Environmental DNA (eDNA) metabarcoding of pond water as a tool to survey conservation and management priority mammals

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Abstract

Environmental DNA (eDNA) metabarcoding can identify terrestrial taxa utilising aquatic habitats alongside aquatic communities, but terrestrial species' eDNA dynamics are understudied. We evaluated eDNA metabarcoding for monitoring semi-aquatic and terrestrial mammals, specifically nine species of conservation or management concern, and examined spatiotemporal variation in mammal eDNA signals. We hypothesised eDNA signals would be stronger for semi-aquatic than terrestrial mammals, and at sites where individuals exhibited behaviours. In captivity, we sampled waterbodies at points where behaviours were observed ('directed' sampling) and at equidistant intervals along the shoreline ('stratified' sampling). We surveyed natural ponds (N = 6) where focal species were present using stratified water sampling, camera traps, and field signs. eDNA samples were metabarcoded using vertebrate-specific primers. All focal species were detected in captivity. eDNA signal strength did not differ between directed and stratified samples across or within species, between semi-aquatic or terrestrial species, or according to behaviours. eDNA was evenly distributed in artificial waterbodies, but unevenly distributed in natural ponds. Survey methods deployed at natural ponds shared three species detections. Metabarcoding missed badger and red fox recorded by cameras and field signs, but detected small mammals these tools overlooked, e.g. water vole. Terrestrial mammal eDNA signals were weaker and detected less frequently than semi-aquatic mammal eDNA signals. eDNA metabarcoding could enhance mammal monitoring through large-scale, multi-species distribution assessment for priority and difficult to survey species, and provide early indication of range expansions or contractions. However, eDNA surveys need high

spatiotemporal resolution and metabarcoding biases require further investigation before routine implementation.

Key-words: camera traps, field signs, lentic, monitoring, semi-aquatic mammals, terrestrial mammals

1. Introduction

Mammals are among the most threatened vertebrates globally, with 25% of species at risk of extinction due to harvesting, habitat degradation/loss, non-native species or perception as pests (Visconti et al., 2011). Most species lack long-term, systematic monitoring, with survey efforts biased towards rare species (Massimino, Harris, & Gillings, 2018). Data deficiency prevents robust estimation of mammalian range expansions/declines and population trends (Bland, Collen, Orme, & Bielby, 2015). Therefore, effective and evidence-based strategies for mammal conservation and management are urgently needed (Mathews et al., 2018).

Many mammals are nocturnal and elusive thus monitoring requires non-invasive, observational methods, e.g. camera traps and field signs (Caravaggi et al., 2018; Harris & Yalden, 2004; Sadlier, Webbon, Baker, & Harris, 2004). Camera trapping is cost-efficient, standardised, reproducible, and produces data suited to site occupancy modelling, but only surveys a fraction of large, heterogeneous landscapes. Trap placement can substantially influence species detection probabilities, and traps often miss small species (Burton et al., 2015; Caravaggi et al., 2018; Ishige et al., 2017; Stat et al., 2019). Field sign surveys are inexpensive, but resource-intensive for broad geographic coverage (Sadlier et al., 2004). Species can have similar footprints and scat, increasing the potential for misidentification (Franklin et al., 2019; Harris & Yalden, 2004). Mammal survey methods can be species-specific, thus multiple methods are necessary for large-scale, multi-species monitoring schemes (Massimino et al., 2018).

Environmental DNA (eDNA) analysis is a recognised tool for rapid, non-invasive, cost-

efficient biodiversity assessment across aquatic and terrestrial ecosystems (Deiner et al., 2017). Organisms transfer genetic material to their environment via secretions, excretions, gametes, blood, or decomposition, which can be isolated from environmental samples (Thomsen & Willerslev, 2015). Studies using eDNA analysis to target specific semi-aquatic and terrestrial mammals have employed PCR or quantitative PCR (qPCR) (e.g. Franklin et al., 2019; Lugg, Griffiths, van Rooyen, Weeks, & Tingley, 2017; Rodgers & Mock, 2015; Thomsen et al., 2012; Williams, Huyvaert, Vercauteren, Davis, & Piaggio, 2018). eDNA metabarcoding can screen entire communities using PCR combined with high-throughput sequencing (Deiner et al., 2017; Thomsen & Willerslev, 2015), but mammalian assessments are uncommon (Klymus, Richter, Thompson, & Hinck, 2017; Ushio et al., 2017). Tropical mammal assemblages have been obtained by metabarcoding invertebrate blood meals (e.g. Tessler et al., 2018) and salt licks (Ishige et al., 2017), but samples from the physical environment have tremendous potential to reveal mammal biodiversity over broad spatiotemporal scales (Ushio et al., 2017).

In aquatic ecosystems, eDNA metabarcoding has predominantly been applied to characterise fish (e.g. Evans et al., 2017; Hänfling et al., 2016; Lawson Handley et al., 2018; Valentini et al., 2016) and amphibian (e.g. Bálint et al., 2018; Valentini et al., 2016) communities. However, mammals also leave eDNA signatures in water that metabarcoding can detect (Harper et al., 2019; Klymus et al., 2017; Ushio et al., 2017). Ponds in particular provide drinking, foraging, dispersive, and reproductive opportunities for semi-aquatic and terrestrial mammals (Klymus et al., 2017). Samples from these waterbodies could uncover biodiversity present in the wider environment (Deiner et al., 2017; Harper et al., 2019). Drinking is a major source of eDNA deposition due to the release of saliva, but mammals may also swim, wallow,

urinate or defecate in water (Rodgers & Mock, 2015; Ushio et al., 2017; Williams et al., 2018). Furthermore, arboreal mammals may use ponds less than semi-aquatic and ground-dwelling species, non-territorial mammals may visit ponds less than territorial species, and group-living species may deposit more eDNA than solitary species (Williams et al., 2018). Despite evidence for eDNA deposition by semi-aquatic and terrestrial mammals in freshwater ecosystems, little is known about the influence of mammal behaviour on the distribution and strength of the eDNA signal left behind.

In this study, we conducted two experiments to evaluate eDNA metabarcoding of pond water as a tool for monitoring semi-aquatic, ground-dwelling, and arboreal mammals of conservation or management concern. The first experiment, carried out on nine focal species housed at two wildlife parks, examined the role of sampling strategy, mammal lifestyle, and mammal behaviour on eDNA detection and signal strength. We hypothesised that: (1) eDNA would be unevenly distributed, thus directed sampling would yield stronger eDNA signals (i.e. higher read counts) for mammals than stratified sampling; (2) semi-aquatic mammals would have stronger eDNA signals than ground-dwelling or arboreal mammals; and (3) mammal behaviours involving water contact would generate stronger eDNA signals. The second experiment validated eDNA metabarcoding against camera trapping and field sign searches for mammal identification at natural ponds, and investigated spatiotemporal variation in mammal eDNA signals. We hypothesised that: (1) eDNA metabarcoding would detect more mammals than camera trapping or field signs; (2) semi-aquatic mammals would be readily detected and their eDNA evenly distributed in ponds in comparison to terrestrial mammals; and (3) temporal sampling would reveal that terrestrial mammal eDNA is detectable for short periods in

comparison to fully aquatic vertebrates.

2. Materials and methods

2.1 Study species

We studied nine mammal species that are the focus of European conservation or management (Mathews et al., 2018): European water vole (*Arvicola amphibius*), European otter (*Lutra lutra*), Eurasian beaver (*Castor fiber*), European hedgehog (*Erinaceus europaeus*), European badger (*Meles meles*), red deer (*Cervus elaphus*), Eurasian lynx (*Lynx lynx*), red squirrel (*Sciurus vulgaris*), and European pine marten (*Martes martes*). Water vole, otter, red squirrel, pine marten and hedgehog are UK Biodiversity Action Plan species (Joint Nature Conservation Committee, 2018). Water vole, otter, and beaver are semi-aquatic, red squirrel and pine marten are arboreal, and the other species are ground-dwelling. Badger and red deer live in groups whereas the other species are predominantly solitary.

2.2 Experiment 1: eDNA detection and signal strength in artificial systems

Behavioural observation and eDNA sampling were conducted between 18/09/2017 – 21/09/2017 at Wildwood Trust (WT), Kent, England, and 10/10/2017 – 11/10/2017 at Royal

Zoological Society of Scotland (RZSS) Highland Wildlife Park (HWP), Kingussie, Scotland. Sixteen categories of behaviour were defined based on potential contact with waterbodies and species lifestyle, and the frequency and duration of behaviours recorded (Table A1). The number of individuals in each enclosure was recorded alongside waterbody size (Table 1). Beaver, lynx, red deer, and red squirrel were present at both wildlife parks, whereas other captive species were only present at WT. Each species was observed for one hour on two separate occasions except nocturnal mammals (badger and beaver), which were observed overnight using camera traps (Bushnell Trophy Cam Standard, Bushnell Corporation, KS, USA). One camera trap per enclosure was positioned perpendicular to the ground (1 m height, 2 m from shoreline) to capture water and shoreline. Cameras took 30 s videos (1920 x 1080) when triggered (30 s interval between triggers) at high sensitivity. Behavioural observation was not undertaken for WT water voles as animals were under quarantine or HWP red squirrels as individuals were wild.

