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 (i.e. food, medicine, materials), habitat degradation/loss (e.g. agriculture), non-native species (i.e. competition, hybridisation, disease transmission) or perception as pests (Visconti et al., 2011). The paucity of data for mammals prevents robust estimation of 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 while widespread species receive less attention (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 monitoring tools, such as camera traps and field signs (Caravaggi et al., 2018; Harris & Yalden, 2004; McShea, Forrester, Costello, He, & Kays, 2016; 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; Glen, Cockburn, Nichols, Ekanayake, & Warburton, 2013; Ishige et al., 2017; McShea et al., 2016; 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).

Environmental DNA (eDNA) analysis is a recognised tool for rapid, non-invasive, cost-efficient biodiversity assessment (Deiner et al., 2017). Organisms transfer their genetic material to their environment via secretions, excretions, gametes, blood, or decomposition, which can be isolated from environmental samples (Thomsen & Willerslev, 2015). Targeted (i.e. single-species) eDNA analysis, using PCR, quantitative PCR (qPCR), or droplet digital PCR of eDNA samples, is increasingly applied to survey marine (Baker, Steel, Nieukirk, & Klinck, 2018; Hunter et al., 2018; Parsons, Everett, Dahlheim, & Park, 2018), freshwater (Lugg, Griffiths, van Rooyen, Weeks, & Tingley, 2017; Padgett-Stewart et al., 2016; Thomsen et al., 2012), and terrestrial (Aylward, Johnson, Sullivan, Perry, & Louis, 2018; Franklin et al., 2019; Rodgers & Mock, 2015; Williams, Huyvaert, Vercauteren, Davis, & Piaggio, 2018) mammals. Alternatively, eDNA metabarcoding can screen entire communities using PCR combined with high-throughput sequencing (Deiner et al., 2017; Thomsen & Willerslev, 2015), but eDNA metabarcoding assessments of mammal communities are less common (Klymus, Richter, Thompson, & Hinck, 2017; Ushio et al., 2017). Mammal assemblages have been obtained by metabarcoding invertebrate blood meals (e.g. Tessler et al., 2018) and salt licks (Ishige et al., 2017) in tropical habitats, but samples from the physical environment have tremendous potential to reveal mammal biodiversity over broad spatiotemporal scales (Ushio et al., 2017).

In aquatic ecosystems, eDNA metabarcoding has predominantly been applied to

101 characterise fish (e.g. Evans et al., 2017; Hänfling et al., 2016; Lawson Handley et al., 2018;
102 Valentini et al., 2016) and amphibian (e.g. Bálint et al., 2018; Valentini et al., 2016)
103 communities. However, mammals also leave eDNA signatures in water that metabarcoding can
104 detect (Harper et al., 2018a; Klymus et al., 2017; Port et al., 2016; Ushio et al., 2017). Ponds in
105 particular act as stepping stones for semi-aquatic and terrestrial mammals by providing
106 drinking, foraging, dispersive, and reproductive opportunities (Klymus et al., 2017). Therefore,
107 samples from these waterbodies could provide information on biodiversity present in the wider
108 environment (Deiner et al., 2017; Harper et al., 2018a).

109 Despite evidence for eDNA deposition by semi-aquatic and terrestrial mammals in
110 freshwater systems (Harper et al., 2018a; Klymus et al., 2017; Lugg et al., 2017; Padgett-Stewart
111 et al., 2016; Rodgers & Mock, 2015; Thomsen et al., 2012; Ushio et al., 2017; Williams et al.,
112 2018), little is known about the influence of mammal behaviour on the distribution and
113 strength of the eDNA signal left behind. Drinking is a major source of eDNA deposition in water
114 due to the release of saliva, but mammals may also swim, wallow, urinate or defecate in water
115 (Rodgers & Mock, 2015; Ushio et al., 2017; Williams et al., 2018). Furthermore, arboreal
116 mammals may be expected to use ponds less than semi-aquatic and ground-dwelling species,
117 non-territorial mammals may visit ponds less than territorial species, and species living in
118 groups may leave more DNA in water than solitary species (Williams et al., 2018).

119 In this study, we conducted two experiments to evaluate eDNA metabarcoding of pond
120 water as a tool for monitoring semi-aquatic, ground-dwelling, and arboreal mammals of
121 conservation or management concern. The first experiment, carried out on nine focal species
122 housed at two wildlife parks, was designed to examine the role of sampling strategy, mammal

lifestyle, and mammal behaviour on eDNA detection and signal strength (i.e. read counts). Our hypotheses for Experiment 1 were as follows: (1) eDNA would be unevenly distributed thus directed sampling would yield stronger eDNA signals (i.e. higher read counts) for mammals than stratified sampling; (2) semi-aquatic mammals would have stronger eDNA signals than ground-dwelling or arboreal mammals; and (3) mammal behaviours involving water contact (e.g. swimming, drinking) would be associated with stronger eDNA signals. The second experiment aimed to validate eDNA metabarcoding against camera trapping and field sign searches at natural ponds within sites where focal species were present. For Experiment 2, our hypotheses were: (1) eDNA metabarcoding would detect more mammals than camera trapping or field signs; (2) semi-aquatic mammals would be readily detected and their eDNA evenly distributed in ponds in comparison to terrestrial mammals; and (3) temporal sampling would reveal that terrestrial mammal eDNA is detectable for short periods in comparison to fully aquatic vertebrates.

2. Materials and methods

2.1 Study species

We studied nine mammal species that are the focus of UK conservation or management (Battersby & Tracking Mammals Partnership, 2005; Mathews et al., 2018): European water vole (*Arvicola amphibius*), European otter (*Lutra lutra*), Eurasian beaver (*Castor fiber*), European

145 hedgehog (*Erinaceus europaeus*), European badger (*Meles meles*), red deer (*Cervus elaphus*),
146 Eurasian lynx (*Lynx lynx*), red squirrel (*Sciurus vulgaris*), and European pine marten (*Martes*
147 *martes*). Water vole, otter, red squirrel, pine marten and hedgehog are UK Biodiversity Action
148 Plan species (Joint Nature Conservation Committee, 2018). Lynx is not currently present in the
149 UK, but reintroduction trials have been proposed (Lynx UK Trust, 2018). Water vole, otter, and
150 beaver are semi-aquatic, red squirrel and pine marten are arboreal, and the other species are
151 ground-dwelling. Badger and red deer live in groups whereas the other species are solitary,
152 except when courting, mating, and rearing young.
153

154 **2.2 Experiment 1: eDNA detection and signal strength in artificial systems**

155

156 We performed eDNA metabarcoding for focal species under artificial conditions at two wildlife
157 parks to investigate whether mammal eDNA signal strength (i.e. read counts) depends on
158 sampling strategy, species lifestyle (semi-aquatic, ground-dwelling, arboreal), and behaviour.
159 Behavioural observation and eDNA sampling were conducted between 18th - 21st September
160 2017 at Wildwood Trust (WT), Kent, England, and 10th - 11th October 2017 at Royal Zoological
161 Society of Scotland (RZSS) Highland Wildlife Park (HWP), Kingussie, Scotland. Sixteen categories
162 of behaviour were defined based on potential contact with waterbodies and species lifestyle,
163 and the frequency and duration of behaviours recorded (Table S1). The number of individuals in
164 each enclosure was recorded alongside waterbody size (Table 1). Beaver, lynx, red deer, and
165 red squirrel were present at both wildlife parks, whereas other species were only present at
166 WT. Each species was observed for one hour on two separate occasions with exceptions.

167 Badger and beaver were nocturnal and required overnight observation using camera traps
168 (Bushnell Trophy Cam Aggressor). Camera traps (one per enclosure) were positioned
169 perpendicular to the ground at a height of 1 m and distance of 2 m from the pond shoreline to
170 capture water and shoreline in the field of view. Cameras were set to take 30 s videos (1920 x
171 1080) when triggered (30 s interval between triggers) at high sensitivity. Behavioural
172 observation was not undertaken for WT water voles as animals were under quarantine or HWP
173 red squirrels as individuals were wild and widely distributed.

174 Water samples were collected from enclosures within 3 hrs of the second behavioural
175 observation period. Up to six directed or stratified samples were collected, but sample number
176 varied by species due to waterbody size as well as type and frequency of behaviours observed
177 (Tables S1, S2). Enclosure drinking containers were also sampled and classed as 'other' samples.
178 There were no artificial waterbodies in the WT water vole, red squirrel, or hedgehog
179 enclosures, thus bathing and drinking bowls were sampled. The HWP beaver enclosure was
180 empty for 24 hrs before sampling as animals were moved to RZSS Edinburgh Zoo (EZ), Scotland,
181 for quarantine before wild release. Water from the empty enclosure and the EZ beaver
182 quarantine enclosure was sampled. A sample was also collected from a water bath situated in
183 the HWP woods to capture any wild red squirrels present and classed as 'other'.

