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

50 # 1. Introduction

51

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

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

71 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; Kinoshita et al., 2019; Leempoel et al., 2019; Sales et al., 2019; 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 (Sales et al., 2019; 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; Sales et al., 2019; 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).

94 Drinking is a major source of eDNA deposition due to the release of saliva, but mammals may
95 also swim, wallow, urinate or defecate in water (Rodgers & Mock, 2015; Ushio et al., 2017;
96 Williams et al., 2018). Furthermore, arboreal mammals may use ponds less than semi-aquatic
97 and ground-dwelling species, non-territorial mammals may visit ponds less than territorial
98 species, and group-living species may deposit more eDNA than solitary species (Williams et al.,
99 2018). Despite evidence for eDNA deposition by semi-aquatic and terrestrial mammals in
100 freshwater ecosystems, little is known about the influence of mammal behaviour on the
101 distribution and strength of the eDNA signal left behind (defined here as proportional read
102 counts).

103 In this study, we conducted two experiments under artificial and natural conditions to
104 evaluate eDNA metabarcoding of pond water as a tool for monitoring semi-aquatic, ground-
105 dwelling, and arboreal mammals of conservation or management concern. The first
106 experiment, carried out on nine focal species housed at two wildlife parks, examined the role of
107 sampling strategy, mammal lifestyle, and mammal behaviour on eDNA detection and signal
108 strength under artificial conditions. Mammal eDNA detection is expected from enclosure water
109 that is frequently used by individuals for drinking, swimming and bathing. We hypothesised
110 that: (1) eDNA would be unevenly distributed, thus directed sampling would yield stronger
111 eDNA signals (i.e. higher proportional read counts) for mammals than stratified sampling; (2)
112 semi-aquatic mammals would have stronger eDNA signals than ground-dwelling or arboreal
113 mammals; and (3) mammal behaviours involving water contact would generate stronger eDNA
114 signals. The second experiment validated eDNA metabarcoding against camera trapping and
115 field sign searches for mammal identification at natural ponds, and investigated spatiotemporal

116 variation in mammal eDNA signals. Mammal eDNA detection is unpredictable at natural
117 waterbodies that can be extensive, subject to environmental fluctuations, and used rarely or
118 not at all by individuals. We hypothesised that: (1) eDNA metabarcoding would detect more
119 mammals than camera trapping or field signs; (2) semi-aquatic mammals would be readily
120 detected and their eDNA evenly distributed in ponds in comparison to terrestrial mammals; and
121 (3) temporal sampling would reveal that terrestrial mammal eDNA is detectable for short
122 periods in comparison to fully aquatic vertebrates.
123
124

125 # 2. Materials and methods

126

127 ## 2.1 Study species

128

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

138

139 ## 2.2 Experiment 1: eDNA detection and signal strength in artificial systems

140

141 Behavioural observation and eDNA sampling were conducted between 18th – 21st September
142 2017 at Wildwood Trust (WT), Kent, England, and 10th – 11th October 2017 at Royal Zoological
143 Society of Scotland (RZSS) Highland Wildlife Park (HWP), Kingussie, Scotland. Sixteen categories
144 of behaviour were defined based on potential contact with waterbodies and species lifestyle,
145 and the frequency and duration of behaviours recorded (Table 1, Appendix A: Table A1). The
146 number of individuals in each enclosure was recorded alongside waterbody size (Table 2).
147 Beaver, lynx, red deer, and red squirrel were present at both wildlife parks, whereas other
148 captive species were only present at WT. Each species was observed for one hour on two
149 separate occasions except nocturnal mammals (badger and beaver), which were observed
150 overnight using camera traps (Bushnell Trophy Cam Standard, Bushnell Corporation, KS, USA).
151 One camera trap per enclosure was positioned perpendicular to the ground (1 m height, 2 m
152 from shoreline) to capture water and shoreline. Cameras took 30 s videos (1920 x 1080) when
153 triggered (30 s interval between triggers) at high sensitivity. Behavioural observation was not
154 undertaken for WT water voles as animals were under quarantine or HWP red squirrels as
155 individuals were wild. Photos of waterbodies in animal enclosures are provided in Appendix B.

156 Water samples were collected from enclosures within 3 hrs of the second behavioural
157 observation period. Up to six directed or stratified samples were collected, but sample number
158 varied by species according to waterbody size and observed behaviours (Tables A1, A2).
159 Enclosure drinking containers were also sampled and classed as 'other' samples. Bathing and

160 drinking bowls were sampled where enclosures contained no artificial waterbodies (WT water
161 vole, red squirrel, and hedgehog). The HWP beaver enclosure was empty for 24 hrs before
162 sampling. Water was sampled from a RZSS Edinburgh Zoo (EZ) enclosure containing beavers
163 and classed as 'other'. A sample was collected from a water bath in the HWP woods to capture
164 wild red squirrels and classed as 'other'.

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

175 Samples and blanks were vacuum-filtered within 6 hrs of collection in a UoK wet
176 laboratory and within 24 hrs of collection in an EZ staff room. Surfaces and equipment were
177 sterilised before, during, and after set-up in temporary work areas. Surfaces and vacuum
178 pumps were wiped with 10% v/v chlorine-based commercial bleach (Elliott Hygiene Ltd, UK)
179 solution. Non-electrical equipment was immersed in 10% bleach solution for 10 minutes,
180 followed by 5% v/v MicroSol detergent (Anachem, UK), and rinsed with purified water. Up to
181 500 ml of each 2 L sample was vacuum-filtered through sterile 0.45 µm mixed cellulose ester

182 membrane filters with pads (47 mm diameter; Whatman, GE Healthcare, UK) using Nalgene™
183 filtration units. One hour was allowed for each sample to filter and a second filter used if
184 clogging occurred. A filtration blank (1 L MGW) was processed during each filtration round (*n* =
185 12), and equipment sterilised after each filtration round. After 500 ml had filtered or one hour
186 had passed, filters were removed from pads using sterile tweezers, placed in sterile 47 mm
187 petri dishes (Fisher Scientific UK Ltd, UK), sealed with parafilm (Sigma-Aldrich Company Ltd,
188 UK), and stored at -20 °C. The total water volume filtered per sample was recorded for
189 downstream analysis (Table A2; Fig. A1).
190