Water samples were collected from enclosures within 3 hrs of the second behavioural observation period. Up to six directed or stratified samples were collected, but sample number varied by species according to waterbody size and observed behaviours (Tables S1, S2). Enclosure drinking containers were also sampled and classed as ‘other’ samples. Bathing and drinking bowls were sampled where enclosures contained no artificial waterbodies (WT water vole, red squirrel, and hedgehog). The HWP beaver enclosure was empty for 24 hrs before sampling. Water was sampled from a RZSS Edinburgh Zoo (EZ) enclosure containing beavers and classed as ‘other’. A sample was collected from a water bath in the HWP woods to capture wild red squirrels and classed as ‘other’.

Directed samples (2 L surface water taken approximately where behaviours were

159 observed) were collected before stratified samples (2 L surface water [8 x 250 ml pooled
160 subsamples] taken at equidistant points [access permitting] around the waterbody perimeter)
161 to minimise disturbance to the water column and cross-contamination risk. Samples were
162 collected using sterile Gosselin™ HDPE plastic bottles (Fisher Scientific UK Ltd, UK) and
163 disposable gloves. A field blank (1 L molecular grade water [MGW]) was taken into each species
164 enclosure and opened. Samples (*n* = 80) collected from WT and HWP were transported
165 alongside field blanks (*n* = 13) in sterile coolboxes with ice packs to the University of Kent (UoK)
166 and EZ respectively, where ice was added to coolboxes.

167 Samples and blanks were vacuum-filtered within 6 hrs of collection in a UoK wet
168 laboratory and within 24 hrs of collection in an EZ staff room. Surfaces and equipment were
169 sterilised before, during, and after set-up in temporary work areas. Surfaces and vacuum
170 pumps were wiped with 10% v/v chlorine-based commercial bleach (Elliott Hygiene Ltd, UK)
171 solution. Non-electrical equipment was immersed in 10% bleach solution for 10 minutes,
172 followed by 5% v/v MicroSol detergent (Anachem, UK), and rinsed with purified water. Up to
173 500 ml of each 2 L sample was vacuum-filtered through sterile 0.45 µm mixed cellulose ester
174 membrane filters with pads (47 mm diameter; Whatman, GE Healthcare, UK) using Nalgene™
175 filtration units. One hour was allowed for each sample to filter and a second filter used if
176 clogging occurred. A filtration blank (1 L MGW) was processed during each filtration round (*n* =
177 12), and equipment sterilised after each filtration round. After 500 ml had filtered or one hour
178 had passed, filters were removed from pads using sterile tweezers, placed in sterile 47 mm
179 petri dishes (Fisher Scientific UK Ltd, UK), sealed with parafilm (Sigma-Aldrich Company Ltd,
180 UK), and stored at -20 °C. The total water volume filtered per sample was recorded for

181 downstream analysis (Table A2; Fig. A1).

182

183 **2.3 Experiment 2: eDNA detection and signal strength in natural systems**

184

185 At three sites where focal species were present based on cumulative survey data, we selected
186 two ponds (range 293-5056 m^2, average 1471 m^2) within 4 km of each other. The Bamff Estate
187 (BE), Alyth, Scotland, was selected for beaver, otter, badger, red deer, and red squirrel, but roe
188 deer (*Capreolus capreolus*) and red fox (*Vulpes vulpes*) were also present. Otter, water vole, and
189 badger were present at Tophill Low Nature Reserve (TLNR), Driffield, East Yorkshire, alongside
190 American mink (*Neovison vison*), stoat (*Mustela erminea*), weasel (*Mustela nivalis*), rabbit
191 (*Oryctolagus cuniculus*), brown hare (*Lepus europaeus*), red fox, roe deer, and grey squirrel
192 (*Sciurus carolinensis*). We selected Thorne Moors (TM), Doncaster, South Yorkshire, for red deer
193 and badger, but stoat, weasel, red fox, roe deer, and Reeve's muntjac (*Muntiacus reevesi*) were
194 also present. Camera traps (Bushnell Trophy Cam Standard/Aggressor, Bushnell Corporation,
195 KS, USA) were deployed at TM (one per pond) and BE (three per pond) one week prior to eDNA
196 sampling and collected once sampling was completed. At TLNR, camera traps (two to three per
197 pond) were deployed one day before a 5-day period of eDNA sampling and collected one week
198 after sampling was completed. Camera traps were positioned perpendicular to the ground (1 m
199 height, 0.3-1 m from shoreline) to capture water and shoreline. Cameras took three
200 photographs (5 megapixel) when triggered (3 s interval between triggers) at high sensitivity.

201 Ten stratified samples were collected from the shoreline of each pond (TM: 17/04/2018;
202 BE: 20/04/2018; TLNR: 23/04/2018 -27/04/2018) and a field blank (1 L MGW) included as in

203 Experiment 1. TLNR ponds were sampled every 24 hrs over 5 days to investigate spatiotemporal
204 variation in mammal eDNA signals. TM and TLNR samples were transported on ice in sterile
205 coolboxes to the University of Hull (UoH) eDNA facility, and stored at 4 °C. BE samples were
206 transported in sterile coolboxes with ice packs to BE accommodation. Surfaces and equipment
207 were sterilised before, during, and after set-up as in Experiment 1. Samples (*n* = 140) and field
208 blanks (*n* = 14) were vacuum-filtered within 4 hrs of collection as in Experiment 1 with minor
209 modifications to maximise detection probability as follows. The full 2 L of each sample was
210 vacuum-filtered where possible, two filters were used for each sample, and duplicate filters
211 were stored in one petri dish at -20 °C. A filtration blank (1 L MGW) was processed during each
212 filtration round (*n* = 21). The total water volume filtered per sample was recorded (Table A3).
213

214 **2.4 DNA extraction**

215

216 DNA was extracted within 2 weeks of filtration at the UoH eDNA facility using the Mu-DNA
217 water protocol (Sellers, Di Muri, Gómez, & Hänfling, 2018). Duplicate filters from samples in
218 Experiment 1 were lysed independently and the lysate from each loaded onto one spin column.
219 As more samples were collected in Experiment 2, duplicate filters were co-extracted by placing
220 both in a single tube for bead milling. An extraction blank, consisting only of extraction buffers,
221 was included for each round of DNA extraction (*n* = 17). Eluted DNA (100 µl) was stored at -20
222 °C until PCR amplification.
223

2.5 eDNA metabarcoding

Our eDNA metabarcoding workflow is fully described in Appendix A. Briefly, we performed nested metabarcoding using a two-step PCR protocol, where Multiplex Identification (MID) tags were included in the first and second PCR for sample identification (Kitson et al., 2019). The first PCR amplified eDNA in triplicate with published 12S ribosomal RNA (rRNA) primers 12S-V5-F (5'-ACTGGGATTAGATACCCC-3') and 12S-V5-R (5'-TAGAACAGGCTCCTCTAG-3') (Riaz et al., 2011). Harper et al. (2018) validated these primers *in silico* for UK vertebrates, and found 91/112 mammal species listed on the Natural History Museum Checklist of Mammalia v1 (subspecies excluded) could be distinguished. Nine indistinguishable species lacked reference sequences, whereas 12 had reference sequences but did not amplify. PCR positive controls (two per PCR plate; n = 16) were exotic cichlid (*Maylandia zebra*) DNA (0.05 ng/µl), and PCR negative controls (two per PCR plate; n = 16) were MGW (Fisher Scientific UK Ltd, UK). PCR products were pooled to create sub-libraries (Fig. A2) and purified. The second PCR bound pre-adapters, MID tags, and Illumina adapters to the sub-libraries. PCR products were purified, quantified, and pooled. The library was purified again, quantified by qPCR, and fragment size (330 bp) and removal of secondary product verified. The library (220 eDNA samples, 27 field blanks, 33 filtration blanks, 17 extraction blanks, 16 PCR negative controls, and 16 PCR positive controls) was sequenced on an Illumina MiSeq®. Raw sequence reads were demultiplexed using a custom Python script. metaBEAT v0.97.11 (https://github.com/HullUni-bioinformatics/metaBEAT) was used for quality trimming, merging, chimera removal, clustering, and taxonomic assignment of sequences against our UK vertebrate reference database (Harper et al., 2018) which contains

sequences for 103 UK mammals.

