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Comparison of eDNA metabarcoding to camera trapping for terrestrial vertebrate monitoring highlights the importance of substrate type, frequency of sampling and animal size

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16 **Abstract**

17 Fauna biodiversity assessments often rely on traditional biomonitoring techniques such as
18 camera traps, which may have biases that lead to gaps in biodiversity data. Environmental
19 DNA (eDNA) has emerged as a new source of biodiversity data that may account for these
20 gaps. However, eDNA biodiversity assessment remains relatively untested in terrestrial
21 environments. We compared vertebrate detections using two independent monitoring
22 methods: camera traps and eDNA (n = 160), across two sites in south-western Australia. We
23 also investigated the suitability of tree hollow sediment as a source of eDNA, and the effect of
24 other factors (visitation frequency and timing, animal size) on vertebrate species detectability.
25 We detected 31 taxa with eDNA and 47 with camera traps of which 14 overlapped (12
26 mammals and 2 birds). Tree hollow sediment detected a wider range of biodiversity than did
27 soil at the entrance of the hollow. By comparing camera trap data with eDNA sequence reads,
28 we were able to detect animals with eDNA that had visited the area up to two months prior to
29 sample collection, with a negative correlation between sequence read amount and days since
30 last recorded detection via camera. “Large” animals (>3kg) detected via camera were
31 associated with significantly higher sequence read amounts than smaller animals. Our results
32 show the effect of substrate selection, frequency of sampling and animal size, on eDNA based
33 surveys. If the aim is to detect broad taxon diversity eDNA based approaches need to be
34 complemented by traditional vertebrate survey methods.
35

36 KEYWORDS: Biodiversity, Biomonitoring, Environmental DNA, Metabarcoding,
37 Vertebrates, Tree hollows

Introduction

There is a continuing global deterioration of biodiversity caused by multiple anthropogenic activities including changes in land and sea use, exploitation of organisms, human induced climate change, pollution and invasive species. Since the 1970s, vertebrate abundance has been rapidly declining as a result of these activities and over the last 500 years at least 680 vertebrate species have been driven to extinction (IPBES, 2019). Ecological research and monitoring are important in identifying environmental changes; however, biodiversity assessments are constrained by a reliance on infrequent "snapshot" studies that mask change over time (Lindenmayer et al., 2018) or by a lack of available data (Anderson et al., 2011). The conflict between high expectations of efficacy and the need for high frequency of biodiversity assessments may also stretch resources (Hajibabaei et al., 2016). These resource limitations may result in a narrower focus on specific 'indicators' which may not always be consistently defined or properly representative, reducing their effectiveness as a method of holistic assessment (Yu, Lu, & Fu, 2017).

Numerous methods are used to conduct surveys of animals including field surveys (aerial or ground) and mark-recapture. Camera traps are one of the most favoured methods (Steenweg et al., 2017) and have been used to record species diversity in a wide variety of environments (Ahumada et al., 2011; Kiswayadi et al., 2019; Lijia et al., 2014). They can collect data over long periods of time without the need for continuous visits by researchers and have a smaller impact on animal behaviour compared to trapping (Caravaggi et al., 2017). However, camera traps have some limitations. They may be biased against certain behavioural (cryptic, slow moving) or physiological traits (small, low body temperature) (Glen et al., 2013; Paull et al., 2012; Zylstra et al., 2010), and data interpretation can require extensive taxonomic knowledge, which may be difficult to source (Hajibabaei et al., 2016). Ethical and privacy

concerns also exist over the possibility of "human bycatch" when collecting data in easily accessible areas (Sandbrook et al., 2018).

Environmental DNA (eDNA) metabarcoding is a relatively new method of molecular-based biomonitoring. eDNA refers to a mixture of intracellular and extracellular DNA from a wide range of organisms that can be extracted directly from environmental samples (including soil, water and air) as opposed to a specific target organism. Subsequent metabarcoding of the eDNA can then allow for the simultaneous characterisation of a wide variety of biota using targeted sequences (barcodes) (Taberlet et al., 2012). In practice this allows for the myriad DNA fragments existing within an eDNA sample to be sequenced simultaneously, with the potential to gain a broad measure of the biodiversity from a relatively small environmental sample (Bohmann et al., 2014; Fernandes et al., 2018). As a biodiversity monitoring tool, this provides rapid, non-invasive and cost-efficient data and has enormous potential in policy making and conservation management (Hajibabaei et al., 2016).

However, there are many questions regarding the efficacy of eDNA metabarcoding as a tool for biomonitoring that remain unanswered, or underexplored. While studies have been conducted over a range of environmental conditions (Bohmann et al., 2014), aquatic studies still dominate (Barnes et al., 2014; Strickler et al., 2015; Tsuji et al., 2017; Williams et al., 2018). As terrestrial-focused eDNA studies increase, insights have been made into its application as a biomonitoring tool. For example, the type of substrate used as a source of DNA (soil, scat, arthropod, plant material) affects biodiversity estimates (van der Heyde et al., 2020). However, the effect of abiotic variables on terrestrial eDNA longevity remain mostly unexplored. Studies focusing on water samples suggest UV, pH, temperature and bacterial abundance all impact eDNA longevity (Barnes et al., 2014; Strickler et al., 2015; Tsuji et al.,

2017; Williams et al., 2018). Whilst important, environmental factors only partly explain eDNA detectability, as the biomass, mobility and behaviour of an organism may also impact the rates of eDNA shedding and therefore probability of detection (Andersen et al., 2012; Walker et al., 2017). Specific taxa such as reptiles may have low rates of eDNA shedding due to their scales (Adams et al., 2019). Birds also appear to be underrepresented in terrestrial DNA experiments and may have similar difficulties with detection. For example, in a controlled experiment, ostrich (*Struthio camelus*) was detected less frequently than were mammals from soil samples (Andersen et al, 2012).

When attempting to detect a broad range of vertebrates with eDNA, targeting sample sites with increased relative DNA concentration as a result of organism behaviour or environmental factors may provide greater diversity than would sampling from open areas. Globally, hollows in trees, either created by organisms or reflecting the presence of venerable trees, are recognised as vital for biodiversity (Bryant et al., 2012; Carlson et al., 1998; Grüebler et al., 2013; Schauer et al., 2018; Tatsumi et al., 2017). Tree hollows in standing or fallen trees have a role in providing shelter, foraging, thermoregulation (Gibbons, 2002) and reproduction (Flanagan-Moodie et al., 2018) for a range of taxa. As such, monitoring of tree hollows may be an effective way of assessing biodiversity across habitats, particularly as long-term use and restriction of movement within a log by fauna (Kucherenko et al., 2018) increases DNA release and accumulation (Barnes & Turner, 2016; Klymus et al., 2015; Bylemans et al., 2017). To our knowledge, eDNA has never been sampled from log hollows before and, when compared to external substrates, the difference in environmental conditions such as lower ambient temperatures and reduced light (Gibbons 2002) may reduce eDNA degradation (Tsuji et al., 2017; Gutiérrez-Cacciabue et al., 2016). However, the higher

concentrations of coarse woody debris and other humic substances could inhibit PCR (Matheson et al., 2010).

