820
dNTP Set 100mM (4 × 25µM)	Bioline	Cat# BIO-39025
Bovine serum albumin (BSA)	Fisher Biotec	Cat# BSA-50
AmpliTaq Gold DNA Polymerase	Thermofisher	Cat# 4311820
SYBR Green	Life Technologies	Cat#
Critical commercial assays		
Qiagen PowerLyzer PowerSoil Kit	QIAGEN	Cat# 12855-100
QIAxcel DNA High Resolution Kit	QIAGEN	Cat# 929002
Pippin-Prep 2% Agarose Gel Cassette	Sage Science	Cat# SSC-CDF2010
QuBit 1x dsDNA HS Assay kit	Thermofisher	Cat# Q33231
MiSeq Reagent Kit v2 (300-cycles)	Illumina	Cat# MS-102-2002
MiSeq Reagent Nano Kit v2 (500-cycles)	Illumina	Cat# MS-103-1003
Deposited data		
Raw sequence data	This paper	Available at Mendeley data https://doi.org/10.17632/b4n9vpxzcd.1
Oligonucleotides		
12S-V5 primers – Forward 2: 5′-TAGAACAGGCTCCTCTAG-3′; Reverse 2: 5′-TTAGATACCCCACTATGC-3	Riaz et al.[51]	N/A
16Smam1/2 primers - Forward: 5′-CGGTTGGGGTGACCTCGGA-3′; Reverse: 5′-GCTGTTATCCCTAGGGTAACT-3′	Taylor et al.[52]	N/A
16S Reptile primers - Forward: 5′-AGACNAGAAGACCCTGTG-3′; Reverse: 5′-CCTGATCCAACATCGAGG-3	West et al.[53]	N/A
Software and algorithms		
eDNAFlow	Mousavi-Derazmahalleh et al.[55]	https://github.com/mahsa-mousavi/eDNAFlow
FASTQC	Andrews [56]	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
AdapterRemoval v2	Schubert et al.[57]	https://github.com/MikkelSchubert/adapterremoval
Obitools	Boyer et al.[58]	https://pythonhosted.org/OBITools/welcome.html
USEARCH	Edgar [59]	https://www.drive5.com/usearch/
BLASTN	Altschul et al.[60]	https://blast.ncbi.nlm.nih.gov/Blast.cgi
LULU	Frøslev et al.[61]	https://github.com/tobiasgf/lulu
Geneious Prime 2023.0.3	Geneious	https://www.geneious.com/download/
R version 4.0.2	Team, R.C[62]	https://cran.r-project.org/bin/windows/base/
Interactive Tree of Life	Letunic et al.[64]	https://itol.embl.de/
QGIS v3.22-Białowieża	Team, Q.D.[65]	https://www.qgis.org/en/site/forusers/download.html

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Joshua Newton (joshua.p.newton@postgrad.curtin.edu.au)

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Data: All sequencing data that support the findings of this study is publicly available at Mendeley Data https://doi.org/10.17632/b4n9vpxzcd.1.
- Code: Not applicable.
- Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Vertebrate DNA was obtained from spider webs collected from Perth Zoo and Karakamia Wildlife Sanctuary, and extracted within the TrEnD laboratories at Curtin University, Perth, Western Australia. See method details section for more information.

METHOD DETAILS

Study site

Spider webs were collected from two locations in south-west Western Australia with known differences in vertebrate diversity and abundance: Karakamia Wildlife Sanctuary, and Perth Zoo (February 2023; Figure S1). Karakamia is a 268-ha fenced wildlife sanctuary containing a diversity of habitats including, jarrah forest, marri and wandoo woodland, and riparian areas. Karakamia supports a high number of native Australian vertebrates including both threatened and reintroduced species with 20 mammals, 118 birds, 30 reptiles and nine amphibians recorded to date. Perth Zoo is a 17-ha zoological park housing 136 vertebrate species, many of which are not native to the area. Of these, 98 species (47 mammals, 34 birds, 11 reptiles and five amphibians) are displayed with access to outdoor enclosures. Animals are displayed by geographical region of origin and are grouped into eight main outdoor zones, with the main zones being African Savannah, Australian Bushland, Australian Wetland, Primate Trail, and Asian Rainforest.

