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

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Abstract

1. Environmental DNA (eDNA) metabarcoding is largely used to survey aquatic communities, but can also provide data on terrestrial taxa utilising aquatic habitats. However, the entry, dispersal, and detection of terrestrial species' DNA within waterbodies is understudied.
2. We evaluated eDNA metabarcoding of pond water for monitoring semi-aquatic, ground-dwelling, and arboreal mammals, and examined spatiotemporal variation in mammal eDNA signals using experiments in captive and wild conditions.
3. We selected nine focal species of conservation and management concern: European water vole, European otter, Eurasian beaver, European hedgehog, European badger, red deer, Eurasian lynx, red squirrel, and European pine marten. We hypothesised that eDNA signals (i.e. proportional read counts) would be stronger for semi-aquatic than terrestrial species, and at sites where mammals exhibited behaviours (e.g. swimming, urination). We tested this by sampling waterbodies in enclosures of captive focal species at specific sites where behaviours had been observed ('directed' sampling) and at equidistant intervals along the shoreline ('stratified' sampling). We then 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.
4. All focal species were detected in captivity. eDNA signal strength did not differ between directed and stratified samples across or within species, between species lifestyles (i.e. semi-aquatic, ground-dwelling, arboreal), or according to behaviours. Therefore, eDNA was evenly distributed within artificial waterbodies. Conversely, eDNA was unevenly distributed

in natural ponds. eDNA metabarcoding, camera trapping, and field signs detected beaver, red deer, and roe deer. Badger and red fox were recorded with cameras and field signs, but not eDNA metabarcoding. However, eDNA metabarcoding detected small mammals missed by cameras and field signs, e.g. water vole. Terrestrial mammal eDNA signals were weaker and detected in fewer samples than semi-aquatic mammal eDNA signals.

5. eDNA metabarcoding has potential for inclusion in mammal monitoring schemes by enabling large-scale, multi-species distribution assessment for priority and difficult to survey species, and could provide early indication of range expansions or contractions. However, eDNA surveys need high spatiotemporal resolution and metabarcoding biases require further investigation before this tool is routinely implemented.

Key-words: camera traps, environmental DNA (eDNA), field signs, lentic, monitoring, semi-aquatic mammals, terrestrial mammals

1. Introduction

Mammals are among the most threatened vertebrate groups globally, with at least 25% of species at risk of extinction due to harvesting, habitat degradation/loss, non-native species or perception as pests (Visconti et al., 2011). Lack of data prevents robust estimation of mammalian range expansions or declines and population trends (Bland, Collen, Orme, & Bielby, 2015). Most species lack long-term, systematic monitoring, with survey efforts biased towards rare species (Massimino, Harris, & Gillings, 2018). Consequently, there is a need for more effective and evidence-based strategies for mammal conservation and management (Battersby & Tracking Mammals Partnership, 2005; Mathews et al., 2018).

Many mammals are nocturnal and elusive thus monitoring often requires non-invasive, observational methods, such as 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. However, camera traps can only survey a fraction of large, heterogeneous landscapes, trap placement can substantially influence species detection probabilities and community insights, 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 (i.e. time, volunteers) for broad geographic coverage (Sadlier et al., 2004). Some species also have similar footprints and scat, increasing the potential for misidentification (Franklin et al., 2019; Harris & Yalden, 2004). Mammal survey methods are usually species-specific, and multiple methods are necessary for large-scale, multi-species monitoring schemes (Massimino et al., 2018).

80 Environmental DNA (eDNA) analysis is a recognised tool for rapid, non-invasive, cost-
81 efficient biodiversity assessment across a wide range of aquatic and terrestrial habitats (Deiner
82 et al., 2017). Organisms transfer genetic material to their environment via secretions,
83 excretions, gametes, blood, or decomposition, which can be isolated from environmental
84 samples (Thomsen & Willerslev, 2015). Studies using eDNA analysis for semi-aquatic and
85 terrestrial mammal detection have largely employed PCR or quantitative PCR (qPCR) to target
86 single species (Aylward, Johnson, Sullivan, Perry, & Louis, 2018; Franklin et al., 2019; Lugg,
87 Griffiths, van Rooyen, Weeks, & Tingley, 2017; Padgett-Stewart et al., 2016; Rodgers & Mock,
88 2015; Thomsen et al., 2012; Williams, Huyvaert, Vercauteren, Davis, & Piaggio, 2018).
89 Alternatively, eDNA metabarcoding can screen entire communities using PCR combined with
90 high-throughput sequencing (Deiner et al., 2017; Thomsen & Willerslev, 2015), but such
91 assessments of mammal communities are less common (Klymus, Richter, Thompson, & Hinck,
92 2017; Ushio et al., 2017). Mammal assemblages have been obtained by metabarcoding
93 invertebrate blood meals (e.g. Tessler et al., 2018) and salt licks (Ishige et al., 2017) in tropical
94 habitats, but samples from the physical environment have tremendous potential to reveal
95 mammal biodiversity over broad spatiotemporal scales (Ushio et al., 2017).

96 In aquatic ecosystems, eDNA metabarcoding has predominantly been applied to
97 characterise fish (e.g. Evans et al., 2017; Hänfling et al., 2016; Lawson Handley et al., 2018;
98 Valentini et al., 2016) and amphibian (e.g. Bálint et al., 2018; Valentini et al., 2016)
99 communities. However, mammals also leave eDNA signatures in water that metabarcoding can
100 detect (Harper et al., 2018a; Klymus et al., 2017; Ushio et al., 2017). Ponds in particular provide
101 drinking, foraging, dispersive, and reproductive opportunities for semi-aquatic and terrestrial

102 mammals (Klymus et al., 2017). Therefore, samples from these waterbodies could uncover
103 biodiversity present in the wider environment (Deiner et al., 2017; Harper et al., 2018a).
104 Drinking is a major source of eDNA deposition in water due to the release of saliva, but
105 mammals may also swim, wallow, urinate or defecate in water (Rodgers & Mock, 2015; Ushio et
106 al., 2017; Williams et al., 2018). Furthermore, arboreal mammals may use ponds less than semi-
107 aquatic and ground-dwelling species, non-territorial mammals may visit ponds less than
108 territorial species, and group-living species may leave more DNA in water than solitary species
109 (Williams et al., 2018). Despite evidence for eDNA deposition by semi-aquatic and terrestrial
110 mammals in freshwater systems, little is known about the influence of mammal behaviour on
111 the distribution and strength of the eDNA signal left behind.