184 Directed samples (2 L surface water taken approximately where behaviours were
185 observed) were collected before stratified samples (2 L surface water [8 x 250 ml pooled
186 subsamples] taken at equidistant points around the waterbody perimeter where access
187 permitted) to minimise disturbance to the water column and cross-contamination risk. All
188 samples were collected using sterile Gosselin™ HDPE plastic bottles (Fisher Scientific UK Ltd,

189 UK) and disposable gloves. A field blank (1 L molecular grade water [MGW]) was taken into
190 each species enclosure and opened. Samples (*n* = 80) collected from WT and HWP were
191 transported alongside field blanks (*n* = 13) in sterile coolboxes with ice packs to the University
192 of Kent (UoK) and EZ respectively, where ice was added to coolboxes.

193 Samples and blanks were vacuum-filtered within 6 hrs of collection in a UoK wet
194 laboratory that housed exotic amphibians (Table S3) and within 24 hrs of collection in a EZ staff
195 room. All surfaces and equipment were sterilised before, during, and after set-up in temporary
196 work areas. Surfaces and vacuum pumps were wiped with 10% v/v chlorine-based commercial
197 bleach (Elliott Hygiene Ltd, UK) solution. Non-electrical equipment was immersed in 10% bleach
198 solution for 10 minutes, followed by 5% v/v MicroSol detergent (Anachem, UK), and rinsed with
199 purified water. Up to 500 ml of each 2 L sample was vacuum-filtered through sterile 0.45 µm
200 mixed cellulose ester membrane filters with pads (47 mm diameter; Whatman, GE Healthcare,
201 UK) using Nalgene™ filtration units. One hour was allowed for each sample to filter and a
202 second filter used if clogging occurred. A filtration blank (1 L MGW) was processed during each
203 filtration round (*n* = 12), and equipment sterilised after each filtration round. After 500 ml had
204 filtered or one hour had passed, filters were removed from pads using sterile tweezers, placed
205 in sterile 47 mm petri dishes (Fisher Scientific UK Ltd, UK), sealed with parafilm (Sigma-Aldrich
206 Company Ltd, UK), and stored at -20 °C. The total water volume filtered per sample was
207 recorded for downstream analysis (Table S2; Fig. S1).

208

209 **2.3 Experiment 2: eDNA detection and signal strength in natural systems**

210

211 We performed eDNA metabarcoding for focal species at natural ponds in conjunction with
212 conventional surveys to validate this molecular approach for mammal identification and to
213 investigate spatiotemporal variation in mammal eDNA signals. At three sites where focal
214 species were present based on cumulative survey data from landowners, reserve managers,
215 researchers, and volunteers, we selected two ponds (range 293-5056 m^2, average 1471 m^2)
216 within <4 km of each other. The Bamff Estate (BE), Alyth, Scotland, was selected for beaver,
217 otter, badger, red deer, and red squirrel, but roe deer (*Capreolus capreolus*) and red fox (*Vulpes*
218 *vulpes*) were also present. Otter, water vole, and badger were also present at Tophill Low
219 Nature Reserve (TLNR), Driffield, East Yorkshire, where American mink (*Neovison vison*), stoat
220 (*Mustela erminea*), weasel (*Mustela nivalis*), rabbit (*Oryctolagus cuniculus*), brown hare (*Lepus*
221 *europaeus*), fox, roe deer, and grey squirrel (*Sciurus carolinensis*) additionally occur. We
222 selected Thorne Moors (TM), Doncaster, South Yorkshire, for red deer and badger, but stoat,
223 weasel, red fox, roe deer, and Reeve's muntjac (*Muntiacus reevesi*) were also present. Camera
224 traps (Bushnell Trophy Cam Aggressor) were deployed at TM (one per pond) and BE (three per
225 pond) one week prior to eDNA sampling and collected once sampling was completed. At TLNR,
226 camera traps (two at one pond and three at the other pond) were deployed one day before a 5-
227 day period of eDNA sampling and collected one week after sampling was completed. Camera
228 traps were positioned perpendicular to the ground at a height of 1 m and distance of 0.3-1 m
229 from the pond shoreline to capture water and shoreline in the field of view. Cameras were set
230 to take three photographs (5 megapixel) when triggered (3 s interval between triggers) at high

231 sensitivity.

232 Ten stratified samples (2 L surface water [8 x 250 ml pooled subsamples] taken at
233 equidistant points around the pond perimeter where access permitted) were collected from the
234 shoreline of each pond and a field blank (1 L MGW) included using the same procedures as
235 Experiment 1. TM ponds were sampled on 17th April 2018, and BE ponds on 20th April 2018.
236 TLNR ponds were sampled every 24 hrs between 23rd - 27th April 2018 to investigate
237 spatiotemporal variation in mammal eDNA signals. TM and TLNR samples were transported on
238 ice in sterile coolboxes with ice packs to the University of Hull (UoH) eDNA facility, which is
239 devoted to pre-PCR processes with separate rooms for filtration, DNA extraction and PCR
240 preparation of environmental samples, and stored at 4 °C. BE samples were transported in
241 sterile coolboxes with ice packs to BE accommodation. Surfaces and equipment were sterilised
242 before, during, and after set-up in local accommodation as in Experiment 1. Samples (n = 140)
243 and field blanks (n = 14) were vacuum-filtered within 4 hrs of collection as outlined in
244 Experiment 1 with minor modifications to maximise detection probability as follows. The full 2 L
245 of each sample was vacuum-filtered where possible, two filters were used for each sample, and
246 duplicate filters were stored in one petri dish at -20 °C. A filtration blank (1 L MGW) was
247 processed during each filtration round (n = 21). The total water volume filtered per sample was
248 recorded (Table S4).

249

250 ## 2.4 DNA extraction

251

252 DNA was extracted within 2 weeks of filtration at the UoH eDNA facility using the water variant
253 of mu-DNA, a DNA extraction protocol tailored for complex environmental samples (Sellers, Di
254 Muri, Gómez, & Hänfling, 2018). Duplicate filters from samples in Experiment 1 were lysed
255 independently and the lysate from each loaded onto one spin column. Due to a larger sample
256 size in Experiment 2, duplicate filters from samples were co-extracted by placing both filters in
257 a single tube for bead milling. An extraction blank, consisting only of extraction buffers, was
258 included for each round of DNA extraction (*n* = 17). Eluted DNA (100 µl) was stored at -20 °C
259 until PCR amplification.

260

261 ## 2.5 eDNA metabarcoding

262

263 Our eDNA metabarcoding workflow is fully described in Supporting Information. Briefly, we
264 performed nested metabarcoding using a two-step PCR protocol, where Multiplex Identification
265 (MID) tags were included in the first and second PCR for sample identification (Kitson et al.,
266 2019). In the first PCR, eDNA was amplified in triplicate with published 12S ribosomal RNA
267 (rRNA) primers 12S-V5-F (5'-ACTGGGATTAGATACCCC-3') and 12S-V5-R (5'-
268 TAGAACAGGCTCCTCTAG-3') (Riaz et al., 2011) that were validated *in silico* for UK vertebrates
269 (Harper et al., 2018a, b). PCR positive controls (two per PCR plate; *n* = 16) were exotic cichlid
270 (*Maylandia zebra*) DNA (0.05 ng/µl), and PCR negative controls (two per PCR plate; *n* = 16) were
271 MGW (Fisher Scientific UK Ltd, UK). PCR products were pooled to create sub-libraries (Fig. S2)

272 and purified. The second PCR bound pre-adapters, MID tags, and Illumina adapters to the sub-
273 libraries. PCR products were purified, quantified, and pooled, following which the library was
274 purified, quantified by qPCR, and fragment size (330 bp) and removal of secondary product
275 verified. The library (220 eDNA samples, 27 field blanks, 33 filtration blanks, 17 extraction
276 blanks, 16 PCR negative controls, and 16 PCR positive controls) was sequenced on an Illumina
277 MiSeq®. Raw sequence reads were demultiplexed using a custom Python script, following which
278 metaBEAT (metaBarcoding and Environmental Analysis Tool) v0.97.11
279 (https://github.com/HullUni-bioinformatics/metaBEAT) was used for quality trimming, merging,
280 chimera removal, clustering, and taxonomic assignment of sequences against our UK vertebrate
281 reference database (Harper et al., 2018a, b) which contains sequences for all UK mammals
282 including focal species.
283

284 **2.6 Data analysis**

285

286 Analyses were performed in R v.3.4.3 (R Core Team, 2017). The total unrefined read counts (i.e.
287 raw taxonomically assigned reads) per sample were calculated and retained for downstream
288 analyses. Assignments were corrected and merged, taxon-specific sequence thresholds (i.e. the
289 maximum sequence frequency of each taxon in PCR positive controls) used to mitigate cross-
290 contamination and false positives (Fig. S3), and remnant contaminants and higher taxonomic
291 assignments removed with exceptions. The genus *Anas* (Dabbling ducks) was not removed due
292 to potential for hybridisation reducing confidence in species-level assignments, and the genera
293 *Emberiza* (Buntings) and *Larus* (White-headed gulls) were not removed as reference sequences

294 were missing for several common species. Consequently, taxonomic assignments in the refined
295 dataset (i.e. reads left after assignments were corrected, merged, removed, and had sequence
296 thresholds applied) were predominantly of species resolution and considered true positives.
297 Dataset refinement is fully described in Supporting Information: Appendix 1. We subsetted the
298 data by experiment to generate separate datasets for eDNA samples from artificial waterbodies
299 (Experiment 1) and natural ponds (Experiment 2). Proportional read counts for each species
300 were calculated from the total unrefined read counts per sample. Our proportional read count
301 data were not normally distributed (Shapiro–Wilk normality test: $W = 0.915$, $P < 0.001$), thus we
302 used a Mann-Whitney *U* test to compare the median proportional read count of stratified and
303 directed samples across species.