191 **2.3 Experiment 2: eDNA detection and signal strength in natural systems**

192

193 At three sites where focal species were present based on cumulative survey data, we selected
194 two ponds (range 293-5056 m^2, average 1471 m^2) within 4 km of each other. The Bamff Estate
195 (BE), Alyth, Scotland, was selected for beaver, otter, badger, red deer, and red squirrel, but roe
196 deer (*Capreolus capreolus*) and red fox (*Vulpes vulpes*) were also present. Otter, water vole, and
197 badger were present at Tophill Low Nature Reserve (TLNR), Driffield, East Yorkshire, alongside
198 American mink (*Neovison vison*), stoat (*Mustela erminea*), weasel (*Mustela nivalis*), rabbit
199 (*Oryctolagus cuniculus*), brown hare (*Lepus europaeus*), red fox, roe deer, and grey squirrel
200 (*Sciurus carolinensis*). We selected Thorne Moors (TM), Doncaster, South Yorkshire, for red deer
201 and badger, but stoat, weasel, red fox, roe deer, and Reeve's muntjac (*Muntiacus reevesi*) were
202 also present. Camera traps (Bushnell Trophy Cam Standard/Aggressor, Bushnell Corporation,
203 KS, USA) were deployed at TM (one per pond) and BE (three per pond) one week prior to eDNA

204 sampling and collected once sampling was completed. At TLNR, camera traps (two to three per
205 pond) were deployed one day before a 5-day period of eDNA sampling and collected one week
206 after sampling was completed. Camera traps were positioned perpendicular to the ground (1 m
207 height, 0.3-1 m from shoreline) to capture water and shoreline. Cameras took three
208 photographs (5 megapixel) when triggered (3 s interval between triggers) at high sensitivity.

209 Ten stratified samples were collected from the shoreline of each pond (TM: 17th April
210 2018; BE: 20th April 2018; TLNR: 23rd – 27th April 2018) and a field blank (1 L MGW) included as
211 in Experiment 1. TLNR ponds were sampled every 24 hrs over 5 days to investigate
212 spatiotemporal variation in mammal eDNA signals. TM and TLNR samples were transported on
213 ice in sterile coolboxes to the University of Hull (UoH) eDNA facility, and stored at 4 °C. BE
214 samples were transported in sterile coolboxes with ice packs to BE accommodation. Surfaces
215 and equipment were sterilised before, during, and after set-up as in Experiment 1. Samples (n =
216 140) and field blanks (n = 14) were vacuum-filtered within 4 hrs of collection as in Experiment 1
217 with minor modifications to maximise detection probability as follows. The full 2 L of each
218 sample was vacuum-filtered where possible, two filters were used for each sample, and
219 duplicate filters were stored in one petri dish at -20 °C. A filtration blank (1 L MGW) was
220 processed during each filtration round (n = 21). The total water volume filtered per sample was
221 recorded (Table A3).
222

223 ## 2.4 DNA extraction

224

225 DNA was extracted within 2 weeks of filtration at the UoH eDNA facility using the Mu-DNA
226 water protocol (Sellers, Di Muri, Gómez, & Hänfling, 2018). The full protocol is available at:
227 https://doi.org/10.17504/protocols.io.qn9dvh6. Duplicate filters from samples in Experiment 1
228 were lysed independently and the lysate from each loaded onto one spin column. As more
229 samples were collected in Experiment 2, duplicate filters were co-extracted by placing both in a
230 single tube for bead milling. An extraction blank, consisting only of extraction buffers, was
231 included for each round of DNA extraction (*n* = 17). Eluted DNA (100 µl) was stored at -20 °C
232 until PCR amplification.

233

234 ## 2.5 eDNA metabarcoding

235

236 Our eDNA metabarcoding workflow is fully described in Appendix A. Briefly, we performed
237 nested metabarcoding using a two-step PCR protocol, where Multiplex Identification (MID) tags
238 were included in the first and second PCR for sample identification (Kitson et al., 2019). The first
239 PCR amplified eDNA in triplicate with published 12S ribosomal RNA (rRNA) primers 12S-V5-F (5'-
240 ACTGGGATTAGATACCCC-3') and 12S-V5-R (5'-TAGAACAGGCTCCTCTAG-3') (Riaz et al., 2011).
241 Harper et al. (2018) validated these primers *in silico* for UK vertebrates, and found 91/112
242 mammal species listed on the Natural History Museum Checklist of Mammalia v1 (subspecies
243 excluded) could be distinguished. Nine indistinguishable species lacked reference sequences,
244 whereas 12 had reference sequences but did not amplify. PCR positive controls (two per PCR

245 plate; *n* = 16) were exotic cichlid (*Maylandia zebra*) DNA (0.05 ng/µl), and PCR negative controls
246 (two per PCR plate; *n* = 16) were MGW (Fisher Scientific UK Ltd, UK). PCR products were pooled
247 to create sub-libraries (Fig. A2) and purified with Mag-BIND® RxnPure Plus magnetic beads
248 (Omega Bio-tek Inc, GA, USA), following the double size selection protocol established by
249 Bronner et al. (2009). Ratios of 0.9x and 0.15x magnetic beads to 100 µL of each sub-library
250 were used. Eluted DNA (30 µL) was stored at -20 °C until the second PCR could be performed.
251 The second PCR bound pre-adapters, MID tags, and Illumina adapters to the sub-libraries. PCR
252 products were purified with Mag-BIND® RxnPure Plus magnetic beads (Omega Bio-tek Inc, GA,
253 USA), following the double size selection protocol established by Bronner et al. (2009). Ratios of
254 0.7x and 0.15x magnetic beads to 50 µL of each sub-library were used. Eluted DNA (30 µL) was
255 stored at 4 °C until quantification and normalisation. The library was purified again, quantified
256 by qPCR using the NEBNext® Library Quant Kit for Illumina® (New England Biolabs® Inc., MA,
257 USA), and fragment size (330 bp) and removal of secondary product verified using an Agilent
258 2200 TapeStation and High Sensitivity D1000 ScreenTape (Agilent Technologies, CA, USA). The
259 library (220 eDNA samples, 27 field blanks, 33 filtration blanks, 17 extraction blanks, 16 PCR
260 negative controls, and 16 PCR positive controls) was sequenced on an Illumina MiSeq® using a
261 MiSeq Reagent Kit v3 (600-cycle) (Illumina, Inc, CA, USA). Raw sequence reads were
262 demultiplexed using a custom Python script. metaBEAT v0.97.11 (https://github.com/HullUni-
263 bioinformatics/metaBEAT) was used for quality trimming, merging, chimera removal, clustering,
264 and taxonomic assignment of sequences against our UK vertebrate reference database (Harper
265 et al., 2018) which contains sequences for 103 UK mammals. Taxonomic assignment used a
266 lowest common ancestor approach based on the top 10% BLAST matches for any query that

267 matched a reference sequence across more than 80% of its length at minimum identity of 98%.