It is possible some of the limitations inherent to eDNA metabarcoding can be better identified and addressed when also using camera trapping as a comparative method. During molecular analysis, controlling false positives remains a challenge (Ficetola et al., 2016). In contrast, photographic evidence of a species via camera trap has few potentials for false positives beyond mis-identifying similar-looking species. While eDNA metabarcoding and camera trapping share limitations, particularly for taxa such as reptiles (Adams et al., 2019; Goosem, 2005), overall, the two methods have been found to work well in tandem. For instance, eDNA has been found to detect small/cryptic organisms with greater success than camera trapping (Carla et al., 2020; Leempoel et al., 2020), while camera traps are not reliant on reference databases to generate accurate data, which can be incomplete (Dormontt et al., 2018; Ishige et al. 2017). Camera trapping's ability to record changes over time allowed us to identify sample areas that were highly trafficked during our study, increasing potential eDNA deposition, as well as the exact time between animal interaction and sample collection of a given site, which can be compared with eDNA data to determine appropriate cut-offs for reliable eDNA detection. Historically, degradation has proven to vary greatly between environments and target species (Bohmann et al., 2014).

In this study we compare vertebrate biodiversity assessments using camera traps and two eDNA metabarcoding assays of two different substrates: soil, and hollow sediment, focusing our sampling around likely areas of concentrated biodiversity in log hollows. We aim to improve decision making for terrestrial eDNA surveys by:

1. Examining whether eDNA from terrestrial vertebrates can be recovered from log hollow sediment and soil surface samples and assess diversity within and overlap between these two substrates

2. Comparing DNA sequence-based and traditional biodiversity survey methods. Specifically, we compare a DNA sequence-based approach with camera trap studies to understand the extent to which they complement one another.

3. Using the temporal data obtained by camera trap data to measure how many times an animal visited a hollow during the experiment (increased dispersal of eDNA) and how many days between its last visitation and the date of sample collection (increased degradation from environment), to better understand and rank the factors affecting detection probability via eDNA. We hypothesised that eDNA from log hollow sediment would show broader diversity than from soil, and that eDNA metabarcoding would show higher vertebrate diversity than camera trapping (with an increased presence of small/cryptic organisms). We also predicted the majority of our sequences will derive from animals that visited our hollows recently and/or frequently. While we expect eDNA data will contain many small organisms, we are interested in finding out whether larger animals are more reliably detected as a result of increased dispersal.

Materials and Methods

Study sites and camera trapping

Our study was conducted at two sites in south-western Australia. First, the Dryandra Woodland, a large remnant of open, temperate eucalypt woodlands containing a variety of threatened fauna species including woylie (*Bettongia pencillata*) (Garkaklis, 2001) and numbat (*Myrmecobius fasciatus*) (Friend, 2005). This area has a Mediterranean climate; temperatures have a mean low of 8.5°C, mean high of 23.8°C and annual mean rainfall of 508.1mm. Our sampling location within Dryandra was focused on the largest unfragmented area of the Lol Gray forest (-32.76737, 116.95231). The area is characterised by open woodlands of *Eucalyptus wandoo*, *E. accedens* and *E. calophylla* (Burrows et al., 1987) with minimal understory and high concentrations of leaf litter. The remnant blocks within Dryandra Woodland are among the largest and most diverse of the central western wheat belt, itself a major hub of diverse fauna due to its transitional location between the hydric coast and more mesic west and south-west (*Dryandra Woodland Management Plan No. 70*, 2011). The second site was 600km further east in the Great Western Woodland (GWW), a eucalypt woodland with a similar Mediterranean climate notable for being the world's largest remaining temperate woodland. Our sites were characterised by a eucalypt over storey including *E. salubris*, *E. celastroides* and *E. calycogona* over mixed shrubs, herbs and grasses (*NOVA Nickel Project - EPA Referral Supporting Document*, 2014). Temperatures have a mean low of 10°C, a mean high of 25.2°C and an annual mean rainfall of 293.7mm. Our study site was located within undisturbed habitat (-31.84886, 123.17553). The nearest site of human activity was a mine site 4km from the edge of the study area.

173 *Sample collection*

174 All sites had an abundance of fallen logs with hollows. Logs were selected based on
175 fulfilment of suitability criteria, including one major entrance/exit point, a majority shaded
176 cavity and a diameter large enough to reliably collect samples (15 cm). Twenty logs were
177 selected from each site. For each log, a 50 ml collection tube was filled with soil from the
178 entrance of the hollow and another 50 ml collection tube with sediment from within the log
179 hollow. Soil samples were obtained by randomly subsampling from five points (depth 2 cm)
180 within a 50 cm^2 area directly outside the hollow's entrance (based on availability of
181 recoverable soil), while the interior sediment samples were obtained by subsampling
182 scrapings at five points along the floor of the hollow between 50 cm and 1m past the entrance
183 (with depth ranging from several millimetres to 2 cm depending on amount of sediment in the
184 hollow). Samples (n = 160) were collected from each log at two time points; December 2019
185 and January 2020 for GWW, and January 2020 and March 2020 for Dryandra.

186 *Sample processing and DNA extraction*

187 Samples were pre-mixed in their 50 ml collection tubes using a Qiagen Tissuelyzer II
188 (Qiagen, Germany) for 1 minute. 300 mg of either soil or hollow sediment was weighed out,
189 avoiding large rocks and woody detritus, for extraction. Samples were extracted using a
190 DNeasy PowerLyzer PowerSoil Kit (Qiagen, Germany) and 100 ul elution on an automated
191 Qiacube (Qiagen, Germany). DNA extraction controls were carried out for every 12 samples
192 using extraction reagents only.

193 *DNA amplification and sequencing*

194 Two primers were selected targeting short amplicons due to the degraded nature of eDNA
195 (Ficetola et al., 2010), with one focused on mammals, and one on all vertebrates. The

196 mammal specific primers 16Smam1/2 targeted the mitochondrial 16S ribosomal gene
197 (~130bp, Taylor, 1996) while the vertebrate primers 12Sv5-F/R targeted the mitochondrial
198 12S gene (~98bp, Riaz et al., 2011). Quantitative PCR amplification was carried out with neat
199 extract and dilutions of 1/10 and 1/100 to test for PCR inhibition and the quality and quantity
200 of DNA in the extract using a StepOne Plus (Applied BioSystems), but only neat samples
201 were retained for fusion tagging. Positive and negative controls were included on each PCR
202 plate. A 12Sv5 primer with a human blocker (Boessenkool et al., 2012) was trialled, but was
203 found to limit detections of nonhuman vertebrates too greatly.

204 The PCR mix for amplification was made up to 25 µl and contained 2.5 mM MgCl2 (Applied
205 Biosystems), 1× PCR Gold buffer (Applied Biosystems), 0.25 mM dNTPs (Astral Scientific,
206 Australia), 0.4 mg/ml bovine serum albumin (Fisher Biotec, Australia), 0.4 µmol/L forward
207 and reverse primer, 1 U AmpliTaq Gold DNA polymerase (Applied Biosystems) and 0.6 µl of
208 a 1:10,000 solution of SYBR Green dye (Life Technologies, USA). PCRs were run on a
209 StepOne Plus (Applied BioSystems) real-time qPCR instrument. Cycling conditions for 12sv5
210 were 10 minutes at 95°C, and 55 cycles of 94°C for 30 seconds, 51°C at annealing
211 temperature for 30 seconds and 51°C for 1 minute, ending with 10 minute elongation at 72°C.
212 Cycling conditions for 16S were 10 minutes at 95°C, and 55 cycles of 95°C for 12 seconds,
213 59°C at annealing temperature for 30 seconds and 70°C for 25 second, ending with 10 minute
214 elongation at 72°C.

215 All PCR mixes were prepared in a dedicated clean room to minimise contamination, with
216 samples added in a separate laboratory in specialised UV cabinets. Samples were assigned a
217 unique combination of fusion tag primers that contained a unique multiplex identifier (MID)
218 tag between 6-9 bp, the gene-specific primer and Illumina's sequencing adaptors. Fusion
219 tagged reactions were carried out on samples using the same cycling conditions as the qPCR.