2.6 Data analysis

Analyses were performed in R v.3.4.3 (R Core Team, 2017). The total unrefined read counts (i.e. raw taxonomically assigned reads) per sample were calculated and retained for downstream analyses. Assignments were corrected and merged, taxon-specific sequence thresholds (i.e. maximum sequence frequency of each taxon in PCR positive controls) used to mitigate cross-contamination and false positives (Table A4, Fig. A3), and remnant contaminants and higher taxonomic assignments removed excluding the following genera. *Anas* (Dabbling ducks) was retained because potential for hybridisation reduced confidence in species-level assignments, and *Emberiza* (Buntings) and *Larus* (White-headed gulls) were retained because reference sequences were missing for several common species. Dataset refinement is fully described in Appendix A1. Taxonomic assignments remaining in the refined dataset were predominantly of species resolution and considered true positives. We split the refined dataset by Experiment 1 (artificial waterbodies) and Experiment 2 (natural ponds). Proportional read counts for each species were calculated from the total unrefined read counts per sample. Our proportional read count data were not normally distributed (Shapiro–Wilk normality test: $W = 0.915$, $P < 0.001$), thus we used a Mann-Whitney U test to compare the median proportional read count of stratified and directed samples across species.

We employed binomial Generalized Linear Mixed-effects Models (GLMMs) with the logit link function using the package glmmTMB (development version; Brooks et al., 2017) for the

following tests. First, we compared the eDNA signals from stratified and directed samples for each mammal species using a hierarchical model including sample type nested within species (fixed) and wildlife park (random) as effects. We tested the influence of species lifestyle on mammal eDNA signals using a model with species lifestyle (fixed) and species nested within wildlife park (random) as effects. Using directed samples, we tested the influence of behaviour on mammal eDNA signals using two hierarchical models, including species nested within wildlife park (random) and specific (e.g. swimming, drinking) or generic (i.e. water contact versus no water contact) behaviour(s) respectively (fixed) as effects. We assessed model fit using diagnostic plots and performed validation checks to ensure model assumptions were met and overdispersion was absent (Zuur, Ieno, Walker, Saveliev, & Smith, 2009).

For Experiment 2, we qualitatively compared mammal presence-absence records generated by eDNA metabarcoding, camera trapping, and field signs. TLNR ponds were sampled every 24 hrs for 5 days, thus proportional read counts were averaged across days for comparison to BE and TM ponds (sampled once each). We qualitatively compared the distribution and persistence of eDNA signals between semi-aquatic and terrestrial mammals using tile plots and heat maps of the unaveraged proportional read counts for identified species at TLNR over the 5-day period. All figures were produced using the package ggplot2 v3.0.0 (Wickham, 2016).

3. Results

3.1 eDNA metabarcoding

The sequencing run generated 47,713,656 raw sequence reads, of which 37,590,828 remained following trimming, merging, and length filter application. After removal of chimeras and redundancy via clustering, the library contained 21,127,061 sequences (average read count of 64,215 per sample including controls), of which 16,787,750 (79.46%) were assigned a taxonomic rank. Contamination (Fig. A4) was observed in the field blanks (badger, beaver, lynx, pine marten, red squirrel, and water vole) as well as in the filtration and extraction blanks (human [*Homo sapiens*] and cichlid). PCR negative controls were contaminated to different extents with human, cichlid, beaver, and pine marten as well as non-focal species. After threshold application, contaminants remaining in eDNA samples included Gentoo penguin (*Pygoscelis papua*), reindeer (*Rangifer tarandus*), cichlid, and human. The refined dataset contained 59 vertebrate species, including six amphibians, 10 fish, 19 birds, and 24 mammals (Table A5).

3.2 Experiment 1: eDNA detection and signal strength in artificial systems

All nine focal species were detected in captivity, of which seven were detected in all water samples taken from their respective enclosures. HWP red deer were not detected in 2 of 5

309 stratified samples, and WT hedgehog was not detected in 1 of 2 drinking bowl samples (Fig. 1).
310 'Other' samples (neither directed nor stratified) were excluded from further comparisons, thus
311 hedgehog, red squirrel, and water vole were omitted in downstream analyses. Across species,
312 stratified samples (0.406) had a higher median proportional read count than directed samples
313 (0.373), but this difference was not significant (Mann-Whitney *U* test: $U = 1181.5$, $P = 0.829$).
314 Proportional read counts for directed and stratified samples did not significantly differ ($\chi^2_6 =$
315 0.364, $P = 0.999$) within species either (Fig. 2a; GLMM: $\theta = 0.168$, $\chi^2_{53} = 8.915$, $P = 1.000$,
316 pseudo-$R^2 = 39.21\%$). Otter proportional read counts were lower than other species, but not
317 significantly so. Similarly, species lifestyle (semi-aquatic, ground-dwelling, arboreal) did not
318 influence ($\chi^2_2 = 0.655$, $P = 0.721$) proportional read counts (Fig. 2b; GLMM: $\theta = 0.213$, $\chi^2_{61} =$
319 13.002, $P = 1.000$, pseudo-$R^2 = 11.85\%$). Proportional read counts did not differ ($\chi^2_{11} = 1.369$, P
320 $= 0.999$) according to specific behaviours exhibited by species (Fig. 3a; GLMM: $\theta = 0.355$, $\chi^2_{31} =$
321 11.013, $P = 0.999$, pseudo-$R^2 = 9.17\%$). Likewise, generic behaviour (i.e. water contact versus no
322 water contact) did not influence ($\chi^2_{11} = 0.002$, $P = 0.964$) proportional read counts (Fig. 3b;
323 GLMM: $\theta = 0.217$, $\chi^2_{41} = 8.897$, $P = 1.000$, pseudo-$R^2 = 8.50\%$).
324

325 **3.3 Experiment 2: eDNA detection and signal strength in natural systems**

326

327 At natural ponds, eDNA metabarcoding, camera trapping, and field signs all detected beaver,
328 red deer, and roe deer. Camera traps (Fig. 4) and field signs recorded red fox and badger when
329 eDNA metabarcoding did not (Fig. 5). However, eDNA metabarcoding revealed small mammals
330 missed by cameras and field signs, including water vole, water shrew (*Neomys fodiens*), bank

331 vole (*Myodes glareolus*), common shrew (*Sorex araneus*), brown rat (*Rattus norvegicus*), rabbit,
332 grey squirrel, and common pipistrelle (*Pipistrellus pipistrellus*). We observed mice or vole
333 footprints at BE Pond 1, but could not ascertain species. Fig. 5 summarises mammals recorded
334 by different methods at each site with reference to cumulative survey data. Notably, only
335 beaver was found at the same ponds by all methods. Although methods shared species at site
336 level, species were not always detected at the same pond. Detection rates for species captured
337 by at least one survey method are summarised in Table A6.

338 Sampling of natural ponds revealed spatial patterns in eDNA detection and signal
339 strength. eDNA from non-domestic terrestrial mammals (i.e. mammals excluding dog [*Canis*
340 *lupus familiaris*], pig [*Sus scrofa domesticus*], sheep [*Ovis aries*] and cow [*Bos taurus*]) was
341 unevenly dispersed compared with semi-aquatic mammals (Fig. A5). Semi-aquatic beaver and
342 water vole were detected in at least 90% and 60% respectively of water samples (n = 10)
343 collected from single ponds, albeit water shrew was only detected in 10% of samples. Non-
344 domestic terrestrial mammals were routinely detected in <20% of water samples collected from
345 a pond and left relatively weak eDNA signals. Overall, beaver was the most consistently
346 detected mammal with the highest proportional read counts. However, the strongest and most
347 evenly distributed signals belonged to amphibians, particularly common frog (*Rana temporaria*)
348 and great crested newt (*Triturus cristatus*) (Fig. A5).