268

269 **2.6 Data analysis**

270

271 Analyses were performed in R v.3.4.3 (R Core Team, 2017). The total unrefined read counts (i.e.
272 raw taxonomically assigned reads) per sample were calculated and retained for downstream
273 analyses. Assignments were corrected: family and genera containing a single UK species were
274 reassigned to that species, species were reassigned to domestic subspecies, and
275 misassignments were corrected, e.g. *Lynx pardinus* and *Lynx lynx*. Manual reassignment
276 duplicated some metaBEAT assignments thus the read count data for these assignments were
277 merged. Taxon-specific sequence thresholds (i.e. maximum sequence frequency of each taxon
278 in PCR positive controls) were used to mitigate cross-contamination and false positives (Table
279 A4, Fig. A3), and remnant contaminants and higher taxonomic assignments removed excluding
280 the following genera. *Anas* (Dabbling ducks) was retained because potential for hybridisation
281 reduced confidence in species-level assignments, and *Emberiza* (Buntings) and *Larus* (White-
282 headed gulls) were retained because reference sequences were missing for several common
283 species. Dataset refinement is fully described in Appendix A. Taxonomic assignments remaining
284 in the refined dataset were predominantly of species resolution and considered true positives.
285 We split the refined dataset by Experiment 1 (artificial waterbodies) and Experiment 2 (natural
286 ponds). Proportional read counts for each species were calculated from the total unrefined
287 read counts per sample. Our proportional read count data were not normally distributed
288 (Shapiro–Wilk normality test: $W = 0.915$, $P < 0.001$), thus we used a Mann-Whitney U test to

289 compare the median proportional read count of stratified and directed samples across species.

290 We employed binomial Generalized Linear Mixed-effects Models (GLMMs) with the logit
291 link function using the package glmmTMB (development version; Brooks et al., 2017) for the
292 following tests. First, we compared the eDNA signals from stratified and directed samples for
293 each mammal species using a hierarchical model including sample type nested within species
294 (fixed) and wildlife park (random) as effects. We tested the influence of species lifestyle on
295 mammal eDNA signals using a model with species lifestyle (fixed) and species nested within
296 wildlife park (random) as effects. Using directed samples, we tested the influence of behaviour
297 on mammal eDNA signals using two hierarchical models, including species nested within
298 wildlife park (random) and specific (e.g. swimming, drinking) or generic (i.e. water contact
299 versus no water contact) behaviour(s) respectively (fixed) as effects. We assessed model fit
300 using diagnostic plots and performed validation checks to ensure model assumptions were met
301 and overdispersion was absent (Zuur, Ieno, Walker, Saveliev, & Smith, 2009).

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

310

311

312 # 3. Results

313

314 ## 3.1 eDNA metabarcoding

315

316 The sequencing run generated 47,713,656 raw sequence reads, of which 37,590,828 remained
317 following trimming, merging, and length filter application. After removal of chimeras and
318 redundancy via clustering, the library contained 21,127,061 sequences (average read count of
319 64,215 per sample including controls), of which 16,787,750 (79.46%) were assigned a
320 taxonomic rank. Contamination (Fig. A4) was observed in the field blanks (badger, beaver, lynx,
321 pine marten, red squirrel, and water vole) as well as in the filtration and extraction blanks
322 (human [*Homo sapiens*] and cichlid). PCR negative controls were contaminated to different
323 extents with human, cichlid, beaver, and pine marten as well as non-focal species. After
324 threshold application, contaminants remaining in eDNA samples included Gentoo penguin
325 (*Pygoscelis papua*), reindeer (*Rangifer tarandus*), cichlid, and human. The refined dataset
326 contained 59 vertebrate species, including six amphibians, 10 fish, 19 birds, and 24 mammals
327 (Table A5).

328

329 ## 3.2 Experiment 1: eDNA detection and signal strength in artificial systems

330

331 All nine focal species were detected in captivity, of which seven were detected in all water

332 samples taken from their respective enclosures. HWP red deer were not detected in 2 of 5
333 stratified samples, and WT hedgehog was not detected in 1 of 2 drinking bowl samples (Fig. 1).
334 'Other' samples (neither directed nor stratified) were excluded from further comparisons, thus
335 hedgehog, red squirrel, and water vole were omitted in downstream analyses. Across species,
336 stratified samples (0.406) had a higher median proportional read count than directed samples
337 (0.373), but this difference was not significant (Mann-Whitney *U* test: $U = 1181.5$, $P = 0.829$).
338 Proportional read counts for directed and stratified samples did not significantly differ ($\chi^2_6 =$
339 0.364, $P = 0.999$) within species either (Fig. 2a; GLMM: $\theta = 0.168$, $\chi^2_{53} = 8.915$, $P = 1.000$,
340 pseudo-$R^2 = 39.21\%$). Otter proportional read counts were lower than other species, but not
341 significantly so. Similarly, species lifestyle (semi-aquatic, ground-dwelling, arboreal) did not
342 influence ($\chi^2_2 = 0.655$, $P = 0.721$) proportional read counts (Fig. 2b; GLMM: $\theta = 0.213$, $\chi^2_{61} =$
343 13.002, $P = 1.000$, pseudo-$R^2 = 11.85\%$). Proportional read counts did not differ ($\chi^2_{11} = 1.369$, P
344 $= 0.999$) according to specific behaviours exhibited by species (Fig. 3a; GLMM: $\theta = 0.355$, $\chi^2_{31} =$
345 11.013, $P = 0.999$, pseudo-$R^2 = 9.17\%$). Likewise, generic behaviour (i.e. water contact versus no
346 water contact) did not influence ($\chi^2_{11} = 0.002$, $P = 0.964$) proportional read counts (Fig. 3b;
347 GLMM: $\theta = 0.217$, $\chi^2_{41} = 8.897$, $P = 1.000$, pseudo-$R^2 = 8.50\%$).
348

349 **3.3 Experiment 2: eDNA detection and signal strength in natural systems**

350

351 At natural ponds, eDNA metabarcoding, camera trapping, and field signs all detected beaver,
352 red deer, and roe deer. Camera traps (Fig. 4) and field signs recorded red fox and badger when
353 eDNA metabarcoding did not (Fig. 5). However, eDNA metabarcoding revealed small mammals

354 missed by cameras and field signs, including water vole, water shrew (*Neomys fodiens*), bank
355 vole (*Myodes glareolus*), common shrew (*Sorex araneus*), brown rat (*Rattus norvegicus*), rabbit,
356 grey squirrel, and common pipistrelle (*Pipistrellus pipistrellus*). We observed mice or vole
357 footprints at BE Pond 1, but could not ascertain species. Fig. 5 summarises mammals recorded
358 by different methods at each site with reference to cumulative survey data. Notably, only
359 beaver was found at the same ponds by all methods. Although methods shared species at site
360 level, species were not always detected at the same pond. Detection rates for species captured
361 by at least one survey method are summarised in Table A6.