A single step fusion protocol was used with no reuse of index combinations. MID-tag amplicons were generated in triplicate and pooled together. Pooled amplicons were quantified using the QIAxcel Advanced System (Qiagen, Germany). Pools were combined in approximate equimolar ratios based on quantitation to create a DNA library which was quantified using Qubit Fluorometric Quantitation (Thermo Fisher Scientific) and sequenced as per Illumina sequencing protocols for single-end sequencing with a 300 cycle V2 reagent kit with a standard V2 flow cell.

Sequence filtering and taxonomic assignment

Raw sequence reads were quality filtered and demultiplexed (filtered to minimum length with erroneous barcodes removed) using OBITools (Boyer et al., 2016). Sequences were concatenated, denoised and assigned to a zero-radius Operational Taxonomic Unit (ZOTU) table using Usearch (Edgar et al., 2011) ZOTUs were filtered and clustered using LULU (Frøslev et al., 2017). Taxonomy was assigned by matching ZOTU sequences to a reference database using Basic Local Alignment Search Tool (BLASTn) on a high-performance cluster computer (Pawsey Supercomputing Centre Perth, WA, Australia) against the online reference database Genbank (https://www.ncbi.nlm.nih.gov/genbank/). Sequences with a Genbank alignment of < 95% were removed along with a 90% identity cut off to keep ID, with anything below 90% dropped to family level assignment. When the absolute value for the difference between %identity of ZOTUs was < 0.5, species level taxonomy was not returned and the ZOTU was dropped to the closest common ancestor (LCA).

The results of the LCA script were compared against existing species diversity data for the sites to ensure detected species were accurate. Several species were detected in eDNA that were not recorded as present in that area however, if they had a single sister species that was recorded as present, the ZOTU was reattributed. ZOTUs refined to genus-level that only had a

single species of said genus present at the site were similarly marked as that species. Taxa adjusted in this way included *Dasyurus*, *Bettongia,* and *Trichosurus*, *Antechinus*, *Isoodon*, and *Phascogale*. When there were multiple potential reassignments, the ZOTU was left at the closest taxonomic level and labelled "sp." One ZOTU was reassigned beyond species level, *Zaglossus bruigni* to *Tachyglossus aculeatus.*

Camera traps

Each log was monitored with a wireless Reconyx HyperFire or Hyperfire 2 IR Camera (Reconyx, USA) set to high sensitivity, 5-image rapid-fire starting in November 2019 in Dryandra and October 2019 in GWW (roughly two months before the first associated eDNA collection date). Cameras were set up using nearby logs and tree trunks or the use of portable stakes and focused on the likely point of entry into the associated hollow. The variable landscape meant that the distance between hollow opening and camera varied from <1 m to ~10 m, altering the amount of the surrounding woodland in view. On later visits, cameras were sometimes adjusted if there was an abundance of non-fauna photos due to unwanted movement in the field of view. Camera trap data was processed by logging all species detected with the associated date and level of interaction. Camera trap data was collected at the same time as the eDNA samples, providing two timeframes containing roughly two months of data. SD cards and batteries were replaced during the first collection date.

Statistical Analysis

All statistics were performed using R 4.0.2 (R Core Team, 2020). Samples with low sequencing depth (<642 reads) were removed and within-sample copy numbers were filtered by removing taxa from samples where they made up less than 0.06% of the reads of that sample. Sequences present in the extraction controls, taxa not known to inhabit terrestrial

Australia and taxa not recorded on the site that are common contaminants during PCR (such as ungulates and human sequences) were removed from the dataset using the 'phyloseq' package (McMurdie & Holmes, 2013).

We calculated ZOTU richness for each substrate (soil and hollow sediment) and tested the differences between substrates using a Kruskal-Wallis one-way analysis of variance for each of our assays (16S and 12Sv5) and sites (GWW and Dryandra), with p values adjusted using the BH method (Benjamini & Hochberg, 1995). We calculated accumulation curves of the number of ZOTUs as a function of the number of soil/hollow sediment samples, comparing sites and sample type using the 'accumcomp' function in the 'BiodiversityR' package (Kindt & Coe, 2005). Plots were visualized using 'ggplot2' (Wickham, 2016). Correlation between eDNA and camera traps distance matrices of species presence/absence per site was tested by running a Mantel test (999 permutations).

Specific samples that detected the same taxa through eDNA and camera traps (9 taxa across 32 sample pairs) were collated together. Then, the total number of camera trap detections across a time point (excluding detections where the animal did not come in contact with the hollow or the area in front of it sampled for soil) was tested for correlation with the number of reads in ZOTUs corresponding to taxa of the same genus using Spearman's Rank correlation coefficient (Wissler, 1905). Similar correlations were tested between the sequence read number and the amount of days between the last recorded detection and the date of sample collection. Average body mass for each of these species was recorded using the literature (Strahan, 2002) and categorised into small (<999g) medium (1-3kg) or large (>3kg). Average sequence reads between each species/sample combination arranged into these size categories were compared using a Kruskal-Wallis test with p values again adjusted using the BH method (Benjamini & Hochberg, 1995). Sequence reads were transformed in all comparisons using

291 log10 due to extreme variation, ranging from the hundreds to one ZOTU of over 900,000.
292 Genus-level association between camera trap and eDNA data was only selected to allow the
293 inclusion of *Climacteris*. As *C. rufus* was seen hundreds of times across the majority of
294 hollows, but due to the presence of one other *Climacteris* species historically recorded at the
295 GWW site, our single Climacteris ZOTU could not be conclusively linked to this species. The
296 remaining eight species included in this dataset all matched to species across both records.

297 **Results**

298 A total of 15,849.075 metabarcoding sequences were generated from the two PCR assays
299 prior to filtering (see Table 1 for summary). Non-chordate ZOTUs and ZOTUs that were only
300 resolved to Chordate sp. were removed along with several ZOTUs corresponding to species
301 not known to be present across either site that were likely products of contamination,
302 including a species of Delphinidae, *Gallus gallus, Meleagris gallopavo*, *Sus scrofa,*
303 *Damaliscus lunatus*, *Rupicapra rupicapra* and *Bos Taurus.* Several of these species are
304 known contaminants arising from lab reagents such as BSA (Leempoel et al., 2020) while
305 others likely derive from food items dispersed alongside human eDNA. Three reptile taxa,
306 one bird taxa and four mammal taxa were filtered out entirely because of read abundance
307 below our threshold, four of which had been detected by camera traps (*Varanus, Tiliqua*
308 *rugosa, Sminthopsis dolichura* and *Felis catus*). 12Sv5 samples contained 33 aggregated
309 ZOTUs and 16S contained 23. Collectively, eDNA detected 31 unique taxa which contained
310 19 Families, 23 genera and 21 species across. 5 ZOTUs were only assigned to family and 10
311 to genus. Human OTUs were present in most extraction controls (60% of 16S and 82% of
312 12sV5).

313 In total, 406,365 images were collected across the 40 camera traps, with 154,413 from
314 Dryandra, and 251,952 from GWW. The number of photos between cameras varied

equivalently for both sites, ranging from <100 to >20,000. Two cameras from Dryandra did not function correctly across both timeframes. For both sites, there was a decline in total number of photos between the two timeframes (120,045 to 34,368 for Dryandra, 175,983 to 75,969 for GWW) due to camera position and framing being adjusted when it was clear something had been triggering false positives. There were 1466 discrete detections across the camera traps from 47 species. Dryandra recorded 912 detections and GWW recorded 554. Of these species, 25 were detected less than ten times across either site, 17 were detected less than five times, and eight only detected once across the entire six-month period. The highest number of detections were of *Climacteris rufus* (Dryandra 177, GWW 267). Other frequently seen species were *Tachyglossus aculeatus* (112, 0), *Macropus fuliginosus* (91, 1) and *Varanus gouldii* (64, 26).