112 In this study, we conducted two experiments to evaluate eDNA metabarcoding of pond
113 water as a tool for monitoring semi-aquatic, ground-dwelling, and arboreal mammals of
114 conservation or management concern. The first experiment, carried out on nine focal species
115 housed at two wildlife parks, examined the role of sampling strategy, mammal lifestyle, and
116 mammal behaviour on eDNA detection and signal strength. We hypothesised that: (1) eDNA
117 would be unevenly distributed, thus directed sampling would yield stronger eDNA signals (i.e.
118 higher read counts) for mammals than stratified sampling; (2) semi-aquatic mammals would
119 have stronger eDNA signals than ground-dwelling or arboreal mammals; and (3) mammal
120 behaviours involving water contact (e.g. swimming, drinking) would be associated with stronger
121 eDNA signals. The second experiment validated eDNA metabarcoding against camera trapping
122 and field sign searches at natural ponds within sites where focal species were present. We
123 hypothesised that: (1) eDNA metabarcoding would detect more mammals than camera

124 trapping or field signs; (2) semi-aquatic mammals would be readily detected and their eDNA
125 evenly distributed in ponds in comparison to terrestrial mammals; and (3) temporal sampling
126 would reveal that terrestrial mammal eDNA is detectable for short periods in comparison to
127 fully aquatic vertebrates.
128
129

130 2. Materials and methods

131

132 2.1 Study species

133

134 We studied nine mammal species that are the focus of European conservation or management
135 (Battersby & Tracking Mammals Partnership, 2005; Mathews et al., 2018): European water vole
136 (*Arvicola amphibius*), European otter (*Lutra lutra*), Eurasian beaver (*Castor fiber*), European
137 hedgehog (*Erinaceus europaeus*), European badger (*Meles meles*), red deer (*Cervus elaphus*),
138 Eurasian lynx (*Lynx lynx*), red squirrel (*Sciurus vulgaris*), and European pine marten (*Martes*
139 *martes*). Water vole, otter, red squirrel, pine marten and hedgehog are UK Biodiversity Action
140 Plan species (Joint Nature Conservation Committee, 2018). Water vole, otter, and beaver are
141 semi-aquatic, red squirrel and pine marten are arboreal, and the other species are ground-
142 dwelling. Badger and red deer live in groups whereas the other species are predominantly
143 solitary.
144

145 ## 2.2 Experiment 1: eDNA detection and signal strength in artificial systems

146

147 We performed eDNA metabarcoding for focal species under artificial conditions at two wildlife
148 parks to investigate whether mammal eDNA signal strength (i.e. read counts) depends on
149 sampling strategy, species lifestyle (semi-aquatic, ground-dwelling, arboreal), and behaviour.
150 Behavioural observation and eDNA sampling were conducted between 18/09/2017 –
151 21/09/2017 at Wildwood Trust (WT), Kent, England, and 10/10/2017 – 11/10/2017 at Royal
152 Zoological Society of Scotland (RZSS) Highland Wildlife Park (HWP), Kingussie, Scotland. Sixteen
153 categories of behaviour were defined based on potential contact with waterbodies and species
154 lifestyle, and the frequency and duration of behaviours recorded (Table S1). The number of
155 individuals in each enclosure was recorded alongside waterbody size (Table 1). Beaver, lynx, red
156 deer, and red squirrel were present at both wildlife parks, whereas other captive species were
157 only present at WT. Each species was observed for one hour on two separate occasions except
158 nocturnal mammals (badger and beaver), which were observed overnight using camera traps
159 (Bushnell Trophy Cam Aggressor). One camera trap per enclosure was positioned perpendicular
160 to the ground (1 m height, 2 m from pond shoreline) to capture water and shoreline. Cameras
161 took 30 s videos (1920 x 1080) when triggered (30 s interval between triggers) at high
162 sensitivity. Behavioural observation was not undertaken for WT water voles as animals were
163 under quarantine or HWP red squirrels as individuals were wild.

164 Water samples were collected from enclosures within 3 hrs of the second behavioural
165 observation period. Up to six directed or stratified samples were collected, but sample number
166 varied by species according to waterbody size and observed behaviours (Tables S1, S2).

167 Enclosure drinking containers were also sampled and classed as 'other' samples. Bathing and
168 drinking bowls were sampled where enclosures contained no artificial waterbodies (WT water
169 vole, red squirrel, and hedgehog). The HWP beaver enclosure was empty for 24 hrs before
170 sampling. Water was sampled from an enclosure containing beavers at RZSS Edinburgh Zoo (EZ)
171 and classed as 'other'. A sample was collected from a water bath in the HWP woods to capture
172 wild red squirrels and classed as 'other'.

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

182 Samples and blanks were vacuum-filtered within 6 hrs of collection in a UoK wet
183 laboratory and within 24 hrs of collection in an EZ staff room. Surfaces and equipment were
184 sterilised before, during, and after set-up in temporary work areas. Surfaces and vacuum
185 pumps were wiped with 10% v/v chlorine-based commercial bleach (Elliott Hygiene Ltd, UK)
186 solution. Non-electrical equipment was immersed in 10% bleach solution for 10 minutes,
187 followed by 5% v/v MicroSol detergent (Anachem, UK), and rinsed with purified water. Up to
188 500 ml of each 2 L sample was vacuum-filtered through sterile 0.45 µm mixed cellulose ester

membrane filters with pads (47 mm diameter; Whatman, GE Healthcare, UK) using Nalgene™ filtration units. One hour was allowed for each sample to filter and a second filter used if clogging occurred. A filtration blank (1 L MGW) was processed during each filtration round (*n* = 12), and equipment sterilised after each filtration round. After 500 ml had filtered or one hour had passed, filters were removed from pads using sterile tweezers, placed in sterile 47 mm petri dishes (Fisher Scientific UK Ltd, UK), sealed with parafilm (Sigma-Aldrich Company Ltd, UK), and stored at -20 °C. The total water volume filtered per sample was recorded for downstream analysis (Table S2; Fig. S1).

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

We performed eDNA metabarcoding for focal species at natural ponds in conjunction with conventional surveys to validate this molecular approach for mammal identification and to investigate spatiotemporal variation in mammal eDNA signals. At three sites where focal species were present based on cumulative survey data, we selected two ponds (range 293-5056 m^2, average 1471 m^2) within 4 km of each other. The Bamff Estate (BE), Alyth, Scotland, was selected for beaver, otter, badger, red deer, and red squirrel, but roe deer (*Capreolus capreolus*) and red fox (*Vulpes vulpes*) were also present. Otter, water vole, and badger were present at Tophill Low Nature Reserve (TLNR), Driffield, East Yorkshire, alongside American mink (*Neovison vison*), stoat (*Mustela erminea*), weasel (*Mustela nivalis*), rabbit (*Oryctolagus cuniculus*), brown hare (*Lepus europaeus*), red fox, roe deer, and grey squirrel (*Sciurus carolinensis*). We selected Thorne Moors (TM), Doncaster, South Yorkshire, for red deer and

211 badger, but stoat, weasel, red fox, roe deer, and Reeve's muntjac (*Muntiacus reevesi*) were also
212 present. Camera traps (Bushnell Trophy Cam Aggressor) were deployed at TM (one per pond)
213 and BE (three per pond) one week prior to eDNA sampling and collected once sampling was
214 completed. At TLNR, camera traps (two to three per pond) were deployed one day before a 5-
215 day period of eDNA sampling and collected one week after sampling was completed. Camera
216 traps were positioned perpendicular to the ground (1 m height, 0.3-1 m from pond shoreline)
217 to capture water and shoreline. Cameras took three photographs (5 megapixel) when triggered
218 (3 s interval between triggers) at high sensitivity.

