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Quick detection of a rare species: Forensic swabs of survey tubes for hazel dormouse *Muscardinus avellanarius* urine

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Abstract

1. Effective conservation decisions rely on accurate survey data, but methods can be resource-intensive and risk false negative results. Presence of the threatened hazel dormouse (England, UK) is typically confirmed by looking for its nest in survey tubes, over a 6-month period. As an alternative, environmental DNA (eDNA) surveys have proven benefits in efficiency and accuracy for other taxa, but generally rely on the extraction and amplification of DNA from water, soil or sediment, which are not yet dependable samples for rare terrestrial mammals like the hazel dormouse.
2. At a known occupancy site, paper-lined survey tubes were used to capture a DNA sample. Like other species of rodent, the hazel dormouse excretes urine freely, and this was highlighted by ultraviolet torch, swabbed from the paper, extracted and hazel dormouse eDNA amplified by quantitative polymerase chain reaction (qPCR).
3. Hazel dormouse presence was confirmed in this way in three out of 50 tubes within 8 days. Detection by conventional nest survey occurred on day 63 when a hazel dormouse nest was found in a single survey tube. We calculate that amplification of eDNA left behind in tubes increased survey efficiency here at least 12-fold.
4. *Synthesis and applications*. In this study we demonstrate that eDNA swabbed from a clean substrate placed in survey apparatus can significantly hasten the detection of a rare species. This method has the potential to broaden the application of eDNA to other terrestrial vertebrates, including surveys at large spatiotemporal scales. Beyond presence/absence, the non-invasive DNA sample could also offer insights into sex ratio, abundance, behaviour and population genetics.

KEYWORDS

ecological survey, environmental DNA, forensic swab, hazel dormouse, nest tubes, quantitative PCR, rare mammal, species detection

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1 | INTRODUCTION

Rodents account for approximately 40% of all mammal species (Singleton et al., 2015), are a source of food for larger predators and have a seed dispersal and habitat architecture function. Despite their high fecundity, species-specific requirements mean that they are sensitive to environmental change (Rowe & Terry, 2014). The hazel dormouse *Muscardinus avellanarius* is a nocturnal, arboreal rodent that while native across much of Europe, has suffered a population decline of 72% in Great Britain due to habitat loss and fragmentation (Goodwin et al., 2017). The State of Nature Report (Hayhow et al., 2019) warns that numbers continue to be in decline and the IUCN-compliant British Red List categorises the hazel dormouse as vulnerable to extinction (Matthews et al., 2020).

There are several survey methods for the hazel dormouse, and a Table (S1) outlines the relative merits of each, but the most established method to confirm presence and infer absence is currently by nest tube survey. First devised and tested by Morris and Temple (1998), with larger tubes for the non-native edible dormouse *Glis glis*, nest tubes (Figure 1) are sealed at one end with a rectangular wooden tray to accommodate a hazel dormouse nest. A positive survey result relies on seeing a hazel dormouse within one or more tubes and/or a hazel dormouse nest.

Guidelines (Natural England, 2015) stipulate that 50 nest tubes should be used per survey site and that they be checked by a licensed dormouse surveyor every 2 months. In practice, tubes are typically checked on a monthly basis. Hazel dormouse population cycle, breeding and activity are all seasonal, including periods of torpor when temperatures dip below tolerable levels and hibernation at ground level during winter months. To factor this into surveys, a monthly index of detection probability (Table 1) was derived by Chanin and Woods (2003). The target for a formal survey is a total index score ≥20, which equates to at least 6 months of survey effort between April and November. This lengthy survey protocol is the accepted standard, yet false negative results cannot be ruled out (Chanin & Woods, 2003) as detection relies on considerable investment on the part of the target species, that is, building a nest in a survey tube.