Taxonomic composition within and between soil and hollow sediment

A Kruskal-Wallis test found ZOTU richness in hollow sediment samples was significantly higher than associated soil sample richness with 12Sv5 GWW samples ($t = 4.680$, $df = 1$, $p = 0.03$), 12Sv5 Dryandra samples ($t = 5.799$, $df = 1$, $p = 0.02$), 16S GWW samples ($t = 5.830$. $df = 1$, $p = 0.02$) and 16S Dryandra samples ($t = 5.506$, $df = 1$, $p = 0.02$) (figure 2). In total, taxa were recovered from 119 hollow sediment samples and 79 soil samples. Notably, only a single soil sample in GWW returned taxa using the 16S primer. Richness did not reach an asymptote for any site as number of samples continued to increase (Figure 3). 29 taxa were recovered from hollow sediment samples including 6 birds, 3 reptiles and 20 mammals and 13 from soil samples, including 3 birds, 2 reptiles and 8 mammals (Figure 4).

Taxonomic composition differences between camera traps and eDNA

337 A Mantel test between the presence/absence of taxon detected by eDNA and camera traps
338 found no significant correlation (Mantel statistic = 0.090, p = 0.158). Across both sites eDNA
339 detected 38 taxa (23 in Dryandra and 18 in GWW) and camera traps detected 47 (31 in
340 Dryandra and 26 in GWW). Taxa detected by camera traps comprised 23 birds, 15 mammals
341 and 9 reptiles while eDNA detected 7 birds, 20 mammals and 4 reptiles, with 75% of these
342 eDNA detections in hollow sediment (Figure 5).

343 The number of genera detected by camera traps did not decrease across timeframes for any
344 class in either location, but genera detected by eDNA decreased in GWW for Aves and
345 Mammalia (Figure 4). The loss in diversity did not reflect the absence of a single obvious
346 taxa, as 60% genera detected at GWW via eDNA were only detected in one of the two
347 timeframes (35% in Dryandra). 47% of GWW genera that were only detected in one time
348 point were birds All Dryandra reptiles and 88% of Dryandra mammals were detected across
349 both timeframes with either method but birds were once again unreliably detected (36%
350 across both time points).

351 Of the 21 genera detected across both timeframes by Dryandra camera traps, 10 were detected
352 with eDNA in both timeframes too. *Isoodon* and *Oryctolagus* were only detected by eDNA in
353 the first timeframe, and *Phaps* and *Phascogale* in the second. There were 7 genera that were
354 detected in no eDNA sample despite appearing in both camera trap timeframes, including two
355 mammals, two reptiles and three birds. Of the 11 genera seen on GWW cameras in both
356 timeframes, only four were detected across both respective eDNA samples (*Climacteris*,
357 *Cryptoblepharus, Mus* and *Oryctolagus*).

358 Over half (55%) of taxa detected by both eDNA and camera traps had over 10 discrete
359 detections on camera. The taxa with less than 10 detections on camera traps that were
360 detected by eDNA were the mammal *Phascogale calura,* and the bird *Pardalotus striatus*.

However, taxa with the highest number of camera trap detections were not necessarily present in eDNA. The most frequently detected species on camera traps was the rufous treecreeper (*Climacteris rufus*) which was not represented by any ZOTU. However, a *Climacteris* species not known to inhabit either site was detected (*C. picumnus*) suggesting that *C. rufus*, or *C. affinis* (also present at GWW) eDNA has been misidentified as *C. picumnus*. Similarly, the small lizard *Cryptoblepharus virgatus* was detected with camera traps and an unassigned *Cryptoblepharus* species was detected by eDNA, but the presence of multiple *Cryptoblepharus* species across the sites prevented us from confidently reassigning this as *C virgatus*.

Taxa identified to species level by both methods were overwhelmingly mammals (86%). With only two birds (*Phaps chalcoptera* and *Pardalotus striatus*) and no reptiles (Figure 5). eDNA detected several species of bat, none of which were detected by camera traps. 68% of taxa only detected by camera traps were birds. 58% of said camera-trap taxa were detected less than five times over the period of study.

Correlation between sequence read, tree hollow interactions and animal size

A significant negative correlation was found between the log10 sequence read and number of days since the last recorded interaction via camera ($r = -0.366$, $p = 0.020$). However, no significant correlation was found between the transformed sequence read number and total number of interactions recorded on camera ($p = 0.820$). The longest time between the last camera trap detection and the sample collection date was 64 days but the average number of days was 13 ±2.745 (n=32). 52% of matching camera trap/eDNA detections occurred when the animal had visited the site up to a week ago and 90% occurred when the animal had visited up to a month ago.

384 The Kruskal-Wallis test on size categories against log10 sequence was significant (f = 6.564,
385 df = 2, p = 0.038) and the post-hoc Dunn's test found 'large' animals were associated with
386 higher sequence reads than 'medium' (z = 2.412, p.adj = 0.048) (Figure 6).

387

Discussion

We have demonstrated that eDNA can detect birds, mammals and reptiles from soil and hollow sediment. As eDNA metabarcoding becomes increasingly commonplace in biodiversity monitoring, it is imperative that the use of different substrates and benchmarking against traditional approaches is explored, as our study demonstrates neither biomonitoring method can capture the entire range of diversity at a given site.

Substrate comparison

The two metabarcoding assays detected a wide range of vertebrate species in each substrate. Differences in ZOTU richness for both soil and hollow sediment samples between the two sites is likely to be a true reflection of the higher vertebrate species abundance across Dryandra compared to GWW, particularly of mammals, which were most commonly detected by eDNA. The species accumulation curves suggest that our study timeframe was insufficient to capture the full breadth of diversity present across the site, which is also reflected in the number of taxa that were detected in only one of our timeframes. Many birds were only detected at a single time point, possibly because it was rare that they would descend low enough to be picked up by camera and deposit eDNA compared to mammals and reptiles which had many opportunities for detection by either method due to how they interacted with the environment. The density of target species is likely also a consideration, but is difficult to take into account with our data, particularly at the GWW where the majority of the time-point diversity differences occur. Future investigations will need to conduct surveys for longer periods, more typical of experiments involving camera trap data (Liu et al., 2013; Palmer et al., 2018; Stein et al., 2008).

411 A higher number of filtered sequences and broader range of species was detected from the log
412 hollow sediment than from soil obtained from around the hollow. Our results align with a
413 previous study by van der Heyde et al. (2020), which also demonstrated that the choice of
414 substrate may impact the detection of certain taxa. As tree hollows are capable of functioning
415 as a microclimate, shielding the interior from heat and light (Gibbons, 2002), which may
416 speed eDNA degradation (Gutiérrez-Cacciabue et al., 2016; Tsuji et al., 2017), we suspect the
417 broader diversity observed in hollow sediment samples is reflective of the better conditions
418 for the preservation of eDNA. It is also likely that behaviour of organisms associated with the
419 hollows contributes to the higher number of species detected from hollow sediment. For
420 example, camera trap footage recorded animals targeting hollows for shelter, feeding and
421 thermoregulation as well as simple investigation. These types of behaviour are all associated
422 with increased surface contact resulting in more deposited eDNA in the confined environment
423 (Kucherenko et al., 2018). However, our camera traps were unable to provide consistent
424 information on how long an animal stayed in a hollow, as the interior was too dark for
425 continuous capture and typically the animal would only be seen either entering or exiting the
426 hollow, not both. Whether an increase in species richness detected in hollow sediment reflects
427 better eDNA preservation or a larger initial concentration will need to be investigated in
428 further studies. However, from our results, tree hollows, and other areas sheltered from excess
429 heat and light that are frequently inhabited by species, may be a more efficient way of
430 collecting terrestrial eDNA than open soil.