304 We employed binomial Generalized Linear Mixed Models (GLMMs) with the logit link
305 function from the development version of the R package glmmTMB (Brooks et al., 2017) for the
306 following tests. First, we compared the eDNA signals from stratified and directed samples for
307 each mammal species, with higher proportional read counts expected from directed than
308 stratified samples. A hierarchical model including sample type nested within species (fixed) and
309 wildlife park (random) as effects was used. We tested the hypothesis that species lifestyle
310 would influence mammal eDNA signals, with higher proportional read counts anticipated for
311 semi-aquatic than ground-dwelling or arboreal species. This model included species lifestyle
312 (fixed) and species nested within wildlife park (random) as effects. Using the directed samples,
313 we tested the hypothesis that mammal behaviour influences their eDNA signals. We expected
314 that behaviour(s) involving water contact would be associated with higher proportional read
315 counts. We used two hierarchical models including species nested within wildlife park as a

316 random effect to test the influence of behaviour(s) on proportional read counts. These
317 modelled specific (e.g. swimming, drinking) or generic (i.e. water contact versus no water
318 contact) behaviour(s) as a fixed effect. We did not have enough data on mammal behavioural
319 frequencies or duration to test for an effect on proportional read counts. We assessed model fit
320 using diagnostic plots and performed validation checks to ensure model assumptions were met
321 where possible and absence of overdispersion (Zuur, Ieno, Walker, Saveliev, & Smith, 2009).
322 Predictions for each model were obtained using the *predict* function and 95% confidence
323 intervals were calculated from the standard error of the predictions.

324 For Experiment 2, we tested two hypotheses relating to spatiotemporal variation in
325 mammal eDNA signals at natural ponds. We qualitatively compared mammal presence-absence
326 records generated by eDNA metabarcoding, camera trapping, and field signs, with eDNA
327 metabarcoding expected to identify more species than other survey methods. TLNR samples
328 spanned a 5-day period, where 10 samples were taken from each pond at the same locations
329 every 24 hrs. For the method performance comparison with BE and TM ponds, which were
330 sampled once each at 10 locations, the proportional read count data for TLNR samples were
331 averaged to condense 50 samples to 10 samples per pond. To test the hypothesis that eDNA
332 signals from aquatic species would be more evenly distributed and persist longer than those
333 from terrestrial mammals, the unaveraged data from TLNR ponds were used. We qualitatively
334 assessed change in proportional read counts for identified species over the 5-day period using
335 tile plots and heat maps. All figures were produced using the R package ggplot2 v3.0.0
336 (Wickham, 2016).

337

338

339 # 3. Results

340

341 ## 3.1 eDNA metabarcoding

342

343 The sequencing run generated 47,713,656 raw sequence reads, of which 37,590,828 remained
344 following trimming, merging, and length filter application. After removal of chimeras and
345 redundancy via clustering, the library contained 21,127,061 sequences (average read count of
346 64,215 per sample including controls), of which 16,787,750 (79.46%) were assigned a
347 taxonomic rank. Environmental contamination was observed in the field blanks (badger,
348 beaver, lynx, pine marten, red squirrel, and water vole) as well as environmental and/or
349 laboratory contamination in the filtration and extraction blanks (human [*Homo sapiens*] and
350 cichlid). PCR negative controls were contaminated with human, cichlid, beaver, and pine
351 marten as well as non-focal species (Fig. S4). After threshold application, contaminants
352 remaining in eDNA samples included Gentoo penguin (*Pygoscelis papua*), reindeer (*Rangifer*
353 *tarandus*), cichlid, and human. The refined dataset contained 62 vertebrate species, including
354 six amphibians, 10 fish, 22 birds, and 24 mammals (Table S6).

355

356 ## 3.2 Experiment 1: eDNA detection and signal strength in artificial systems

357

358 All nine focal species were detected in captivity. Seven species (red deer and hedgehog

excluded) were detected in all water samples taken from their respective enclosures. Red deer were not detected in 2 of 5 stratified samples from HWP, and hedgehog was not detected in 1 of 2 WT drinking bowl samples (Fig. 1). 'Other' samples (neither directed nor stratified) were excluded from further comparisons, thus hedgehog, red squirrel, and water vole were omitted in downstream analyses.

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

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

381

382 **3.3 Experiment 2: eDNA detection and signal strength in natural systems**

383

384 At natural ponds, eDNA metabarcoding, camera trapping, and field signs all detected beaver,
385 red deer, and roe deer. Camera traps (Fig. 4) and field signs recorded red fox and badger when
386 eDNA metabarcoding did not (Fig. 5). However, eDNA metabarcoding revealed small mammal
387 species not caught on camera, including water vole, water shrew (*Neomys fodiens*), bank vole
388 (*Myodes glareolus*), common shrew (*Sorex araneus*), brown rat (*Rattus norvegicus*), rabbit, grey
389 squirrel, and common pipistrelle (*Pipistrellus pipistrellus*). Mice or vole footprints were
390 observed at BE Pond 1, but species could not be determined. Fig. 5 summarises mammals
391 recorded by different methods at each site with reference to cumulative survey data. Notably,
392 only beaver presence was captured at the same ponds by all methods. Although methods
393 shared species at site level, species were not always detected at the same pond. Detection
394 rates for species captured by at least one survey method are summarised in Table S7.

395 Sampling of natural ponds revealed spatial patterns in eDNA detection and signal
396 strength. eDNA from non-domestic terrestrial mammals (i.e. mammals excluding dog [*Canis*
397 *lupus familiaris*], pig [*Sus scrofa domesticus*], sheep [*Ovis aries*] and cow [*Bos taurus*]) was
398 unevenly dispersed compared with semi-aquatic mammals (Fig. S5). Semi-aquatic beaver and
399 water vole were detected in at least 90% and 60% respectively of water samples (N = 10)
400 collected from single ponds, whereas water shrew was only detected in 10% of samples.
401 Conversely, non-domestic terrestrial mammals were consistently detected in less than 20% of
402 water samples collected from a pond and left relatively weak eDNA signals. Overall, beaver was

403 the most consistently detected mammal with the highest proportional read counts. However,
404 the strongest and most evenly distributed signals belonged to amphibians, particularly common
405 frog (*Rana temporaria*) and great crested newt (*Triturus cristatus*) (Fig. S5).

406 TLNR samples collected over a 5-day period (D01-05) revealed that mammal detection
407 was highly dependent on the spatial and temporal resolution of eDNA metabarcoding surveys
408 (Fig. S6). Mammal eDNA signals in pond water were ephemeral, often disappearing within 24-
409 48 hrs of initial detection, as opposed to amphibians that were detected for multiple days and
410 whose eDNA signal increased in strength. The majority of semi-aquatic or terrestrial mammals
411 were only detected in a single sample on each day.

412

413

414 4. Discussion

415

416 We have demonstrated that eDNA metabarcoding is a potential monitoring tool for
417 conservation and management priority mammals, but species detection rates are highly
418 variable. We used two experiments to validate this molecular approach and gained new insights
419 that will inform the development and application of eDNA metabarcoding for mammals.
420 Sampling strategy, mammal lifestyle, and mammal behaviour had little influence on eDNA
421 detection and signal strength in captivity, but all played vital roles in natural ponds. Although
422 mammals were detected from pond water, their eDNA signals were temporary and weak in
423 comparison to amphibians. Nonetheless, this would suggest eDNA is representative of
424 contemporary and local mammal diversity in a given area.