349 TLNR samples collected over a 5-day period (D01-05) revealed that mammal detection
350 heavily depends on the spatial and temporal resolution of eDNA metabarcoding surveys (Fig.
351 A6). Mammal eDNA signals in pond water were ephemeral, often disappearing within 24-48 hrs
352 of initial detection, as opposed to amphibians that were detected for multiple days and whose

353 eDNA signal increased in strength. The majority of semi-aquatic or terrestrial mammals were
354 only detected in a single sample on each day.
355
356
357 # 4. Discussion
358
359 We have demonstrated the potential of eDNA metabarcoding for monitoring conservation and
360 management priority mammals, but species detection rates are variable. Our experiments have
361 validated this molecular approach and provided new insights that will inform the development
362 and application of mammal eDNA metabarcoding. Sampling strategy, mammal lifestyle, and
363 mammal behaviour did not influence eDNA detection and signal strength in captivity, but all
364 played vital roles in natural ponds. Although mammals were detected from pond water, their
365 eDNA signals were temporary and weak in comparison to amphibians and fishes. Nonetheless,
366 this suggests that eDNA is representative of contemporary and local mammal diversity.
367
368 ## 4.1 Experimental insights
369
370 In Experiment 1, all nine focal species were detected in captivity, and seven were detected in all
371 water samples taken from their respective enclosures. This demonstrates that our method can
372 successfully detect a variety of mammals from pond and drinking water. Surprisingly, we found
373 that neither sampling strategy, mammal lifestyle or mammal behaviour influenced eDNA
374 detectability and signal strength in captivity. This included behaviours associated with eDNA

375 deposition, e.g. swimming, drinking, urination, and defecation (Rodgers & Mock, 2015; Ushio et
376 al., 2017; Williams et al., 2018). Enclosures were permanently occupied and artificial
377 waterbodies likely saturated with eDNA, which possibly masked behavioural signals. Modest
378 replication may have limited experimental power, preventing patterns being detected
379 statistically. Nonetheless, our results show that mammal contact with water enables eDNA
380 deposition and detection.

381 Unsurprisingly, given the nature of wild mammal interactions with natural systems
382 versus those in captivity, Experiment 2 results highlight the challenges of mammal eDNA
383 detection. We recorded 17 mammals using three monitoring tools, comparable to the 17
384 mammals expected from cumulative survey data despite discordance. Field signs and camera
385 trapping detected red fox and badger where eDNA metabarcoding did not, but eDNA
386 metabarcoding identified water vole and other small mammals missed on camera or with
387 ambiguous field signs, i.e. mice, voles, shrews. Importantly, camera trap deployment period,
388 height, and positioning may have influenced small mammal detection by this method
389 (Caravaggi et al., 2018). Ishige et al. (2017) achieved comparable mammal detection at salt licks
390 with eDNA metabarcoding and camera trapping, but species presence was inconsistent
391 between salt licks surveyed. Currently, no published studies have compared eDNA
392 metabarcoding of water to camera trapping for mammal identification. However, our results
393 reflect those of Stat et al. (2019) who found highest marine fish species richness using both
394 baited remote underwater video systems and eDNA metabarcoding. Each method identified
395 unique species, but eDNA metabarcoding detected smaller and cryptic taxa.

Notably, no survey method captured semi-aquatic otter despite presence at study sites and successful detection in eDNA metabarcoding studies of UK ponds (Harper et al., 2019) and lakes (Hänfling et al., 2017). Captive otter also had a weaker eDNA signal than other semi-aquatic mammals studied here. Lower eDNA detection rates for otter, badger, and red fox may stem from species' ecologies. These mammals are wide-ranging (Gaughran et al., 2018; Thomsen et al., 2012) and may not readily release DNA in water. Otters often spraint on grass or rock substrata outside water and use latrines associated with caves and dens (Ruiz-Olmo & Gosálbez, 1997). As terrestrial mammals, red fox and badger must drink from or enter ponds for eDNA deposition to occur (Rodgers & Mock, 2015; Ushio et al., 2017; Williams et al., 2018). Otter, badger, and red fox detection may require greater spatiotemporal resolution of eDNA sampling. This is reinforced by other eDNA metabarcoding studies where mammal detection was highly variable across sites surveyed (Ishige et al., 2017; Klymus et al., 2017; Ushio et al., 2017). False negatives may instead be symptomatic of metabarcoding bias, but this is unlikely in our study (section 4.2).

eDNA from other semi-aquatic mammals was evenly distributed, being found in most or all samples collected on fine spatial scales within natural ponds, whereas terrestrial mammal eDNA was highly localised and detected in few (<20%) samples. Mammal eDNA signals varied temporally, being detectable for two consecutive days maximum. Depending on the species, mammal eDNA may be spatially and temporally clumped in lentic ecosystems due to the nature and frequency of water contact. Unless non-domestic mammals exhibit behaviours involving prolonged water contact (e.g. swimming, wallowing), they may only be detected at drinking sites (Klymus et al., 2017; Ushio et al., 2017; Williams et al., 2018). Conversely, domestic

mammals may have elevated detection rates in ponds due to high occurrence of these waterbodies in agricultural landscapes as well as eDNA transport by rainfall and run-off (Staley et al., 2018). eDNA detection and persistence are further influenced by group size, where eDNA from multiple individuals endures for longer periods in water than eDNA from single individuals (Williams et al., 2018). Detailed investigations incorporating biotic (e.g. population size, body mass, behaviour) and abiotic (e.g. temperature, pH, rainfall) factors are needed to understand the longevity of mammal eDNA signals in aquatic ecosystems (Rodgers & Mock, 2015; Williams et al., 2018).

Our two experiments have shown that sampling strategy influences mammal eDNA detection. Mammal eDNA was evenly distributed in closed, artificial waterbodies, but locally distributed in open, natural ponds. Captive mammal enclosures contained one species (excluding HWP red deer) and a drinking container(s) and/or small waterbody (range 0.01-162 m^2, mean 27.4 m^2). Some enclosures housed more individuals of a species than others, thereby increasing eDNA deposition and detection probability (Williams et al., 2018). Wild mammals have an array of freshwater habitats at their disposal and can hold vast territories. Therefore, rates of pond visitation and eDNA deposition are more irregular (Klymus et al., 2017; Ushio et al., 2017), possibly leading to between-sample variation (Williams et al., 2018).

4.2 Technical pitfalls of eDNA metabarcoding for mammal monitoring

eDNA metabarcoding has potential for inclusion in mammal monitoring schemes (section 4.3), but like existing monitoring tools, may produce false negatives or false positives. Our process

440 controls identified low-level contamination at all stages of metabarcoding, but primarily during
441 sampling or PCR (Appendix A1). We applied taxon-specific sequence thresholds to our data to
442 mitigate false positives as in Harper et al. (2019). Remnant contaminants were cichlid
443 (laboratory), Gentoo penguin (environment), reindeer (environment), and human
444 (environment/laboratory). Gentoo penguin is housed at EZ and was identified from EZ beaver
445 enclosure water. The WT red squirrel and reindeer enclosures are in close proximity. DNA
446 transport by wildlife (e.g. waterfowl [Hänfling et al., 2016]) and park staff/visitors may explain
447 this environmental contamination. Human DNA was present across process controls
448 corresponding to artificial and natural waterbodies. Human DNA may be amplified and
449 sequenced instead of focal species, potentially resulting in false negative detections for rare
450 and/or less abundant species. Human DNA blocking primers can prevent this bias, but may
451 impair PCR amplification efficiency (Klymus et al., 2017; Ushio et al., 2017; Valentini et al.,
452 2016).

453 In our study, eDNA metabarcoding produced false negatives for otter, badger, and red
454 fox at natural ponds. We selected a 12S metabarcode designed to amplify vertebrate DNA (Riaz
455 et al., 2011). There were no primer mismatches with otter, badger or red fox reference
456 sequences (Harper et al., 2018), thus amplification bias was not responsible for these false
457 negatives. DNA from aquatic and more abundant species may have overwhelmed otter, badger,
458 and red fox DNA during amplification and sequencing, i.e. species-masking (Kelly, Port,
459 Yamahara, & Crowder, 2014; Klymus et al., 2017). Metabarcoding primers targeting mammals
460 (Ushio et al., 2017) or multi-marker (e.g. 12S, 16S, COI) investigations (Evans et al., 2017;
461 Hänfling et al., 2016; Kelly et al., 2014; Klymus et al., 2017) may improve mammal detection in

systems with competition from aquatic species. Similarly, more biological and technical replication may improve species detection probabilities (Evans et al., 2017; Lawson Handley et al., 2019; Stat et al., 2019; Valentini et al., 2016). Importantly, otter also had lower qPCR detection than amphibians and fish (Thomsen et al., 2012). A metabarcoding and qPCR comparison (e.g. Harper et al., 2018; Lacoursière-Roussel, Dubois, Normandeau, & Bernatchez, 2016) would confirm whether poor amplification efficiency for otter arises from technical bias or species ecology, and whether eDNA metabarcoding can reliably monitor otter alongside the wider mammalian community.