219 Ten stratified samples were collected from the shoreline of each pond (TM: 17/04/2018;
220 BE: 20/04/2018; TLNR: 23/04/2018 -27/04/2018) and a field blank (1 L MGW) included as in
221 Experiment 1. TLNR ponds were sampled every 24 hrs over 5 days to investigate spatiotemporal
222 variation in mammal eDNA signals. TM and TLNR samples were transported on ice in sterile
223 coolboxes to the University of Hull (UoH) eDNA facility, and stored at 4 °C. BE samples were
224 transported in sterile coolboxes with ice packs to BE accommodation. Surfaces and equipment
225 were sterilised before, during, and after set-up as in Experiment 1. Samples (n = 140) and field
226 blanks (n = 14) were vacuum-filtered within 4 hrs of collection as in Experiment 1 with minor
227 modifications to maximise detection probability as follows. The full 2 L of each sample was
228 vacuum-filtered where possible, two filters were used for each sample, and duplicate filters
229 were stored in one petri dish at -20 °C. A filtration blank (1 L MGW) was processed during each
230 filtration round (n = 21). The total water volume filtered per sample was recorded (Table S3).
231

232 ## 2.4 DNA extraction

233

234 DNA was extracted within 2 weeks of filtration at the UoH eDNA facility using the mu-DNA
235 water protocol (Sellers, Di Muri, Gómez, & Hänfling, 2018). Duplicate filters from samples in
236 Experiment 1 were lysed independently and the lysate from each loaded onto one spin column.
237 As more samples were collected in Experiment 2, duplicate filters were co-extracted by placing
238 both in a single tube for bead milling. An extraction blank, consisting only of extraction buffers,
239 was included for each round of DNA extraction (*n* = 17). Eluted DNA (100 µl) was stored at -20
240 °C until PCR amplification.

241

242 ## 2.5 eDNA metabarcoding

243

244 Our eDNA metabarcoding workflow is fully described in Supporting Information. Briefly, we
245 performed nested metabarcoding using a two-step PCR protocol, where Multiplex Identification
246 (MID) tags were included in the first and second PCR for sample identification (Kitson et al.,
247 2019). In the first PCR, eDNA was amplified in triplicate with published 12S ribosomal RNA
248 (rRNA) primers 12S-V5-F (5'-ACTGGGATTAGATACCCC-3') and 12S-V5-R (5'-
249 TAGAACAGGCTCCTCTAG-3') (Riaz et al., 2011) that were validated *in silico* for UK vertebrates
250 (Harper et al., 2018a, b). The *in silico* analysis found that 91/112 mammal species listed on the
251 Natural History Museum Checklist of Mammalia v1 (subspecies excluded) can be distinguished
252 using this 12S metabarcode. Nine indistinguishable species lacked reference sequences,
253 whereas the other 12 had reference sequences but did not amplify. PCR positive controls (two

254 per PCR plate; *n* = 16) were exotic cichlid (*Maylandia zebra*) DNA (0.05 ng/µl), and PCR negative
255 controls (two per PCR plate; *n* = 16) were MGW (Fisher Scientific UK Ltd, UK). PCR products
256 were pooled to create sub-libraries (Fig. S2) and purified. The second PCR bound pre-adapters,
257 MID tags, and Illumina adapters to the sub-libraries. PCR products were purified, quantified,
258 and pooled. The library was then purified, quantified by qPCR, and fragment size (330 bp) and
259 removal of secondary product verified. The library (220 eDNA samples, 27 field blanks, 33
260 filtration blanks, 17 extraction blanks, 16 PCR negative controls, and 16 PCR positive controls)
261 was sequenced on an Illumina MiSeq®. Raw sequence reads were demultiplexed using a custom
262 Python script, then metaBEAT v0.97.11 (https://github.com/HullUni-bioinformatics/metaBEAT)
263 was used for quality trimming, merging, chimera removal, clustering, and taxonomic
264 assignment of sequences against our UK vertebrate reference database (Harper et al., 2018a, b)
265 which contains sequences for 103 UK mammals.
266

267 **2.6 Data analysis**

268

269 Analyses were performed in R v.3.4.3 (R Core Team, 2017). The total unrefined read counts (i.e.
270 raw taxonomically assigned reads) per sample were calculated and retained for downstream
271 analyses. Assignments were corrected and merged, taxon-specific sequence thresholds (i.e.
272 maximum sequence frequency of each taxon in PCR positive controls [Table S4]) used to
273 mitigate cross-contamination and false positives (Fig. S3), and remnant contaminants and
274 higher taxonomic assignments removed excluding the following genera. *Anas* (Dabbling ducks)
275 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 Supporting Information: Appendix 1. 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

298 generated by eDNA metabarcoding, camera trapping, and field signs. TLNR ponds were
299 sampled every 24 hrs for 5 days, thus proportional read counts were averaged across days for
300 comparison to BE and TM ponds (sampled once each). We qualitatively compared the
301 distribution and persistence of eDNA signals between semi-aquatic and terrestrial mammals
302 using tile plots and heat maps of the unaveraged proportional read counts for identified species
303 at TLNR over the 5-day period. All figures were produced using the package ggplot2 v3.0.0
304 (Wickham, 2016).
305
306

307 3. Results

308

309 3.1 eDNA metabarcoding

310

311 The sequencing run generated 47,713,656 raw sequence reads, of which 37,590,828 remained
312 following trimming, merging, and length filter application. After removal of chimeras and
313 redundancy via clustering, the library contained 21,127,061 sequences (average read count of
314 64,215 per sample including controls), of which 16,787,750 (79.46%) were assigned a
315 taxonomic rank. Contamination (Fig. S4) was observed in the field blanks (badger, beaver, lynx,
316 pine marten, red squirrel, and water vole) as well as in the filtration and extraction blanks
317 (human [*Homo sapiens*] and cichlid). PCR negative controls were contaminated to different
318 extents with human, cichlid, beaver, and pine marten as well as non-focal species. After
319 threshold application, contaminants remaining in eDNA samples included Gentoo penguin

320 (*Pygoscelis papua*), reindeer (*Rangifer tarandus*), cichlid, and human. The refined dataset
321 contained 59 vertebrate species, including six amphibians, 10 fish, 19 birds, and 24 mammals
322 (Table S5).
323

324 **3.2 Experiment 1: eDNA detection and signal strength in artificial systems**

325

326 All nine focal species were detected in captivity. Seven species (red deer and hedgehog
327 excluded) were detected in all water samples taken from their respective enclosures. Red deer
328 were not detected in 2 of 5 stratified samples from HWP, and hedgehog was not detected in 1
329 of 2 WT drinking bowl samples (Fig. 1). 'Other' samples (neither directed nor stratified) were
330 excluded from further comparisons, thus hedgehog, red squirrel, and water vole were omitted
331 in downstream analyses.

332 Across species, stratified samples (0.406) had a higher median proportional read count
333 than directed samples (0.373), but this difference was not significant (Mann-Whitney *U* test: *U*
334 = 1181.5, $P = 0.829$). Within species, sample type also had no effect ($\chi^2_6 = 0.364$, $P = 0.999$) on
335 proportional read counts (GLMM: $\theta = 0.168$, $\chi^2_{53} = 8.915$, $P = 1.000$, pseudo-R^2 = 39.21%).
336 Proportional read counts for directed and stratified samples belonging to each species did not
337 significantly differ. Otter proportional read counts were lower than other species, but not
338 significantly so (Fig 2a). Similarly, species lifestyle had no influence ($\chi^2_2 = 0.655$, $P = 0.721$) on
339 proportional read counts (GLMM: $\theta = 0.213$, $\chi^2_{61} = 13.002$, $P = 1.000$, pseudo-R^2 = 11.85%).
340 Proportional read counts for semi-aquatic, ground-dwelling, and arboreal species did not
341 significantly differ (Fig. 2b).