FIGURE 1 Hazel dormouse nest tube, with paper insert to capture DNA shed by visiting animals (V. Priestley)

TABLE 1 Index of detection probability for nest tube surveys (Natural England, 2015)

Month	Index for 50 nest tubes (score ≥ 20 is required)
April	1
May	4
June	2
July	2
August	5
September	7
October	2
November	2

Environmental DNA (eDNA) surveys detect single species or profile entire communities by looking for genetic clues in environmental samples, such as soil or water. Presence can be confirmed, absence inferred and in some instances a measure of relative abundance or biomass calibrated (Doi et al., 2017; Lacoursiere-Roussel et al., 2016; Tillotson et al., 2018; Yates et al., 2019). Crucially, eDNA methods obviate the requirement for a confirmed sighting and are therefore particularly powerful for the accurate detection of rare, elusive or cryptic species at large spatial scales (Fukumoto et al., 2015; Nevers et al., 2018; Pfleger et al., 2016). The consensus is that eDNA analysis can be more accurate and efficient than traditional survey methods, though this assumption should be tested by direct comparisons with traditional methods (Rose et al., 2019 and McInerney & Rees, 2018). Despite such promise, there is just one formally approved eDNA protocol (Great Britain), for surveys of the great crested newt *Triturus cristatus* (Biggs et al., 2014). Consistent, considerable and reliable advantage of eDNA methods over traditional surveys needs to be proved, which may explain the current research-application gap. For example, a soil or water-based eDNA survey of a terrestrial mammal could significantly narrow down the search area for traditional methods (Ushio et al., 2017; Yonezewa et al., 2020) but for solitary or very rare species the eDNA sampling effort necessary would negate any perceived eDNA efficiency savings (Harper, Lawson, et al., 2019; Leempoel et al., 2019; Sales et al., 2020). Either way, from a practitioner's perspective the eDNA method for surveys of terrestrial species is not yet a reliable standalone tool.

In this study we integrate a paper sampler with dormouse survey apparatus to simultaneously get the best from both eDNA and conventional methods (Taberlet et al., 2012). Survey nest tubes were lined with a clean paper substrate and the DNA shed by visiting animals, most obviously in the form of urine, was swabbed, extracted and hazel dormouse provenance confirmed by quantitative polymerase chain reaction (qPCR).

2 | MATERIALS AND METHODS

Three physically separate laboratories were used for DNA extraction, pre-PCR and post-PCR (the latter under positive air pressure). Clean laboratory coats and sterile gloves were worn and changed if they came into contact with contaminants. Benches were cleaned using DNA Away™ (Thermo Fisher Scientific™) and tweezers, scalpels or surfaces in direct contact with samples cleaned using a dilution of 50% commercial bleach (Goldberg et al., 2016). Filter pipette tips and DNA-free tubes were used throughout. All custom oligonucleotides were synthesised by Sigma-Aldrich® (Sigma-Aldrich).

2.1 | Assay design, optimisation and validation

A primer pair (5′-CCCTTACACGGTTTTTCGCA-3′ and 5′-TAGGCC TGATGGGTTGTTGG-3′) was designed using Primer3 and BLAST (NCBI Primer-BLAST, Ye et al., 2012) with a dual-labelled hydrolysis probe 5′-6FAM-TACCTTTCATCGTTGCAGCCCTAGTAATAGT-BHQ-1-3′ (S2 assay design parameters, Supporting Information) to amplify a 113 base-pair fragment of hazel dormouse mitochondrial cytochrome b (cyt*b*), with no mismatches.

Gradient PCR was used to validate the primers and identify the optimum annealing temperature in the range 55–65°C (25 µl reaction volume) with Verity™ 96-well thermal cycler (Thermo Fisher Scientific™): 12.5 µl Taq MasterMix (Thermo Fisher Scientific™), 1 µl forward primer (10 µM working stock), 1 µl reverse primer (10 µM working stock), 9.5 µl nuclease-free water and 1 µl template DNA. Thermal cycling conditions of 95°C for 3 min, followed by 40 cycles of 95°C for 30 s and 55–65°C for 30 s and 72°C for 30 s. Final extension of 72°C for 5 min. A negative control (nuclease-free water in place of template DNA) was run at each temperature.