425

426 **4.1 Experimental insights**

427

428 In Experiment 1, all nine focal species were detected in captivity, and seven of those species
429 were detected in all water samples taken from their respective enclosures. This demonstrates
430 that our method can successfully detect a wide range of mammal species from pond and
431 drinking water. Perhaps surprisingly, we found that none of sampling strategy, mammal
432 lifestyle or mammal behaviour influenced eDNA detectability and signal strength in enclosures.
433 We found no significant differences in eDNA detection or signal strength between stratified and
434 directed samples collected from artificial waterbodies across or within species. There were no
435 differences in eDNA signal strength between semi-aquatic, ground-dwelling, and arboreal
436 species. No relationships between proportional read counts and specific or generic behaviour(s)
437 were found. This included behaviours associated with eDNA deposition, e.g. swimming,
438 drinking, urination, and defecation (Rodgers & Mock, 2015; Ushio et al., 2017; Williams et al.,
439 2018). Enclosures were permanently occupied and artificial waterbodies likely saturated with
440 eDNA, which possibly masked behavioural signals. Our sample size was constrained as most
441 focal species were housed by WT only. Modest replication may have limited experimental
442 power, preventing patterns being detected statistically. Nonetheless, our results have
443 demonstrated that mammal contact with water enables eDNA deposition and detection.

444 Unsurprisingly, given the nature of wild mammal interactions with natural systems
445 versus those in captivity, results from Experiment 2 highlighted the challenges of mammal
446 eDNA detection. We detected 17 mammals using three monitoring tools, comparable to the 17

447 mammals expected from cumulative survey data despite some differences. Field signs and
448 camera trapping performed better than eDNA metabarcoding for red fox and badger detection,
449 but only eDNA metabarcoding identified water vole and other small mammals missed on
450 camera or with ambiguous field signs, e.g. mice, voles, shrews. Importantly, the deployment
451 period, height, and positioning of camera traps may have influenced small mammal detection
452 by this method (Caravaggi et al., 2018; Glen et al., 2013). Ishige et al. (2017) achieved
453 comparable mammal detection at salt licks with eDNA metabarcoding and camera trapping, but
454 species presence was inconsistent between salt licks surveyed. Currently, there are no
455 published studies comparing eDNA metabarcoding of water to camera trapping for mammal
456 identification. However, Stat et al. (2019) found highest marine fish species richness using both
457 baited remote underwater video systems and eDNA metabarcoding, but each method
458 identified unique species.

459 Notably, no survey method captured the semi-aquatic otter despite known presence at
460 study sites and successful detection in eDNA metabarcoding studies of UK ponds (Harper et al.,
461 2018a) and lakes (Hänfling et al., 2017) as well as Experiment 1 here; although, the eDNA signal
462 for otter was weaker than other semi-aquatic mammals studied. eDNA metabarcoding may
463 have performed poorly for otter, badger, and red fox due to species' ecologies. These mammals
464 are wide-ranging (Gaughran et al., 2018; Thomsen et al., 2012) and may not readily release DNA
465 in water. Otters often spraint on grass or rock substrata outside water and use latrines
466 associated with caves and dens (Ruiz-Olmo & Gosálbez, 1997). As terrestrial mammals, red fox
467 and badger must drink from or enter ponds for eDNA deposition to occur (Rodgers & Mock,
468 2015; Ushio et al., 2017; Williams et al., 2018). Otter, badger, and red fox detection may require

469 greater spatiotemporal resolution of eDNA sampling. This is reinforced by other eDNA
470 metabarcoding studies where mammal detection was highly variable across sites surveyed
471 (Ishige et al., 2017; Klymus et al., 2017; Ushio et al., 2017). Alternatively, false negatives may be
472 symptomatic of metabarcoding bias (section 4.2).

473 eDNA from other semi-aquatic mammals (e.g. beaver, water vole) was evenly
474 distributed, being found in most or all samples collected on fine spatial scales within natural
475 ponds, whereas terrestrial mammal (e.g. red and roe deer) eDNA was highly localised and often
476 detected in less than 20% of samples. Mammal eDNA signals varied temporally, being
477 detectable for two consecutive days maximum. Depending on the species, mammal eDNA may
478 be spatially and temporally clumped in lentic ecosystems due to the nature and frequency of
479 contact that individuals have with water. Unless behaviours involving prolonged water contact
480 (e.g. swimming, wallowing) are exhibited, non-domestic mammals may only be detected at
481 drinking sites (Klymus et al., 2017; Ushio et al., 2017; Williams et al., 2018). In contrast,
482 domestic mammals (e.g. cow, sheep, pig) may have elevated detection rates in ponds due to
483 high occurrence of these waterbodies in agricultural landscapes as well as eDNA transport by
484 rainfall and run-off (Staley et al., 2018). eDNA detection and persistence are further influenced
485 by group size, where eDNA from multiple individuals endures for longer time periods in water
486 than eDNA from single individuals (Williams et al., 2018). Detailed investigations incorporating
487 biotic (e.g. population size, sex, body mass, behaviour) and abiotic (e.g. temperature, pH,
488 ultraviolet light, rainfall) factors are needed to understand the longevity of mammal eDNA
489 signals in aquatic ecosystems (Parsons et al., 2018; Rodgers & Mock, 2015; Williams et al.,
490 2018).

491 Both of our experiments have shown that sampling strategy drastically influences
492 mammal eDNA detection probability. Mammal eDNA was evenly distributed in closed, artificial
493 waterbodies, but locally distributed in open, natural ponds. In captivity, mammal species were
494 housed individually (excluding HWP red deer) with a drinking container(s) and/or small
495 waterbody (range 0.01-162 m^2, mean 27.4 m^2) in their enclosure. Some enclosures housed
496 more individuals of a species than others, thereby increasing eDNA deposition and detection
497 probability (Williams et al., 2018). Wild mammals have an array of freshwater habitats at their
498 disposal and can hold vast territories. Therefore, rates of pond visitation and eDNA deposition
499 are more random and irregular (Klymus et al., 2017; Ushio et al., 2017) which may lead to
500 between-sample variation (Williams et al., 2018).
501

502 **4.2 Technical pitfalls of eDNA metabarcoding for mammal monitoring**

503

504 eDNA metabarcoding has strong potential for inclusion in mammal monitoring schemes
505 (section 4.3), but suffers from biases that may result in false negatives or false positives. Our
506 process controls identified low-level contamination at all stages of metabarcoding, but
507 contaminants primarily occurred in field and PCR negative controls (Supporting Information:
508 Appendix 2). We applied taxon-specific sequence thresholds to our data to mitigate false
509 positives as in Harper *et al*. (2018a, b). Remnant contaminants were cichlid (laboratory), Gentoo
510 penguin (environment), reindeer (environment), and human (environment/laboratory). Gentoo
511 penguin is housed at EZ and was identified from beaver quarantine enclosure water. The WT
512 red squirrel and reindeer enclosures are in close proximity. DNA transport by wildlife (e.g.

513 waterfowl [Hänfling et al., 2016]) and park staff or visitors may explain this environmental
514 contamination. Human DNA was present across process controls corresponding to artificial and
515 natural waterbodies. Human DNA may be amplified and sequenced instead of focal species,
516 potentially resulting in false negative detections for rare and/or less abundant species. Human
517 DNA blocking primers can prevent this bias, but may impair PCR amplification efficiency
518 (Klymus et al., 2017; Port et al., 2016; Ushio et al., 2017; Valentini et al., 2016).

519 In our study, eDNA metabarcoding produced false negatives for otter, badger, and red
520 fox at natural ponds. DNA from purely aquatic and more abundant species may have
521 overwhelmed otter, badger, and red fox DNA during amplification and sequencing, i.e. species-
522 masking (Kelly, Port, Yamahara, & Crowder, 2014; Klymus et al., 2017). More biological and
523 technical replication could help mitigate amplification bias and improve species detection
524 probabilities (Evans et al., 2017; Lawson Handley et al., 2018; Stat et al., 2019; Valentini et al.,
525 2016), but potential for primer bias remains. We selected a 12S rRNA primer pair designed to
526 amplify vertebrate DNA (Riaz et al., 2011) for metabarcoding. There were no primer
527 mismatches with otter, badger or red fox reference sequences. These species also amplified *in*
528 *silico* with up to three mismatches between each primer and reference sequence allowed.
529 However, other species may have been preferentially amplified *in vitro* due to greater primer
530 binding affinity (Evans et al., 2017; Kelly et al., 2014; Klymus et al., 2017; Stat et al., 2019).
531 Metabarcoding primers targeting mammals (Ushio et al., 2017), or multi-marker (e.g. 12S, 16S,
532 COI) investigations (Evans et al., 2017; Hänfling et al., 2016; Kelly et al., 2014; Klymus et al.,
533 2017) may improve mammal detection in systems with competition from aquatic species. Yet,
534 fish and amphibians also had higher qPCR detection than otter (Thomsen et al., 2012). A

535 comparison of metabarcoding and qPCR would confirm whether poor amplification efficiency
536 for otter is due to technical bias or species ecology, and whether eDNA metabarcoding can
537 reliably monitor otter alongside the wider mammal community. Similar comparisons have been
538 made for the great crested newt (Harper et al., 2018b) and wood turtle (*Glyptemys insculpta*)
539 (Lacoursière-Roussel, Dubois, Normandeau, & Bernatchez, 2016).
540