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

373 TLNR samples collected over a 5-day period (D01-05) revealed that mammal detection
374 heavily depends on the spatial and temporal resolution of eDNA metabarcoding surveys (Fig.
375 A6). Mammal eDNA signals in pond water were ephemeral, often disappearing within 24-48 hrs

376 of initial detection, as opposed to amphibians that were detected for multiple days and whose
377 eDNA signal increased in strength. The majority of semi-aquatic or terrestrial mammals were
378 only detected in a single sample on each day.
379
380
381 # 4. Discussion
382
383 We have demonstrated the potential of eDNA metabarcoding for monitoring conservation and
384 management priority mammals, but species detection rates are variable. Our experiments have
385 validated this molecular approach and provided new insights that will inform the development
386 and application of mammal eDNA metabarcoding. Sampling strategy, mammal lifestyle, and
387 mammal behaviour did not influence eDNA detection and signal strength in captivity, but all
388 played vital roles in natural ponds. Although semi-aquatic and terrestrial mammals were
389 detected from pond water, their eDNA signals were temporary and weak in comparison to
390 aquatic amphibians and fishes. Nonetheless, this suggests that eDNA is representative of
391 contemporary and local mammal diversity.
392
393 ## 4.1 Influence of sampling strategy and mammal behaviour on eDNA detection
394
395 In Experiment 1, all nine focal species were detected in captivity, and seven were detected in all
396 water samples taken from their respective enclosures. This demonstrates that our method can
397 successfully detect a variety of mammals from pond and drinking water. Surprisingly, we found

398 that neither sampling strategy nor mammal lifestyle nor mammal behaviour influenced eDNA
399 detectability and signal strength in captivity. This included behaviours associated with eDNA
400 deposition, e.g. swimming, drinking, urination, and defecation (Rodgers & Mock, 2015; Ushio et
401 al., 2017; Williams et al., 2018). Enclosures were permanently occupied and artificial
402 waterbodies likely saturated with eDNA, which possibly masked behavioural signals. Modest
403 replication may have limited experimental power, preventing patterns being detected
404 statistically. Nonetheless, our results show that mammal contact with water enables eDNA
405 deposition and detection.

406 Unsurprisingly, given the nature of wild mammal interactions with natural systems
407 versus those in captivity, Experiment 2 results highlight the challenges of mammal eDNA
408 detection. We recorded 17 mammals using three monitoring tools, comparable to the 17
409 mammals expected from cumulative survey data despite discordance. Field signs and camera
410 trapping detected red fox and badger where eDNA metabarcoding did not, but eDNA
411 metabarcoding identified water vole and other small mammals missed on camera or with
412 ambiguous field signs, i.e. mice, voles, shrews. Importantly, camera trap deployment period,
413 height, and positioning may have influenced small mammal detection by this method
414 (Caravaggi et al., 2018). Ishige et al. (2017) achieved comparable mammal detection at salt licks
415 with eDNA metabarcoding and camera trapping, but species presence was inconsistent
416 between salt licks surveyed. Using multi-species occupancy modelling for three mammal
417 species, Sales et al. (2019) observed water-based eDNA metabarcoding provided comparable
418 detection probabilities to conventional survey methods and actually outperformed camera
419 trapping. Similarly, Leempoel et al. (2019) found soil-based eDNA metabarcoding identified the

420 same mammals as camera trapping as well as small mammals rarely seen on camera, albeit the
421 methods differed between sites. Our own results echo all three studies, where despite some
422 inconsistencies, eDNA metabarcoding enhanced species inventories and identified smaller,
423 cryptic taxa.

424 Notably, no survey method captured semi-aquatic otter despite presence at study sites
425 and successful detection in eDNA metabarcoding studies of UK ponds (Harper et al., 2019),
426 lakes (Hänfling et al., 2017), and rivers/streams (Sales et al., 2019). Captive otter also had a
427 weaker eDNA signal than other semi-aquatic mammals studied here. Lower eDNA detection
428 rates for otter, badger, and red fox may stem from species' ecologies (Sales et al., 2019). These
429 mammals are wide-ranging (Gaughran et al., 2018; Thomsen et al., 2012) and may not readily
430 release DNA in water. Otters often spraint on grass or rock substrata outside water and use
431 latrines associated with caves and dens (Ruiz-Olmo & Gosálbez, 1997). As terrestrial mammals,
432 red fox and badger must drink from or enter ponds for eDNA deposition to occur (Rodgers &
433 Mock, 2015; Ushio et al., 2017; Williams et al., 2018). Otter, badger, and red fox detection may
434 require greater spatiotemporal resolution of eDNA sampling. This is reinforced by other eDNA
435 metabarcoding studies where mammal detection was highly variable across sites surveyed
436 (Ishige et al., 2017; Klymus et al., 2017; Leempoel et al., 2019; Sales et al., 2019; Ushio et al.,
437 2017). False negatives may instead be symptomatic of metabarcoding bias, but this is unlikely in
438 our study (section 4.2).

439 eDNA from other semi-aquatic mammals was evenly distributed, being found in most or
440 all samples collected on fine spatial scales within natural ponds, whereas terrestrial mammal
441 eDNA was highly localised and detected in few (<20%) samples. Mammal eDNA signals varied

442 temporally, being detectable for two consecutive days maximum. Depending on the species,
443 mammal eDNA may be spatially and temporally clumped in lentic ecosystems due to the nature
444 and frequency of water contact. Unless non-domestic mammals exhibit behaviours involving
445 prolonged water contact (e.g. swimming, wallowing), they may only be detected at drinking
446 sites (Klymus et al., 2017; Ushio et al., 2017; Williams et al., 2018). Conversely, domestic
447 mammals may have elevated detection rates in ponds due to high occurrence of these
448 waterbodies in agricultural landscapes as well as eDNA transport by rainfall and run-off (Staley
449 et al., 2018). eDNA detection and persistence are further influenced by group size, where eDNA
450 from multiple individuals endures for longer periods in water than eDNA from single individuals
451 (Williams et al., 2018). Detailed investigations incorporating biotic (e.g. population size, body
452 mass, behaviour) and abiotic (e.g. temperature, pH, rainfall) factors are needed to understand
453 the longevity of mammal eDNA signals in aquatic ecosystems (Rodgers & Mock, 2015; Sales et
454 al., 2019; Williams et al., 2018).

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

464

465 **4.2 Accounting for false positives and false negatives in metabarcoding**

466

467 eDNA metabarcoding has potential for inclusion in mammal monitoring schemes (section 4.3),
468 but like existing monitoring tools, may produce false negatives or false positives. Our process
469 controls identified low-level contamination at all stages of metabarcoding, but primarily during
470 sampling or PCR (Appendix A). We applied taxon-specific sequence thresholds to our data to
471 mitigate false positives as in Harper et al. (2019). Remnant contaminants were cichlid
472 (laboratory), Gentoo penguin (environment), reindeer (environment), and human
473 (environment/laboratory). Gentoo penguin is housed at EZ and was identified from EZ beaver
474 enclosure water. The WT red squirrel and reindeer enclosures are in close proximity. DNA
475 transport by wildlife (e.g. waterfowl [Hänfling et al., 2016]) and park staff/visitors may explain
476 this environmental contamination. Human DNA was present across process controls
477 corresponding to artificial and natural waterbodies. Human DNA may be amplified and
478 sequenced instead of focal species, potentially resulting in false negative detections for rare
479 and/or less abundant species. Human DNA blocking primers can prevent this bias, but may
480 impair PCR amplification efficiency (Klymus et al., 2017; Ushio et al., 2017; Valentini et al.,
481 2016). Sequence thresholds are one method of accounting for contamination in metabarcoding
482 datasets, but this is a topic that warrants deeper investigation aimed at researching and
483 refining standardised methods for false positive identification and mitigation, e.g. the R
484 package microDecon (McKnight et al., 2019).

