Received: 19 December 2021 | Revised: 15 April 2022 | Accepted: 22 April 2022

DOI: 10.1002/edn3.313

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

WILEY

ORIGINAL ARTICLE

Home is where the hollow is: Revealing vertebrate tree hollow user biodiversity with eDNA metabarcoding

Joshua P. Newton[1,2] | Philip W. Bateman[1,3] | Matthew J. Heydenrych[2] | Mahsa Mousavi-Derazmahalleh[2] | Paul Nevill[1,2]

[1]Terrestrial Ecology and eDNA Research Group, School of Life and Molecular Sciences, Curtin University, Perth, Western Australia, Australia

[2]Trace and Environmental DNA Laboratory, School of Life and Molecular Sciences, Curtin University, Perth, Western Australia, Australia

[3]Behavioural Ecology Lab, School of Molecular and Life Sciences, Curtin University, Perth, Western Australia, Australia

Correspondence
Joshua P. Newton, Terrestrial Ecology and eDNA Research Group, School of Life and Molecular Sciences, Curtin University, GPO Box U1987, Perth, WA, 6845 Australia.
Email: joshua.p.newton@postgrad.curtin.edu.au

Funding information
Department of Biodiversity, Conservation and Attractions

Abstract

Tree hollows are essential for many vertebrate species, providing both nesting sites and shelter. Globally, old hollow-bearing trees are in decline resulting in many dependent species being under threat. It is, therefore, imperative that vital hollow-bearing trees are preserved, but it is logistically difficult to rapidly determine which hollows are being used and by which taxa. Here, we investigate the efficacy of eDNA metabarcoding as a survey tool for vertebrate hollow users. We compared the identity and richness of hollow-inhabiting vertebrate taxa using eDNA metabarcoding of both sediment from the tree hollows, and material collected using roller swabs. Samples (n = 138) were collected from hollow-bearing tuart trees (*Eucalyptus gomphocephala*; N = 28), within both an urban remnant and a relatively undisturbed forested area of South-West Western Australia. We detected a wide range of vertebrate taxa, including cryptic species such as the brush-tailed phascogale (*Phascogale tapoatafa*), while also providing ecologically informative data, such as hollow use by invasive Rainbow Lorikeet (*Trichoglossus moluccanus*) within the study areas. Our results showed variation in the species detected between methods, with the roller swab method detecting a greater number of species and a higher mean species richness per sample than hollow sediment did. The species detected from both methods did not perfectly overlap, highlighting the value of using multiple methods or substrates to detect a greater number of taxa. Our results suggest eDNA metabarcoding from tree hollow samples offers a sensitive and resource-efficient method of monitoring vertebrate hollow users, if enough hollows are sampled. This provides not only a broad biodiversity assessment tool, but also an effective method for detecting taxa that may be elusive using other methods.

KEYWORDS
biomonitoring, environmental DNA, metabarcoding, terrestrial, tree hollows, vertebrate

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
© 2022 The Authors. *Environmental DNA* published by John Wiley & Sons Ltd.

1 | INTRODUCTION

Tree hollows or cavities, providing nesting sites and shelter, are a vital habitat component worldwide for a range of vertebrate fauna including mammals, birds, reptiles, and amphibians (Cockle et al., 2011; van der Hoek et al., 2017). While some of these taxa (e.g., woodpeckers) are excavators capable of creating their own cavities, the vast majority of hollow users are non-excavators, using either pre-existing hollows constructed by excavator species or those formed through fungal decay and environmental damage (Cockle et al., 2011, 2012; Gibbons & Lindenmayer, 2002). Hollows suitable for many non-excavator species are, therefore, predominantly found in large, older trees due to these processes occurring over long periods—over 100 years in some eucalypt species (Gibbons et al., 2000; Lindenmayer et al., 2017; Wormington & Lamb, 1999). For this reason, non-excavator hollow-dependent species are highly vulnerable to disturbances that result in the loss of hollow-bearing trees (Gibbons & Lindenmayer, 2002).

Globally, anthropogenic activities such as urbanization, forestry, and agriculture have resulted in a decline in large, old, hollow-bearing trees such that many hollow-dependent species are under threat (Gibbons & Lindenmayer, 2002; Mansfield et al., 2017; van der Hoek et al., 2017). These threats are exacerbated by increased competition associated with limited availability of nesting hollows (Davis et al., 2013; Newton, 1994). Retention forestry practices where key biodiversity structures within production forests such as hollow-bearing trees are retained have been shown to increase the abundance of hollow-dependent species (Gutzat & Dormann, 2018). Similarly, urban developments where remnant trees are preserved have been shown to have a higher number and total abundance of species present than where the trees are removed (Barth et al., 2015). For practices like these to be most effective, it is vital to know which taxa use, or have used, hollows in particular places across an environment and which trees are used by the widest diversity of taxa, allowing for the identification of critical hollow-bearing trees. Effective management strategies can then be designed to preserve trees that will maximize the conservation of hollow-dependent species.

A number of methods are currently available to determine the ecological importance of hollow-bearing trees: physical inspection through climbing to the hollow or using a camera on a pole (e.g., Terry et al., 2021), camera traps, plus ground visual surveys, and tree characteristic analysis to estimate hollow presence. While physical inspection only provides data for a single point in time, camera traps provide useful insights into ongoing hollow use and the functional role of hollows (Cotsell & Vernes, 2016; Gregory et al., 2014). They also record more species than do physical inspection of hollows (Honey et al., 2021). However, their use in the monitoring of tree hollows has some limitations. Camera traps involve a considerable time commitment, not only in the field through the installation of cameras in a complex environment, but also through the screening process of photographs which can be complicated by false triggers (Krauss et al., 2018). Lower detections may also be seen in species with certain physiological (small, low body temperature) and behavioral (cryptic, slow-moving) traits (Glen et al., 2013; Paull et al., 2012; Zylstra et al., 2010). Therefore, while data retrieved from camera trap surveys may be highly beneficial, where long-term monitoring is inappropriate, a method to assess tree hollows rapidly is needed to identify biodiversity in hollows and contribute to the conservation of hollow-dependent species.

The current methods used to determine the ecological importance of these trees (i.e., ground visual surveys and tree characteristic analysis) often make assumptions about which trees and hollows could be used by various taxa, based on certain tree characteristics (e.g., abundance of hollows, hollow characteristics, and width at breast height). This is fraught with uncertainty as not only can animals exhibit strong preferences for hollows with particular characteristics (Goldingay, 2009, 2012; Stojanovic et al., 2020) but many individuals commonly use multiple hollows (Goldingay, 2012). Ground surveys can both over and underestimate tree hollow availability (Stojanovic et al., 2012). Furthermore, unless the dimensions of hollows can be measured directly to determine if they exhibit appropriate characteristics for particular species, hollows seen from the ground may in fact be unsuitable habitats (Cockle et al., 2010; Stojanovic et al., 2020). As counting hollows or characterizing trees does not accurately inform surveyors of the taxa using hollows, when used in the development of conservation actions targeting highly specialized species, the potential for error may result in inappropriate management strategies (Fedrowitz et al., 2014; Koch, 2008).

With the demand for more effective biomonitoring, new technologies have been developed to expand the survey toolkit. The analysis of environmental DNA (eDNA) has been used to resolve many ecological questions related to biodiversity, diet, the detection of invasive and cryptic species, and population analysis (Bohmann et al., 2014; Creer et al., 2016; Fernandes et al., 2018; Taberlet et al., 2012). eDNA metabarcoding is the process of sequencing a taxonomically informative genomic region, isolated from DNA that has been extracted from environmental samples (e.g., water, soil, and air; Taberlet et al., 2012). This technique has several benefits, including the ability to gather data quickly that can be audited by third parties (Ji et al., 2013), reduce reliance on specialist taxonomic or biological expertise, increase the ability to sample in complex and even inhospitable environments, increase the range of biodiversity detected, including elusive species, and reduce labor costs (Bohmann et al., 2014).

The recovery of eDNA within a sampling location is dependent on the rate it is deposited and its persistence in the environment (Barnes & Turner, 2016). When animals interact with their environment, such as tree hollows, they inadvertently deposit DNA. However, deposition rates differ among taxa due to variations such as biomass, seasonal activity, shedding rate, and behavior (Andersen et al., 2012; Barnes & Turner, 2016; Klymus et al., 2015; Kucherenko et al., 2018). In terrestrial systems, due to limited dispersal capabilities, DNA is more likely to be detected in locations where a species has spent enough time to deposit recoverable quantities of genetic material (Burns et al., 2020). However, once deposited, the persistence of DNA is dependent on a variety of factors that can

affect the rate of DNA decay such as temperature, UV, substrate type, pH, and microbial activity. Accordingly, the use of multiple substrates and the selection of sampling locations needs to be carefully considered to increase the chances of accurately detecting diversity (Dickie et al., 2018; van der Heyde et al., 2020). Tree hollows have the potential to provide an ideal environment to detect eDNA as they have many characteristics likely to reduce DNA decay and increase the probability of species detection. Hollow use is commonly long-term (e.g., nesting), and animal movement within hollows is restricted, likely contributing to an increase in the deposition of DNA (Kucherenko et al., 2018). The internal conditions that make hollows highly desirable to many species, such as lower ambient temperature, reduced light and shelter from rainfall (Gibbons & Lindenmayer, 2002) are also likely to reduce the rate of DNA decay.

Our study area was the southwest of Western Australia (SWWA). Native vegetation in SWWA suffers from habitat fragmentation and diverse levels of disturbance, from largely cleared urban areas, to national parks containing areas of old-growth forest. This in turn has impacted the taxon richness and community structure of vertebrate hollow users throughout this area (Ruthrof et al., 2002). Tuarts (*Eucalyptus gomphocephala*) are an endemic tree species restricted to a 400 km long belt along the Swan Coastal Plan in SWWA (Keighery et al., 2002). Occurring in both woodland and open forest, tuarts range in height from 10–15 m in their northern distribution to up to 40 m in a unique tall open forest at their southern extent (Keighery et al., 2002). They are known to support substantial populations of hollow-dependent species, including the critically endangered western ringtail possum (*Pseudocheirus occidentalis*) and the threatened Carnaby's Black-Cockatoo (*Calyptorhynchus latirostris*; Anderson et al., 2019; Boland et al., 2006; Dell et al., 2002). However, since European colonization, the tuart's range has been reduced to <35% of its original distribution, predominantly due to urbanization and agriculture (Tuart Response Group, 2003).