541 **4.3 Scope of eDNA metabarcoding for mammal monitoring**

542

543 The most recent assessment of UK mammal populations emphasised the lack of data and
544 systematic monitoring for many species (Mathews et al., 2018). Distribution and occupancy
545 data are poor for most species, with ongoing survey effort biased toward rare species. Surveys
546 are also heavily reliant on citizen science and casual records (Massimino et al., 2018). Tools that
547 can provide standardised, systematic monitoring of UK mammal populations are needed
548 (Mathews et al., 2018). Despite issues inherent to metabarcoding for biodiversity monitoring
549 (Deiner et al., 2017), this tool has enormous potential to enhance mammal monitoring,
550 conservation, and management. eDNA metabarcoding generates distribution data for multiple
551 species, whether rare, invasive, or abundant, and could be a powerful tool for tracking species
552 in conflict with one another, e.g. water vole, American mink, and otter (Bonesi & Macdonald,
553 2004) or red squirrel, grey squirrel, and pine marten (Sheehy, Sutherland, O'Reilly, & Lambin,
554 2018). Different sample types (e.g. water, soil, snow, salt licks, feeding traces, faeces, hair, and
555 blood meals) may also offer new insights to mammal biodiversity in a given area (Aylward et al.,
556 2018; Franklin et al., 2019; Ishige et al., 2017; Tessler et al., 2018; Ushio et al., 2017).

557 eDNA metabarcoding can rapidly survey multitudes of aquatic sites at landscape-scale
558 where camera traps might be resource-intensive, cost-inefficient, and susceptible to theft or
559 damage (Glen et al., 2013; Stat et al., 2019; Ushio et al., 2017). Field signs can be employed at
560 comparable spatial scales to eDNA metabarcoding but depend on volunteer time and skill
561 (McShea et al., 2016). eDNA metabarcoding could provide accurate data for species
562 misidentified from field signs, e.g. mice and voles, otter and mink (Franklin et al., 2019; Harris &
563 Yalden, 2004). However, both camera traps and field signs recorded species that eDNA
564 metabarcoding did not. Therefore, eDNA metabarcoding is complementary and should be
565 incorporated into, not replace, existing monitoring schemes. This tool could be most effective in
566 mammal monitoring if deployed at the edges of known species distributions, in areas where
567 species presence is unknown, and in areas with isolated species records (Mathews et al., 2018).
568 Mammal eDNA detection probabilities from natural ponds will likely be high when areas
569 with dense populations are studied. However, rigorous sampling strategies are required to
570 track mammals in areas that are sparsely populated by individuals. Multiple ponds must be
571 sampled repeatedly, and samples taken at multiple locations within ponds. Ideally, samples and
572 subsamples would not be pooled to allow inferences about site occupancy to be made.
573 Importantly, we sampled natural ponds in spring but sampling in other seasons may produce
574 different results. For example, Lawson Handley et al. (2018) found high repeatability in eDNA
575 metabarcoding detection and abundance estimates across seasons, but strong spatial
576 differences reflective of species ecology. We recommend that researchers and practitioners
577 using eDNA metabarcoding for mammal monitoring channel efforts into extensive sampling of
578 numerous waterbodies in a given area over larger timescales. This will account for differential

579 mammal visitation rates and maximise eDNA detection probabilities.

580

581

582 ## Data accessibility

583

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

588

589

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591

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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.

References

Aylward, M. L., Johnson, S. E., Sullivan, A. P., Perry, G. H., & Louis, E. E. (2018). A novel environmental DNA (eDNA) sampling method for aye-ayes from their feeding traces. *bioRxiv, 272153*. https://doi.org/10.1101/272153

Baker, C. S., Steel, D., Nieukirk, S., & Klinck, H. (2018). Environmental DNA (eDNA) From the Wake of the Whales: Droplet Digital PCR for Detection and Species Identification. *Frontiers in Marine Science*, *5*, 133.

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

Battersby, J. & Tracking Mammals Partnership (2005) *UK Mammals: Species Status and Population Trends. First Report by the Tracking Mammals Partnership*. Joint Nature Conservation Committee /Tracking Mammals Partnership, Peterborough.

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

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

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

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

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

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

654 Evans, N. T., Li, Y., Renshaw, M. A., Olds, B. P., Deiner, K., Turner, C. R., Jerde, C. L., Lodge, D. M.,
655 Lamberti, G. A. & Pfrender, M. E (2017). Fish community assessment with eDNA
656 metabarcoding: effects of sampling design and bioinformatic filtering. *Canadian Journal of*
657 *Fisheries and Aquatic Sciences*, *74*, 1362–1374. https://doi.org/10.1139/cjfas-2016-0306
658 Franklin, T. W., McKelvey, K. S., Golding, J. D., Mason, D. H., Dysthe, J. C., Pilgrim, K. L., Squires,
659 J. R., Aubry, K. B., Long, R. A., Greaves, S. E., Raley, C. M., Jackson, S., MacKay, P., Lisbon, J.,
660 Sauder, J. D., Pruss, M. T., Heffington, D. & Schwartz, M. K (2019). Using environmental DNA
661 methods to improve winter surveys for rare carnivores: DNA from snow and improved
662 noninvasive techniques. *Biological Conservation*, *229*, 50–58.
663 https://doi.org/10.1016/j.biocon.2018.11.006
664 Gaughran, A., Kelly, D. J., MacWhite, T., Mullen, E., Maher, P., Good, M., & Marples, N. M.
665 (2018). Super-ranging. A new ranging strategy in European badgers. *PLoS ONE*, *13*,
666 e0191818. https://doi.org/10.1371/journal.pone.0191818
667 Glen, A. S., Cockburn, S., Nichols, M., Ekanayake, J., & Warburton, B. (2013). Optimising camera
668 traps for monitoring small mammals. *PLoS ONE*, *8*, e67940.
669 https://doi.org/10.1371/journal.pone.0067940
670 Hänfling, B., Lawson Handley, L., Lunt, D., Shum, P., Winfield, I., & Read, D. S. (2017). *A Review*
671 *of Recent Advances in Genetic Methods to Identify Improvements in CAMERAS Partners*
672 *Monitoring Activities*. University of Hull, Hull, UK.
673 Hänfling, B., Lawson Handley, L., Read, D. S., Hahn, C., Li, J., Nichols, P., Blackman, R. C., Oliver,
674 A. & Winfield, I. J. (2016). Environmental DNA metabarcoding of lake fish communities
675 reflects long-term data from established survey methods. *Molecular Ecology*, *25*, 3101–3119.
676 https://doi.org/10.1111/mec.13660
677 Harper, L. R., Handley, L. L., Hahn, C., Boonham, N., Rees, H. C., Lewis, E., Adams, I. P.,
678 Brotherton, P., Phillips, S. & Hänfling, B. (2018a) Ground-truthing environmental DNA (eDNA)
679 metabarcoding for ecological hypothesis testing at the pondscape. *bioRxiv*, *278309*.
680 https://doi.org/10.1101/278309
681 Harper, L. R., Lawson Handley, L., Hahn, C., Boonham, N., Rees, H. C., Gough, K. C., Lewis, E.,
682 Adams, I. P., Brotherton, P., Phillips, S. & Hänfling, B. (2018b). Needle in a haystack? A
683 comparison of eDNA metabarcoding and targeted qPCR for detection of the great crested
684 newt (*Triturus cristatus*). *Ecology and Evolution*, *8*, 6330–6441.
685 https://doi.org/10.1002/ece3.4013
686 Harris, S., & Yalden, D. W. (2004). An integrated monitoring programme for terrestrial mammals
687 in Britain. *Mammal Review*, *34*, 157–167. https://doi.org/10.1046/j.0305-1838.2003.00030.x
688 Hunter, M. E., Meigs-Friend, G., Ferrante, J. A., Takoukam Kamla, A., Dorazio, R. M., Keith-
689 Diagne, L., Luna, F., Lanyon, J. M. & Reid, J. P. (2018). Surveys of environmental DNA (eDNA):
690 a new approach to estimate occurrence in Vulnerable manatee populations. *Endangered*
691 *Species Research*, *35*, 101–111. https://doi.org/10.3354/esr00880