Primer concentrations (varying both forward and reverse) of 0.25, 0.5 and 1 µM were tested by qPCR with hazel dormouse DNA (diluted to 10^{-4} ng/µl to reflect likely concentration of eDNA) with three technical replicates and three non-template controls ('*x*' here reflects a variable, according to primer optimisation matrix): 12.5 µl TaqMan™ Environmental Master Mix 2.0 (Applied Biosystems®) *x*µl (10 µM working stock) forward and *x*µl (10 µM working stock) reverse primer, 1 µl of hydrolysis probe (2.5 µM working stock solution) and *x*µl of nuclease-free water with 2 µl of template DNA, combined to give a total reaction volume per well of 20 µl. Thermal cycling conditions for qPCR with MicroAmp™ optical 96-well plate run in an ABI Prism™ 7000 sequence detection system (Applied Biosystems™): 50°C for 5 min, 95°C for 10 min followed by 55 cycles of 95°C for 30 s and 57°C for 1 min.

Primers and probe were first tested for species specificity in silico (NCBI Primer-BLAST, Ye et al., 2012) against the full *nr* database. Specificity was then tested in vivo with DNA extracted from hazel dormouse tissue and six other closely related and/or co-occurring small mammal species (distribution maps at S3, Supporting Information): edible dormouse *Glis glis*; house mouse *Mus musculus*; grey squirrel *Sciurus carolinensis*; common shrew *Sorex araneus*; wood mouse *Apodemus sylvaticus*; and yellow-necked mouse *Apodemus flavicollis*. DNA was extracted in a laboratory dedicated to DNA extraction using the DNeasy Blood and Tissue Kit (Qiagen®) and following the manufacturer's guidelines.

The quality of extracted DNA was confirmed by gel electrophoresis (1% agarose at 100 v for 45 min) and amplification with universal cyt*b* mammal primers, designed by Verma and Singh (2002): mcb398 5′-TACCATGAGGACAAATATCATTCTG-3′ and mcb869 5′-CCTCCTAGTTTGTTAGGGATTGATCG-3′; 25 µl Taq MasterMix (Thermo Fisher Scientific™), 1 µl forward primer (10 µM working stock), 1 µl reverse primer (10 µM working stock), 22 µl nuclease-free water and 1 µl template DNA, diluted to mirror typical DNA yields from urine on paper, 15–25 ng/µl. Thermal cycling conditions as per Verma & Singh, 2002): 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 51°C for 30 s and 72°C for 30 s. Final extension of 72°C for 7 min.

Non-specific amplification of co-occurring and/or related species by hazel dormouse primers was first tested by PCR: total reaction volume of 25 µl: 12.5 µl Taq MasterMix (Thermo Fisher Scientific™), 1 µl forward primer (10 µM working stock), 1 µl reverse primer (10 µM working stock), 9.5 µl nuclease-free water and 1 µl template DNA (diluted to within the range 15–25 ng/µl). Thermal cycling conditions of 95°C for 3 min, followed by 40 cycles of 95°C for 30 s and 57°C for 30 s and 72°C for 30 s. Final extension of 72°C for 5 min. PCR product, positive control (hazel dormouse DNA) non-template and extraction controls were visualised by gel electrophoresis with 1% agarose at 100 v for 45 min.

Non-specific amplification was then tested by qPCR, with six technical replicates per DNA sample, a standard dilution series/positive control of hazel dormouse DNA (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} ng/µl) in triplicate, six non-template controls (nuclease-free water) and six DNA extraction controls. Component volumes for each well of a 96-well plate were: 12.5 µl TaqMan™ Environmental Master Mix 2.0 (Applied Biosystems®), 2 µl (10 µM working stock) forward and 2 µl (10 µM working stock) reverse primer, 1 µl of hydrolysis probe (2.5 µM working stock solution) and 0.5 µl of nuclease-free water with 2 µl of template DNA, combined to give a total reaction volume of 20 µl per well. Thermal cycling conditions for qPCR using MicroAmp™ optical 96-well plate and ABI Prism™ 7000 sequence detection system (Applied Biosystems™): 50°C for 5 min, 95°C for 10 min followed by 55 cycles of 95°C for 30 s and 57°C for 1 min.