431 Interestingly, our results suggest that interaction with a hollow is not a necessity to ensure
432 eDNA deposition, as species such as *Phaps chalcoptera* and *Macropus fuliginosus* were never
433 seen entering target hollows (in the case of *M. fuliginosus* this would be impossible) yet were
434 detected by eDNA. Both these species were seen moving and feeding in very close proximity

435 to the hollow's entrance, suggesting that eDNA deposited outside a hollow is capable of
436 movement further in. This movement of genetic material could be the result of transportation
437 through the soil medium by invertebrates (Prosser & Hedgpeth, 2018) or environmental
438 factors such as wind and rain, as well as soil movement by foragers.

439 *Camera trap and eDNA comparison*

440 The species detected by eDNA did not perfectly overlap with the species detected by camera
441 traps. Both methods had unique detections, which is consistent with a previous comparative
442 study by Leempoel et al., (2020), which found that small mammals were less represented in
443 camera trap data. We detected the burrowing skink *Hemiergis initialis* as well as another
444 likely burrower in the genus *Lerista* with eDNA, as further examples of small, cryptic species
445 better suited to detection by eDNA than camera trapping. However, other skinks such as
446 *Cryptoblepharus* were detected by both methods, and there were small mammals such as *Mus*
447 *musculus* in Dryandra and *Sminthopsis dolichura* in GWW that were not detected in eDNA
448 (in high enough abundance to not be filtered) despite appearing in camera traps, suggesting
449 there are further factors to take into consideration.

450 Taxa detected by both eDNA and camera traps were overwhelmingly mammals. Reasons for
451 this likely include larger body mass, which increases likelihood of detection via camera
452 (Silveira et al., 2003) and more frequent shedding of genetic material through saliva, waste,
453 mucus, shed skin and hair and (Taberlet et al., 2018). Mucus as a source of eDNA may be the
454 explanation as to why the short-beak echidna *Tachyglossus aculeatus* was detected in GWW
455 soils and sediments without any associated camera trap detections, even though echidnas can
456 be reliably detected by camera trap based on Dryandra data, as foraging with "nose pokes"
457 may leave copious amounts of mucus preserved deep in the soil. However, there were other
458 species that could be assumed to be conspicuous enough for camera traps that were only

459 picked up by eDNA, including medium-sized organisms such as *Canis lupus familiaris*. High
460 temperatures can decrease the sensitivity of camera traps (Apps & McNutt, 2018) and our
461 study sites had limited cover available, with temperatures in excess of 40 degrees recorded
462 during the experiment. This may have limited detections, but other possible explanations are
463 that the eDNA is derived from the faeces of a roaming predator (Boyer et al., 2015) or other
464 mode of secondary transportation, or has been retained in the environment from a visit prior
465 to our initial camera trap records. Several species of bats were also detected with eDNA
466 across both sites with zero detection via camera traps. Other diversity assessments involving
467 bats, such as that performed by Taylor et al., (2018) suggest camera traps do not reliably
468 detect them, thus eDNA may represent a new avenue for bat biomonitoring to be explored in
469 future studies.

470 Most species detected by camera traps but not by eDNA had very minimal interaction with
471 site hollows and adjacent soil (for example, birds seen roosting on branches near the hollow
472 without coming into contact), but there were some notable absences. No numbat reads were
473 detected even prior to filtration even though they were seen on camera foraging around and
474 entering many sample hollows. As numbats are a derived monophyletic group (Zemann et al.,
475 2013), it is possible there was an issue with primer binding/targeting, but the broad range of
476 other mammals detected makes this unlikely. Several bird species with many captured
477 interactions such as *Strepera versicolor*, *Ptilotula ornata* and *Anthochaera carunculata* also
478 were not detected by eDNA. Previous studies have amplified avian eDNA less reliably than
479 mammal (Andersen et al., 2012), but overall studies on terrestrial avian eDNA are limited. It
480 is possible that due to low genome size (Kapusta et al., 2017), avian eDNA degrades at a
481 faster rate than other species, but more research is needed to test this. A similar trend was seen
482 among reptiles, with frequently seen species such as *Ctenophorus cristatus* and *Varanus*

483 *gouldii* not detected with eDNA. Detecting reptiles with eDNA has previously been found to
484 be difficult (Kucherenko et al., 2018), it is possible that the presence of scales and lack of
485 mucus integument may reduce the shedding of eDNA by reptiles and thus their detectability
486 (Adams et al., 2019).

487 Both methods of species detection have the potential for false positives and false negatives
488 arising from different sources. Comparisons between available species lists and our results
489 show an absence of many canopy-dwelling birds, which is likely to be because our cameras
490 were positioned at low elevation facing hollows that these animals were unlikely to visit,
491 which also explains the lack of eDNA from these species. We were able, however, to detect
492 the vast majority of Dryandra mammals with one or both of the available methods, though
493 many ground-dwelling reptiles were also undetected by either method, likely as a result of
494 lack of eDNA as with birds, as well as a comparatively smaller thermal signature that cameras
495 did not detect. False positives can be detected by referring to existing species records in and
496 around the target site, though camera traps are already resistant to this issue (apart from the
497 risk of wrongly identifying species)

498 One noticeable downside to eDNA diversity data was the unreliable species-level assignment
499 of our ZOTUs. We found that many eDNA assignments did not provide a reliable
500 identification down to species level, and often needed to be reassigned to a species present at
501 the site or left at a higher taxonomic level. Using existing biodiversity surveys for the study
502 sites allows for easy detection of probable contaminants. However, problems may arise when
503 contaminant species are also present across study sites. It is also possible that the detection of
504 taxa not previously recorded on a site is not an error at all, and the species may have had a
505 recent range expansion, or had always been present, yet undetected by previous surveys.

506 The species-level misattributions likely come from the choice of assay as 12SV5 and 16S
507 target short sequences (a necessity when amplifying degraded eDNA) that simply lack the
508 fidelity for perfect species-level resolution, although they reliably identified down to genus-
509 level for birds, mammals and reptiles. However, even short assays can be capable of
510 unambiguous species identification (Bienert, 2012). Precise species identification may require
511 more specific assays for several of our groups, which increases the time and cost of
512 biodiversity assessments targeting a wide variety of organisms such as this.

513 *Correlation between sequence reads, number of days since interaction, length of interaction*
514 *and animal size*

515 Our transformed sequence read amount did not correlate with total number of recorded
516 interactions with the sample location but did show negative correlation with days since last
517 recorded interaction, which suggests that while a single batch of samples works well to
518 provide a snapshot of site diversity, generating comprehensive lists will require continuous
519 sampling to account for eDNA degradation rather than waiting long periods of time for
520 deposited eDNA to build up. We were able to detect species with eDNA from hollows they
521 had visited up to two months ago, but not reliably. Our results would suggest that eDNA
522 typically lasts about a month in our environments. However, this is only based off sample
523 data that could successfully be timestamped with matching camera trap data, which was
524 limited. This data set was entirely mammal except for a single avian genera, *Climacteris*
525 which was the most frequently detected taxa across both sites. Previous studies indicate both
526 camera traps and eDNA can reliably detect mammals (Leempoel et al., 2020), while reptile
527 eDNA can disappear from an environment quickly (Kucherenko et al., 2018) on top of
528 already being difficult to detect via camera without accommodation (Hobbs & Brehme,
529 2017), which may explain this skewed representation.