4.3 Scope of eDNA metabarcoding for mammal monitoring

Mammal population assessments are hindered by lack of data and systematic monitoring for many species (Mathews et al., 2018). Distribution and occupancy data are poor for most species, with ongoing survey effort biased toward rare species. Surveys heavily rely on citizen science and casual records (Massimino et al., 2018). Tools that provide standardised, systematic monitoring of mammal populations are needed (Mathews et al., 2018). Despite issues inherent to metabarcoding for biodiversity monitoring (Deiner et al., 2017), this tool has enormous potential to enhance mammal monitoring, conservation, and management. eDNA metabarcoding generates distribution data for multiple species, whether rare, invasive, or abundant, and could track conflicting species simultaneously, e.g. water vole, American mink, and otter (Bonesi & Macdonald, 2004) or red squirrel, grey squirrel, and pine marten (Sheehy, Sutherland, O'Reilly, & Lambin, 2018). Different sample types (e.g. water, soil, snow, salt licks,

feeding traces, faeces, hair, and blood meals) may also offer new insights to mammal biodiversity (Franklin et al., 2019; Ishige et al., 2017; Tessler et al., 2018; Ushio et al., 2017).

eDNA metabarcoding can rapidly survey multitudes of aquatic sites at landscape-scale where camera traps might be resource-intensive, cost-inefficient, and susceptible to theft/damage (Stat et al., 2019; Ushio et al., 2017). Field signs require volunteer time and skill (Sadlier et al., 2004) to be employed at comparable spatial scales to eDNA metabarcoding which could provide accurate data for species misidentified from field signs, e.g. mice and voles, otter and mink (Franklin et al., 2019; Harris & Yalden, 2004). However, camera traps and field signs both recorded species that eDNA metabarcoding missed. Therefore, eDNA metabarcoding is complementary and should be incorporated into, not replace, existing monitoring schemes. This tool could be most effective in mammal monitoring if deployed at the edges of known species distributions, in areas where species presence is unknown, and in areas with isolated species records (Mathews et al., 2018).

Mammal eDNA detection probabilities from natural ponds will likely be high when areas with dense populations are studied. However, rigorous sampling strategies are required to track mammals in areas sparsely populated by individuals. Multiple ponds must be sampled repeatedly, and samples taken at multiple locations within ponds without pooling to enable site occupancy inferences. Importantly, we sampled natural ponds in spring but sampling in other seasons may produce different results, reflective of species' ecologies (Lawson Handley et al., 2019). We recommend that researchers and practitioners using eDNA metabarcoding for mammal monitoring channel efforts into extensive sampling of numerous waterbodies in a given area over larger timescales. This will account for differential mammal visitation rates and

maximise eDNA detection probabilities.

Data accessibility

Raw sequence reads have been archived on the NCBI Sequence Read Archive (Study: SRP164740; BioProject: PRJNA495011; BioSamples: SAMN10195928 - SAMN10196255; SRA accessions: SRR7986451 - SRR7986778). Jupyter notebooks, R scripts and corresponding data are archived online (https://doi.org/10.5281/zenodo.2561415).

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Author contributions

L.R.H, B.H, and L.L.H conceived and designed the study. A.I.C and M.G coordinated sampling at Wildwood Trust and RZSS Highland Wildlife Park respectively. L.R.H, C.D.M, C.J.M, and T.L collected and filtered water samples. A.L, T.L, and T.B helped select natural ponds to be surveyed using eDNA, camera trapping, and field signs, and provided camera traps for the study. L.R.H, A.L, and T.L deployed camera traps, which were then collected and footage analysed by L.R.H. L.R.H processed samples in the laboratory with advice from C.D.M and A.M. D.S.R sequenced the final library. L.R.H completed bioinformatic processing of samples, and subsequent data analysis. L.R.H wrote the manuscript, which all authors contributed critically to drafts of and gave final approval for publication.

References

Bálint, M., Nowak, C., Márton, O., Pauls, S. U., Wittwer, C., Aramayo, J. L., Schulze, A., Chambert, T., Cocchiararo, B. & Jansen, M. (2018). Accuracy, limitations and cost efficiency of eDNA-based community survey in tropical frogs. *Molecular Ecology Resources*, *18*, 1415–1426. https://doi.org/10.1111/1755-0998.12934

Bland, L. M., Collen, B., Orme, C. D. L., & Bielby, J. (2015). Predicting the conservation status of data-deficient species: Predicting Extinction Risk. *Conservation Biology*, *29*, 250–259. https://doi.org/10.1111/cobi12372

Bonesi, L., & Macdonald, D. W. (2004). Differential habitat use promotes sustainable coexistence between the specialist otter and the generalist mink. *Oikos* , *106*, 509–519. https://doi.org/10.1111/j.0030-1299.2004.13034.x

Brooks, M. E., Kristensen, K., van Benthem, K. J., Magnusson, A., Berg, C. W., Nielsen, A., Skaug, H. J., Machler, M. & Bolker, B. M. (2017). glmmTMB Balances Speed and Flexibility Among Packages for Zero-inflated Generalized Linear Mixed Modeling. *The R Journal*, *9*, 378–400. https://doi.org/10.32614/RJ-2017-066

Burton, A. C., Neilson, E., Moreira, D., Ladle, A., Steenweg, R., Fisher, J. T., Bayne, E. & Boutin, S. (2015). REVIEW: Wildlife camera trapping: a review and recommendations for linking surveys to ecological processes. *Journal of Applied Ecology*, *52*, 675–685. https://doi.org/10.1111/1365-2664.12432

Caravaggi, A., Gatta, M., Vallely, M. -C., Hogg, K., Freeman, M., Fadaei, E., Dick, J. T. A., Montgomery, W. I., Reid, N. & Tosh, D. G. (2018). Seasonal and predator-prey effects on circadian activity of free-ranging mammals revealed by camera traps. *PeerJ*, *6*, e5827. https://doi.org/10.7717/peerj.5827

Deiner, K., Bik, H. M., Mächler, E., Seymour, M., Lacoursière-Roussel, A., Altermatt, F., Creer, S., Bista, I., Lodge, D. M., de Vere, N., Pfrender, M. E. & Bernatchez, L. (2017). Environmental DNA metabarcoding: transforming how we survey animal and plant communities. *Molecular Ecology*, *26*, 5872–5895. https://doi.org/10.1111/mec.14350

Evans, N. T., Li, Y., Renshaw, M. A., Olds, B. P., Deiner, K., Turner, C. R., Jerde, C. L., Lodge, D. M., Lamberti, G. A. & Pfrender, M. E (2017). Fish community assessment with eDNA metabarcoding: effects of sampling design and bioinformatic filtering. *Canadian Journal of Fisheries and Aquatic Sciences*, *74*, 1362–1374. https://doi.org/10.1139/cjfas-2016-0306

Franklin, T. W., McKelvey, K. S., Golding, J. D., Mason, D. H., Dysthe, J. C., Pilgrim, K. L., Squires, J. R., Aubry, K. B., Long, R. A., Greaves, S. E., Raley, C. M., Jackson, S., MacKay, P., Lisbon, J., Sauder, J. D., Pruss, M. T., Heffington, D. & Schwartz, M. K (2019). Using environmental DNA methods to improve winter surveys for rare carnivores: DNA from snow and improved noninvasive techniques. *Biological Conservation*, *229*, 50–58. https://doi.org/10.1016/j.biocon.2018.11.006

Gaughran, A., Kelly, D. J., MacWhite, T., Mullen, E., Maher, P., Good, M., & Marples, N. M. (2018). Super-ranging. A new ranging strategy in European badgers. *PLoS ONE*, *13*, e0191818. https://doi.org/10.1371/journal.pone.0191818