In this study, we investigate the use of eDNA metabarcoding as a survey tool for vertebrate hollow users. We compared the identity and richness of hollow-inhabiting vertebrate taxa using eDNA metabarcoding of hollow substrate collected in two ways—sediment collected directly, and a relatively easier and faster, roller swab method. We further evaluated survey design options by testing the relationship between the number of trees sampled and taxon richness detected. The ultimate aim of this work is to inform decision-making for the application of eDNA metabarcoding surveys of vertebrates that use hollows.

2 | METHODS

2.1 | Study sites and sample collection

Samples were collected between September 2020 and January 2021 from a total of 28 tuart trees, including 14 trees from two sites in the Tuart Forest National Park, Ludlow (hollows = 37, total samples = 58, roller = 37, sediment = 21), and 14 trees from two urban remnants within the City of Perth, Kings Park and Bold Park (hollows = 56, total samples = 80, roller = 56, sediment = 24; Figure 1).

At each site, seven hollow-bearing trees were chosen due to their accessibility and likelihood of containing at least one potential hollow based on visual surveys, resulting in a total of 93 hollows sampled. Within the urban remnants of Kings Park (September/November 2020) and Bold Park (December 2020), an elevated work platform was used to access tree hollows. Single rope access techniques were used to access trees in the Tuart Forest National Park (January 2021). For all hollows (n = 93), a roller swab sample, adapted from Valentin et al. (2020), was taken using a 40 mm polyester roller dampened with distilled water and stored in a zip lock bag. Prior to sampling, each hollow was inspected, and if an animal was present, only the opening and immediate inside walls were swabbed. For all other hollows, the opening, inside walls, and across the hollow floor were all sampled. Where hollow sediment was present and accessible (n = 45), a 50-ml sterile container was used to collect surface sediment randomly from multiple locations across each hollow floor, creating one bulk sediment sample per hollow. Care was taken not to sample sediment disturbed by the roller sampling. Samples were then stored on ice, frozen within 48 h of sampling, and stored at −18°C prior to extraction. Visual vertebrate activity was also recorded (e.g., animal present, feathers, fur, and eggs). Finally, the location, height to hollow opening, hollow opening diameter, depth of hollow, and tree width at breast height were recorded.

To prevent contamination of samples, sterile gloves were worn during sampling and single-use disposable, pre-sterilized collection containers were used. Single-use polyester rollers were decontaminated prior to use by submerging in a 10% bleach solution (made using White King Premium Bleach, ~5% sodium hypochlorite) for a minimum of 10 min before being thoroughly rinsed with purified distilled water and UV-sterilized for a further 10 min. All multi-use field sampling equipment such as roller frames was decontaminated on-site prior to use by submerging in 100% bleach (White King Premium Bleach, ~5% sodium hypochlorite) for a minimum of 10 min before being thoroughly rinsed with distilled water. Multi-use equipment was also swabbed prior to use before being preserved and processed in the same way as field samples as a negative control allowing for the detection of possible contamination of samples.

2.2 | Sample processing and DNA extraction

Sample processing and extraction was undertaken in dedicated laboratories at the Trace & Environmental DNA (TrEnD) Laboratory, Curtin University, Perth, Western Australia. Each individual roller swab was placed in a plastic container decontaminated with 10% bleach solution as outlined above, covered with purified distilled water (~500 ml) for 10 min and manually agitated twice for 30 s at 5-min intervals. Water samples were then individually filtered across Pall 0.45 μm GN-6 Metricel® mixed cellulose ester membranes using a peristaltic Pall Sentino® Microbiology pump (Pall Corporation, Port, WA, USA) and frozen at −18°C prior

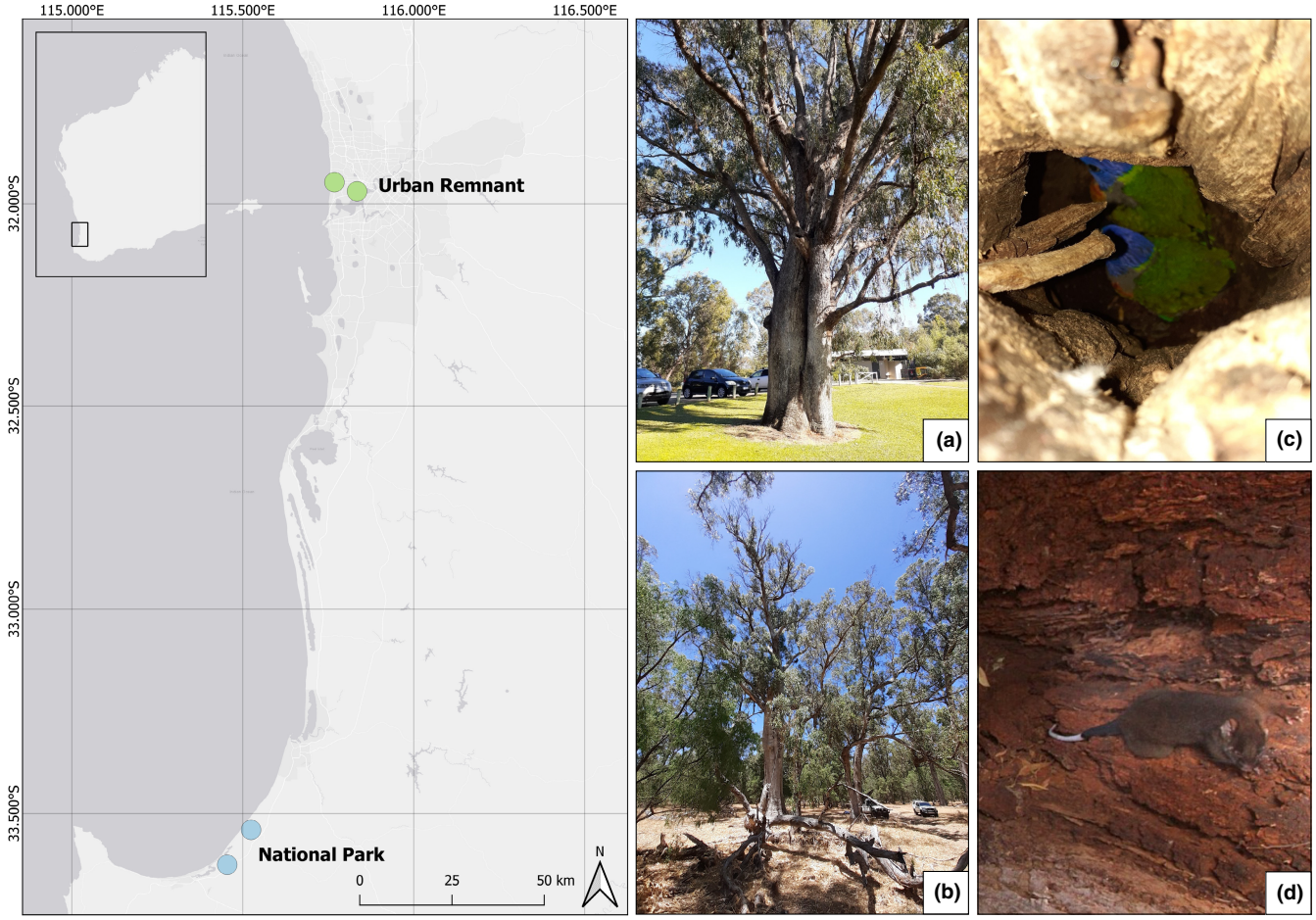


FIGURE 1 Map of two study sites in South-West Western Australia. Photographs show Perth City urban remnant bushland (a), the Tuart National Park, Ludlow (b) and hollows containing a pair of Rainbow Lorikeets (c) and ringtail possum (d; credit S. Cherriman)

to extraction. DNA was extracted from half of the filter membrane using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), with the following modifications to the standard protocol: 60 µl of Proteinase K, 540 µl of ATL lysis buffer and a 17-h digestion at 56°C. Sediment samples were manually shaken for 30 s to homogenize and DNA extracted from 0.25 g of sample using a DNeasy PowerLyzer PowerSoil Kit (Qiagen) following the manufacturer's instructions. Blank extraction controls (n = 6) containing reagents only were processed concurrently with samples to detect any cross-contamination, and eDNA extracts were then stored at −18°C.

2.3 | Assessment of DNA extracts

In silico studies were conducted using DNA sequences from the 12S and 16S rRNA marker regions (Othman et al., 2021) to identify the assay able to amplify the widest range of vertebrate taxa in the study region. The primer set 12S-V5 F2/R2 (Riaz et al., 2011), previously used to successfully amplify vertebrate taxa within the study region (Ryan et al., 2022; Van Der Heyde et al., 2020, 2021), was broad enough to amplify avian, mammalian and reptilian DNA, and short enough to pick up degraded DNA. As such, we amplified DNA with the general vertebrate primer 12S-V5 (Riaz et al., 2011), designed to amplify a 98 bp fragment which includes the V5 variable region of the 12S rRNA gene with the primers F2 (5′-TAGAACAGGCTCCTCTAG-3′) and R2 (5′-TTAGATACCCCACTATGC-3′).