Large animals were associated with significantly higher sequence read numbers after transformation compared to medium animals, while small animals showed no clear trends. This can be explained by a larger body mass allowing for greater eDNA deposition, especially as all large animals were mammals capable of shedding large amounts of hair, a good source of eDNA (Andersen et al., 2012). However, as small animals are capable of physically entering the hollow in ways larger animals may not, the density of eDNA can match that of larger animals interacting with the same hollow in a less obtrusive manner. Future tests with a larger pool of samples may better demonstrate relations between these factors.

Conclusion

We conducted vertebrate biodiversity assessments using two different eDNA substrates and camera traps at two sites. This study confirmed that eDNA can be sequenced from log sediments, and that this substrate may be a better source of eDNA than is soil due to a higher proportion of animal interaction and/or a microclimate that better preserves genetic material. Neither biomonitoring method was able to capture the full range of diversity across either site within our study timeframe, as evidenced by the unique detections from each method. As expected, eDNA detected several small/cryptic species that went undetected by camera traps, but it often failed to detect reptiles and birds, which are known to have lower eDNA shedding rates than most mammals. We suggest that a broad taxa target requires multiple, complementary biomonitoring methods to detect the full range of species diversity. We recommend the use of log hollow sediment for molecular-based terrestrial biomonitoring, particularly if mammals are the target taxa. In order to maximise taxa detection by eDNA, monthly sample collection is also recommended to account for environmental degradation, but future eDNA studies will need to focus on further understanding the relationship between

553 the likelihood of detection and the amount of interaction/time since interaction between
554 organism and environment to better utilise molecular-based vertebrate biomonitoring.

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781 Author contributions
782 E.R. conducted the study and wrote the manuscript. P.N., B.B., M.v.H. and K.F. were
783 involved in the experimental design; samples were collected by E.R., P.N., and B.B., and
784 processed by E.R., M.v.H. and K.F.; molecular and bioinformatics work was performed by
785 E.R., M.v.H. and K.F.; all data were analysed and processed by E.R., M.v.H. and K.F.;
786 statistical analysis was done by E.R. and M.v.H.; the manuscript was edited by all authors.
787

788 Data availability statement
789 Sequencing data and dada2 script are available at the Dryad Digital Repository (will be
790 submitted prior to publication.

791

792 **Table 1:** Summary of sequencing results for each assay

Assay	Sequences	Samples >642 reads	ZOTUs	Substrate
12Sv5-F/R	2,774,884	49	15	Soil
	4,036,920	63	28	Hollow Sediment
16Smam1/2	4,273,381	50	6	Soil
	4,172,767	56	21	Hollow Sediment

793

794 **Table 2:** Taxa detected across sites and sampling method.

	Dryandra			GWW		
Class	Sediment	Soil	Camera	Sediment	Soil	Camera
Aves	2	3	13	6	1	13
Mammalia	17	8	13	8	2	5
Reptilia	1	1	3	3	1	7

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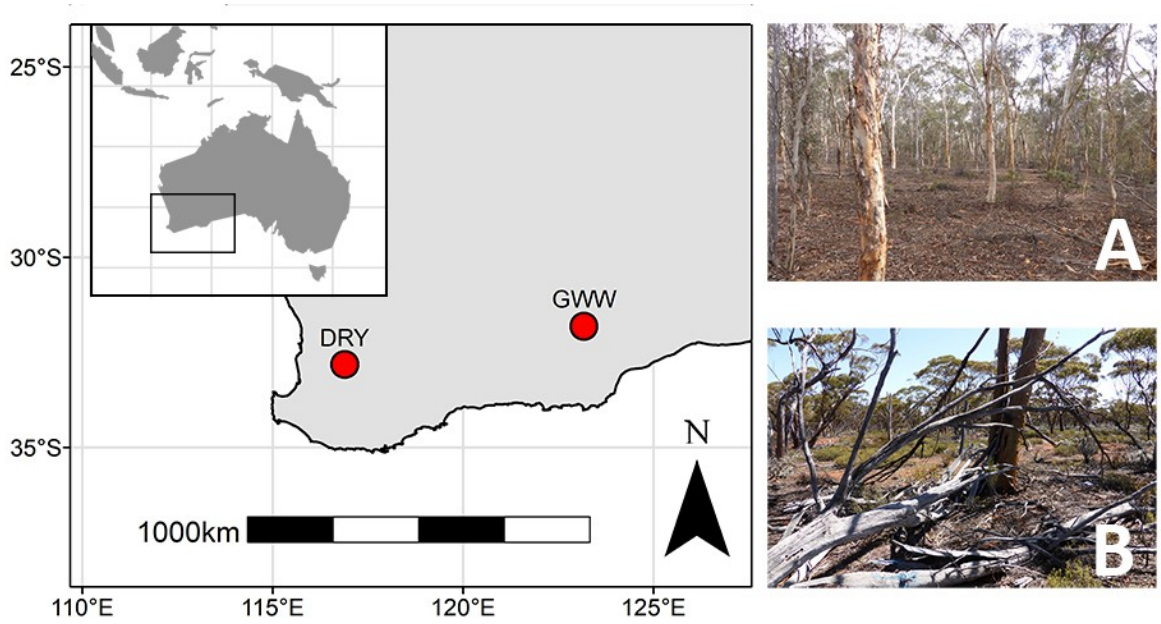
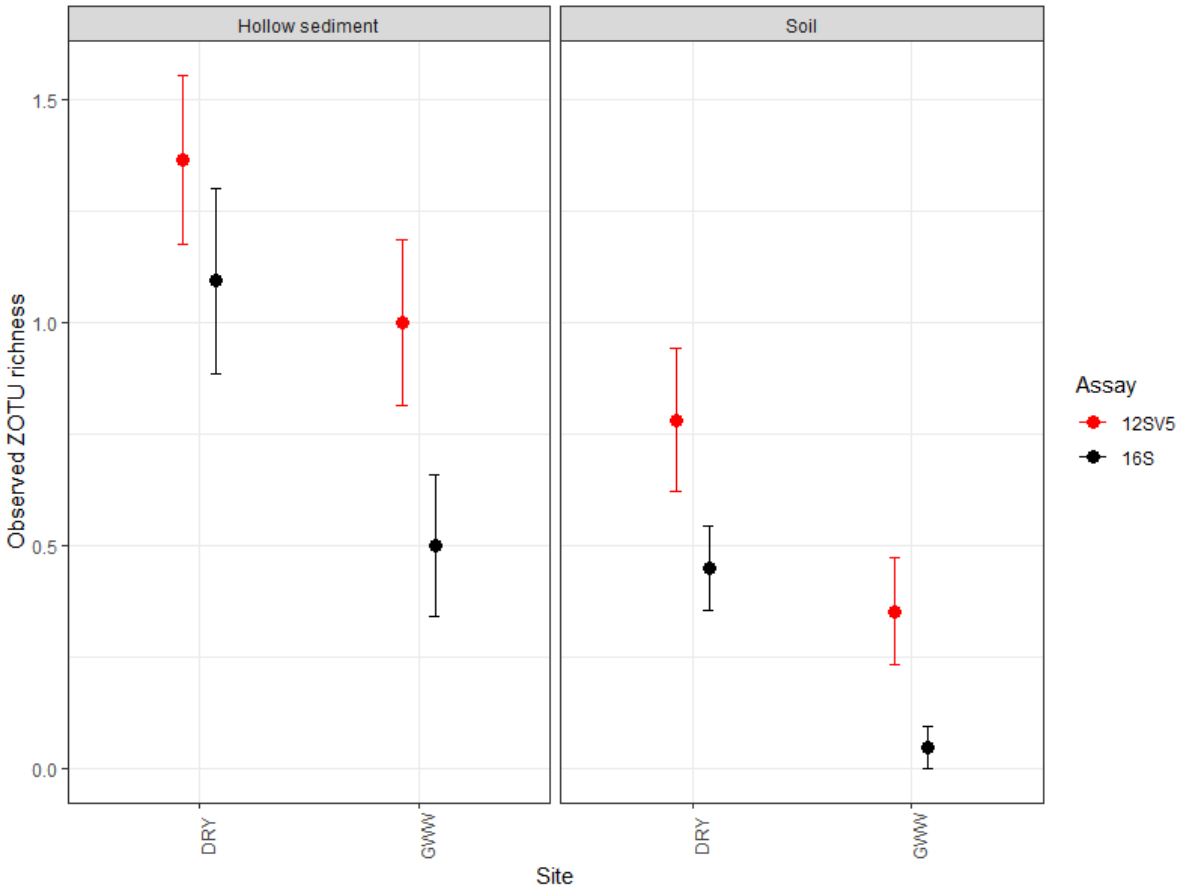