586 Hänfling, B., Lawson Handley, L., Read, D. S., Hahn, C., Li, J., Nichols, P., Blackman, R. C., Oliver,
587 A. & Winfield, I. J. (2016). Environmental DNA metabarcoding of lake fish communities
588 reflects long-term data from established survey methods. *Molecular Ecology*, *25*, 3101–3119.
589 https://doi.org/10.1111/mec.13660
590 Hänfling, B., Lawson Handley, L., Lunt, D., Shum, P., Winfield, I., & Read, D. S. (2017). *A Review*
591 *of Recent Advances in Genetic Methods to Identify Improvements in CAMERAS Partners*
592 *Monitoring Activities*. University of Hull, Hull, UK.
593 Harper, L. R., Lawson Handley, L., Hahn, C., Boonham, N., Rees, H. C., Gough, K. C., Lewis, E.,
594 Adams, I. P., Brotherton, P., Phillips, S. & Hänfling, B. (2018). Needle in a haystack? A
595 comparison of eDNA metabarcoding and targeted qPCR for detection of the great crested
596 newt (*Triturus cristatus*). *Ecology and Evolution*, *8*, 6330–6441.
597 https://doi.org/10.1002/ece3.4013
598 Harper, L.R., Handley, L.L., Hahn, C., Boonham, N., Rees, H.C., Lewis, E., Adams, I.P., Brotherton,
599 P., Phillips, S. & Hänfling, B. (2019) Testing ecological hypotheses at the pondscape with
600 environmental DNA metabarcoding: a case study on a threatened amphibian. *bioRxiv*,
601 278309. https://doi.org/10.1101/278309
602 Harris, S., & Yalden, D. W. (2004). An integrated monitoring programme for terrestrial mammals
603 in Britain. *Mammal Review*, *34*, 157–167. https://doi.org/10.1046/j.0305-1838.2003.00030.x
604 Ishige, T., Miya, M., Ushio, M., Sado, T., Ushioda, M., Maebashi, K., Yonechi, R., Lagan, P. &
605 Matsubayashi, H. (2017). Tropical-forest mammals as detected by environmental DNA at
606 natural saltlicks in Borneo. *Biological Conservation*, *210*, 281–285.
607 https://doi.org/10.1016/j.biocon.2017.04.023
608 Joint Nature Conservation Committee (2018) UK BAP priority terrestrial mammal species.
609 Accessed on 1st December 2018. http://jncc.defra.gov.uk/page-5170
610 Kelly, R. P., Port, J. A., Yamahara, K. M., & Crowder, L. B. (2014). Using environmental DNA to
611 census marine fishes in a large mesocosm. *PLoS ONE*, *9*, e86175.
612 https://doi.org/10.1371/journal.pone.0086175
613 Kitson, J. J. N., Hahn, C., Sands, R. J., Straw, N. A., Evans, D. M., & Lunt, D. H. (2019). Detecting
614 host-parasitoid interactions in an invasive Lepidopteran using nested tagging DNA-
615 metabarcoding. *Molecular Ecology*, *28*, 471–483. https://doi.org/10.1111/mec.14518
616 Klymus, K. E., Richter, C. A., Thompson, N., & Hinck, J. E. (2017). Metabarcoding of
617 Environmental DNA Samples to Explore the Use of Uranium Mine Containment Ponds as a
618 Water Source for Wildlife. *Diversity*, *9*, 54. https://doi.org/10.3390/d9040054
619 Lacoursière-Roussel, A., Dubois, Y., Normandeau, E., & Bernatchez, L. (2016). Improving
620 herpetological surveys in eastern North America using the environmental DNA method.
621 *Genome*, *59*, 991–1007. https://doi.org/10.1139/gen-2015-0218

Lawson Handley, L., Read, D.S., Winfield, I.J., Kimbell, H., Johnson, H., Li, J., Hahn, C., Blackman, R., Wilcox, R., Donnelly, R., Szitenberg, A. & Hänfling, B. (2019) Temporal and spatial variation in distribution of fish environmental DNA in England's largest lake. *Environmental DNA*, **12**, e0176343. https://doi.org/10.1002/edn3.5

Lugg, W. H., Griffiths, J., van Rooyen, A. R., Weeks, A. R., & Tingley, R. (2017). Optimal survey designs for environmental DNA sampling. *Methods in Ecology and Evolution*, *9*, 1049–1059. https://doi.org/10.1111/2041-210X.12951

Massimino, D., Harris, S. J., & Gillings, S. (2018). Evaluating spatiotemporal trends in terrestrial mammal abundance using data collected during bird surveys. *Biological Conservation*, *226*, 153–167. https://doi.org/10.1016/j.biocon.2018.07.026

Mathews, F., Kubasiewicz, L. M., Gurnell, J., Harrower, C. A., McDonald, R. A. & Shore, R. F. (2018) *A Review of the Population and Conservation Status of British Mammals: Technical Summary*. Natural England, Peterborough.

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*, e145. https://doi.org/10.1093/nar/gkr732

Rodgers, T. W., & Mock, K. E. (2015). Drinking water as a source of environmental DNA for the detection of terrestrial wildlife species. *Conservation Genetics Resources*, *7*, 693–696. https://doi.org/10.1007/s12686-015-0478-7

Ruiz-Olmo, J., & Gosálbez, J. (1997). Observations on the sprainting behaviour of the otter *Lutra lutra* in the NE Spain. *Acta Theriologica*, *42*, 259–270.

Sadlier, L. M. J., Webbon, C. C., Baker, P. J., & Harris, S. (2004). Methods of monitoring red foxes Vulpes vulpes and badgers Meles meles: are field signs the answer? *Mammal Review*, *34*, 75–98. https://doi.org/10.1046/j.0305-1838.2003.00029.x

Sellers, G. S., Di Muri, C., Gómez, A., & Hänfling, B. (2018). Mu-DNA: a modular universal DNA extraction method adaptable for a wide range of sample types. *Metabarcoding and Metagenomics*, *2*, e24556. https://doi.org/10.3897/mbmg.2.24556

Sheehy, E., Sutherland, C., O'Reilly, C. & Lambin, X. (2018) The enemy of my enemy is my friend: native pine marten recovery reverses the decline of the red squirrel by suppressing grey squirrel populations. *Proceedings of the Royal Society of London B*, *285*, 20172603. https://doi.org/10.1098/rspb.2017.2603

Staley, Z. R., Chuong, J. D., Hill, S. J., Grabuski, J., Shokralla, S., Hajibabaei, M., & Edge, T. A. (2018). Fecal source tracking and eDNA profiling in an urban creek following an extreme rain event. *Scientific Reports*, *8*, 14390. https://doi.org/10.1038/s41598-018-32680-z

Stat, M., John, J., DiBattista, J. D., Newman, S. J., Bunce, M. & Harvey, E. S. (2019) Combined use of eDNA metabarcoding and video surveillance for the assessment of fish biodiversity. *Conservation Biology*, *33*, 196–205. https://doi.org/10.1111/cobi.13183

Tessler, M., Weiskopf, S. R., Berniker, L., Hersch, R., Mccarthy, K. P., Yu, D. W., & Siddall, M. E. (2018). Bloodlines: mammals, leeches, and conservation in southern Asia. *Systematics and Biodiversity*, *16*, 488–496. https://doi.org/10.1080/14772000.2018.1433729

Thomsen, P. F., Iversen, L. L., Wiuf, C., Rasmussen, M., Gilbert, M. T. P., Orlando, L., & Willerslev, E. (2012). Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology*, *21*, 2565–2573. https://doi.org/10.1111/j.1365-294X.2011.05418.x

Thomsen, P. F., & Willerslev, E. (2015). Environmental DNA–an emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation*, *183*, 4–18. https://doi.org/10.1016/j.biocon.2014.11.019

Ushio, M., Fukuda, H., Inoue, T., Makoto, K., Kishida, O., Sato, K., Murata, K., Nikaido, M., Sado, T., Sato, Y., Takeshita, M., Iwasaki, W., Yamanaka, H., Kondoh, M. & Miya, M. (2017). Environmental DNA enables detection of terrestrial mammals from forest pond water. *Molecular Ecology Resources*, *17*(6), e63–e75. https://doi.org/10.1111/1755-0998.12690

Valentini, A., Taberlet, P., Miaud, C., Civade, R., Herder, J., Thomsen, P. F., Bellemain, E., Besnard, A., Coissac, E., Boyer, F., Gaboriaud, C., Jean, P., Poulet, N., Roset, N., Copp, G. H., Geniez, P., Pont, D., Argillier, C., Baudoin, J. -M., Peroux, T., Crivelli, A. J., Olivier, A., Acqueberge, M., Le Brun, M., Møller, P. R., Willerslev, E. & Dejean, T. (2016). Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. *Molecular Ecology*, *25*, 929–942. https://doi.org/10.1111/mec.13428

Visconti, P., Pressey, R. L., Giorgini, D., Maiorano, L., Bakkenes, M., Boitani, L., Alkemade, R., Falcucci, A., Chiozza, F. & Rondinini, C. (2011). Future hotspots of terrestrial mammal loss. *Philosophical Transactions of the Royal Society B*, *366*, 2693–2702. https://doi.org/10.1098/rstb.2011.0105

Wickham, H. (2016). *ggplot2: elegant graphics for data analysis*. Springer, New York, USA. http://ggplot2.org

Williams, K. E., Huyvaert, K. P., Vercauteren, K. C., Davis, A. J., & Piaggio, A. J. (2018). Detection and persistence of environmental DNA from an invasive, terrestrial mammal. *Ecology and Evolution*, *8*, 688–695. https://doi.org/10.1002/ece3.3698

Zuur, A. F., Ieno, E. N., Walker, N., Saveliev, A. A. & Smith, G. M. (2009) *Mixed effects models and extensions in ecology with R*. Springer, New York, USA.