Quantitative PCR (qPCR) was used to assess the quality and quantity of DNA in the extracts, as well as potential inhibition to determine the optimal dilution level of DNA input (Murray et al., 2015). All qPCR reactions were prepared in dedicated clean rooms at the TrEnD Laboratories and performed on a StepOnePlusTM Real-Time PCR system (Applied Biosystems, Massachusetts, USA). Reaction volumes totaled 25 µl containing 1× PCR Gold buffer (Applied Biosystems), 2.5 mM MgCl2 (Applied Biosystems), 0.4 mg/ml BSA (Fisher Biotec, Australia), 0.25 mM of each dNTPs (Astral Scientific Australia), 0.4 µM forward and reverse primer, 1 U AmpliTaq Gold (Applied Biosystems), 0.6 µl SYBR Green (Life Technologies, USA), and 2 µl of template eDNA (3 PCR reactions per extract at Neat, 1:10, and 1:100 dilutions). 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 the annealing temperature of 60°C, and 45 s at 72°C, ending with 10 min of elongation at 72°C. For each qPCR run, a non-template control (reagents only) and positive control (*Gallus gallus*,

100 ng) were used to detect contamination and to ensure primers attached to DNA strands.

2.4 | DNA amplification

Samples containing a sufficient quantity of amplifiable DNA ($n = 76$; Table S1) were assigned a unique 6–8 bp multiplex identifier tag (MID tag), 12S-V5 specific primers and Illumina sequencing adaptors. The unique combinations of MID tag (fusion) primers were then used in qPCR which was prepared in a physically separate ultra-clean laboratory with 4 µl of template eDNA for reaction volumes totaling 27 µl, which contained the same reagents, controls, and cycling conditions described above. A single-step fusion protocol was used with no reuse of index combinations. MID-tag amplicons were generated in duplicate to reduce the effects of PCR stochasticity (Murray et al., 2015) and combined before being pooled with other MID-tag amplicons (i.e., minipool) according to qPCR ΔRn values. Minipool concentrations (ng/µl) were quantified using a QiAxcel Advanced System (Qiagen, Hilden, Germany) before being combined in approximate equimolar ratios based on this quantification to create a DNA sequencing library which also contained one unrelated project. Amplicons in this library were size selected to 150–300 bp using a PippinPrep 2% ethidium bromide cassette (Sage Science, Beverly, MA, USA), purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), and re-quantified using a Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA). Sequencing was then undertaken on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) using a single lane flow cell as per Illumina protocols for single-end sequencing (MiSeq® v2 Reagent Kit 300 Cycles PE), with a final library molarity of 6 pM containing 7% PhiX.

2.5 | Sequence analysis (filtering and taxonomic assignment)

Conservative filtering steps were employed while also attempting to retain as much of the "true" eDNA diversity as possible. All sequence filtering, ZOTU formation, and taxonomic assignment were conducted utilizing the eDNAFlow bioinformatics pipeline (Mousavi-Derazmahalleh et al., 2021) via Zeus, an SGI cluster, based at the Pawsey Supercomputing Centre in Kensington, Western Australia. Sequence reads were quality checked using FASTQC (Andrews, 2010) and quality filtered (Phred quality score < 20), including trimming sequences with Ns as implemented in eDNAFlow source code by --trimns & --trimqualities employing AdapterRemoval v2 (Schubert et al., 2016). Demultiplexing with obitools (ngsfilter; Boyer et al., 2016) and sequences under 60 bp removed (obigrep). Dereplication and the creation of zero-radius operational taxonomic units (ZOTUs; min sequence abundance = 4) and ZOTU tables were then undertaken using the USEARCH unoise3 algorithm (Edgar, 2016). The ZOTUs were queried using BLASTN (Altschul et al., 1990) against NCBI's GenBank nucleotide database using the following parameters: % identity ≥ 95, evalue ≤ 1e−3, % query cover 97, max target sequences = 10. Erroneous ZOTUs derived by high-throughput sequencing were identified and removed using the post clustering curation method LULU with the minimum threshold of sequence similarity at 95% (Frøslev et al., 2017). Finally, curated ZOTUs were taxonomically assigned by the lowest common ancestor (LCA) approach using eDNAFlow custom python script (Mousavi-Derazmahalleh et al., 2021), with taxonomic assignment collapsed to the LCA if the percent identity of two hits with 97% query cover and 95% identity, differed by <1% (Table S2).

Further sample filtering was performed in R v.4.0.2 (R Core Team, 2018) using the "phyloseq" package (McMurdie & Holmes, 2013). To mitigate against cross-contamination and false positives, analysis of contamination present in negative controls, and rarefaction curve (Figure S1), resulted in taxa with sequence counts fewer than 3 and samples with low sequencing depth (<50 reads), being removed. Following this, sequences present in negative/extraction controls, taxa not known to inhabit or interact with hollows or be the prey of those that may, common PCR contaminants (e.g., human, and ungulate sequences) and taxonomic assignments higher than family level were removed from the dataset.

Taxonomic assignments were then corrected based on existing species distribution data for each site. Species Red-Collared Lorikeet (*Trichoglossus rubritorquis*), short-eared possum (*Trichosurus caninus*) and New Zealand Fantail (*Rhipidura fuliginosa*), which are not known to occur within SWWA, were reassigned to local taxa Rainbow Lorikeet (*Trichoglossus moluccanus*), brushtail possum (*Trichosurus vulpecula*), and Gray Fantail (*Rhipidura albiscapa*), respectively. The genus *Tadorna* and family Pseudocheiridae were reassigned to Australian Shelduck (*Tadorna tadornoides*) and western ringtail possum (*Pseudocheirus occidentalis*), respectively, due to these taxa containing only a single species in SWWA. The family Strigidae was reassigned to genus *Ninox* again due to known species distributions; however, this could not be assigned to a lower level as two *Ninox* species are known to occur within the SWWA. The Malayan field rat (*Rattus tiomanicus*) and Himalayan field rat (*Rattus nitidus*) were downgraded to genus level, due to species distributions outside of the study area. ZOTUs with multiple possible assignments were left at the closest taxonomic level and labeled "sp." Of these, Pstittacidae sp was reassigned to Psittaculidae sp in line with species distribution data.

2.6 | Statistical analysis

All statistical analyses were performed using R v.4.0.2 (R Core Team, 2018). Initially, the Shannon diversity index was used to calculate the ZOTU richness for each substrate (sediment and roller swabs) from hollows from which both sediment and roller swab samples were collected ($n = 17$) and the difference between substrates tested using a paired samples Wilcoxon test. Total filtered hollow data were also merged into tree samples ($N = 23$) and used to calculate the ZOTU richness at both sites (urban remnant

and National Park), again using the Shannon diversity index, with differences between sites tested with a Wilcoxon test. For both datasets (sites and substrates), differences in community composition were tested by first converting data into a presence–absence matrix which included species and the samples in which they were detected. A Jaccard dissimilarity matrix was then calculated between samples, and a permutational analysis of variance (PERMANOVA) with 999 permutations was used to assess differences in community composition between sites (merged tree sites dataset; $N = 23$) and the effect of substrate, site, and their interaction (hollow substrate dataset; $n = 17$) using the R package "vegan" v.2.5–7 (Okasen, 2019). A pairwise PERMINOVA with 999 permutations was then run between all individual sample sites ($n = 4$) to determine whether significant differences in community composition were due to differences in individual sample sites, using the "pairwise. Adonis" function in the "vegan" package v.2.5–7 (Okasen, 2019), with p values adjusted using the BH method (Benjamini & Hochberg, 1995). Results were visualized using nonmetric multidimensional scaling (NMDS) again using the R package "vegan" v.2.5–7 (Okasen, 2019). Species associations between sites were then accomplished using the "indicspecies" package v.1.7.9 (De Caceres et al., 2016) on presence–absence data with 999 permutations. Finally, species accumulation curves were calculated using the number of ZOTUs and tree samples, between urban remnant and National Park sites using the "accumcomp" function in the "BiodiversityR" package v.2.14–1 (Kindt & Coe, 2005) and all data were visualized using the package "ggplot2" v.3.3.5 (Wickham, 2016).

3 | RESULTS

The 12S-V5 assay generated a total of 4,288,734 metabarcoding sequences of which 3,112,527 remained after quality filtering (mean = 40,422; $SE = 3473$). Contamination was observed in field negative controls (*Trichoglossus moluccanus*, *Homo sapiens*, and *Canis lupus familiaris*) as well as in the filtration and extraction controls (*Homo sapiens*, *Eolophus roseicapilla*) and PCR-negative controls (*Rattus* sp). Within samples, common ungulate contaminates (*Bos taurus*, *Ovis* sp, and *Sus scrofa)* were detected likely due to lab reagents (e.g., BSA), as well as other species not known to inhabit or interact with hollows or be the prey of those that do including human (*Homo sapiens*), dog (*Canis lupus familiaris*), fish (*Coptodon zillii*), and kangaroo (*Macropus fuliginosus*). After filtering applications, the sole contaminant remaining was Rainbow Lorikeet (*Trichoglossus moluccanus)*, detected within one field negative control (Kings Park, tree 7, negative control). Accordingly, all Kings Park, tree 7, roller swab samples were considered affected and all Rainbow Lorikeet (*Trichoglossus moluccanus)* ZOTUs detected within these samples were removed prior to further analysis. Due to the use of extensive field and laboratory negative controls, it was considered appropriate for all other Rainbow Lorikeet (*Trichoglossus moluccanus)* detections to be kept within the dataset. In total, 56% of the 138 samples collected amplified successfully and were used for further analysis. This included 58% of roller swabs ($n = 54$) and 51% of sediment samples ($n = 23$). Four trees failed to amplify any taxa resulting in a total of 13 trees from urban remnants and 11 from National Park used for further analysis. After curation, the final dataset included 34 vertebrate species including 10 mammals, 24 birds, and no reptiles or amphibians, with 18 ZOTUs assigned to species, 5 to genus, and 11 to family.