342 Specific behaviours exhibited by species had no influence (χ^2_{11} = 1.369, P = 0.999) on
343 proportional read counts (GLMM: θ = 0.355, χ^2_{31} = 11.013, P = 0.999, pseudo-R^2 = 9.17%),
344 where proportional read counts did not differ across species' behaviours (Fig. 3a). Likewise,
345 generic behaviour (i.e. water contact versus no water contact) had no effect on proportional
346 read counts (χ^2_{11} = 0.002, P = 0.964). Proportional read counts did not differ between
347 behaviour that involved or lacked water contact (Fig. 3b, GLMM: θ = 0.217, χ^2_{41} = 8.897, P =
348 1.000, pseudo-R^2 = 8.50%).
349

350 **3.3 Experiment 2: eDNA detection and signal strength in natural systems**

351

352 At natural ponds, eDNA metabarcoding, camera trapping, and field signs all detected beaver,
353 red deer, and roe deer. Camera traps (Fig. 4) and field signs recorded red fox and badger when
354 eDNA metabarcoding did not (Fig. 5). However, eDNA metabarcoding revealed small mammal
355 species not caught on camera, including water vole, water shrew (*Neomys fodiens*), bank vole
356 (*Myodes glareolus*), common shrew (*Sorex araneus*), brown rat (*Rattus norvegicus*), rabbit, grey
357 squirrel, and common pipistrelle (*Pipistrellus pipistrellus*). Mice or vole footprints were
358 observed at BE Pond 1, but species could not be determined. Fig. 5 summarises mammals
359 recorded by different methods at each site with reference to cumulative survey data. Notably,
360 only beaver presence was captured at the same ponds by all methods. Although methods
361 shared species at site level, species were not always detected at the same pond. Detection
362 rates for species captured by at least one survey method are summarised in Table S6.
363 Sampling of natural ponds revealed spatial patterns in eDNA detection and signal

364 strength. eDNA from non-domestic terrestrial mammals (i.e. mammals excluding dog [*Canis*
365 *lupus familiaris*], pig [*Sus scrofa domesticus*], sheep [*Ovis aries*] and cow [*Bos taurus*]) was
366 unevenly dispersed compared with semi-aquatic mammals (Fig. S5). Semi-aquatic beaver and
367 water vole were detected in at least 90% and 60% respectively of water samples (n = 10)
368 collected from single ponds, whereas water shrew was only detected in 10% of samples.
369 Conversely, non-domestic terrestrial mammals were consistently detected in less than 20% of
370 water samples collected from a pond and left relatively weak eDNA signals. Overall, beaver was
371 the most consistently detected mammal with the highest proportional read counts. However,
372 the strongest and most evenly distributed signals belonged to amphibians, particularly common
373 frog (*Rana temporaria*) and great crested newt (*Triturus cristatus*) (Fig. S5).

374 TLNR samples collected over a 5-day period (D01-05) revealed that mammal detection
375 was highly dependent on the spatial and temporal resolution of eDNA metabarcoding surveys
376 (Fig. S6). Mammal eDNA signals in pond water were ephemeral, often disappearing within 24-
377 48 hrs of initial detection, as opposed to amphibians that were detected for multiple days and
378 whose eDNA signal increased in strength. The majority of semi-aquatic or terrestrial mammals
379 were only detected in a single sample on each day.

380

381

382 **4. Discussion**

383

384 We have demonstrated that eDNA metabarcoding is a potential monitoring tool for
385 conservation and management priority mammals, but species detection rates are variable. We

386 used two experiments to validate this molecular approach and gained new insights that will
387 inform the development and application of eDNA metabarcoding for mammals. Sampling
388 strategy, mammal lifestyle, and mammal behaviour had little influence on eDNA detection and
389 signal strength in captivity, but all played vital roles in natural ponds. Although mammals were
390 detected from pond water, their eDNA signals were temporary and weak in comparison to
391 amphibians and fish. Nonetheless, this would suggest eDNA is representative of contemporary
392 and local mammal diversity in a given area.
393

394 **4.1 Experimental insights**

395

396 In Experiment 1, all nine focal species were detected in captivity, of which seven were detected
397 in all water samples taken from their respective enclosures. This demonstrates that our method
398 can successfully detect a wide range of mammal species from pond and drinking water. Perhaps
399 surprisingly, we found that neither sampling strategy, mammal lifestyle or mammal behaviour
400 influenced eDNA detectability and signal strength in enclosures. This included behaviours
401 associated with eDNA deposition, e.g. swimming, drinking, urination, and defecation (Rodgers
402 & Mock, 2015; Ushio et al., 2017; Williams et al., 2018). Enclosures were permanently occupied
403 and artificial waterbodies likely saturated with eDNA, which possibly masked behavioural
404 signals. Modest replication may also have limited experimental power, preventing patterns
405 being detected statistically. Nonetheless, our results have demonstrated that mammal contact
406 with water enables eDNA deposition and detection.

407 Unsurprisingly, given the nature of wild mammal interactions with natural systems

408 versus those in captivity, results from Experiment 2 highlighted the challenges of mammal
409 eDNA detection. We detected 17 mammals using three monitoring tools, comparable to the 17
410 mammals expected from cumulative survey data despite some differences. Field signs and
411 camera trapping performed better than eDNA metabarcoding for red fox and badger detection,
412 but only eDNA metabarcoding identified water vole and other small mammals missed on
413 camera or with ambiguous field signs, e.g. mice, voles, shrews. Importantly, the deployment
414 period, height, and positioning of camera traps may have influenced small mammal detection
415 by this method (Caravaggi et al., 2018). Ishige et al. (2017) achieved comparable mammal
416 detection at salt licks with eDNA metabarcoding and camera trapping, but species presence was
417 inconsistent between salt licks surveyed. Currently, there are no published studies comparing
418 eDNA metabarcoding of water to camera trapping for mammal identification. However, our
419 results reflect those of Stat et al. (2019) who found highest marine fish species richness using
420 both baited remote underwater video systems and eDNA metabarcoding, but each method
421 identified unique species.

422 Notably, no survey method captured the semi-aquatic otter despite known presence at
423 study sites and successful detection in eDNA metabarcoding studies of UK ponds (Harper et al.,
424 2018a) and lakes (Hänfling et al., 2017). Furthermore, the eDNA signal for captive otter was
425 weaker than that of other semi-aquatic mammals studied here. Lower eDNA detection rates for
426 otter, badger, and red fox may stem from species' ecologies. These mammals are wide-ranging
427 (Gaughran et al., 2018; Thomsen et al., 2012) and may not readily release DNA in water. Otters
428 often spraint on grass or rock substrata outside water and use latrines associated with caves
429 and dens (Ruiz-Olmo & Gosálbez, 1997). As terrestrial mammals, red fox and badger must drink

430 from or enter ponds for eDNA deposition to occur (Rodgers & Mock, 2015; Ushio et al., 2017;
431 Williams et al., 2018). Otter, badger, and red fox detection may require greater spatiotemporal
432 resolution of eDNA sampling. This is reinforced by other eDNA metabarcoding studies where
433 mammal detection was highly variable across sites surveyed (Ishige et al., 2017; Klymus et al.,
434 2017; Ushio et al., 2017). False negatives may instead be symptomatic of metabarcoding bias,
435 but this is unlikely in our study (section 4.2).