485 In our study, eDNA metabarcoding produced false negatives for otter, badger, and red

486 fox at natural ponds. We selected a 12S metabarcode designed to amplify vertebrate DNA (Riaz
487 et al., 2011). One of four fox reference sequences (NCBI Accession: KF387633.1) possessed one
488 mismatch to the forward primer, and one of three otter reference sequences (NCBI Accession:
489 EF672696.1) possessed one mismatch to the reverse primer. These mismatches did not occur
490 within the first or last four bases of either primer sequence, and there were no primer
491 mismatches with the badger reference sequences (Harper et al., 2018). Therefore, amplification
492 bias was not responsible for these false negatives. DNA from aquatic and more abundant
493 species may have overwhelmed otter, badger, and red fox DNA during amplification and
494 sequencing, i.e. species-masking (Kelly, Port, Yamahara, & Crowder, 2014; Klymus et al., 2017).
495 Metabarcoding primers targeting mammals (Ushio et al., 2017) or multi-marker (e.g. 12S, 16S,
496 COI) investigations (Evans et al., 2017; Hänfling et al., 2016; Kelly et al., 2014; Klymus et al.,
497 2017) may improve mammal detection in systems with competition from non-target aquatic
498 species. Similarly, more biological and technical replication may improve species detection
499 probabilities (Evans et al., 2017; Lawson Handley et al., 2019; Sales et al., 2019; Valentini et al.,
500 2016). Importantly, otter also had lower qPCR detection than amphibians and fish (Thomsen et
501 al., 2012). A metabarcoding and qPCR comparison (e.g. Harper et al., 2018; Lacoursière-Roussel,
502 Dubois, Normandeau, & Bernatchez, 2016) would confirm whether poor amplification
503 efficiency for otter arises from technical bias or species ecology, and whether eDNA
504 metabarcoding can reliably monitor otter alongside the wider mammalian community.
505

506 ## 4.3 Scope of eDNA metabarcoding for mammal monitoring

507

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

519 eDNA metabarcoding can rapidly survey multitudes of aquatic sites at landscape-scale
520 where camera traps might be resource-intensive, cost-inefficient, and susceptible to
521 theft/damage (Ushio et al., 2017). Field signs require volunteer time and skill (Sadlier et al.,
522 2004) to be employed at comparable spatial scales to eDNA metabarcoding which could
523 provide accurate data for species misidentified from field signs, e.g. mice and voles, otter and
524 mink (Franklin et al., 2019; Harris & Yalden, 2004). However, camera traps and field signs both
525 recorded species that eDNA metabarcoding missed. Therefore, eDNA metabarcoding is
526 complementary and should be incorporated into, not replace, existing monitoring schemes
527 (Leempoel et al., 2019; Sales et al., 2019). This tool could be most effective in mammal

528 monitoring if deployed at the edges of known species distributions, in areas where species
529 presence is unknown, and in areas with isolated species records (Mathews et al., 2018).
530

531 **4.4 Recommendations for mammal survey using eDNA metabarcoding**

532

533 Water-based eDNA metabarcoding shows great promise for mammal monitoring encompassing
534 conservation and management priority species (Sales et al., 2019). However, there are factors
535 to be considered when designing and conducting mammal eDNA surveys that may not be
536 problematic for surveys of fishes or amphibians. Mammal eDNA detection probabilities from
537 natural ponds will likely be high when areas with dense populations are studied, but rigorous
538 sampling strategies will be required to track mammals in areas sparsely populated by
539 individuals. Multiple ponds must be sampled repeatedly, and samples taken at multiple
540 locations within ponds without pooling to enable site occupancy inferences. Importantly, we
541 sampled natural ponds in spring but sampling in other seasons may produce different results,
542 reflective of species' ecologies (Lawson Handley et al., 2019). To account for differential
543 mammal visitation rates and maximise eDNA detection probabilities, we recommend that
544 researchers and practitioners using eDNA metabarcoding for mammal monitoring channel their
545 efforts into extensive sampling of numerous waterbodies in a given area over prolonged
546 timescales. Water-based eDNA appears to be indicative of contemporary mammal presence,
547 with most mammal eDNA signals lost within 1-2 days. Therefore, eDNA metabarcoding could
548 provide valuable mammalian community "snapshots" that may not be obtained with other
549 survey methods (Ushio et al., 2017). Different sample types (e.g. water, soil, snow, salt licks,

550 feeding traces, faeces, hair, and blood meals) may also offer new insights to mammal
551 biodiversity (Franklin et al., 2019; Ishige et al., 2017; Kinoshita et al., 2019; Leempoel et al.,
552 2019; Sales et al., 2019; Tessler et al., 2018; Ushio et al., 2017).
553
554

555 ## Data accessibility

556

557 Raw sequence reads have been archived on the NCBI Sequence Read Archive (Study:
558 SRP164740; BioProject: PRJNA495011; BioSamples: SAMN10195928 - SAMN10196255; SRA
559 accessions: SRR7986451 - SRR7986778). Jupyter notebooks, R scripts and corresponding data
560 are archived online (https://doi.org/10.5281/zenodo.2561415).
561
562

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564

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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.

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752

753 **Table 1.** Ethogram used to catalogue mammal behaviours that occur in or near artificial
754 waterbodies in captive enclosures. Importantly, this ethogram was designed to catalogue
755 mammal behaviours potentially leading to eDNA deposition. Therefore, it may not be
756 comparable to ethograms typically used in study of captive animals.

757

Behaviour	Definition
Swimming	Mammal completely submerged in and moving through waterbody using limbs
Bodypart in water	Mammal partially submerged in waterbody, e.g. foot or tail in water
Drinking	Water taken into mouth and swallowed by mammal
Feeding	Food taken into mouth and swallowed by mammal in or near waterbody, e.g. otter and fish
Scratching	Bodypart or external object in enclosure used by mammal to relieve itch near waterbody
Urinating/scent-marking	Liquid excretion passed by mammal in or near waterbody
Pooing	Solid excretion passed by mammal in or near waterbody
Sniffing	Air visibly drawn through nose of mammal to detect a smell around waterbody, possibly involving contact with water
Standing	Mammal motionless in or near waterbody
Walking	Mammal moving around waterbody at a regular pace by lifting and setting down each foot in turn, never having both feet off the ground at once
Running	Mammal moving around waterbody at a speed faster than a walk, never having both or all the feet on the ground at the same time
Vocalising	Mammal producing sound while in or near waterbody
Grooming	Mammal cleaning fur or skin with its tongue while in or near waterbody
Resting	Mammal lying down or sitting in or near waterbody
Other	Behaviour exhibited in or near waterbody that does not conform to other categories, e.g. chasing tail
Not visible	Mammal moved to part of enclosure not visible to the observer

758

759 **Table 2.** Summary of focal species studied at wildlife parks and their lifestyle. The number of

760 individuals present and waterbody size in enclosures is provided.