Figure 1: Map of study sites in Western Australia. Photos show Dryandra Woodland (DRY) (A) and the Great Western Woodland (GWW) (B).



802 **Figure 2:** ZOTU richness hollow sediment and soil samples between the two sample
803 locations. Error bars indicate standard error. Red points are 12sV5 assay samples and black
804 points are 16Smam.

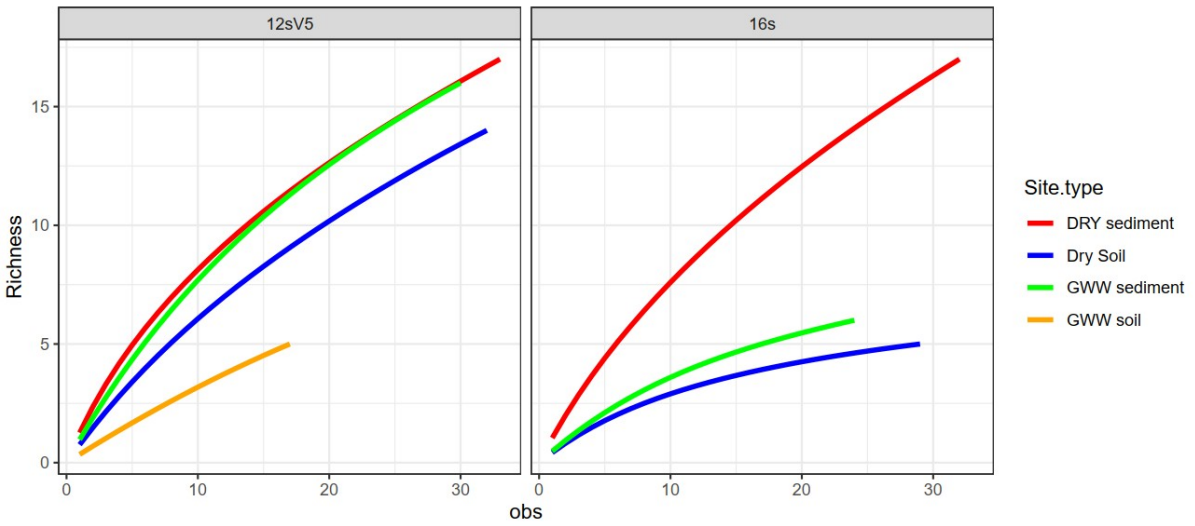


Figure 3: ZOTU richness by Number of samples between the two assays for hollow sediment (deep) and soil samples (entrance) between locations. The difference in proportion of samples that successfully produced detectable eDNA is reflected here. As only one sample from GWW soils was retained, a curve is not shown.