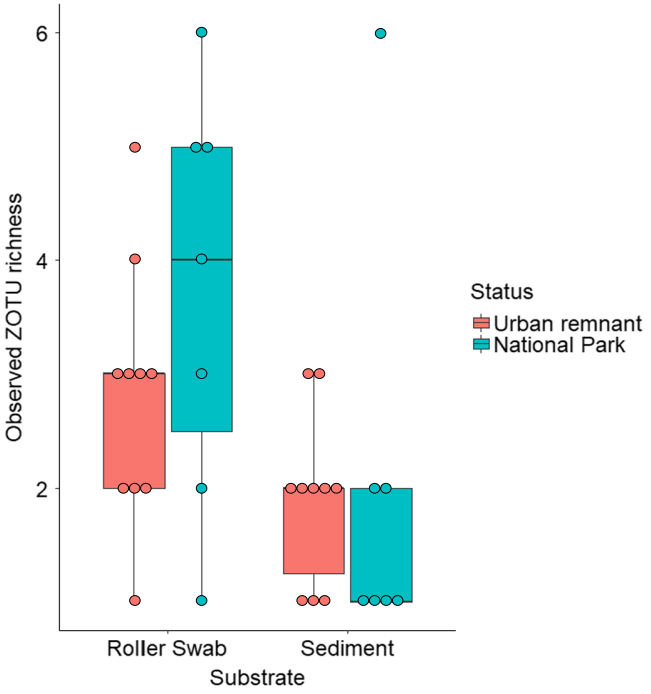


FIGURE 2 Box plot showing observed ZOTU richness between sediment and roller swab substrates detected within hollow samples for urban remnant and National Park study sites that contained both sediment and roller swab samples and successfully amplified taxa ($n = 17$). Overlaid points for individual samples

3.1 | Substrate diversity and richness

When directly comparing hollows that contained both sediment and roller swab samples that successfully amplified taxa ($n = 17$), a paired samples Wilcoxon test ($\alpha = 0.05$) showed ZOTU richness of roller swab samples to be significantly higher compared with sediment samples ($p = 0.008$; Figure 2). A PERMANOVA ($\alpha = 0.05$) showed no significant interaction between sample type and sites ($F = 0.611$, $df = 1$, $R^2 = 0.015$, $p = 0.815$). Community composition was significantly difference between sediment and roller swab samples ($F = 2.196$, $df = 1$, $R^2 = 0.053$, $p = 0.033$) and between sites ($F = 4.845$, $df = 1$, $R^2 = 0.116$, $p = 0.001$; Figure S2). Pairwise comparison showed a significant difference between urban remnant samples sites and National Park sample sites only (Table S3). Within the 17 hollows, 20 taxa were recovered, 12 of which were detected in both substrate types. Roller samples successfully recovered 19 taxa including 15 birds and 4 mammals, and sediment

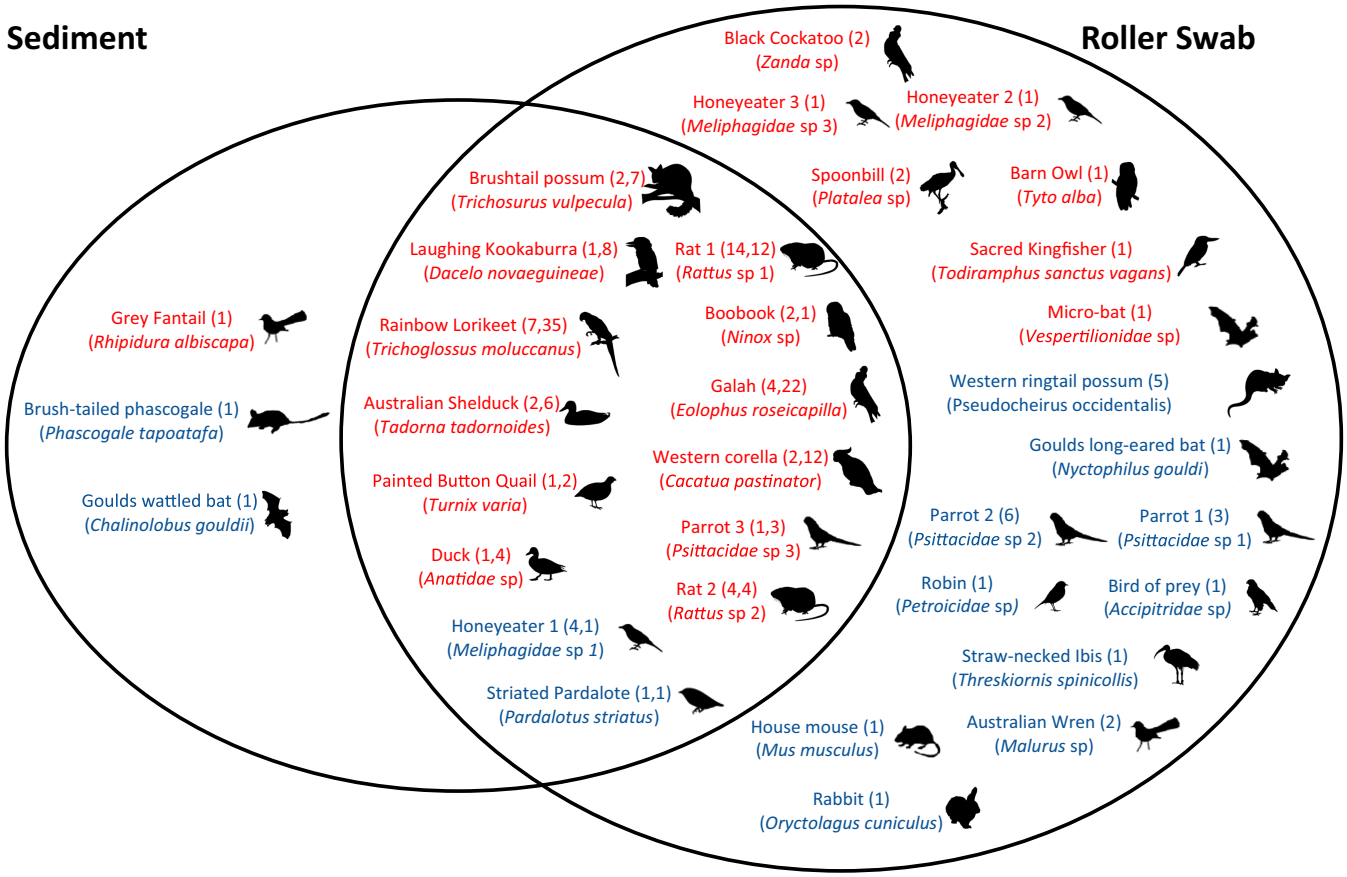


FIGURE 3 Total species detected from sediment ($n = 24$) and roller swab substrates ($n = 53$). Numbers in parentheses represent number of hollows where ZOTUs were detected (sediment, roller). Where ZOTUs had multiple possible assignments, the higher taxonomic order was retained and labeled "sp." Red names indicate species detected within hollows from both sediment and roller swabs (n = 17); blue names represent remaining taxa detected from a single substrate type

samples recovered 13 taxa including 10 birds and 3 mammals (Figure 3).

When comparing all samples (sediment = 24, roller swabs = 52), eDNA metabarcoding of roller samples detected a greater number of ZOTUs compared with sediment samples with 31 and 17 ZOTUs, respectively. Where eDNA metabarcoding of sediment detected a ZOTU within a hollow, rollers successfully detected the same ZOTU on 14 out of 17 instances (82%), while eDNA metabarcoding of sediment was only able to detect 14 out of 31 ZOTUs that were detected by rollers (45%). Of the 34 vertebrate species detected, 14 were detected within both substrates, 17 in roller swab samples only and 3 detected in sediment samples only (Figure 3).

3.2 | Hollow detection verifications

At the time of sampling, 15 hollows were identified as having positive vertebrate activity (e.g., animal present, feathers, fur, and eggs) totaling 17 visual detections due to two hollows showing signs of multiple species activity. Of these, eDNA metabarcoding provided 10 positive detections resulting in a 62% success rate when

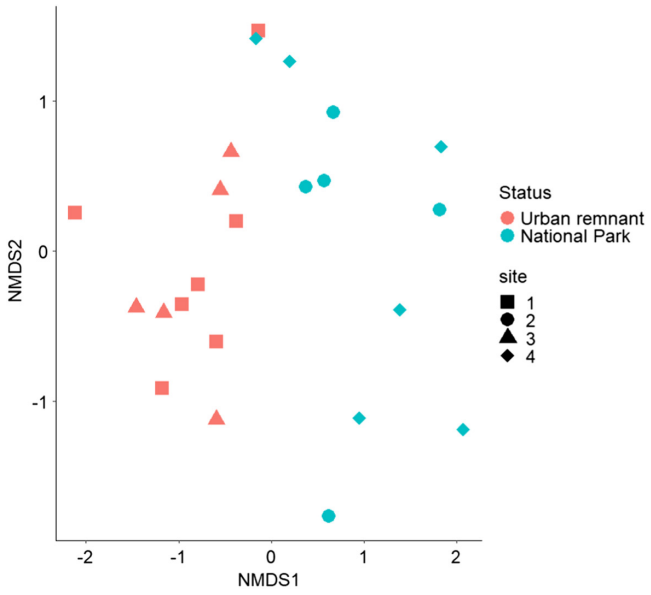


FIGURE 4 Nonmetric multidimensional scaling (NMDS) ordination of a presence–absence matrix of species detected in each tree sampled (stress = 0.167, dissimilarity matrix = Jaccard)

considering individual hollows. However, under the assumption that species interacting with trees may interact with multiple hollows, when total taxa detected per tree is used, the increase in hollows resulted in all taxa besides reptiles being detected (88% detection rate). For hollows where bird species were present and only roller swab sample of the hollow opening was taken ($n = 3$), we were still able to detect the species within the hollow.

3.3 | Site comparison

A Wilcoxon rank-sum test ($\alpha = 0.05$) found no significant difference in the ZOTU richness between urban remnants and National Park sites ($W_1 = 42$, $p = 0.143$), with mean ZOTU richness of 4.1 (+/− 0.5) and 6.3 (+/− 1.1), respectively. However, a PERMANOVA detected a significant difference in the species composition between urban remnants and National Park sites ($F = 4.144$, $df = 1$, $R^2 = 0.164$, $p = 0.002$). Pairwise comparison showed a significant difference between urban remnant sample sites and National Park sample sites only (Table S4). Through NMDS ordination (stress = 0.158), clear spatial separation could be seen (Figure 4) and eight significant indicator species were detected (Table 1). The bird indicator taxon Psittaculidae sp. (Old World Parrots) could not be assigned to species level.