Sample collection

A total of 49 spider webs were opportunistically collected at both sites with the exact location and height of spider webs documented prior to sampling (Karakamia = 23, Perth Zoo = 26; average height = 1.5m; Table S1). Spiders were identified based on web type, resulting in the collection of webs from seven genera within six families (Table S1). Of the webs collected, *Phonognatha, Austracantha, Trichonephila* and *Nephila* species construct typical orb webs with variations in size and web building behaviors; however, all consist of sticky, as well as non-sticky threads.[48] The sticky capture threads within these webs play a pivotal role in capturing a wide array of flying insects, including those studied in previous iDNA research, such as carrion flies[24,39,43] and mosquitoes.[49] The exceptional adhesive properties[50] likely allow for the capture of not only these insects, but also airborne bioaerosols and other particles containing DNA. Conversely, *Pholcus* sp. fashion thin, non-sticky, irregular dome-shaped horizontal webs designed to capture wandering insects that come into contact with the web. *Badumna* and *Latrodectus* species share commonalities in their webs, characterized by long lived, irregular web structures and funnel-shaped retreats. However, while *Latrodectus* sp. contain numerous prey catching sticky threads again likely allowing for the adhesion of bioaerosols, *Badumna* sp. utilise cribellate silk, composite 'wool-like' threads effective at tangling insects but not sticky in the traditional sense. While the majority of webs were collected from outdoor locations, three samples from the Perth Zoo were collected from within a dedicated reptile room which houses 39 reptiles, 12 of which are housed in open air enclosures. Sterile plastic inoculation loops were used to collect individual webs,[38] including trapped arthropods, which were then stored in sterile 15mL Falcon tubes. To prevent contamination of samples, sterile gloves were worn during sampling. All web samples were stored at −80°C within 8 hrs of collection until DNA extraction.

DNA extraction

Sample processing and extraction was undertaken in the Trace & Environmental DNA (TrEnD) Laboratories, Curtin University, Perth, Western Australia, which are dedicated for working with samples of low DNA concentration. All spider webs were extracted using adapted protocol of the Qiagen PowerLyzer PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany). Lysis was performed by adding 540, 810, 1080 or 1350 μL ATL lysis buffer and 60, 19, 120 or 150 μL proteinase K, respectively to plastic tubes containing individual spider webs, depending on the size and absorbent properties of individual webs (Table S1). Samples were lysed at 56°C for 72 hr with a gentle rotation. After lysis, samples were vortexed at 10,000 g for one minute and 650 μL lysis mixture retrieved. DNA was then isolated using the QIAcube automated extraction platform (Qiagen, Hilden, Germany) following manufacturers protocols. To detect cross-contamination, digest (n = 1) and extraction (n = 3) controls were processed with each batch of samples. DNA extracts were stored at −20°C.

DNA amplification and sequencing

Three assays were used to amplify vertebrate DNA from all spider webs collected outdoors, 12S-V5 (F2: 5′-TAGAACAGGCTCCTCTAG-3′; R2: 5′-TTAGATACCCCACTATGC-3′)[51] general vertebrate primer, designed to amplify a 98 bp fragment that includes the V5 variable region of the 12S rRNA gene; 16Smam1/2 assay (F: 5′-CGGTTGGGGTGACCTCGGA-3′; R: 5′-GCTGTTATCCCTAGGGTAACT-3′),[52] hereafter referred to as 16Smam, designed to amplify a ~ 130 bp fragment of mammalian 16S ribosomal gene region; and 16S Reptile assay (F: 5′-AGACNA GAAGACCCTGTG-3′; R: 5′-CCTGATCCAACATCGAGG-3),[53] designed to amplify a ~245 bp fragment of reptilian 16S ribosomal gene region. Only the 16S Reptile assay was used to amplify vertebrate DNA from the spider webs collected within the dedicated reptile room (n = 3).