Table 1. Summary of focal species studied at wildlife parks and their lifestyle. The number of individuals present and waterbody size in enclosures is provided.

Site	Species	Lifestyle	Enclosure	Number of individuals	Waterbody size (m^2)
Wildwood Trust	European otter (*Lutra lutra*)	Semi-aquatic	1	2	162
	European water vole (*Arvicola amphibius*)	Semi-aquatic	1	4	0.09
			2	1	0.09
	European beaver (*Castor fiber*)	Semi-aquatic	1	2	100
			2	1	100
	European hedgehog (*Erinaceus europaeus*)	Ground-dwelling	1	1	0.04
			2	2	0.04
	European badger (*Meles meles*)	Ground-dwelling	1	4	1.73
	Red deer (*Cervus elaphus*)	Ground-dwelling	1	8	100
	Eurasian lynx (*Lynx lynx*)	Ground-dwelling	1	2	2
	Red squirrel (*Sciurus vulgaris*)	Arboreal	1	2	0.01
			2	3	0.01
			3	3	0.01
			4	2	0.01
	European pine marten (*Martes martes*)	Arboreal	1	1	2
			2	1	0.375
Highland Wildlife Park	Red squirrel (*Sciurus vulgaris*)	Arboreal	NA	NA	0.25
	Eurasian lynx (*Lynx lynx*)	Ground-dwelling	1	8	2
	European beaver (*Castor fiber*)	Semi-aquatic	1	2	50
	Red deer (*Cervus elaphus*)	Ground-dwelling	1	30	NA

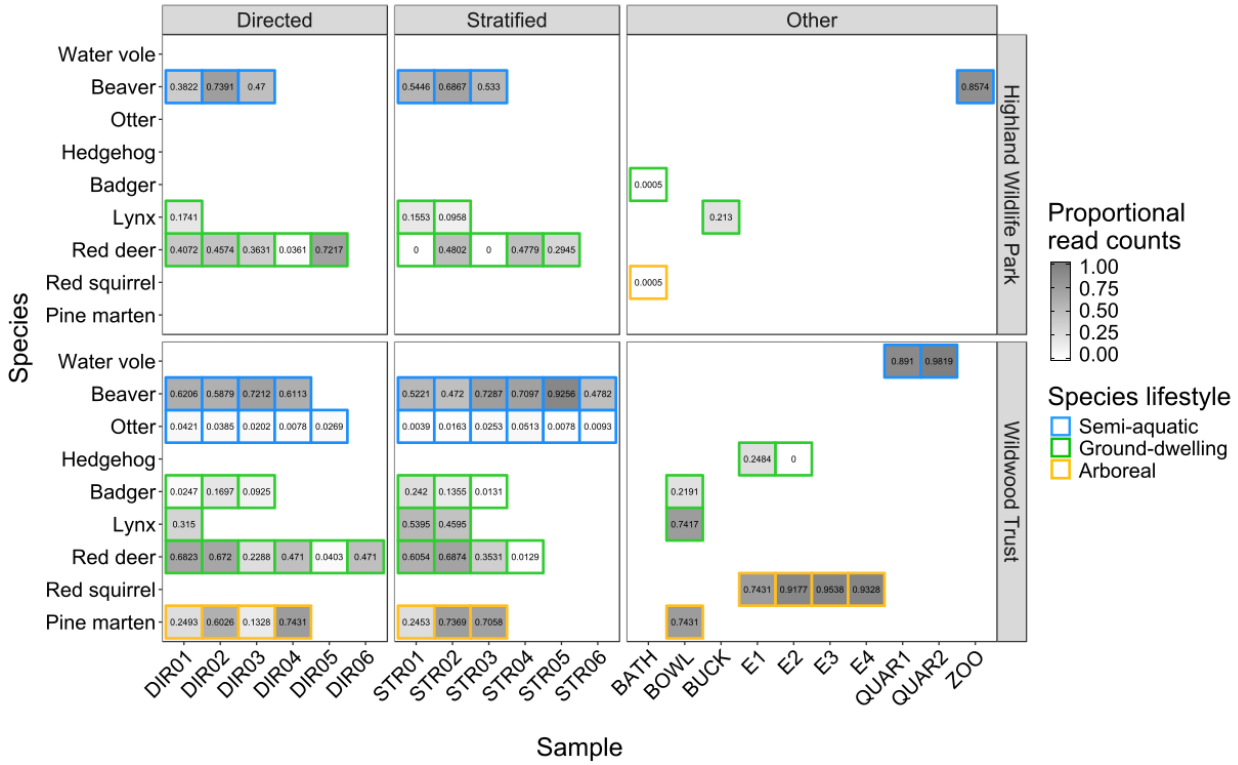


Figure 1. Heatmap showing proportional read counts for eDNA samples. Each square represents a sample taken from a wildlife park enclosure. Directed (DIR01-DIR06) and stratified (STR01-STR06) samples were collected from artificial waterbodies. Samples were collected from drinking containers (E1, E2, E3, E4, BOWL, BUCK), water vole (QUAR1, QUAR2) and RZSS Edinburgh Zoo beaver (ZOO) enclosures, and a water bath (BATH) in RZSS Highland Wildlife Park woods.

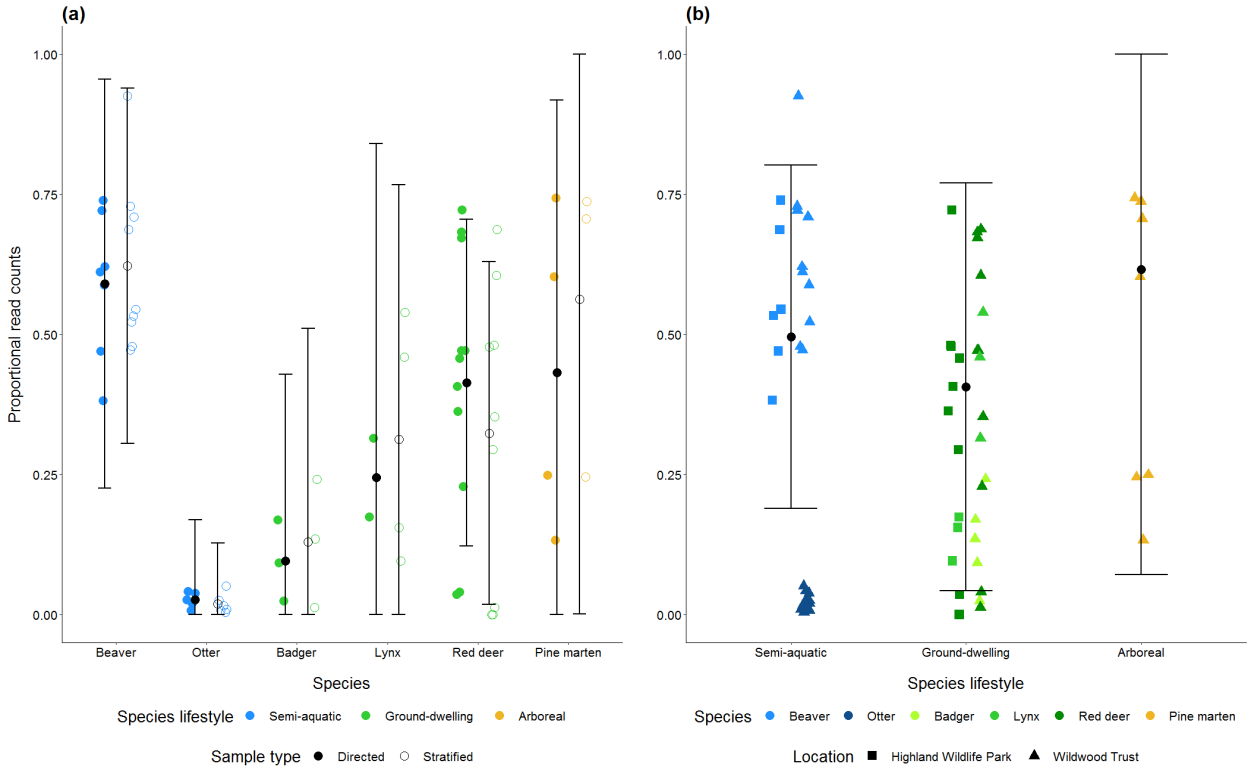


Figure 2. Relationships predicted by the binomial GLMMs between proportional read counts and sample type nested within species **(a)** or species lifestyle **(b)**. The observed data (coloured points) are displayed against the predicted relationships (black points with error bars) for each species **(a)** or species lifestyle **(b)**. Points are shaped by sample type **(a)** or wildlife park **(b)**, and coloured by species lifestyle. Error bars represent the standard error around the predicted means.