761

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

762

763

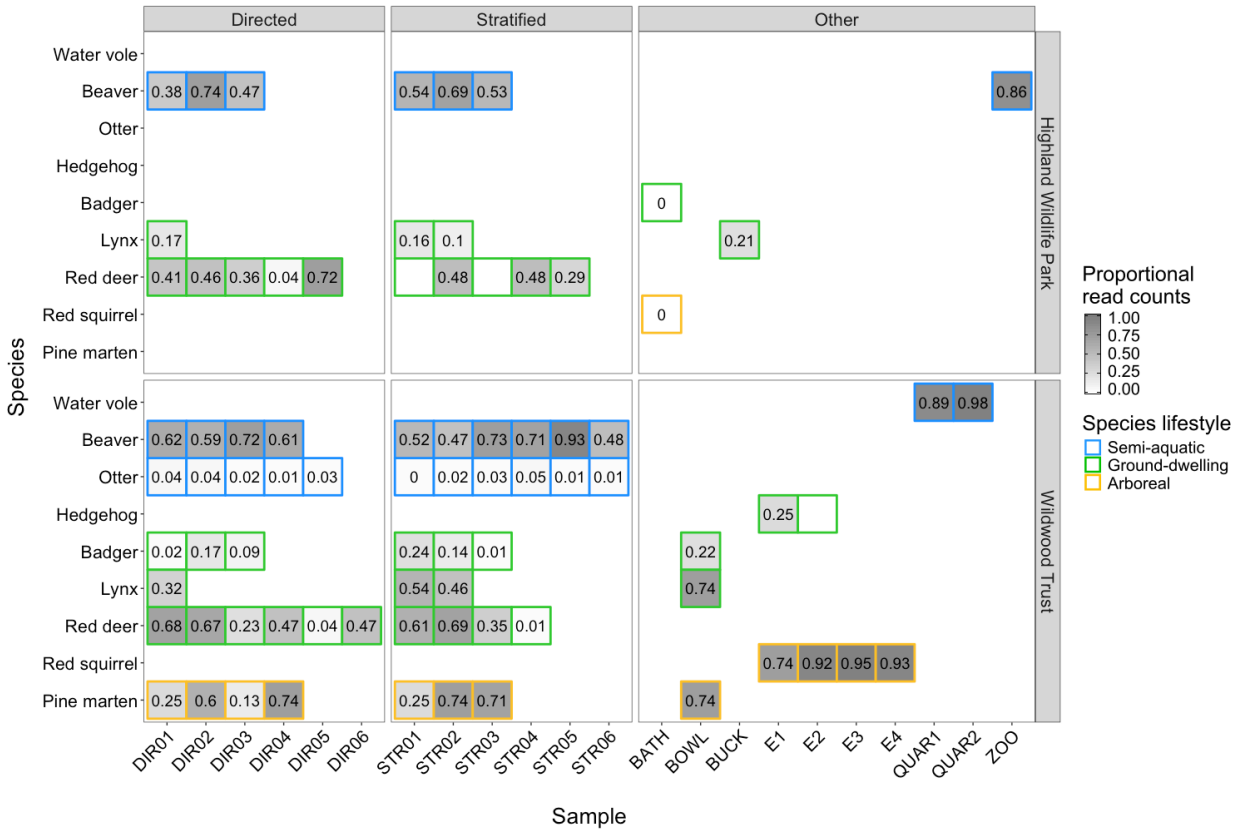
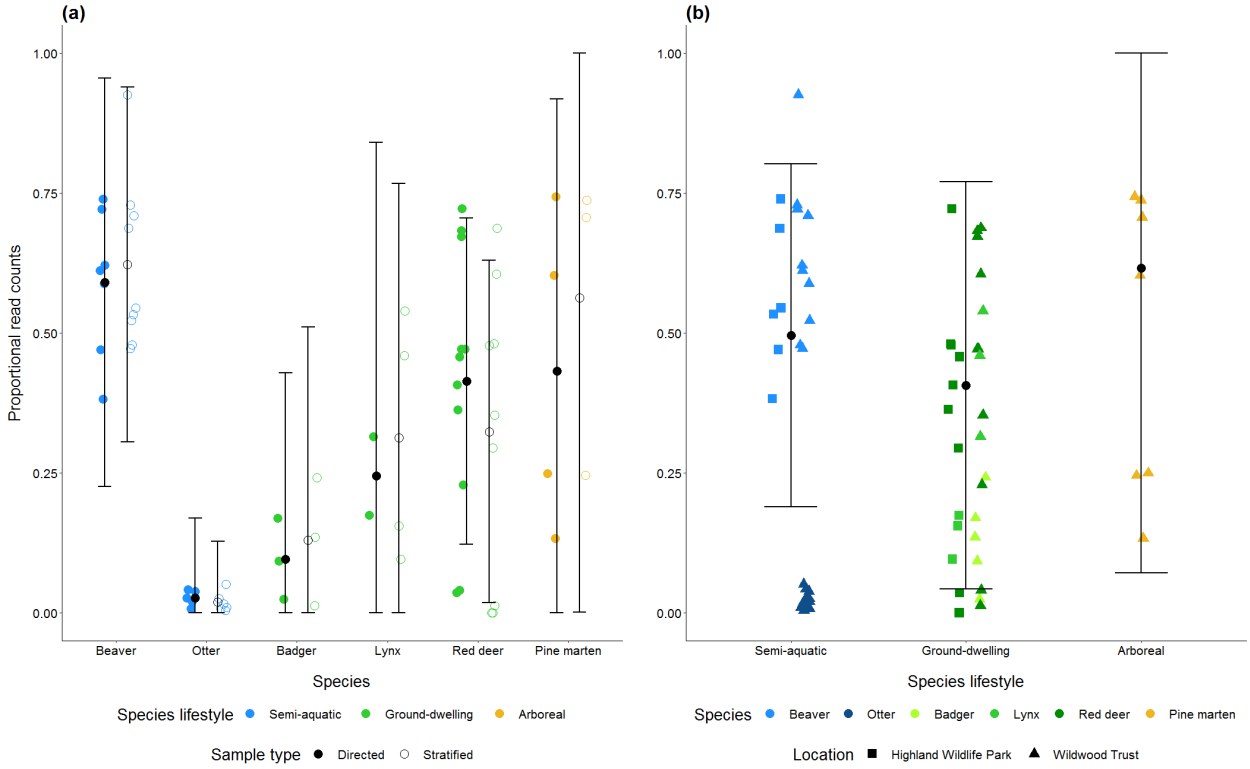


Figure 1. Heatmap showing proportional read counts for eDNA samples from Experiment 1. Each square represents a sample taken from a wildlife park enclosure. Directed (DIR01-DIR06) and stratified (STR01-STR06) samples were collected from artificial waterbodies. Samples were collected from drinking containers (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. Samples containing a 0 in the corresponding cell had proportional read counts greater than two decimal places. Samples with proportional read counts of 0 are represented by empty cells.