To determine the optimal dilution level of DNA input, for each sample quantitative PCR (qPCR) was performed on neat DNA extracts (undiluted) and 1:10 dilutions, to see if samples exhibited inhibition.[54] The qPCRs were run on a StepOnePlusTM Real-Time PCR system (Applied BioSystems, Waltham, USA) with PCR reaction volumes totalling 25 μL containing: 14.45 μL ddH_20, 2 μL $MgCl_2$ (2.5 mM), 2.5 μL 1× PCR Gold buffer, 0.25 μL dNTPs (0.25 mM of each), 1 μL BSA (0.4 mg/ml), 0.6 μL 5X SYBR® Green, 0.2 μL AmpliTaq Gold® DNA Polymerase, 1 μL of each primer (10 μM) and 2 μL of template eDNA. The cycling conditions were initial denaturation for 5 min at 95°C, followed by 50 cycles of 30 s at 95°C, then 30 s at primer specific annealing temperature (52°C for 12S-V5, 55°C for 16Smam and 52°C for 16S reptile), and 45 s at 72°C, ending with 10 min of elongation at 72°C. All qPCR runs included both a non-template control (reagents only) used to detect possible contamination and positive controls (12S-V5 and 16Smam = *Tursiops truncates; 16Srep = Hydrophis peronii*) to ensure efficacy of PCR reagents and cycling conditions.

For DNA metabarcoding, sample dilutions showing optimal levels of amplifiable DNA underwent a single step qPCR, using fusion tagged primers consisting of Illumina compatible sequencing adaptors, a unique index (6-8bp in length) and a respective primer sequence for each assay. All qPCR reactions were prepared in dedicated clean room facilities with 4 μL of template eDNA for reaction volumes totalling 27 μL, which contained the same reagents, controls, and cycling conditions described above. Each eDNA sample was amplified in duplicate PCRs with the same index tag before amplicons were pooled in approximate equimolar concentration based on qPCR ΔRn values resulting in two Amplicon libraries, a single end library containing pooled 12S-V5 and 16Smam samples and a paired end library containing 16Srep samples. Amplicons in the single end library were then size-selected to 150–300 bp and the paired end library size-selected to 200–600 bp, using a Pippin-Prep 2% Agarose Gel Cassette (Sage Science, Beverly, USA) to remove any amplicons outside of the target range. Size-selected libraries were then quantified using a Qubit 4.0 Fluorometer (Invitrogen, Carlsbad, USA) and diluted to 2nM. The single end library was loading onto a 300 cycle MiSeq V2 Standard Flow cell for single end sequencing, and paired end library loaded onto a 500 cycle MiSeq Reagent Nano Kit V2 Standard flow cell for paired end sequencing. Sequencing for both libraries was conducted separately on an Illumina MiSeq platform (Illumina, San Diego, USA), with a final single end library molarity of 10 pM containing 7% PhiX, and paired end library molarity of 7.5 pM containing 5% PhiX.

Sequence analysis (filtering and taxonomic assignment)