436 eDNA from other semi-aquatic mammals (e.g. beaver, water vole) was evenly
437 distributed, being found in most or all samples collected on fine spatial scales within natural
438 ponds, whereas terrestrial mammal (e.g. red and roe deer) eDNA was highly localised and often
439 detected in less than 20% of samples. Mammal eDNA signals varied temporally, being
440 detectable for two consecutive days maximum. Depending on the species, mammal eDNA may
441 be spatially and temporally clumped in lentic ecosystems due to the nature and frequency of
442 contact that individuals have with water. Unless behaviours involving prolonged water contact
443 (e.g. swimming, wallowing) are exhibited, non-domestic mammals may only be detected at
444 drinking sites (Klymus et al., 2017; Ushio et al., 2017; Williams et al., 2018). In contrast,
445 domestic mammals (e.g. cow, sheep, pig) may have elevated detection rates in ponds due to
446 high occurrence of these waterbodies in agricultural landscapes as well as eDNA transport by
447 rainfall and run-off (Staley et al., 2018). eDNA detection and persistence are further influenced
448 by group size, where eDNA from multiple individuals endures for longer time periods in water
449 than eDNA from single individuals (Williams et al., 2018). Detailed investigations incorporating
450 biotic (e.g. population size, sex, body mass, behaviour) and abiotic (e.g. temperature, pH,
451 ultraviolet light, rainfall) factors are needed to understand the longevity of mammal eDNA

452 signals in aquatic ecosystems (Rodgers & Mock, 2015; Williams et al., 2018).

453 Both of our experiments have shown that sampling strategy drastically influences
454 mammal eDNA detection probability. Mammal eDNA was evenly distributed in closed, artificial
455 waterbodies, but locally distributed in open, natural ponds. In captivity, mammal species were
456 housed individually (excluding HWP red deer) with a drinking container(s) and/or small
457 waterbody (range 0.01-162 m^2, mean 27.4 m^2) in their enclosure. Some enclosures housed
458 more individuals of a species than others, thereby increasing eDNA deposition and detection
459 probability (Williams et al., 2018). Wild mammals have an array of freshwater habitats at their
460 disposal and can hold vast territories. Therefore, rates of pond visitation and eDNA deposition
461 are more irregular (Klymus et al., 2017; Ushio et al., 2017) which may lead to between-sample
462 variation (Williams et al., 2018).

463

464 4.2 Technical pitfalls of eDNA metabarcoding for mammal monitoring

465

466 eDNA metabarcoding has strong potential for inclusion in mammal monitoring schemes
467 (section 4.3), but like existing monitoring tools, may produce false negatives or false positives.
468 Our process controls identified low-level contamination at all stages of metabarcoding, but
469 contaminants primarily occurred in field and PCR negative controls (Supporting Information:
470 Appendix 2). We applied taxon-specific sequence thresholds to our data to mitigate false
471 positives as in Harper *et al*. (2018a, b). Remnant contaminants were cichlid (laboratory), Gentoo
472 penguin (environment), reindeer (environment), and human (environment/laboratory). Gentoo
473 penguin is housed at EZ and was identified from EZ beaver enclosure water. The WT red squirrel

474 and reindeer enclosures are in close proximity. DNA transport by wildlife (e.g. waterfowl
475 [Hänfling et al., 2016]) and park staff or visitors may explain this environmental contamination.
476 Human DNA was present across process controls corresponding to artificial and natural
477 waterbodies. Human DNA may be amplified and sequenced instead of focal species, potentially
478 resulting in false negative detections for rare and/or less abundant species. Human DNA
479 blocking primers can prevent this bias, but may impair PCR amplification efficiency (Klymus et
480 al., 2017; Ushio et al., 2017; Valentini et al., 2016).

481 In our study, eDNA metabarcoding produced false negatives for otter, badger, and red
482 fox at natural ponds. We selected a 12S metabarcode designed to amplify vertebrate DNA (Riaz
483 et al., 2011). There were no primer mismatches with otter, badger or red fox reference
484 sequences (Harper et al., 2018a, b), thus amplification bias was not responsible for these false
485 negatives. DNA from purely aquatic and more abundant species may have overwhelmed otter,
486 badger, and red fox DNA during amplification and sequencing, i.e. species-masking (Kelly, Port,
487 Yamahara, & Crowder, 2014; Klymus et al., 2017). Metabarcoding primers targeting mammals
488 (Ushio et al., 2017), or multi-marker (e.g. 12S, 16S, COI) investigations (Evans et al., 2017;
489 Hänfling et al., 2016; Kelly et al., 2014; Klymus et al., 2017) may improve mammal detection in
490 systems with competition from aquatic species. Similarly, more biological and technical
491 replication may improve species detection probabilities (Evans et al., 2017; Lawson Handley et
492 al., 2018; Stat et al., 2019; Valentini et al., 2016). Importantly, fish and amphibians also had
493 higher qPCR detection than otter (Thomsen et al., 2012). A comparison of metabarcoding and
494 qPCR (e.g. Harper et al., 2018b; Lacoursière-Roussel, Dubois, Normandeau, & Bernatchez, 2016)
495 would confirm whether poor amplification efficiency for otter is due to technical bias or species

496 ecology, and whether eDNA metabarcoding can reliably monitor otter alongside the wider
497 mammal community.
498

499 ## 4.3 Scope of eDNA metabarcoding for mammal monitoring

500

501 Assessments of UK mammal populations are hindered by lack of data and systematic
502 monitoring for many species (Mathews et al., 2018). Distribution and occupancy data are poor
503 for most species, with ongoing survey effort biased toward rare species. Surveys are also
504 heavily reliant on citizen science and casual records (Massimino et al., 2018). Tools that can
505 provide standardised, systematic monitoring of UK mammal populations are needed (Mathews
506 et al., 2018). Despite issues inherent to metabarcoding for biodiversity monitoring (Deiner et
507 al., 2017), this tool has enormous potential to enhance mammal monitoring, conservation, and
508 management. eDNA metabarcoding generates distribution data for multiple species, whether
509 rare, invasive, or abundant, and could be a powerful tool for tracking species in conflict with
510 one another, e.g. water vole, American mink, and otter (Bonesi & Macdonald, 2004) or red
511 squirrel, grey squirrel, and pine marten (Sheehy, Sutherland, O'Reilly, & Lambin, 2018).
512 Different sample types (e.g. water, soil, snow, salt licks, feeding traces, faeces, hair, and blood
513 meals) may also offer new insights to mammal biodiversity in a given area (Aylward et al., 2018;
514 Franklin et al., 2019; Ishige et al., 2017; Tessler et al., 2018; Ushio et al., 2017).