692 Ishige, T., Miya, M., Ushio, M., Sado, T., Ushioda, M., Maebashi, K., Yonechi, R., Lagan, P. &
693 Matsubayashi, H. (2017). Tropical-forest mammals as detected by environmental DNA at
694 natural saltlicks in Borneo. *Biological Conservation*, *210*, 281–285.
695 https://doi.org/10.1016/j.biocon.2017.04.023
696 Joint Nature Conservation Committee (2018) UK BAP priority terrestrial mammal species.
697 Accessed on 1st December 2018. http://jncc.defra.gov.uk/page-5170
698 Kelly, R. P., Port, J. A., Yamahara, K. M., & Crowder, L. B. (2014). Using environmental DNA to
699 census marine fishes in a large mesocosm. *PLoS ONE*, *9*, e86175.
700 https://doi.org/10.1371/journal.pone.0086175
701 Kitson, J. J. N., Hahn, C., Sands, R. J., Straw, N. A., Evans, D. M., & Lunt, D. H. (2019). Detecting
702 host-parasitoid interactions in an invasive Lepidopteran using nested tagging DNA-
703 metabarcoding. *Molecular Ecology*, *28*, 471–483. https://doi.org/10.1111/mec.14518
704 Klymus, K. E., Richter, C. A., Thompson, N., & Hinck, J. E. (2017). Metabarcoding of
705 Environmental DNA Samples to Explore the Use of Uranium Mine Containment Ponds as a
706 Water Source for Wildlife. *Diversity*, *9*, 54. https://doi.org/10.3390/d9040054
707 Lacoursière-Roussel, A., Dubois, Y., Normandeau, E., & Bernatchez, L. (2016). Improving
708 herpetological surveys in eastern North America using the environmental DNA method.
709 *Genome*, *59*, 991–1007. https://doi.org/10.1139/gen-2015-0218
710 Lawson Handley, L. J., Read, D., Winfield, I., Kimbell, H., Johnson, H., Li, J., Hahn, C., Blackman,
711 R., Wilcox, R., Donnelly, R., Szitenberg, A. & Hänfling, B. (2018) Temporal and spatial
712 variation in distribution of fish environmental DNA in England's largest lake. *bioRxiv*, *376400*.
713 https://doi.org/10.1101/376400
714 Lugg, W. H., Griffiths, J., van Rooyen, A. R., Weeks, A. R., & Tingley, R. (2017). Optimal survey
715 designs for environmental DNA sampling. *Methods in Ecology and Evolution*, *9*, 1049–1059.
716 https://doi.org/10.1111/2041-210X.12951
717 Lynx UK Trust (2018) Accessed on 1st December 2018. http://www.lynxuk.org/
718 Massimino, D., Harris, S. J., & Gillings, S. (2018). Evaluating spatiotemporal trends in terrestrial
719 mammal abundance using data collected during bird surveys. *Biological Conservation*, *226*,
720 153–167. https://doi.org/10.1016/j.biocon.2018.07.026
721 Mathews, F., Kubasiewicz, L. M., Gurnell, J., Harrower, C. A., McDonald, R. A. & Shore, R. F.
722 (2018) *A Review of the Population and Conservation Status of British Mammals: Technical*
723 *Summary*. Natural England, Peterborough.
724 McShea, W. J., Forrester, T., Costello, R., He, Z., & Kays, R. (2016). Volunteer-run cameras as
725 distributed sensors for macrosystem mammal research. *Landscape Ecology*, *31*, 55–66.
726 https://doi.org/10.1007/s10980-015-0262-9

Padgett-Stewart, T. M., Wilcox, T. M., Carim, K. J., McKelvey, K. S., Young, M. K., & Schwartz, M. K. (2016). An eDNA assay for river otter detection: a tool for surveying a semi-aquatic mammal. *Conservation Genetics Resources*, *8*, 5–7. https://doi.org/10.1007/s12386-015-0511-s

Parsons, K. M., Everett, M., Dahlheim, M., & Park, L. (2018). Water, water everywhere: environmental DNA can unlock population structure in elusive marine species. *Royal Society Open Science*, *5*, 180537. https://doi.org/10.1098/rsos.180537

Port, J. A., O'Donnell, J. L., Romero-Maraccini, O. C., Leary, P. R., Litvin, S. Y., Nickols, K. J., Yamahara, K. M. & Kelly, R. P. (2016). Assessing vertebrate biodiversity in a kelp forest ecosystem using environmental DNA. *Molecular Ecology*, *25*, 527–541. https://doi.org/10.1111/mec.13481

Riaz, T., Shehzad, W., Viari, A., Pompanon, F., Taberlet, P., & Coissac, E. (2011). ecoPrimers: inference of new DNA barcode markers from whole genome sequence analysis. *Nucleic Acids Research*, *39*, e145. https://doi.org/10.1093/nar/gkr732

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

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

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

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

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

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

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

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

765 Thomsen, P. F., Iversen, L. L., Wiuf, C., Rasmussen, M., Gilbert, M. T. P., Orlando, L., &
766 Willerslev, E. (2012). Monitoring endangered freshwater biodiversity using environmental
767 DNA. *Molecular Ecology*, *21*, 2565–2573. https://doi.org/10.1111/j.1365-294X.2011.05418.x
768 Thomsen, P. F., & Willerslev, E. (2015). Environmental DNA–an emerging tool in conservation
769 for monitoring past and present biodiversity. *Biological Conservation*, *183*, 4–18.
770 https://doi.org/10.1016/j.biocon.2014.11.019
771 Ushio, M., Fukuda, H., Inoue, T., Makoto, K., Kishida, O., Sato, K., Murata, K., Nikaido, M., Sado,
772 T., Sato, Y., Takeshita, M., Iwasaki, W., Yamanaka, H., Kondoh, M. & Miya, M. (2017).
773 Environmental DNA enables detection of terrestrial mammals from forest pond water.
774 *Molecular Ecology Resources*, *17*(6), e63–e75. https://doi.org/10.1111/1755-0998.12690
775 Valentini, A., Taberlet, P., Miaud, C., Civade, R., Herder, J., Thomsen, P. F., Bellemain, E.,
776 Besnard, A., Coissac, E., Boyer, F., Gaboriaud, C., Jean, P., Poulet, N., Roset, N., Copp, G. H.,
777 Geniez, P., Pont, D., Argillier, C., Baudoin, J. -M., Peroux, T., Crivelli, A. J., Olivier, A.,
778 Acqueberge, M., Le Brun, M., Møller, P. R., Willerslev, E. & Dejean, T. (2016). Next-
779 generation monitoring of aquatic biodiversity using environmental DNA metabarcoding.
780 *Molecular Ecology*, *25*, 929–942. https://doi.org/10.1111/mec.13428
781 Visconti, P., Pressey, R. L., Giorgini, D., Maiorano, L., Bakkenes, M., Boitani, L., Alkemade, R.,
782 Falcucci, A., Chiozza, F. & Rondinini, C. (2011). Future hotspots of terrestrial mammal loss.
783 *Philosophical Transactions of the Royal Society B*, *366*, 2693–2702.
784 https://doi.org/10.1098/rstb.2011.0105
785 Wickham, H. (2016). *ggplot2: elegant graphics for data analysis*. Springer, New York, USA.
786 http://ggplot2.org
787 Williams, K. E., Huyvaert, K. P., Vercauteren, K. C., Davis, A. J., & Piaggio, A. J. (2018). Detection
788 and persistence of environmental DNA from an invasive, terrestrial mammal. *Ecology and*
789 *Evolution*, *8*, 688–695. https://doi.org/10.1002/ece3.3698
790 Zuur, A. F., Ieno, E. N., Walker, N., Saveliev, A. A. & Smith, G. M. (2009) *Mixed effects models*
791 *and extensions in ecology with R*. Springer, New York, USA.
792

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

794 individuals present and waterbody size in enclosures is provided.