The sequence data from each assay were processed separately. Initial sequence filtering, ZOTU formation, and taxonomic assignment were conducted utilizing the eDNAFlow bioinformatics pipeline[55] via Setonix, a HPE Cray EX supercomputer, based at the Pawsey Supercomputing Centre in Kensington, Western Australia. Sequence reads were quality checked using FASTQC,[56] and quality filtered using AdapterRemoval v2[57] with a minimum Phred quality score set at 20, and minimum alignment of 12 for the paired-end reads. Sequences were then demultiplexed using obitools (ngsfilter),[58] and those under 60 bp removed (obigrep). Zero-radius operational taxonomic units (ZOTUs) with a minimum abundance of 4 were then created from demultiplexed sequences using the USEARCH unoise3 algorithm, in order to filter out low-abundance sequences prone to contain errors.[59] ZOTUs were then queried against the GenBank nucleotide reference database using BLASTN,[60] with the following parameters: 97% query coverage, 95% identity, evalue $\leq$ 1e−3, and max target sequences = 10. Erroneous ZOTUs were removed using the LULU post clustering curation method with the minimum threshold of sequence similarity at 95%.[61] A custom python script was then used to assign the ZOTUs to their lowest common ancestor (LCA),[55] with taxonomic assignment collapsed to the LCA if the percent identity between two hits with 97% query cover and 95% identity, differed by $\leq$ 1%.

The taxonomic identification of all ZOTUs assigned to species level were manually validated based on species distribution data (Tables S2–S4). In cases where species detected with eDNA metabarcoding were know with certainty not to be present within the area, but instead represented by a single closely related sister species, the ZOTU was reassigned to the latter. In cases where multiple species were assigned to a ZOTU, resulting in the taxonomic classification being dropped to genus or family level, the corresponding DNA sequence was visually checked in Geneious Prime 2023.0.3 to assess whether it could be assigned to species level based on known occupants of both the Perth Zoo and Karakamia Sanctuary. Only ZOTUs that could be assigned to species level were retained. In addition, domestic species such as cow, pig, chicken, mouse, and rabbit known to be potential laboratory contaminants (Leonard et al., 2007) were also retained, as they are known to exist within the study area and/or be used as feed for zoo animals, and because we did not detect any of these taxa in negative controls.

Further sample filtering was performed in R v.4.0.2 Team[62] using the "phyloseq" package.[63] Initially, rarefaction curves were used to identify samples with low sequencing depth (12S-V5 <1000 reads; 16Smam <1000 reads; 16srep <50 reads) based on where curves appeared to asymptote (Figure S2); all samples were above the identified read count threshold. The removal of taxa from samples with less than 15 reads was then undertaken, based on, and resulting in the removal of all taxa considered false positives (detection of species that do not reside within the study area), and those found within negative controls. Finally, prior to further analysis all ZOTUs identified as human were removed

and ZOTUs from all assays were combined. To illustrate the taxa diversity detected, we constructed a phylogenetic tree using phyloT (Phylogenetic Tree Generator) based on the National Center for Biotechnology Information (NCBI) taxonomy, and visualized the tree with the Interactive Tree of Life.[64]

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical tests were performed in R v.4.0.2[62] on samples collected from outdoor locations only (Perth Zoo = 23, Karakamia = 23). We calculated the observed ZOTU richness at both sites (Perth Zoo and Karakamia) and tested the difference between sites using a Wilcoxon test. For further analysis utilizing Perth Zoo data, all detected non-zoo animals were removed from the dataset, as it was not possible to assess location and biomass of those species. Biomass of all zoo animals was calculated based on the number of individual animals multiplied by the average body weight, calculated based on a literature survey and online databases (Table S5). While these estimates are not direct measurements of the exact biomass within the Perth Zoo, we emphasize that they represent informed and reasonable approximations derived from available information. A generalized linear model with binomial distribution (detection = 1, non-detection = 0) was then employed to test association between detection probability and biomass (log transformed). The distance from sampled spider webs to the enclosures of all zoo animals detected from eDNA was measured using QGIS v3.22-Białowieża,[65] using a central point of reference for animals with a large enclosure. A generalized linear model with binomial distribution (detection = 1, non-detection = 0) was then employed to test association between detection probability and distance to spider web (log transformed). Finally, for both biomass and distance, the "predict" function in the "car" package v.3.1–2,[66] was used to generate the predicted probabilities for each observation in the dataset based on the generalized linear models and plotted using the package "ggplot2" v.3.4.2.[67]