Of the 34 vertebrate species recovered, 11 were detected in both urban remnant and National Park environments; the number of species detections were higher in National Park than urban remnant sites (Figure 5). Of these species, 23 are confirmed hollow users, 3 are potential prey species, and 8 non-hollow-using birds. Of the prey species, only the Painted Button Quail (*Turnix varia*) was identified within hollows that also contained potential predator DNA (*Ninox* sp, *Trichosurus vulpecula*, Accipitridae sp). All but two of the 14 samples that detected non-hollow-using birds were roller swab samples. Richness did not level off for either site as the number of samples increased (Figure 6).

4 | DISCUSSION

We have demonstrated that eDNA metabarcoding can be used as a survey tool for vertebrate hollow users. In this study, we detected common (e.g., *Eolophus roseicapilla*) and cryptic (e.g., *Phascogale tapoatafa*) vertebrate species from hollows that would have been difficult to survey using conventional techniques. Our data indicate that a relatively easy and rapid roller sampling technique worked best, but sampling multiple substrates and many trees will increase the number of vertebrate hollow users detected. As novel applications of eDNA metabarcoding for biodiversity assessment are identified, it is critical that the many factors affecting biodiversity profiles, including substrate type, sampling method, and sample number, are examined.

4.1 | Substrate evaluation

Roller swabs detected a greater number of species with higher mean ZOTU richness per sample than did sediment taken from within tree hollows. While sediment within tree hollows is sheltered from environmental factors that can degrade or disperse DNA such as high temperatures (Strickler et al., 2015), UV (Ravanat et al., 2001; Strickler et al., 2015), and rainfall (Lee et al., 2019), the composition of sediment samples may impact extraction efficiency and PCR amplification. For example, hollow sediment is high in organic matter, in particular humic substances. Humic acids, even at small amounts, inhibit Taq polymerase activity, a vital enzyme for PCR amplification (Tebbe & Vahjen, 1993; Tsai & Olson, 1992). While multiple steps were taken to reduce the effects of PCR inhibition (i.e., soil specific extraction kit with inhibitor removal technology, and sample screening to determine appropriate working dilutions containing sufficient DNA free of inhibition), further DNA extract purification steps may increase the ZOTU richness detected in sediment samples.

Another major benefit of roller swab samples is that they could be collected from all hollows, while sediment was only present or accessible in 47% of hollows. However, the species detected by roller swabs did not perfectly overlap with the species detected in sediment samples, with significant differences in community composition seen and 9% of species only detected within sediment, including the cryptic brush-tailed phascogale (*P. tapoatafa*). This supports the findings of previous studies, which show that substrate choice can have an effect on taxa detected, with different substrates demonstrating different species diversity (e.g., van der Heyde et al., 2020). If the intent is to capture total vertebrate hollow user diversity, collecting samples from multiple substrate types is likely to increase the number of taxa detected.

While this study suggests that eDNA metabarcoding of samples from tree hollows is a useful vertebrate survey tool, it also indicates that eDNA metabarcoding data should always be interpreted in the

TABLE 1 Indicator value analysis of ZOTUs indicating significant ($p < 0.05$) species-specific characterization between urban remnant and National Park sites

Site status	Species	Common name	Stat	*p* Value
Urban remnant	*Eolophus roseicapilla*	Galah	0.609	<0.001
	Trichoglossus moluccanus	Rainbow Lorikeet	0.544	<0.001
	Cacatua pastinator	Western Corella	0.329	0.011
National Park	*Tadorna tadornoides*	Australian Shelduck	0.400	<0.001
	Trichosurus vulpecula	Brushtail possum	0.359	0.004
	Psittaculidae sp	Parrot species	0.340	0.003
	Pseudocheirus occidentalis	Western ringtail possum	0.307	0.003
	Psittaculidae sp	Parrot species	0.272	0.024

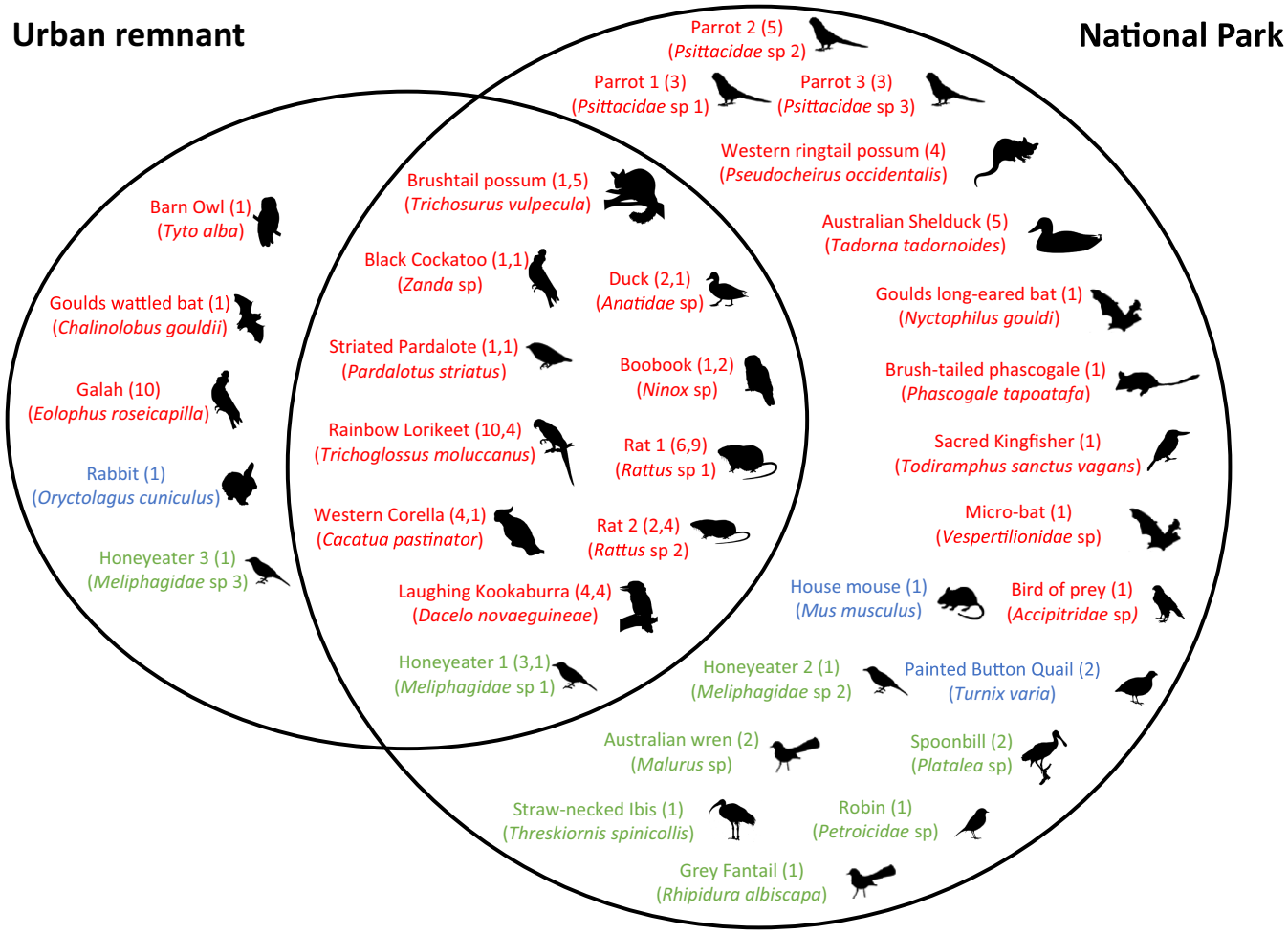


FIGURE 5 Species detected within urban remnant ($N = 12$) and National Park ($N = 11$) tree samples. Numbers in parenthesis represent number of trees where a ZOTU was detected (urban remnant, National Park). Where a ZOTUs had multiple possible assignments, the higher taxonomic order was retained and labeled "sp." Colors of names denote possible hollow interaction (red: Known hollow user, green: Non-hollow-using bird, and blue: Possible prey species)

context of substrate sampled and sampling location. For example, roller swabs identified a proportion of non-hollow users (e.g., honeyeaters), which may have only interacted with the hollow entrance, or taken refuge in the hollow for short periods of time, and very infrequently. A further consideration is that eDNA metabarcoding of hollow samples may detect species that are no longer using this habitat. eDNA metabarcoding of soil has shown that while only recently present species can be detected in surface samples, species absent from a zoo enclosure for 6 years were detected at a depth of 10 cm (Andersen et al., 2012). Therefore, if the survey aim is to detect recent users of a hollow, only surface sediment should be sampled, with the depth to be determined by further research.