For qPCR reactions, the efficiency, limit of detection (concentration of DNA that any technical replicate amplified, LoD) and limit of quantification (concentration that all technical replicates amplified, LoQ) are calculated. For this purpose, six technical replicates of template DNA (starting concentration of 16 ng/µl) were run (qPCR) in serial dilutions, ranging from 10^{-0} to 10^{-8} ng/µl (method described at S4, Supporting Information) with six non-template negative controls (2 µl of nuclease-free water in place of template DNA). qPCR component volumes and thermal cycling conditions as per specificity check.

To achieve high sensitivity at low target DNA concentration, amplification efficiency (*E*) should ideally fall within the range 90%–110% (Bustin et al., 2009). Efficiency (*E*) is calculated using the formula at Equation 1 (Arezi et al., 2003), with *E* representing

amplification efficiency; slope representing the slope of a curve with the quantity of DNA on the *x* and threshold cycle (Ct) on the *y* axis.

$$E = (10^{-1/\text{slope}}) - 1. \quad (1)$$

2.2 | Hazel dormouse eDNA samples

Using sterile gloves, two different types of paper were placed under and around feeding bowls in three hazel dormouse enclosures at the British Wildlife Centre, Lingfield U.K., to identify the most appropriate single-use medium to capture eDNA: (a) Grade 2, 70-mm diameter nitrocellulose filter paper circles (Whatman™) to absorb the urine for direct extraction (samples A–C); (b) Standard paper (300 gsm) to provide a clean surface to swab (samples D–F). After 7 days, all paper samples were removed using sterile gloves and stored individually at −20°C, in separate envelopes to avoid cross-contamination.

Filter papers were cut in half with a sterile scalpel (cleaned by soaking in 50% commercial bleach for 10 min and rinsed in nuclease-free water) and one half carefully folded so that it would fit a nuclease-free 2-ml flip-top plastic tube. DNA was extracted directly from the filter paper using the DNeasy Blood and Tissue Kit (Qiagen®) and associated protocol with the modification of increasing lysis buffer (ATL) to completely submerge the sample (230 μl ATL instead of 180 μl). Proteinase K volume was increased by the same factor to 25.6 μl (Qiagen protocol recommends 20 μl). Filter papers, together with ATL and Proteinase K, were incubated for 24 hr at 56°C in an orbital shaker set to 200 rpm. The samples were vortexed intermittently for 15 s.

The standard paper was swabbed using sterile forensic Omni Swabs (Whatman™). The swabs were first dipped in nuclease-free water for 5 s and then with a circular motion, used to lift dry urine from the standard paper. A long wave commercial ultraviolet torch (Wolfwill LED 395 nm, Shenzhen, China) and safety glasses were used for a few seconds to help pinpoint the five most fluorescent areas of paper to swab (indicative of the most recent urine spots). DNA was extracted using the QIAamp® DNA Investigator Kit (Qiagen®) and associated 'surface Omni Swab' protocol. Adamowicz et al. (2014) observed an increase in DNA yield from cotton swabs with a 3- to 18-hr incubation period at 56°C (in place of the manufacturer's recommendation of at least 1 hr), so this modification and the incubator's orbital shaker (200 rpm) were included here (15 hr).

Extracted DNA and extraction controls for both methods were stored at −20°C before amplification by qPCR: qPCR methods described under 'assay design, optimisation and validation', with six technical replicates per DNA sample and six non-template controls. A dilution series of DNA (S4, Supporting Information) was run with the samples to calibrate DNA quantification and serve as a positive control.