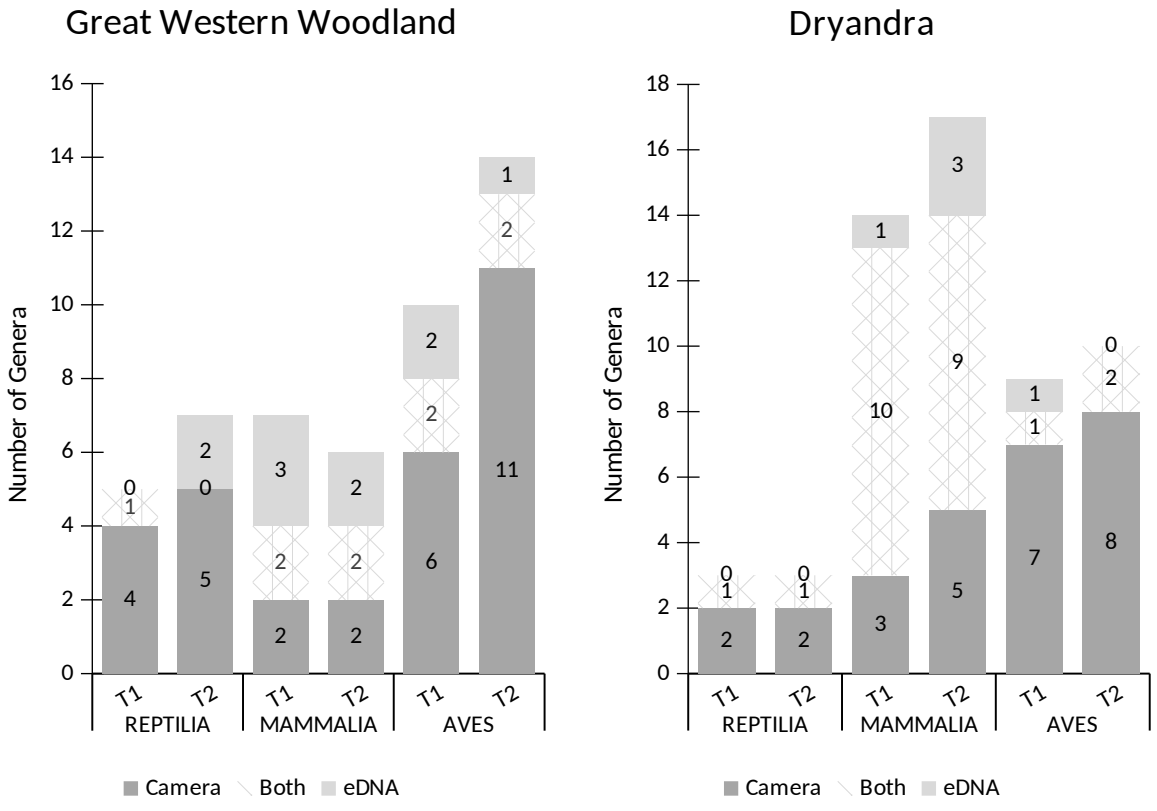
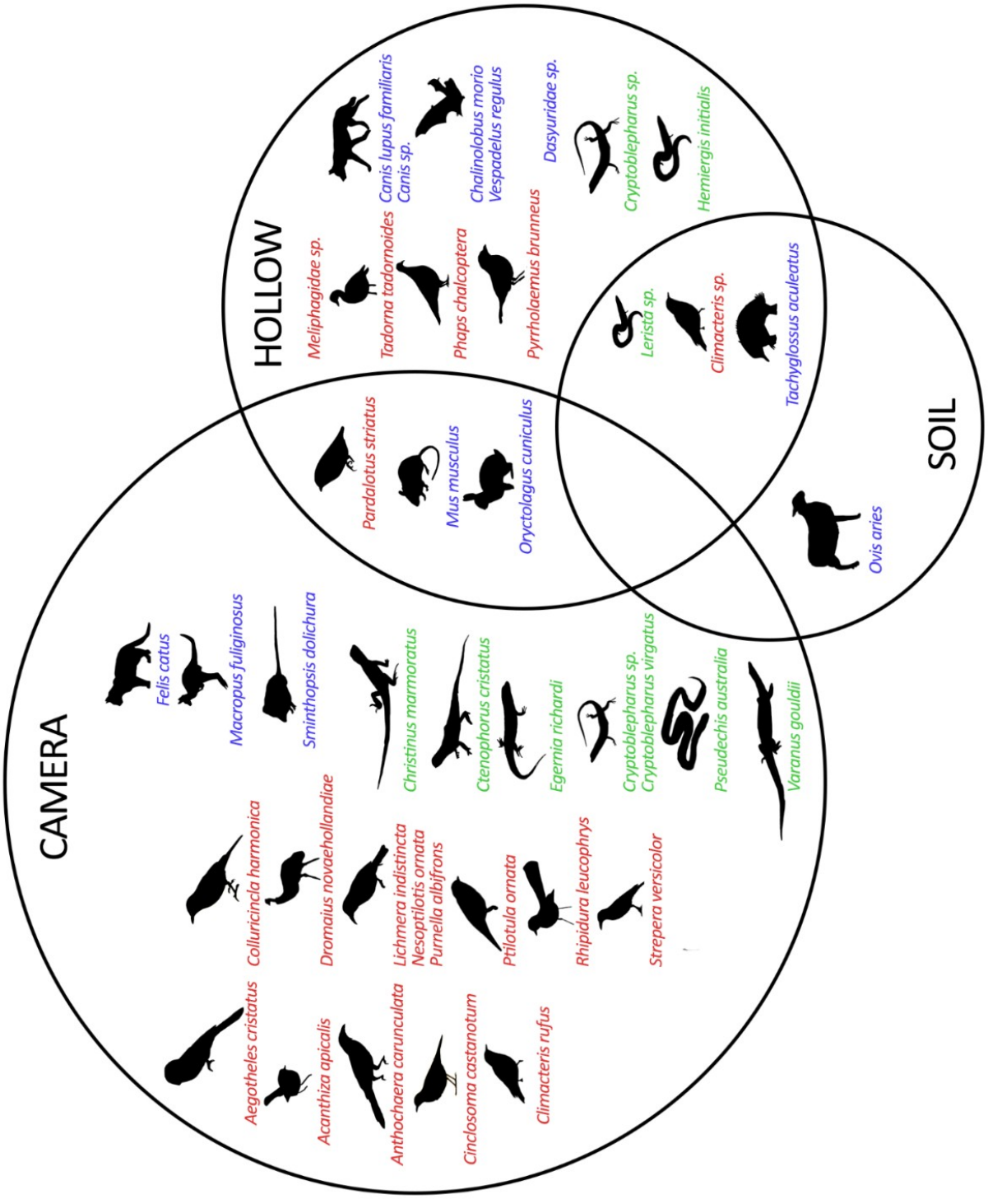


Figure 4: Number of genera detected by eDNA and/or camera traps during timeframe one (T1) and two (T2) across study sites. Although total genera detected appears to accumulate, many were only detected at one time point with the exception of Dryandra mammals and reptiles.

Figure 5: Taxa detected in soil, hollow sediment and camera trap photos in GWW (left) and Dryandra (next page). Colour of names denotes class (green: Reptilia, blue: Mammalia and red: Aves). When eDNA ZOTU could not specify a species level, the higher taxonomic order was retained



CAMERA
HOLLOW
SOIL
Tiliqua rugosa
Cryptoblephaus sp.
Cryptoblepharus virgatus
Varanus gouldii
Aquila audax
Podargus strigoides
Barnadius zonarius
Rhipidura leucophrys
Climacteris rufus
Sericornis frontalis
Corvus coronoides
Smicrornis brevirostris
Dacelo novaguineae
Strepera versicolor
Gymnorhina tibicen
Felis catus
Pardalotus punctatus
Mus musculus
Myrmecobius fasciatus
Tadorna tadornoides
Canis lupus familiaris
Chalinolobus gouldii
Dasyuridae sp.
Macropodidae sp.
Sminthopsis sp.
Cryptoblepharus sp.
Antechinus flavipes
Isoodon fusciventer
Oryctolagus cuniculus
Phascogale calura
Bettongia penicillata
Tachyglossus aculeatus
Dasyurus geoffroii
Trichosurus vulpecula
Macropus fuliginosus
Vulpes vulpes
Ovis aries
Canis sp.
Climacteris sp.
Phaps chalcoptera
Scincidae sp.
Psittacidae sp.
Dryandra Wooodland

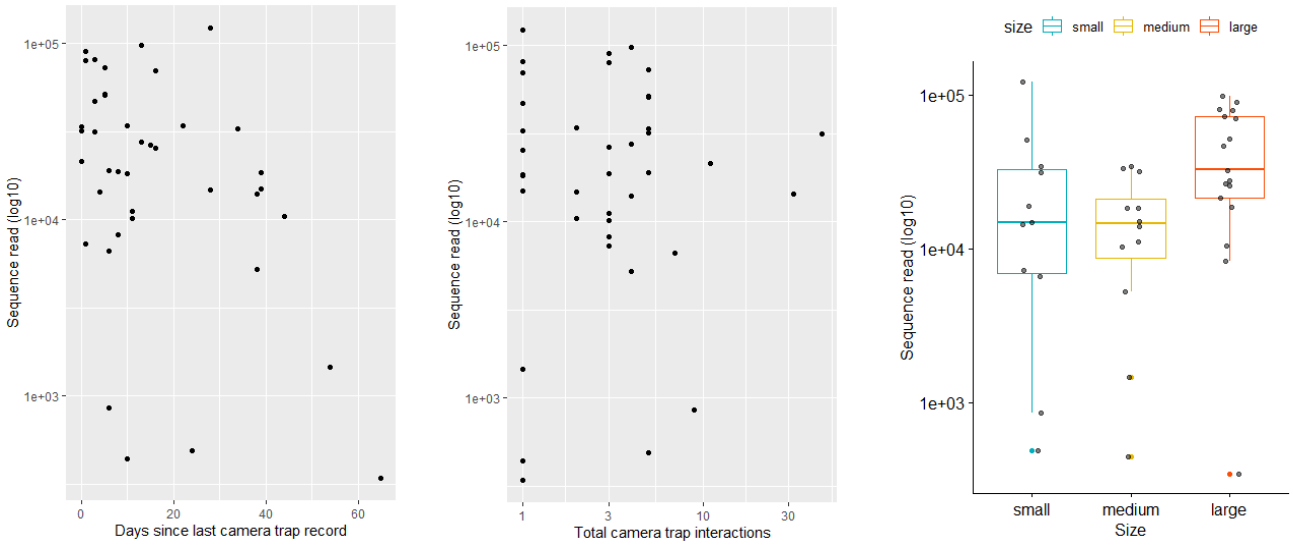


Figure 6: Sequence read amount plotted against camera trap data (left, middle) and size category of target taxa (right).