795

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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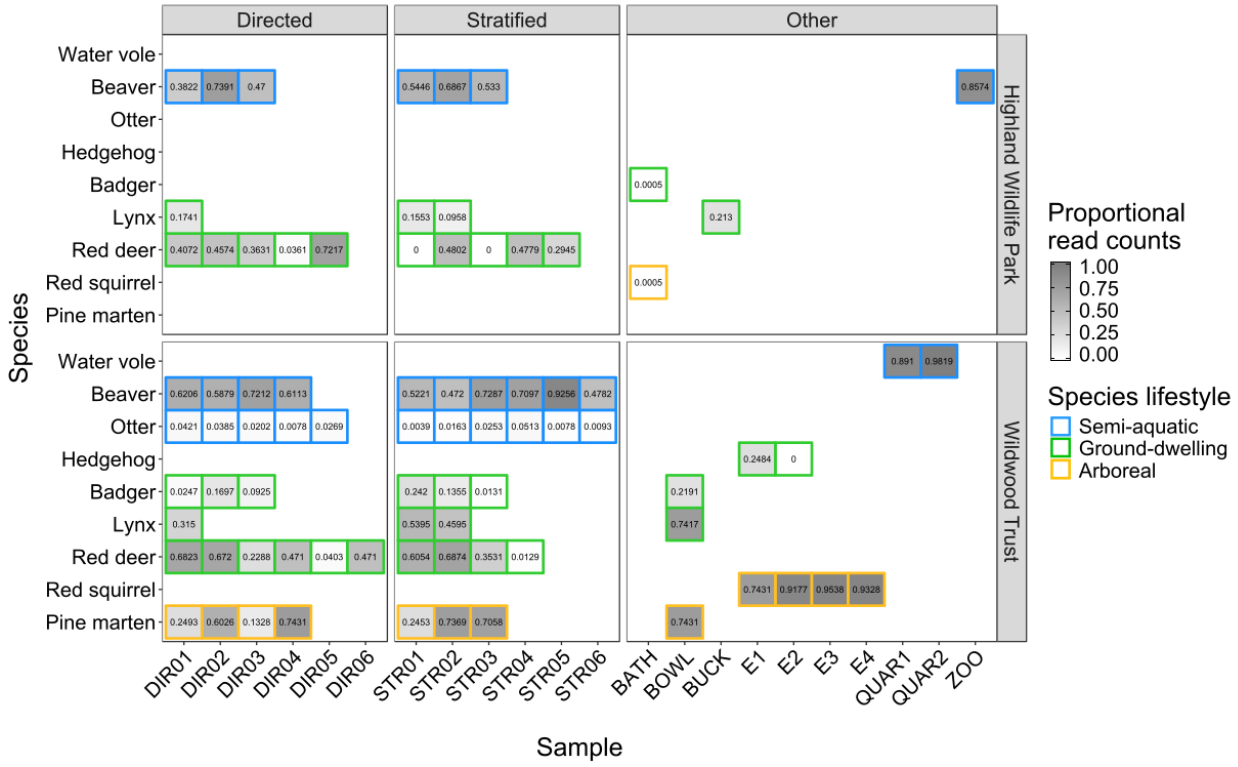

Figure 1. Heatmap showing proportional read counts for eDNA samples. Each square represents a sample taken from a wildlife park enclosure. Directed (DIR01-DIR06) and stratified (STR01-STR06) samples were collected from artificial waterbodies. Samples were collected from drinking containers (E1, E2, E3,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

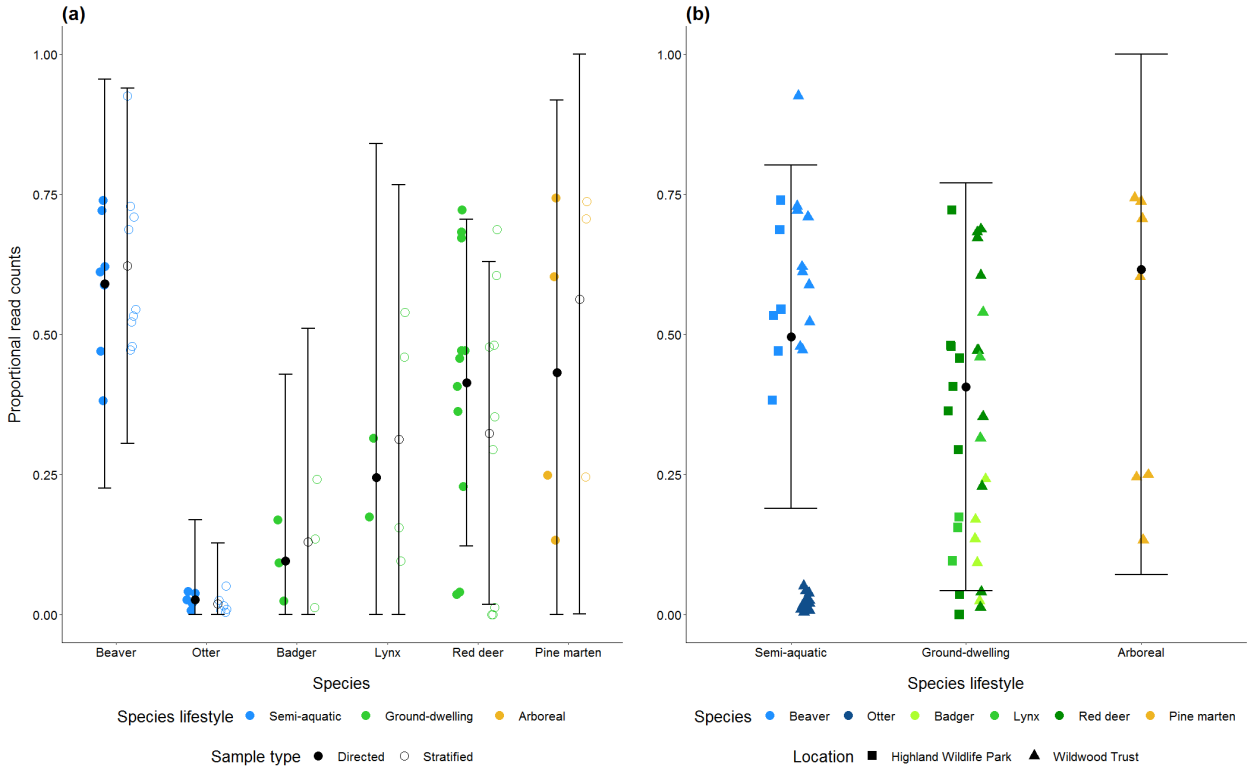

Figure 2. Relationships predicted by the binomial GLMMs between proportional read counts and sample type nested within species **(a)** or species lifestyle **(b)**. The observed data (coloured points) are displayed against the predicted relationships (black points with error bars) for each species **(a)**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

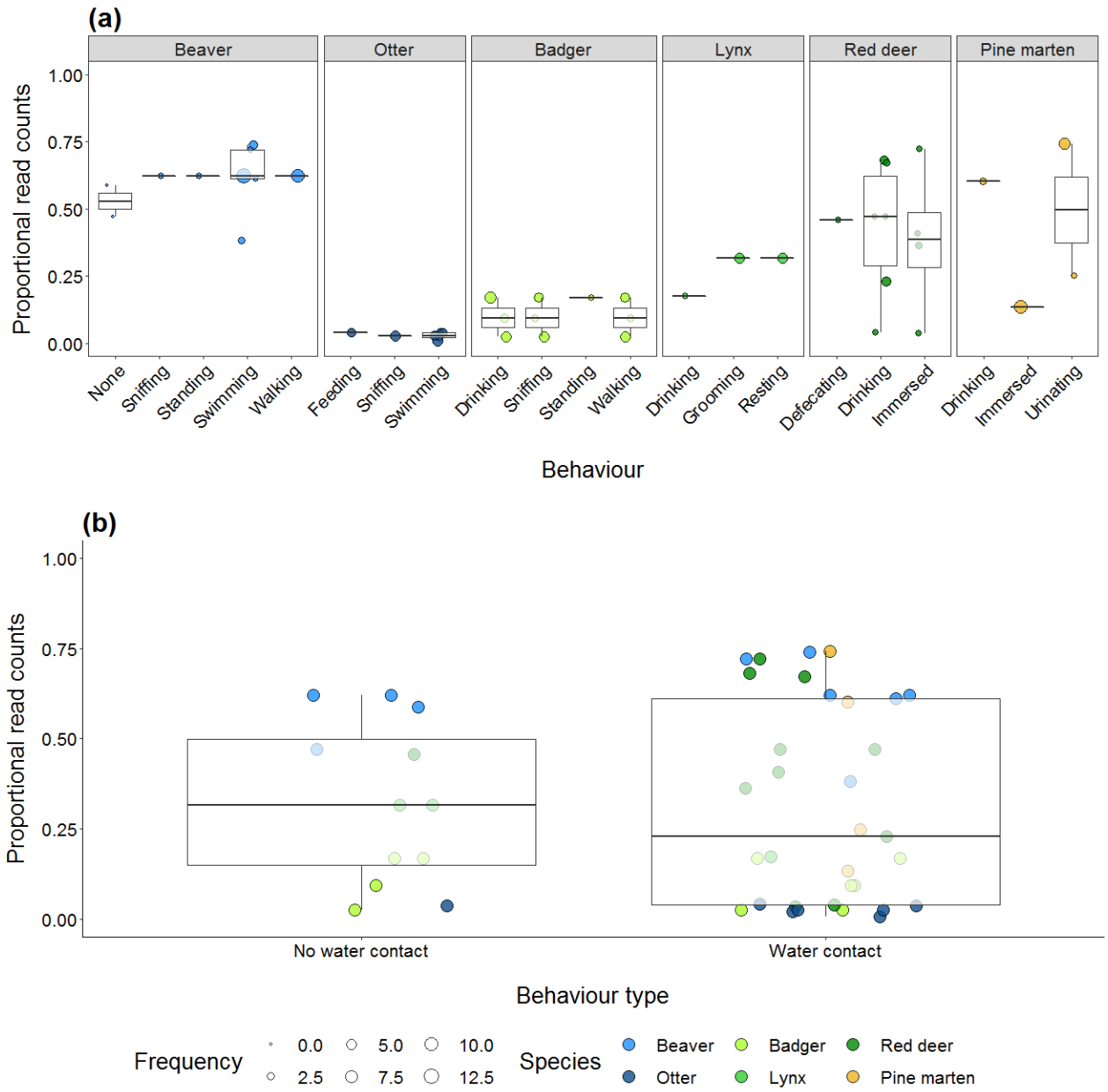

Figure 3. Boxplots showing the mean proportional read counts for specific **(a)** and generic **(b)** behaviour(s) exhibited by focal species. Boxes show 25th, 50th, and 75th percentiles, and whiskers show 5th and 95th percentiles. Points are coloured by species lifestyle, and each point in **(a)** represents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