4.2 | Reducing false negatives and poor resolution assignments

Although the 12S-V5 assay detected many ZOTUs, and from a broad range of species, it did not detect all taxa known to occur at the study sites; taxa observed visually during sample collection, or all ZOTUs to species-level resolution. In our study system, collecting samples from additional trees and hollows is likely to have increased the number of species detected. The inclusion of multiple assays targeting different loci (Alberdi et al., 2018), the development of new assays (Langlois et al., 2021), and the completion of reference databases (Dormontt et al., 2018; Margaryan et al., 2021) would also reduce false negatives and poor resolution assignments. While universal assays such as 12S-V5 have been used on eDNA extracted from a variety of substrates including scat (van der Heyde et al., 2021), soil (van der Heyde et al., 2020), water (Klymus et al., 2017), and carrion flies' gut contents (Rodgers et al., 2017), our data show that when the objective is to detect as many species as possible, they may not be fit for purpose. However, they may be valuable as an initial assessment tool to identify the taxonomic families present, which can then be targeted with refined assays where finer taxonomic resolution is necessary. For example, the inability to identify multiple birds to species level, including the important indicator taxon the Psittaculidae, a family containing Old World Parrots of which there

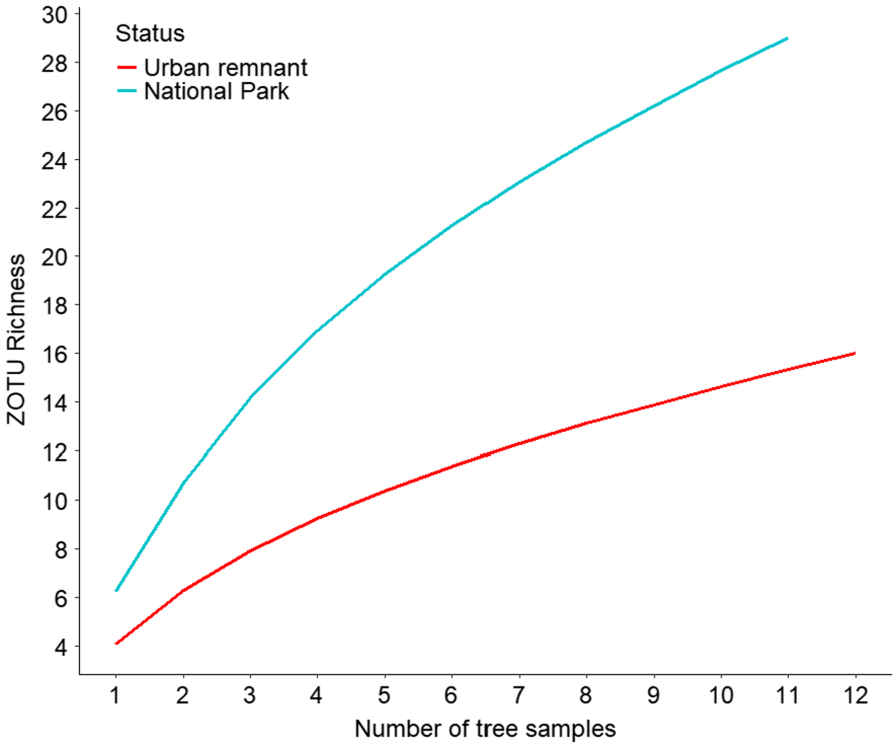


FIGURE 6 ZOTU accumulation curve showing number of ZOTUs by number of tree samples for urban remnant and National Park areas

are eight potential species within the study region, highlights the need for the development of an assay able to distinguish a greater number of avian ZOTUs to species level. Similarly, as the DNA deposition rate of reptiles is considered much lower compared with other vertebrates (Adams et al., 2019; West et al., 2021), the inability to detect reptile DNA may be due to the preferential amplification of taxa with more abundant DNA within samples. Therefore, a reptile-specific assay may increase the ability to detect their DNA within hollows. Primer binding site mutations are another possible explanation for failure to detect taxa (Freeland, 2017), especially reptiles given they show a greater number of mismatches than other taxa for the 12S-V5 primers (Riaz et al., 2011). Incomplete databases and poorly performing assays are longstanding issues of eDNA-based biodiversity surveys (Fernandes et al., 2018), and they are likely to become more prevalent as studies are conducted in new ecosystems and on an ever-wider range of taxa.

4.3 | eDNA metabarcoding surveys of tree hollows provide important information for ecosystem management

While not an objective of this study, we detected significant differences in vertebrate hollow user diversity and assemblages between urban remnants and National Parks with our eDNA metabarcoding survey, providing important information for the management of such species. While differences in community composition may be due to spatial distribution, disturbance, and fragmentation between sites, our results indicate that this technique can provide ecologically informative data. For example, our findings suggest SWWA native parrots may face competition for available hollows in urban areas by the invasive Rainbow Lorikeet (*Trichoglossus moluccanus*). Competition due to limited suitable hollows can result in the exclusion of hollow-dependent species in urban areas (Davis et al., 2013). We detected no native parrot species within urban tree hollows, even though many parrots, including the Australian Ringneck (*Barnardius zonarius*) and Red-Capped Parrot (*Purpureicephalus spurius*), are historically common and known to use tuart trees for both feeding and nesting throughout Perth Metropolitan area (Department of Environment and Energy, 2017). Conversely, the introduced and now highly invasive Rainbow Lorikeet was the most dominant detection within the urban remnant study sites, found in 83% of trees surveyed. This species is known to aggressively protect both its feeding and nesting sites which closely overlap with native parrots (Chapman, 2005). Therefore, with current evidence suggesting a decline in many native urban parrot species and an increase in both the density and abundance of Rainbow Lorikeets in greater Perth Metropolitan Area (BirdLife Western Australia, 2019), these results suggest that Rainbow Lorikeets may be excluding less-competitive parrots through the domination of suitable feeding and nesting sites.

4.4 | Future directions

While the use of eDNA metabarcoding for vertebrate hollow user surveys shows great promise, with vertebrate taxa detected in only 56% of 138 samples, our study suggests that there is a need to increase sampling effort to detect the full range of vertebrate hollow users. Further studies are required, in our system and others,

to determine the number of trees and hollows that need to be sampled, ideally in conjunction with camera trapping (e.g., Leempoel et al., 2020; Ryan et al., 2022), to determine the accuracy of this new survey method.

In addition, further refinement of sampling and laboratory methods to account for the distribution of DNA within hollows, and inhibition within samples, may also increase the number of samples where vertebrate eDNA was detected, and potentially, the number of taxa identified. The analysis of extrinsic conditions controlling the fate of eDNA within hollows such as hollow sediment mineralogy, organic material, and pH (Levy-Booth et al., 2007; Saeki et al., 2010) would also be of value in order to understand the degradation dynamics of hollow sediment eDNA. Such research into the persistence of eDNA would increase confidence in the detection of recent occupation of hollows.

While it was beyond the scope of this study, it is also suggested that multiple assays are considered for future surveys if the aim is to capture most biota. Using multiple assays will reduce the effects of primer bias and the incompleteness of reference database and potentially improve the accuracy of eDNA-based surveys (Alberdi et al., 2018; Leempoel et al., 2020). It is also highly recommended that in silico and in vivo studies are conducted prior to each new study to determine the ability of assays to detect target taxa in each new study system (Elbrecht & Leese, 2017). This will ensure that either appropriate assays are chosen or highlight the need for the development of new assays (Elbrecht & Leese, 2017).

5 | CONCLUSION

We conducted an eDNA metabarcoding survey of vertebrate tree hollow users comparing the species richness and identity detected by samples collected in two ways – sediment collected directly from hollows and roller swabs of hollows. A wide range of vertebrate taxa from tuart trees were detected, while also providing ecologically informative data including insight into the regional use of hollows by the invasive Rainbow Lorikeet in SWWA. Our data confirm the power of eDNA metabarcoding as a biomonitoring tool for this difficult to survey environment. However, our findings suggest that in our study system there is a need to increase the number of trees sampled to detect the full range of vertebrate hollow users. While it is unclear whether our findings extrapolate to other environments with tree hollows, this method potentially provides not only a broad biodiversity assessment tool, but also a sensitive method for detecting individuals that may be elusive using other methods. eDNA metabarcoding of tree hollows could be used to improve species distribution models of hollow users, identify vital habitat trees in need of conservation, and detect range expansion of invasive hollow-using species. However, the persistence of eDNA within tree hollows needs to be further investigated.

While we recommend the use of roller swab samples for eDNA-based monitoring of tree hollows, consistent with other studies (e.g., Koziol et al., 2019; van der Heyde et al., 2020), our findings support the use of multiple substrates where possible to increase the number of taxa detected. This molecular tool for researchers, industry, and government bodies will enhance understanding of the ecology of vertebrate hollow users and support the development of informed management strategies that preserve vital hollow-bearing trees and the species that rely on them.

AUTHOR CONTRIBUTIONS

JN conducted the study and wrote the initial manuscript under the supervision of PB and PN. JN, PB, and PN were involved in the experimental design. Samples were collected by JN and processed by JN and MH; bioinformatics work was performed by JN and MM-D; JN analyzed data assisted by PB and PN; the manuscript was edited by all authors.

ACKNOWLEDGMENTS

We would like to thank the Botanic Gardens and Parks Authority and Simon Cherriman from iNSiGHT Ornithology for logistical support and tree hollow access during sampling. We would also like to thank all of the members of the TrEnD laboratory for their assistance during the metabarcoding workflow and bioinformatics phases of this project. Tree hollow samples were collected with the permission of the Department of Biodiversity, Conservation and Attractions Blackwood division and the Botanic Gardens and Parks Authority, and bioinformatic resources were supplied by the Pawsey Supercomputing Centre.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

Sequencing and sample data are available at the Dryad Digital Repository: https://doi.org/10.5061/dryad.c59zw3r9r.

ORCID

Joshua P. Newton https://orcid.org/0000-0002-6269-1789
Paul Nevill https://orcid.org/0000-0001-8238-0534

REFERENCES

Adams, C. I., Hoekstra, L. A., Muell, M. R., & Janzen, F. J. (2019). A brief review of non-avian reptile environmental DNA (eDNA), with a case study of painted turtle (*Chrysemys picta*) eDNA under field conditions. *Diversity*, *11*(4), 50.

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.

Alberdi, A., Aizpurua, O., Gilbert, M. T. P., & Bohmann, K. (2018). Scrutinizing key steps for reliable metabarcoding of environmental samples. *Methods in Ecology and Evolution*, *9*(1), 134–147.

Andersen, K., Bird, K. L., Rasmussen, M., Haile, J., Breuning-Madsen, H., Kjaer, K. H., Orlando, L., Gilbert, M. T. P., & Willerslev, E. (2012). Meta-barcoding of 'dirt'DNA from soil reflects vertebrate biodiversity. *Molecular Ecology*, *21*(8), 1966–1979.

Anderson, H., Valentine, L. E., Hardy, G. E. S. J., & Fleming, P. A. (2019). Relationship between the common brushtail possum (*Trichosurus*

vulpecula) and tuart (*Eucalyptus gomphocephala*) tree decline in Western Australia. *Australian Mammalogy*, *42*(1), 67–76.

Andrews, S. (2010). *FastQC: A quality control tool for high throughput sequence data*. Babraham Bioinformatics, Babraham Institute.