515 eDNA metabarcoding can rapidly survey multitudes of aquatic sites at landscape-scale
516 where camera traps might be resource-intensive, cost-inefficient, and susceptible to
517 theft/damage (Stat et al., 2019; Ushio et al., 2017). Field signs require volunteer time and skill

518 to be employed at comparable spatial scales to eDNA metabarcoding (Sadlier et al., 2004).
519 eDNA metabarcoding could provide accurate data for species misidentified from field signs, e.g.
520 mice and voles, otter and mink (Franklin et al., 2019; Harris & Yalden, 2004). However, both
521 camera traps and field signs recorded species that eDNA metabarcoding did not. Therefore,
522 eDNA metabarcoding is complementary and should be incorporated into, not replace, existing
523 monitoring schemes. This tool could be most effective in mammal monitoring if deployed at the
524 edges of known species distributions, in areas where species presence is unknown, and in areas
525 with isolated species records (Mathews et al., 2018).

526 Mammal eDNA detection probabilities from natural ponds will likely be high when areas
527 with dense populations are studied. However, rigorous sampling strategies are required to
528 track mammals in areas that are sparsely populated by individuals. Multiple ponds must be
529 sampled repeatedly, and samples taken at multiple locations within ponds without pooling so
530 that inferences about site occupancy can be made. Importantly, we sampled natural ponds in
531 spring but sampling in other seasons may produce different results, reflective of species'
532 ecologies (Lawson Handley et al., 2018). We recommend that researchers and practitioners
533 using eDNA metabarcoding for mammal monitoring channel efforts into extensive sampling of
534 numerous waterbodies in a given area over larger timescales. This will account for differential
535 mammal visitation rates and maximise eDNA detection probabilities.

536

537

538 ## Data accessibility

539

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

544

545

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547

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558

Author contributions

L.R.H, B.H, and L.L.H conceived and designed the study. A.I.C and G.M-D 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.

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727

728 **Table 1.** Summary of focal species studied at wildlife parks and their lifestyle. The number of

729 individuals present and waterbody size in enclosures is provided.

730

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

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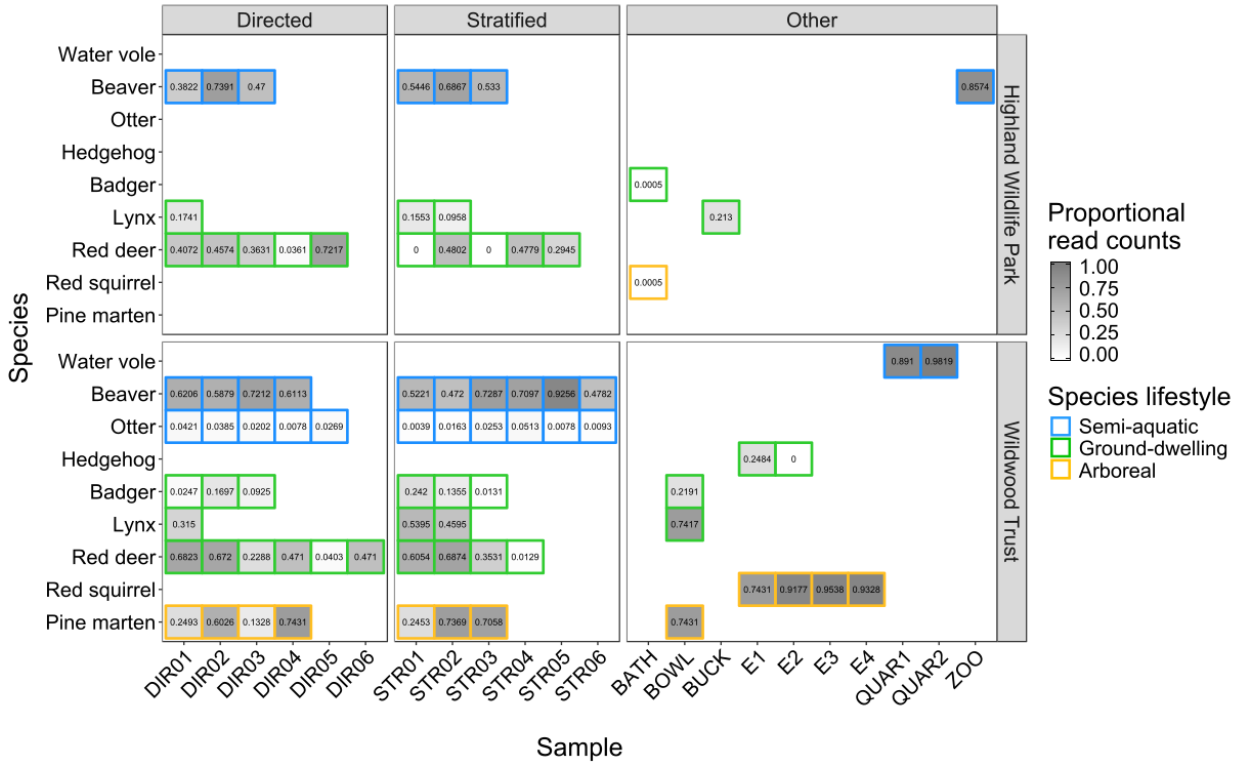
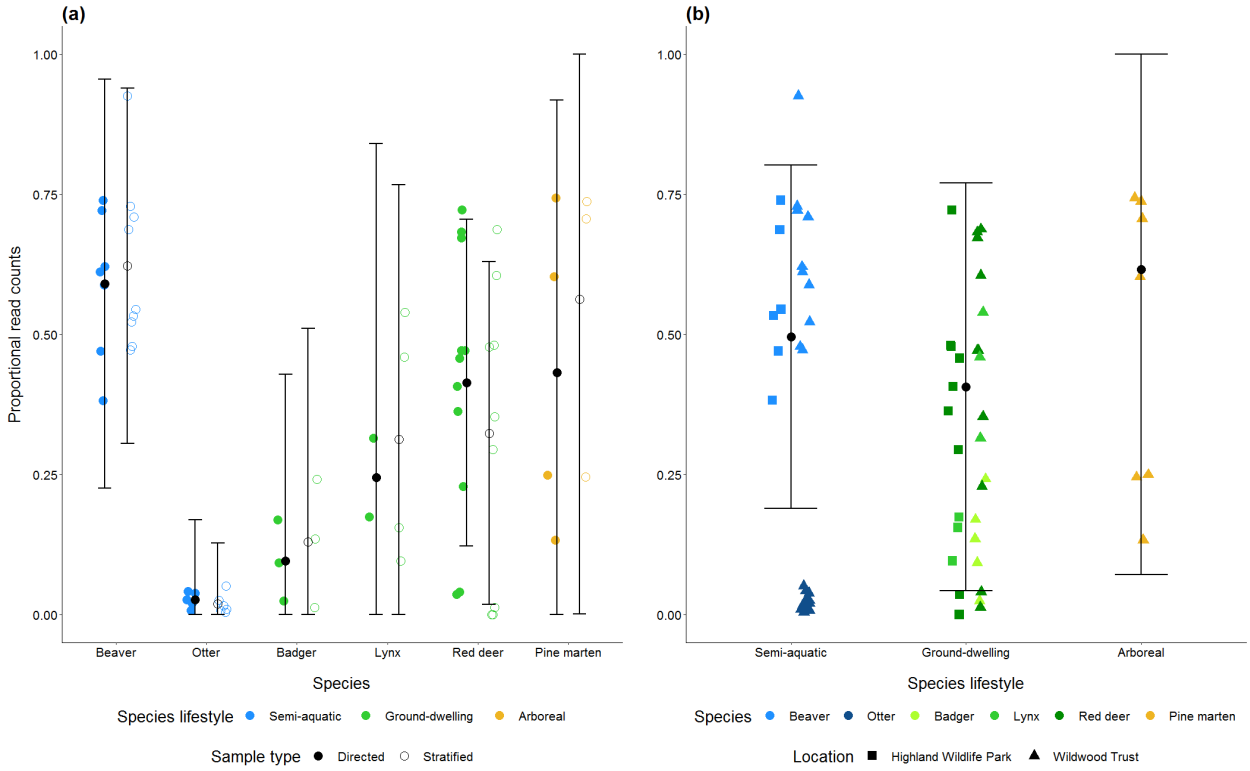