2.3 | Rate of eDNA decay

Informed by results of the 'hazel dormouse eDNA sample' method, four A5-size sheets of standard paper were left adjacent to feeding bowls in three hazel dormouse enclosures (two hazel dormice per enclosure). A maximum of four sheets could be positioned on a feeding shelf within the enclosure. After 24 hr, the paper was removed, labelled, put into separate envelopes and stored at room temperature on a clean laboratory bench in a room dedicated to DNA extraction. Twenty-four hours post-collection, one sheet from each enclosure was highlighted by UV torch, all visible urine swabbed and extracted as per the method described under 'hazel dormouse eDNA sample'. With a limit of four samples per enclosure, this was repeated at an order of hours (24 hr), days (3 days), weeks (13 days) and a month (33 days). The final sample on day 33 mirrors a typical sampling interval for nest tube checks (monthly). Clean paper was stored in a separate envelope but in the same location as the samples. This extraction control was swabbed and DNA extracted alongside the samples. DNA was extracted (as per methods described for swabs of standard paper under 'hazel dormouse eDNA sample') and stored at −20°C before amplification by qPCR, as per methods described for qPCR under 'assay design, optimisation and validation', with six technical replicates per sample and six non-template controls. A dilution series of DNA (S4, Supporting Information) was run with the samples to calibrate DNA quantification and provide a positive control.

2.4 | Field survey

With the guidance of a Natural England-licensed ecologist, 50 new (Wildcare), numbered and paper-lined nest tubes (Figure 1) were fixed with cable ties in proximity to permanent nest boxes (September 2018) at a National Dormouse Monitoring Programme site (Surrey, UK). After 8 days, a sampling interval informed by results of the 'rate of eDNA decay' method, all paper inserts were recovered with sterile gloves and placed individually into separate, numbered (according to nest tube number) envelopes. A fresh paper insert was added to each tube using clean gloves, to replace the samples taken. A negative field control (clean paper insert) was put into a separate envelope, transported, stored and swabbed for DNA together with the samples. All samples were transported to a DNA extraction-dedicated laboratory within 3 hr and stored at −20°C.

The routine monthly nest check ran in parallel, led by a licensed ecologist. The end of the survey comparison would be marked by a positive result from both eDNA analysis and finding a hazel dormouse nest, or the end of the survey season (November, Table 1).

DNA was extracted as per methods described for swabs under 'hazel dormouse eDNA sample' and stored at −20°C before amplification by qPCR. The qPCR method is described under 'assay design, optimisation and validation', with six technical replicates per sample, six extraction controls and six non-template controls. A dilution series of hazel dormouse DNA (S4, Supporting Information) was amplified alongside the samples to calibrate DNA quantity and serve as a positive control. Where there were negative results, qPCR was repeated with double the technical replicates (12, instead of six) to accommodate the stochasticity of low copy DNA and reduce the risk of false negative results.

Negative samples were tested for inhibition by duplex qPCR (FAM and VIC reporter dyes): as per methods described for qPCR under 'assay design, optimisation and validation' with the addition of 1 μl (10,000 copies) of VetMAX™ Xeno™ (Cole-Parmer) DNA per 9 μl of template DNA (1:10 dilution) and 1 μl of VIC™ assay (Cole-Parmer) per well, to give a total reaction volume of 21 μl per well. MicroAmp™ optical 96-well plate contained three technical replicates per eDNA sample, three positive controls (VetMAX™ Xeno™ DNA diluted 1:10 with nuclease-free water) and three non-template controls (nuclease-free water in place of template). Samples with an amplification shift of ≥2 threshold cycles (Ct) were considered inhibited and diluted twofold before retesting by qPCR (Biggs et al., 2014).