774

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

776 and sample type nested within species **(a)** or species lifestyle **(b)** for Experiment 1. The

777 observed data (coloured points) are displayed against the predicted relationships (black points

778 with error bars) for each species **(a)** or species lifestyle **(b)**. Points are shaped by sample type **(a)**

779 or wildlife park **(b)**, and coloured by species lifestyle. Error bars represent the standard error

780 around the predicted means.

781

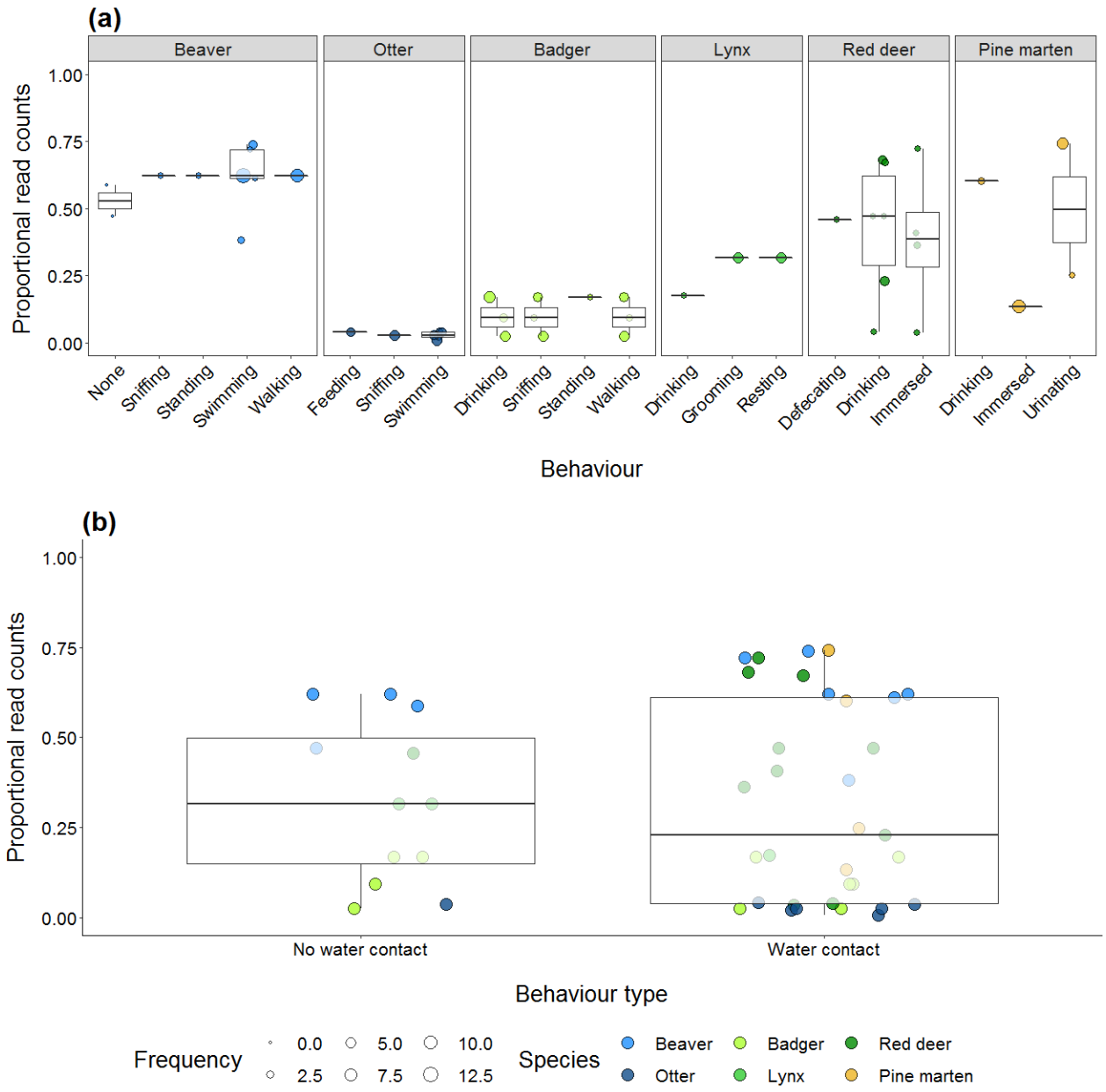


Figure 3. Boxplots showing the mean proportional read counts for specific **(a)** and generic **(b)** behaviour(s) exhibited by focal species in Experiment 1. Boxes show 25th, 50th, and 75th percentiles, and whiskers show 5th and 95th percentiles. Points are coloured by species lifestyle, and each point 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.