Figure 1. Heatmap showing proportional read counts for eDNA samples. Each square represents a sample taken from a wildlife park enclosure. Directed (DIR01-DIR06) and stratified (STR01-STR06) samples were collected from artificial waterbodies. Samples were collected from drinking containers (E1, E2, E3, 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.



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742 **Figure 2.** Relationships predicted by the binomial GLMMs between proportional read counts

743 and sample type nested within species **(a)** or species lifestyle **(b)**. The observed data (coloured

744 points) are displayed against the predicted relationships (black points with error bars) for each

745 species **(a)** or species lifestyle **(b)**. Points are shaped by sample type **(a)** or wildlife park **(b)**, and

746 coloured by species lifestyle. Error bars represent the standard error around the predicted

747 means.

748

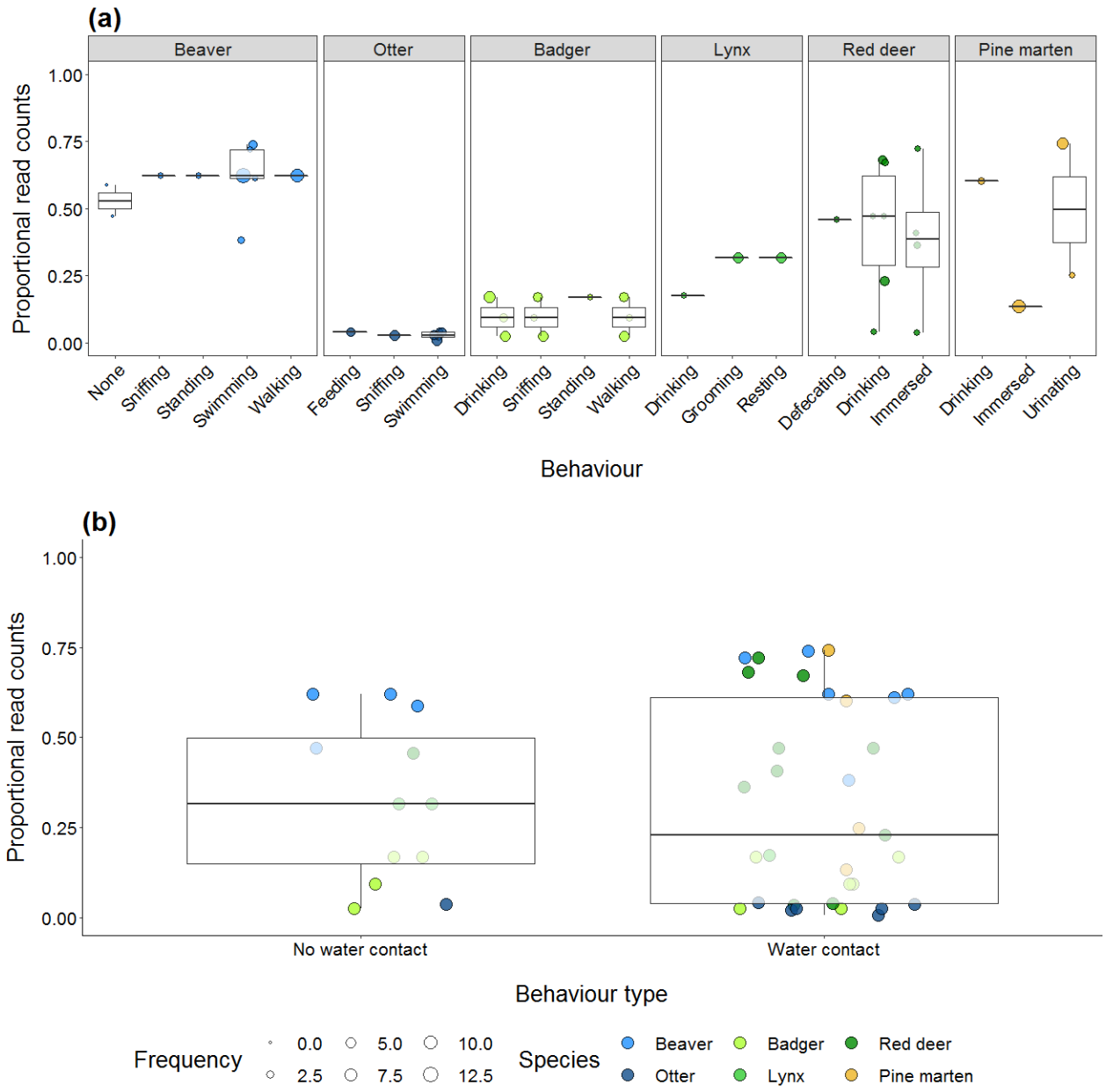

Figure 3. Boxplots showing the mean proportional read counts for specific **(a)** and generic **(b)** behaviour(s) exhibited by focal species. Boxes show 25th, 50th, and 75th percentiles, and whiskers show 5th and 95th percentiles. Points are coloured by species lifestyle, and each point in **(a)** represents 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.