Barnes, M. A., & Turner, C. R. (2016). The ecology of environmental DNA and implications for conservation genetics. *Conservation Genetics*, *17*(1), 1–17. https://doi.org/10.1007/s10592-015-0775-4

Barth, B. J., FitzGibbon, S. I., & Wilson, R. S. (2015). New urban developments that retain more remnant trees have greater bird diversity. *Landscape and Urban Planning*, *136*, 122–129.

Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal statistical society: series B (Methodological)*, *57*(1), 289–300.

BirdLife Western Australia (2019). *Operation rainbow roost, Aug 2021*. https://birdlife.org.au/documents/ORR-Report-_FINAL-4Oct2021.pdf

Bohmann, K., Evans, A., Gilbert, M. T. P., Carvalho, G. R., Creer, S., Knapp, M., Yu, D. W., & De Bruyn, M. (2014). Environmental DNA for wildlife biology and biodiversity monitoring. *Trends in Ecology & Evolution*, *29*(6), 358–367.

Boland, D. J., Brooker, M. I. H., Chippendale, G., Hall, N., Hyland, B., Johnston, R., Kleinig, D. A., McDonald, M. W., & Turner, J. (2006). *Forest trees of Australia*. CSIRO publishing.

Boyer, F., Mercier, C., Bonin, A., Le Bras, Y., Taberlet, P., & Coissac, E. (2016). Obitools: A unix-inspired software package for DNA metabarcoding. *Molecular Ecology Resources*, *16*(1), 176–182.

Burns, T. J., Clemann, N., van Rooyen, A. R., Scheele, B. C., Weeks, A. R., & Driscoll, D. A. (2020). Environmental DNA sampling in a terrestrial environment: Methods to detect a critically endangered frog and a global pathogen. *bioRxiv*.

Chapman, T. (2005). The status and impact of the rainbow lorikeet (*Trichoglossus haematodus moluccanus*) in South-West Western Australia. Department of Primary Industries and Regional Development, Western Australia, Perth. Report 04/2005.

Cockle, K. L., Martin, K., & Drever, M. C. (2010). Supply of tree-holes limits nest density of cavity-nesting birds in primary and logged subtropical Atlantic forest. *Biological Conservation*, *143*(11), 2851–2857.

Cockle, K. L., Martin, K., & Robledo, G. (2012). Linking fungi, trees, and hole-using birds in a neotropical tree-cavity network: Pathways of cavity production and implications for conservation. *Forest Ecology and Management*, *264*, 210–219.

Cockle, K. L., Martin, K., & Wesołowski, T. (2011). Woodpeckers, decay, and the future of cavity-nesting vertebrate communities worldwide. *Frontiers in Ecology and the Environment*, *9*(7), 377–382.

Cotsell, N., & Vernes, K. (2016). Camera traps in the canopy: Surveying wildlife at tree hollow entrances. *Pacific Conservation Biology*, *22*(1), 48–60.

Creer, S., Deiner, K., Frey, S., Porazinska, D., Taberlet, P., Thomas, W. K., Potter, C., & Bik, H. M. (2016). The ecologist's field guide to sequence-based identification of biodiversity. *Methods in Ecology and Evolution*, *7*(9), 1008–1018.

Davis, A., Major, R. E., & Taylor, C. E. (2013). Housing shortages in urban regions: Aggressive interactions at tree hollows in forest remnants. *PLoS One*, *8*(3), e59332.

De Caceres, M., Jansen, F., & De Caceres, M. M. (2016). Package 'indicspecies'. *Indicators*, *8*, 1.

Dell, J., How, R. A., & Burbidge, A. H. (2002). Vertebrate fauna of tuart woodlands. In B. J. Keighery & V. M. Longman (Eds.), *Tuart (Eucalyptus gomphocephala) and Tuart Communities* (pp. 254–276). Perth Branch Wildflower Society of Western Australia Inc.

Department of Environment and Energy. (2017). *Tuart (Eucalyptus gomphocephala) woodlands and forests of the swan coastal plain ecological community draft conservation advice including draft listing advice*. https://www.environment.gov.au/biodiversity/threatened/nominations/comment/tuart-woodlands-forests-swan-coastal-plain

Dickie, I. A., Boyer, S., Buckley, H. L., Duncan, R. P., Gardner, P. P., Hogg, I. D., Holdaway, R. J., Lear, G., Makiola, A., Morales, S. E., Powell, J. R., & Weaver, L. (2018). Towards robust and repeatable sampling methods in eDNA-based studies. *Molecular Ecology Resources*, *18*(5), 940–952.

Dormontt, E. E., Van Dijk, K.-J., Bell, K. L., Biffin, E., Breed, M. F., Byrne, M., Caddy-Retalic, S., Encinas-Viso, F., Nevill, P. G., & Shapcott, A. (2018). Advancing DNA barcoding and metabarcoding applications for plants requires systematic analysis of herbarium collections—An Australian perspective. *Frontiers in Ecology and Evolution*, *6*, 138. https://doi.org/10.3389/fevo.2018.00134

Edgar, R. C. (2016). UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing. *bioRxiv*, 081257.

Elbrecht, V., & Leese, F. (2017). PrimerMiner: An R package for development and in silico validation of DNA metabarcoding primers. *Methods in Ecology and Evolution*, *8*(5), 622–626.

Fedrowitz, K., Koricheva, J., Baker, S. C., Lindenmayer, D. B., Palik, B., Rosenvald, R., Beese, W., Franklin, J. F., Kouki, J., Macdonald, E., Messier, C., Sverdrup-Thygeson, A., & Macdonald, E. (2014). Can retention forestry help conserve biodiversity? A meta-analysis. *Journal of Applied Ecology*, *51*(6), 1669–1679.

Freeland, J. R. (2017). The importance of molecular markers and primer design when characterizing biodiversity from environmental DNA. *Genome*, *60*(4), 358–374.

Fernandes, K., van der Heyde, M., Bunce, M., Dixon, K., Harris, R. J., Wardell-Johnson, G., & Nevill, P. G. (2018). DNA metabarcoding—A new approach to fauna monitoring in mine site restoration. *Restoration Ecology*, *26*(6), 1098–1107.

Frøslev, T. G., Kjøller, R., Bruun, H. H., Ejrnæs, R., Brunbjerg, A. K., Pietroni, C., & Hansen, A. J. (2017). Algorithm for post-clustering curation of DNA amplicon data yields reliable biodiversity estimates. *Nature Communications*, *8*(1), 1–11.

Gibbons, P., & Lindenmayer, D. (2002). *Tree hollows and wildlife conservation in Australia*. CSIRO publishing.

Gibbons, P., Lindenmayer, D., Barry, S., & Tanton, M. (2000). Hollow formation in eucalypts from temperate forests in southeastern Australia. *Pacific Conservation Biology*, *6*(3), 218.

Glen, A. S., Cockburn, S., Nichols, M., Ekanayake, J., & Warburton, B. (2013). Optimising camera traps for monitoring small mammals. *PLoS One*, *8*(6), e67940.

Goldingay, R. L. (2009). Characteristics of tree hollows used by Australian birds and bats. *Wildlife Research*, *36*(5), 394–409.

Goldingay, R. L. (2012). Characteristics of tree hollows used by Australian arboreal and scansorial mammals. *Australian Journal of Zoology*, *59*(5), 277–294.

Gregory, T., Carrasco Rueda, F., Deichmann, J., Kolowski, J., & Alonso, A. (2014). Arboreal camera trapping: Taking a proven method to new heights. *Methods in Ecology and Evolution*, *5*(5), 443–451.

Gutzat, F., & Dormann, C. F. (2018). Decaying trees improve nesting opportunities for cavity-nesting birds in temperate and boreal forests: A meta-analysis and implications for retention forestry. *Ecology and Evolution*, *8*(16), 8616–8626.

Honey, R., McLean, C. M., Murray, B. R., Callan, M. N., Webb, J. K., & Cooper, P. (2021). Choice of monitoring method can influence estimates of usage of artificial hollows by vertebrate fauna. *Australian Journal of Zoology*, *69*(1), 18–25.

Ji, Y., Ashton, L., Pedley, S. M., Edwards, D. P., Tang, Y., Nakamura, A., Kitching, R., Dolman, P. M., Woodcock, P., Edwards, F. A., Larsen, T. H., Hsu, W. W., Benedick, S., Hamer, K. C., Wilcove, D. S., Bruce, C., Wang, X., Levi, T., Lott, M., … Yu, D. W. (2013). Reliable, verifiable and efficient monitoring of biodiversity via metabarcoding. *Ecology Letters*, *16*(10), 1245–1257.

Keighery, B. J., Keighery, G. J., & Shepherd, D. (2002). The distribution and conservation of tuart and the community with which it lives. In B. J. Keighery & V. M. Longman (Eds.), *Tuart (Eucalyptus*

gomphocephala) and Tuart Communities (pp. 6–86). Perth Branch Wildflower Society of Western Australia Inc.

Kindt, R., & Coe, R. (2005). *Tree diversity analysis: A manual and software for common statistical methods for ecological and biodiversity studies*. World Agroforestry Centre.

Klymus, K. E., Richter, C. A., Chapman, D. C., & Paukert, C. (2015). Quantification of eDNA shedding rates from invasive bighead carp Hypophthalmichthys nobilis and silver carp Hypophthalmichthys molitrix. *Biological Conservation*, *183*, 77–84.

Klymus, K. E., Richter, C. A., Thompson, N., & Hinck, J. E. (2017). Metabarcoding of environmental DNA samples to explore the use of uranium mine containment ponds as a water source for wildlife. *Diversity*, *9*(4), 54.

Koch, A. J. (2008). Errors associated with two methods of assessing tree hollow occurrence and abundance in Eucalyptus obliqua forest, Tasmania. *Forest Ecology and Management*, *255*(3–4), 674–685.