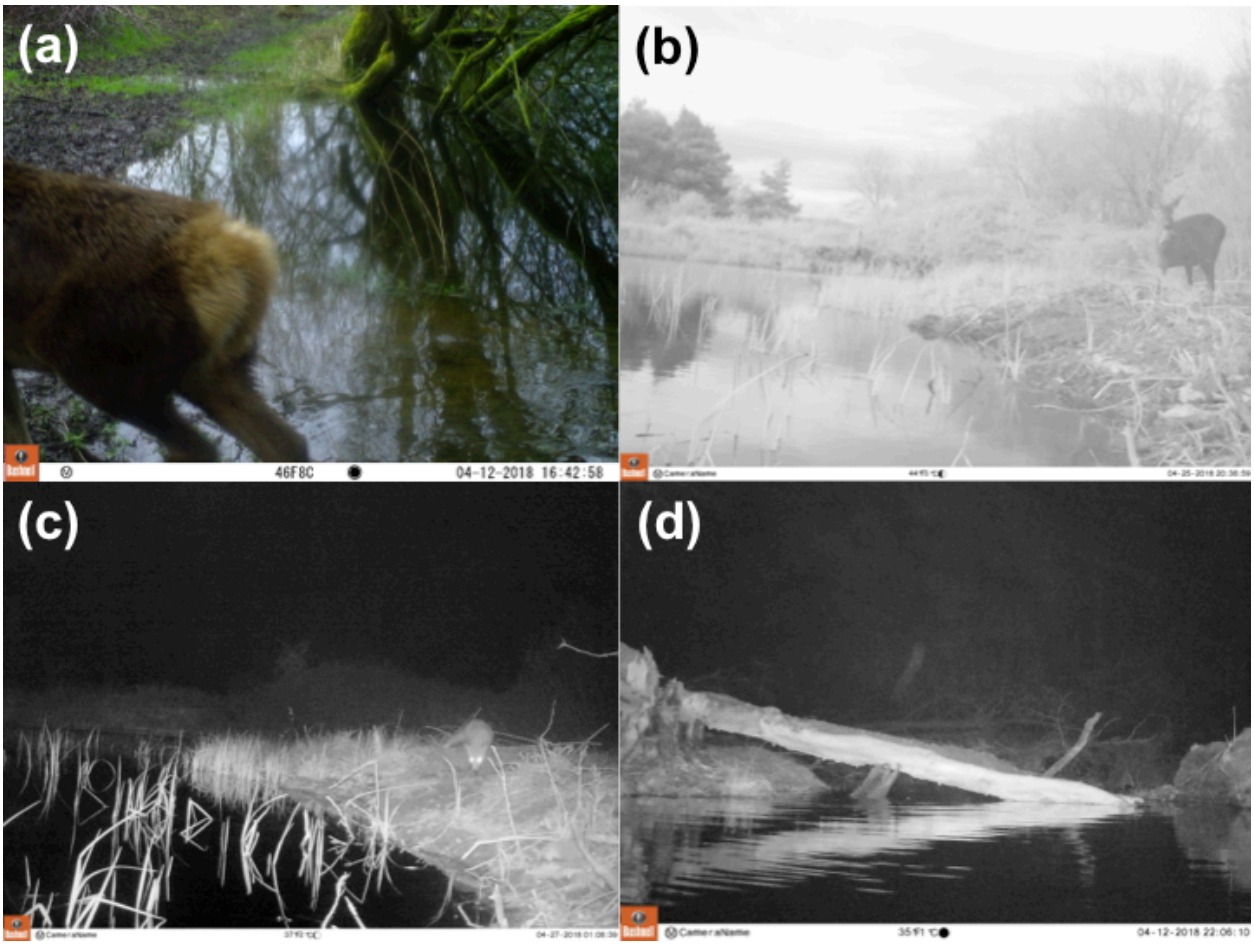

Figure 4. Exemplar camera trap photos taken at natural ponds where focal species were present. Red deer was recorded at Thorne Moors **(a)**, roe deer **(b)** and red fox **(c)** were recorded at Tophill Low Nature Reserve, and beaver was recorded at the Bamff Estate **(d)**.

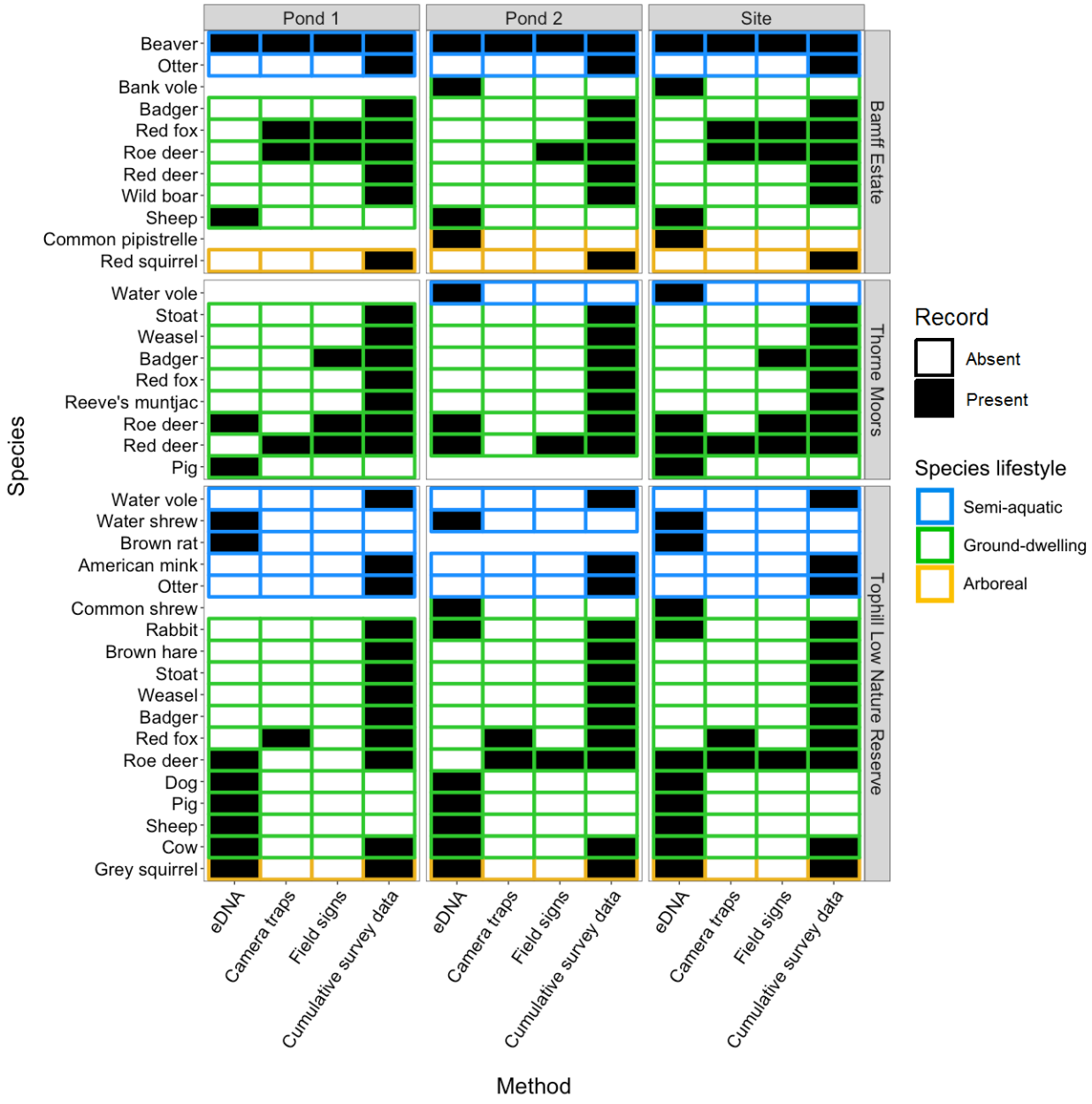

Figure 5. Tile plot showing species presence-absence at individual pond and site-level as indicated by field signs, camera trapping, and eDNA metabarcoding at sites with focal species presence confirmed by cumulative survey data.