Positive results were confirmed by Sanger sequencing (Genewiz) the qPCR product. Poor quality sequencing reads are typical for the first 50 bp, so with an amplicon of just 113 bp (including primers), the qPCR product was sequenced in both directions. Sequences were viewed and concatenated with SnapGene (v5.1.6) and species provenance validated by BLAST (somewhat similar sequences, blastn algorithm) against the full nr database (NCBI Primer-BLAST, Ye et al., 2012). Where sequencing results were too short to return significant similarity across the full database, they were directly compared against both hazel dormouse (GenBank accession GCA_004027005.1) and edible dormouse (GCA_004027185.1) mitochondrial genomes (somewhat similar sequences, blastn algorithm) to validate specificity.

2.5 | Wider applicability: House mouse and wood mouse eDNA samples

To test the broader applicability of the method, standard office paper was left overnight inside house mouse *Mus musculus* and wood mouse *Apodemus sylvaticus* enclosures (British Wildlife Centre, Lingfield). Urine was swabbed and DNA extracted as per the hazel dormouse eDNA swab protocol under 'hazel dormouse eDNA samples'. DNA concentration was measured by NanoDrop™ 2000c Spectrophotometer and amplified by universal mammal cyt*b* primers, as per methods for 'assay design, optimisation and validation' and the PCR product was Sanger sequenced (GeneWiz). Sanger sequencing results were viewed and edited in SnapGene (v5.1.6) and species provenance validated by BLAST using the blastn algorithm and searching against the full nr database (NCBI Primer-BLAST, Ye et al., 2012) for significant similarity.

3 | RESULTS

3.1 | Assay design, optimisation and validation

Assay optimisation by PCR demonstrated that the primers work at the full range of temperatures tested with no amplification in non-template controls, but a balance between sensitivity and specificity (sensitivity decreasing and specificity increasing at higher annealing temperatures), confirmed by qPCR, resulted in an optimum annealing temperature of 57°C.

In silico analysis showed that the hazel dormouse assay could amplify DNA from the northwestern deer mouse *Peromyscus keeni*, though this species is native to North America and not found in the United Kingdom.

DNA extraction of tissue from closely related and/or co-occurring species was quality-checked and validated by gel electrophoresis of DNA and universal mammal cyt*b* PCR product (S5 and S6, Supporting Information). Tissue type and DNA yield are detailed at S7, Supporting Information, with DNA from yellow-necked mouse requiring the post-extraction ethanol precipitation step (S8, Supporting Information).

In vivo validation of the hazel dormouse assay by PCR confirmed that it successfully amplifies hazel dormouse DNA but does not amplify DNA from the co-occurring and/or closely related species tested here (S9, Supporting Information). In vivo validation by qPCR did show non-target amplification of the closely related edible dormouse *Glis glis* in one of 12 technical replicates at a high threshold (Ct 47) cycle, with 11 assay mismatches. The survey tubes are too small to accommodate adult edible dormice and the species does not co-occur at this study site, so does not affect results here but could at other sites. No other non-target DNA was amplified.

The assay achieved an efficiency of 105% (S10, Supporting Information) with a limit of detection of 10^{-7} ng/μl and quantification of 10^{-4} ng/μl.

3.2 | Hazel dormouse eDNA samples

Hazel dormouse eDNA was amplified by qPCR from both types of paper (Figure 2), with no amplification in non-template and extraction controls (NTC). While filter paper achieved a high concentration (10^{-4} ng/μl) for sample B by direct extraction across all six technical replicates, samples A and C failed to amplify. Swabs from standard paper consistently amplified, with at least two of six positive