Koziol, A., Stat, M., Simpson, T., Jarman, S., DiBattista, J. D., Harvey, E. S., Marnane, M., McDonald, J., & Bunce, M. (2019). Environmental DNA metabarcoding studies are critically affected by substrate selection. *Molecular ecology resources*, *19*(2), 366–376. https://doi.org/10.1111/1755-0998.12971

Krauss, S. L., Roberts, D. G., Phillips, R. D., & Edwards, C. (2018). Effectiveness of camera traps for quantifying daytime and nighttime visitation by vertebrate pollinators. *Ecology and Evolution*, *8*(18), 9304–9314.

Kucherenko, A., Herman, J. E., Everham, E. M., III, & Urakawa, H. (2018). Terrestrial snake environmental DNA accumulation and degradation dynamics and its environmental application. *Herpetologica*, *74*(1), 38–49.

Langlois, V. S., Allison, M. J., Bergman, L. C., To, T. A., & Helbing, C. C. (2021). The need for robust qPCR-based eDNA detection assays in environmental monitoring and species inventories. *Environmental DNA*, *3*(3), 519–527.

Lee, L. Y. C., Wong, H. Y., Lee, J. Y., Waffa, Z. B. M., Aw, Z. Q., Fauzi, S. N. A. B. M., Hoe, S. Y., Lim, M.-L., & Syn, C. K.-C. (2019). Persistence of DNA in the Singapore context. *International Journal of Legal Medicine*, *133*(5), 1341–1349.

Leempoel, K., Hebert, T., & Hadly, E. A. (2020). A comparison of eDNA to camera trapping for assessment of terrestrial mammal diversity. *Proceedings of the Royal Society B*, *287*(1918), 20192353.

Levy-Booth, D. J., Campbell, R. G., Gulden, R. H., Hart, M. M., Powell, J. R., Klironomos, J. N., Pauls, K. P., CJSwanton, J. T. T., & Dunfield, K. E. (2007). Cycling of extracellular DNA in the soil environment. *Soil Biology and Biochemistry*, *39*(12), 2977–2991.

Lindenmayer, D. B., Blanchard, W., Blair, D., McBurney, L., & Banks, S. C. (2017). Relationships between tree size and occupancy by cavity-dependent arboreal marsupials. *Forest Ecology and Management*, *391*, 221–229.

Margaryan, A., Noer, C. L., Richter, S. R., Restrup, M. E., Bülow-Hansen, J. L., Leerhøi, F., Langkjær, E. M. R., Gopalakrishnan, S., Carøe, C., & Gilbert, M. T. P. (2021). Mitochondrial genomes of Danish vertebrate species generated for the national DNA reference database, DNAmark. *Environmental DNA*, *3*(2), 472–480.

Mansfield, C., Arnold, A., Bell, T., & York, A. (2017). Habitat characteristics of a threatened arboreal marsupial and its resource use in a degraded landscape: The brush-tailed phascogale (*Phascogale tapoatafa tapoatafa*) in Central Victoria, Australia. *Wildlife Research*, *44*(2), 153–164.

McMurdie, P. J., & Holmes, S. (2013). Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One*, *8*(4), e61217.

Mousavi-Derazmahalleh, M., Stott, A., Lines, R., Peverley, G., Nester, G., Simpson, T., Zawierta, M., De La Pierre, M., Bunce, M., & Christophersen, C. T. (2021). eDNAFlow, an automated, reproducible and scalable workflow for analysis of environmental DNA sequences exploiting Nextflow and singularity. *Molecular Ecology Resources*, *21*(5), 1697–1704.

Murray, D. C., Coghlan, M. L., & Bunce, M. (2015). From benchtop to desktop: Important considerations when designing amplicon sequencing workflows. *PLoS One*, *10*(4), e0124671.

Newton, I. (1994). The role of nest sites in limiting the numbers of hole-nesting birds: A review. *Biological Conservation*, *70*(3), 265–276.

Okasen, J. (2019). *vegan: community ecology package R package version 2.5–5*. https://CRAN.R-project.org/package=vegan

Othman, N., Haris, H., Fatin, Z., Najmuddin, M., Sariyati, N., Md-Zain, B., & Abdul-Latiff, M. (2021). A review on environmental DNA (eDNA) metabarcoding markers for wildlife monitoring research. In *Paper presented at the IOP conference series: Earth and environmental science*.

Paull, D. J., Claridge, A. W., & Cunningham, R. B. (2012). Effective detection methods for medium-sized ground-dwelling mammals: A comparison between infrared digital cameras and hair tunnels. *Wildlife Research*, *39*(6), 546–553.

R Core Team. (2018). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. https://www.R-project.org

Ravanat, J.-L., Douki, T., & Cadet, J. (2001). Direct and indirect effects of UV radiation on DNA and its components. *Journal of Photochemistry and Photobiology B: Biology*, *63*(1–3), 88–102.

Riaz, T., Shehzad, W., Viari, A., Pompanon, F., Taberlet, P., & Coissac, E. (2011). ecoPrimers: Inference of new DNA barcode markers from whole genome sequence analysis. *Nucleic Acids Research*, *39*(21), e145.

Rodgers, T. W., Xu, C. C., Giacalone, J., Kapheim, K. M., Saltonstall, K., Vargas, M., Yu, D. W., Somervuo, P., McMillan, W. O., & Jansen, P. A. (2017). Carrion fly-derived DNA metabarcoding is an effective tool for mammal surveys: Evidence from a known tropical mammal community. *Molecular Ecology Resources*, *17*(6), e133–e145.

Ruthrof, K., Yates, C., & Loneragan, W. A. (2002). *The biology of tuart*. Wildflower Society of Western Australia Inc.

Ryan, E., Bateman, P., van der Heyde, M., Fernandes, K., & Nevill, P. (2022). eDNA metabarcoding of log hollow sediments and soils highlights the importance of substrate type, frequency of sampling and animal size, for vertebrate species detection. *Environmental DNA*.

Saeki, K., Kunito, T., & Sakai, M. (2010). Effects of pH, ionic strength, and solutes on DNA adsorption by andosols. *Biology and Fertility of Soils*, *46*(5), 531–535.

Schubert, M., Lindgreen, S., & Orlando, L. (2016). AdapterRemoval v2: Rapid adapter trimming, identification, and read merging. *BMC Research Notes*, *9*(1), 1–7.

Stojanovic, D., Rayner, L., Cobden, M., Davey, C., Harris, S., Heinsohn, R., Owens, G., & Manning, A. D. (2020). Suitable nesting sites for specialized cavity dependent wildlife are rare in woodlands. *Forest Ecology and Management*, *483*(3), 118718.

Stojanovic, D., Webb, M., Roshier, D., Saunders, D., & Heinsohn, R. (2012). Ground-based survey methods both overestimate and underestimate the abundance of suitable tree-cavities for the endangered swift parrot. *Emu-Austral Ornithology*, *112*(4), 350–356.

Strickler, K. M., Fremier, A. K., & Goldberg, C. S. (2015). Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. *Biological Conservation*, *183*, 85–92.

Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C., & Willerslev, E. (2012). Towards next-generation biodiversity assessment using DNA metabarcoding. *Molecular Ecology*, *21*(8), 2045–2050.

Tebbe, C. C., & Vahjen, W. (1993). Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. *Applied and Environmental Microbiology*, *59*(8), 2657–2665.

Terry, W., Goldingay, R. L., & van der Ree, R. (2021). Can chainsaw carved hollows provide an effective solution to the loss of natural tree

cavities for arboreal mammals? *Forest Ecology and Management*, *490*, 119122.

Tsai, Y.-L., & Olson, B. H. (1992). Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. *Applied and Environmental Microbiology*, *58*(7), 2292–2295.

Tuart Response Group (2003). *An atlas of Tuart woodlands on the Swan Coastal Plain in Western Australia*. Government of Western Australia. https://www.dpaw.wa.gov.au/images/documents/conservation-management/forests/tuart_atlas/tuart_atlas_full300.pdf

Valentin, R. E., Fonseca, D. M., Gable, S., Kyle, K. E., Hamilton, G. C., Nielsen, A. L., & Lockwood, J. L. (2020). Moving eDNA surveys onto land: Strategies for active eDNA aggregation to detect invasive forest insects. *Molecular Ecology Resources*, *20*(3), 746–755.

van der Heyde, M., Bateman, P., Bunce, M., Wardell-Johnson, G., White, N., & Nevill, P. (2021). Scat DNA provides important data for effective monitoring of mammal and bird biodiversity. *Biodiversity and Conservation*, *30*(12), 3585–3602.

van der Heyde, M., Bunce, M., Wardell-Johnson, G., Fernandes, K., White, N. E., & Nevill, P. (2020). Testing multiple substrates for terrestrial biodiversity monitoring using environmental DNA metabarcoding. *Molecular Ecology Resources*, *20*(3), 732–745.

van der Hoek, Y., Gaona, G. V., & Martin, K. (2017). The diversity, distribution and conservation status of the tree-cavity-nesting birds of the world. *Diversity and Distributions*, *23*(10), 1120–1131.

West, K. M., Heydenrych, M., Lines, R., Tucker, T., Fossette, S., Whiting, S., & Bunce, M. (2021). Development of a 16S metabarcoding assay for the environmental DNA (eDNA) detection of aquatic reptiles across northern Australia. *Marine and Freshwater Research*.

Wickham, H. (2016). *ggplot2: Elegant graphics for data analysis* (p. 260). Springer-Verlag.

Wormington, K., & Lamb, D. (1999). Tree hollow development in wet and dry sclerophyll eucalypt forest in south-East Queensland, Australia. *Australian Forestry*, *62*(4), 336–345.

Zylstra, E. R., Steidl, R. J., & Swann, D. E. (2010). Evaluating survey methods for monitoring a rare vertebrate, the Sonoran desert tortoise. *The Journal of Wildlife Management*, *74*(6), 1311–1318.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Newton, J. P., Bateman, P. W., Heydenrych, M. J., Mousavi-Derazmahalleh, M. & Nevill, P. (2022). Home is where the hollow is: Revealing vertebrate tree hollow user biodiversity with eDNA metabarcoding. *Environmental DNA*, *4*, 1078–1091. https://doi.org/10.1002/edn3.313