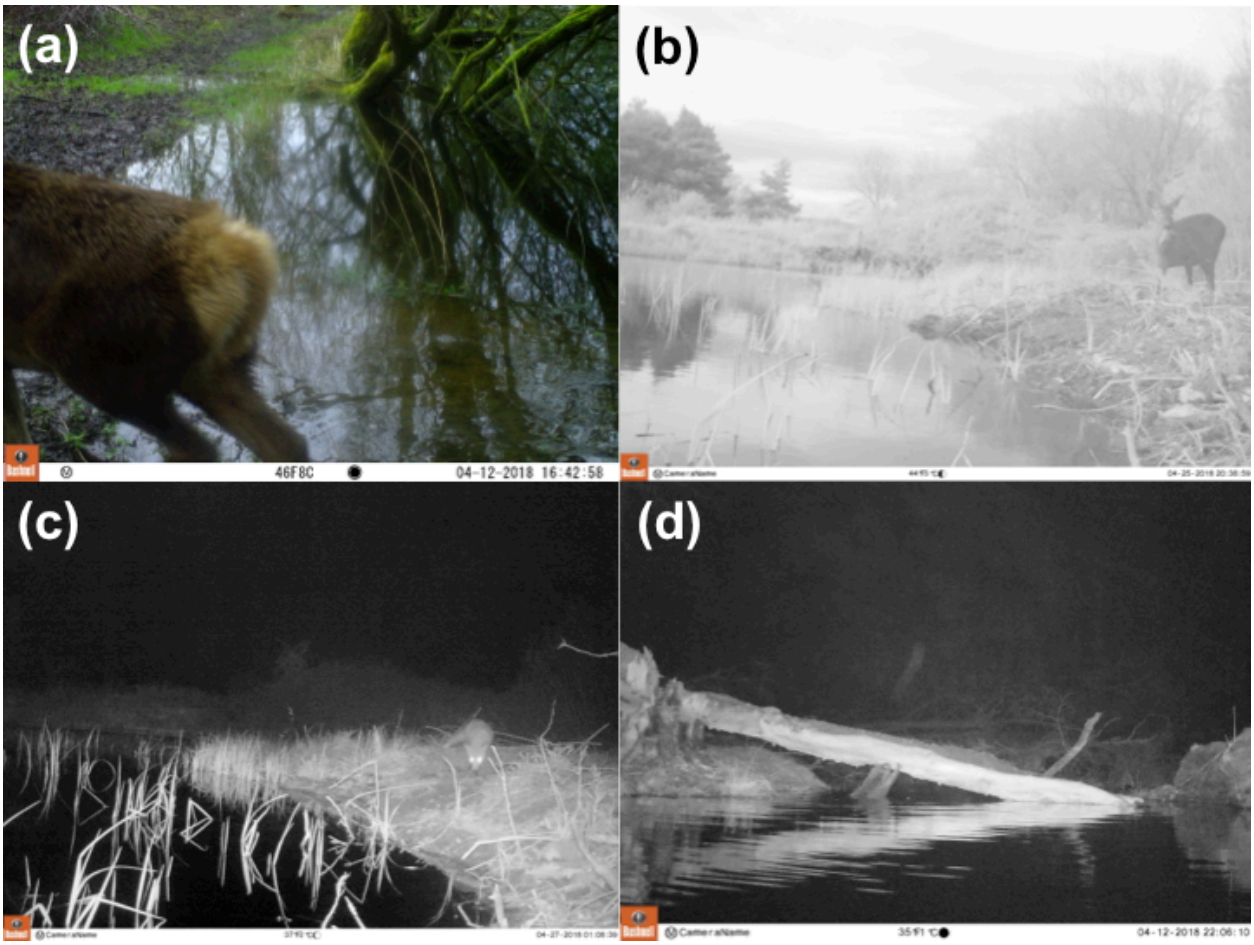

Figure 4. Exemplar camera trap photos taken at natural ponds where focal species were present. Red deer was recorded at Thorne Moors **(a)**, roe deer **(b)** and red fox **(c)** were recorded at Tophill Low Nature Reserve, and beaver was recorded at the Bamff Estate **(d)**.

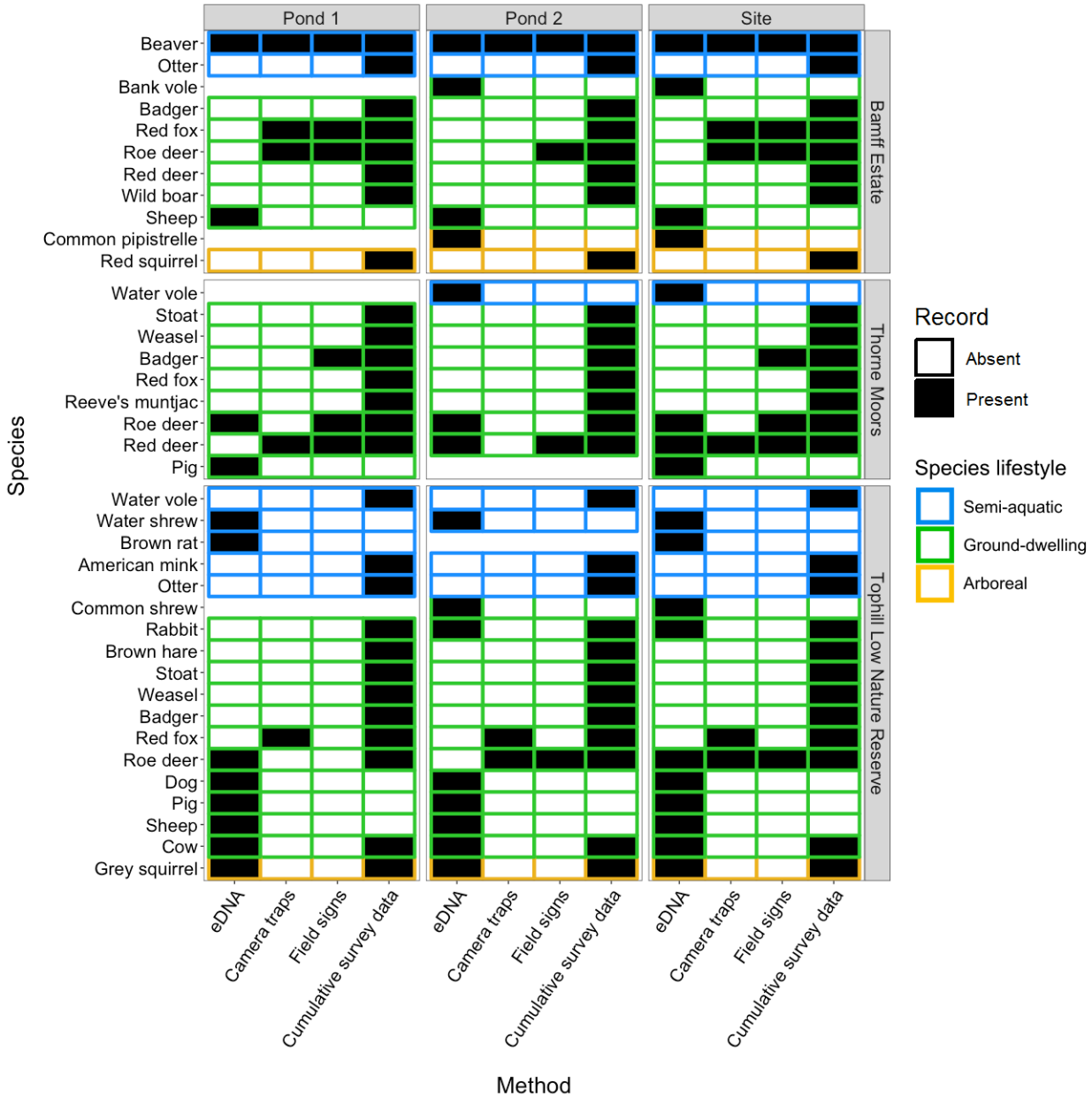

Figure 5. Tile plot showing species presence-absence at individual pond and site-level as indicated by field signs, camera trapping, and eDNA metabarcoding at sites with focal species presence confirmed by cumulative survey data.