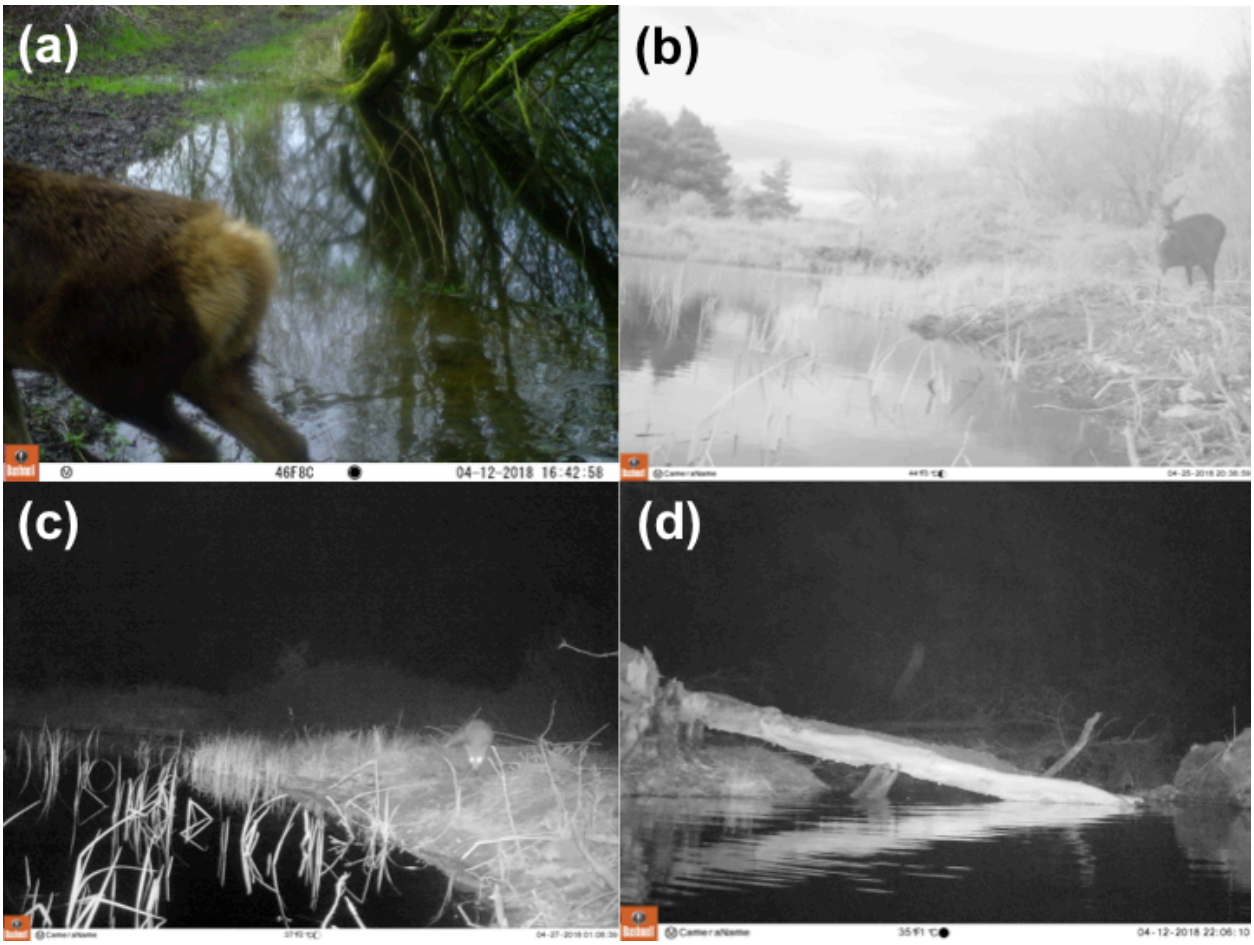


Figure 4. Exemplar camera trap photos taken at natural ponds where focal species were present in Experiment 2. Red deer was recorded at Thorne Moors **(a)**, roe deer **(b)** and red fox **(c)** were recorded at Tophill Low Nature Reserve, and beaver was recorded at the Bamff Estate **(d)**.

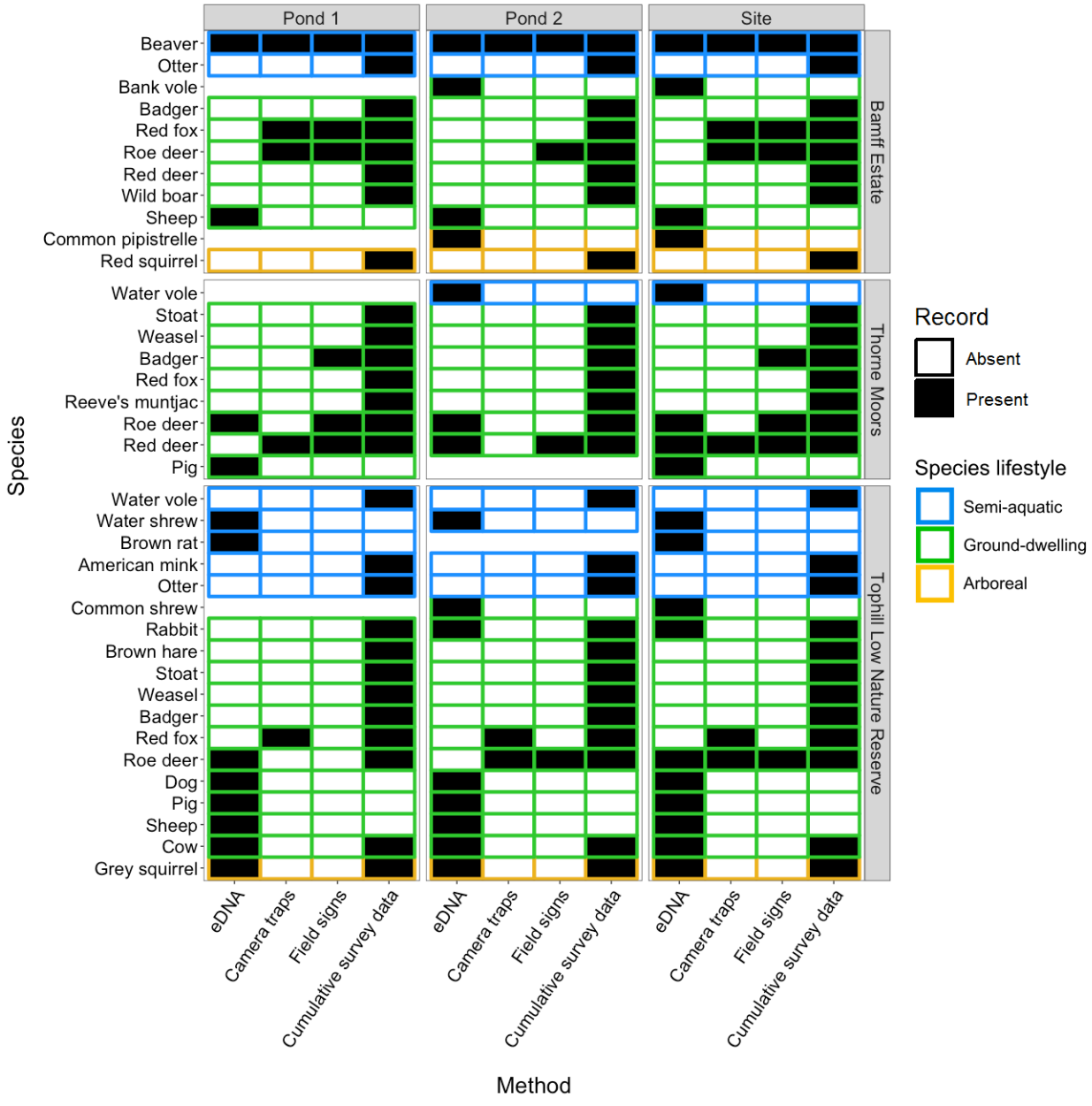


Figure 5. Tile plot showing species presence-absence at individual pond and site-level as indicated by field signs, camera trapping, and eDNA metabarcoding in Experiment 2. Surveys were performed at sites where focal species presence was confirmed by cumulative survey data.