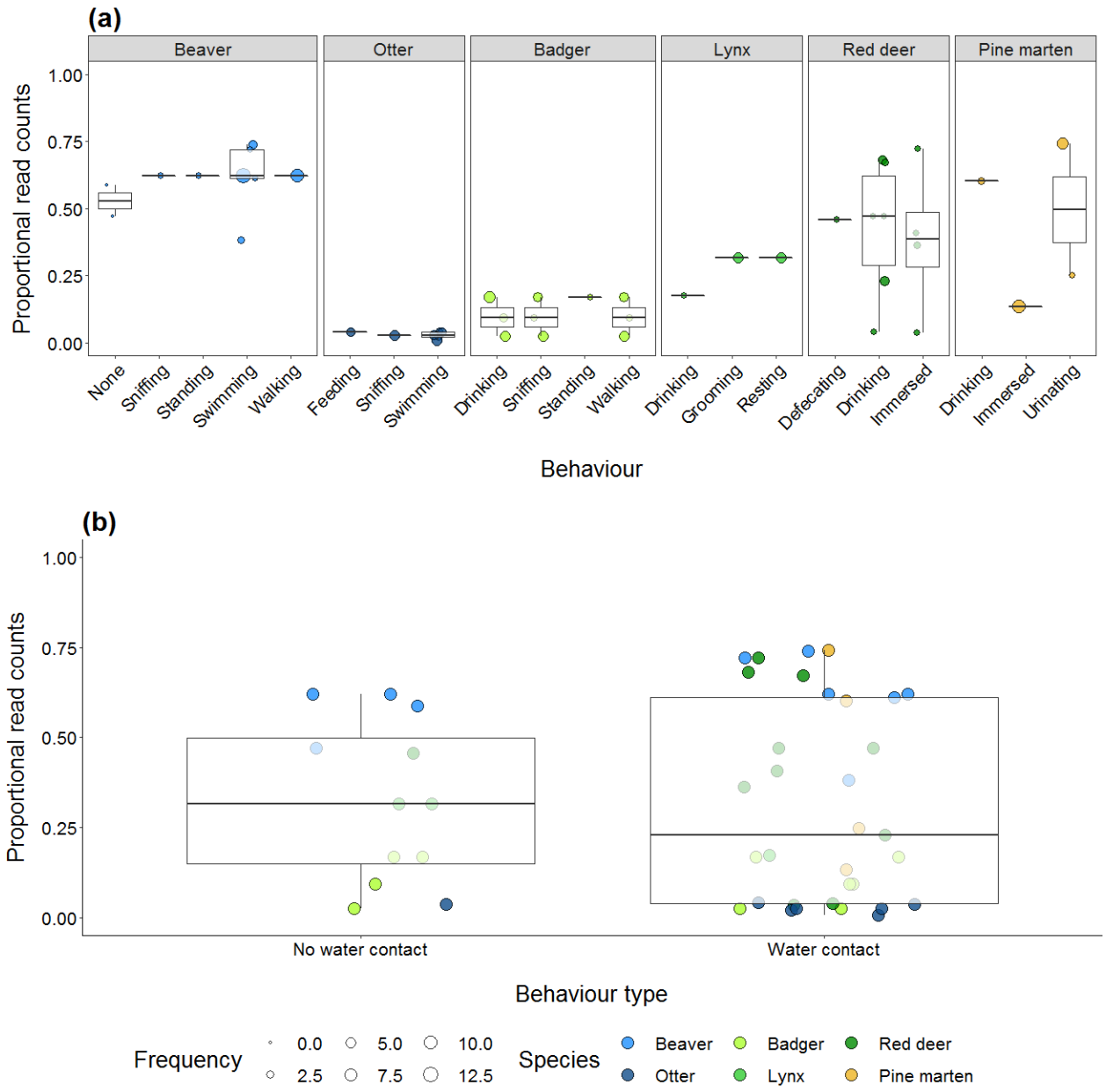
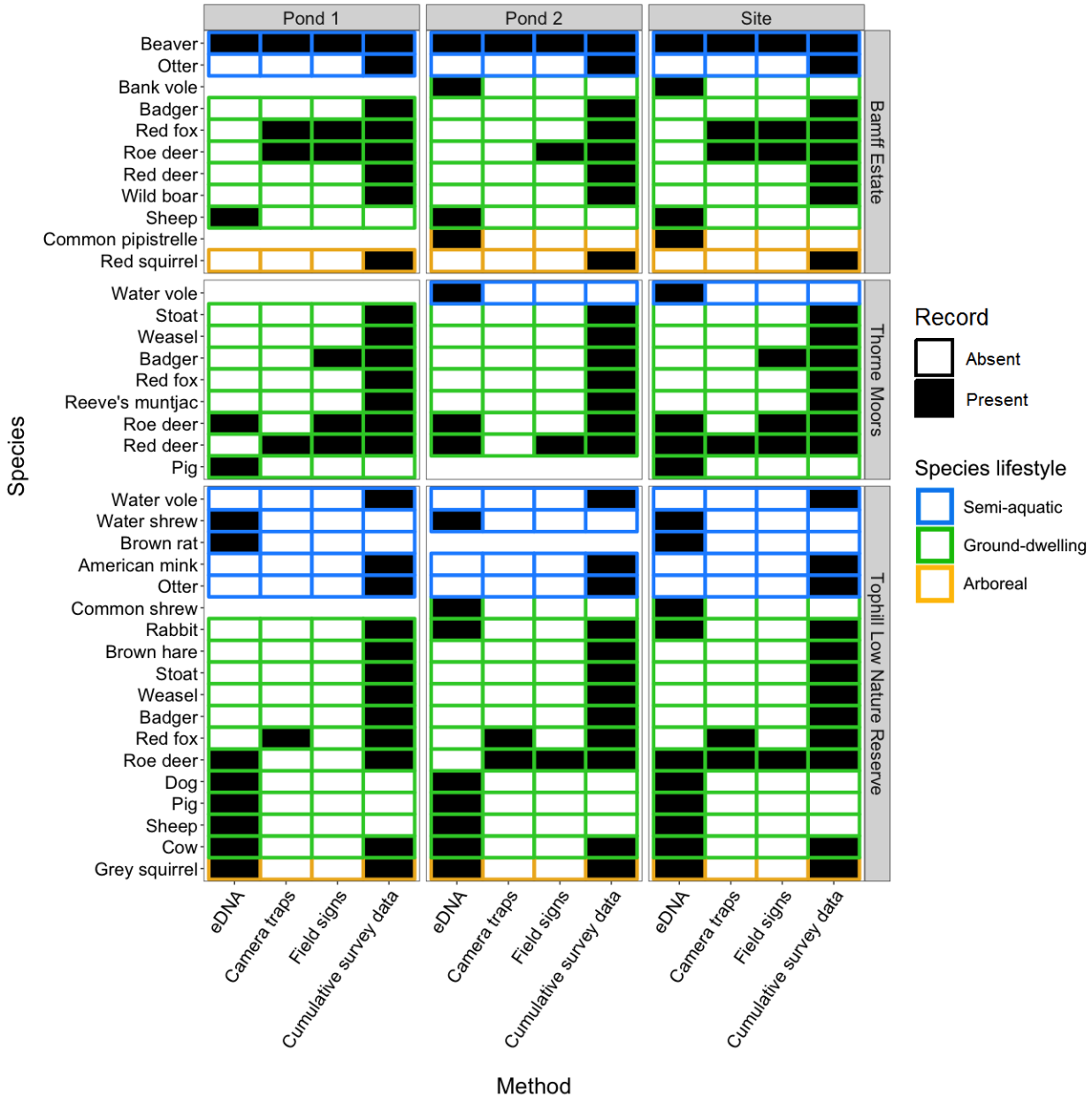


Figure 3. Boxplots showing the mean proportional read counts for specific **(a)** and generic **(b)** behaviour(s) exhibited by focal species. Boxes show 25th, 50th, and 75th percentiles, and whiskers show 5th and 95th percentiles. Points are coloured by species lifestyle, and each point in **(a)** represents a directed sample sized by frequency of behaviour. The behaviour 'none' for beaver represents occurrences of beaver in water but out of view of camera traps.

Figure 4. Exemplar camera trap photos taken at natural ponds where focal species were present. Red deer was recorded at Thorne Moors **(a)**, roe deer **(b)** and red fox **(c)** were recorded at Tophill Low Nature Reserve, and beaver was recorded at the Bamff Estate **(d)**.



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724 **Figure 5.** Tile plot showing species presence-absence at individual pond and site-level as

725 indicated by field signs, camera trapping, and eDNA metabarcoding at sites with focal species

726 presence confirmed by cumulative survey data.

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