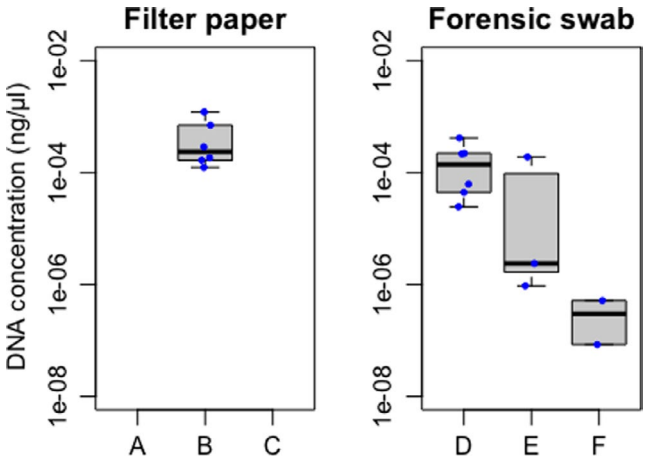


FIGURE 2 Samples A–C represent DNA extracted directly from filter paper and D–F, DNA extracted from swabs of standard paper (six technical replicates per sample). No amplification in extraction and non-template concentration for samples E and F fall outside the limit of quantification (LoQ) for this assay, but within the limit of detection (LoD) and are included here for completeness

replicates, indicating greater consistency by swabbing standard paper over direct extraction from filter paper.

3.3 | Rate of eDNA decay

Hazel dormouse eDNA was amplified from all three swab samples on days 1 and 3 and from two of the three samples on days 13 and 33 (Figure 3). There is no quantifiable trend in the rate of eDNA decay observed in this study, but with a presence/absence focus, results here indicate a sampling interval of <13 days to maximise the probability of detection.

3.4 | Field survey

Of the 50 nest tubes checked on day 8, seven had visible urine spots under UV light, and hazel dormouse eDNA was successfully amplified by qPCR and validated by Sanger sequencing (S11, Supporting Information) from three tubes (Figure 4). The number of positive technical replicates is low (no more than three out of six) compared to other enclosure results (under 'rate of eDNA decay') though not unsurprising given the artificially high number of individuals in a small area in captivity. With just six technical replicates, hazel dormouse presence would have been missed in one of the three tubes. An increase from six to 12 replicates changed this false negative result to a positive. This mirrors guidelines by Biggs et al. (2014) for great crested newt, with one positive replicate out of 12 sufficient to confirm presence. Inhibition was not detected in negative samples.

During a routine nest tube check in November, a single hazel dormouse nest was identified in one of the 50 nest tubes, marking the point at which a standalone nest tube survey would have confirmed hazel dormouse presence for this site (Figure 4). We know that samples on day 8 were positive for eDNA in three of 50 nest tubes, but that hazel dormouse urine could have been deposited at any point between days 1 and 8. The tubes were negative for nests on day 36 and one tube out of a possible 50 was positive on day 63. Nest

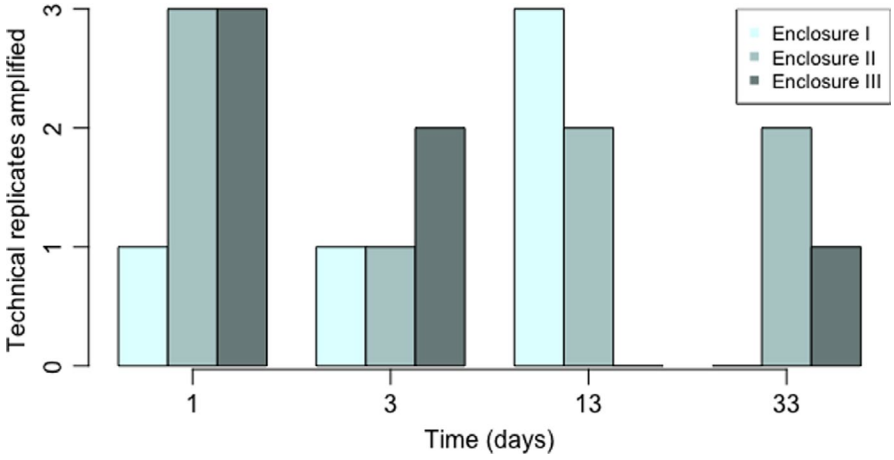


FIGURE 3 Paper samples ($n = 4$) removed from three hazel dormouse enclosures (I, II and III) after 24 hr and urine spots swabbed for DNA at time intervals: 1, 3, 13 and 33 days. Bars represent the number of positive technical replicates ($x/6$)

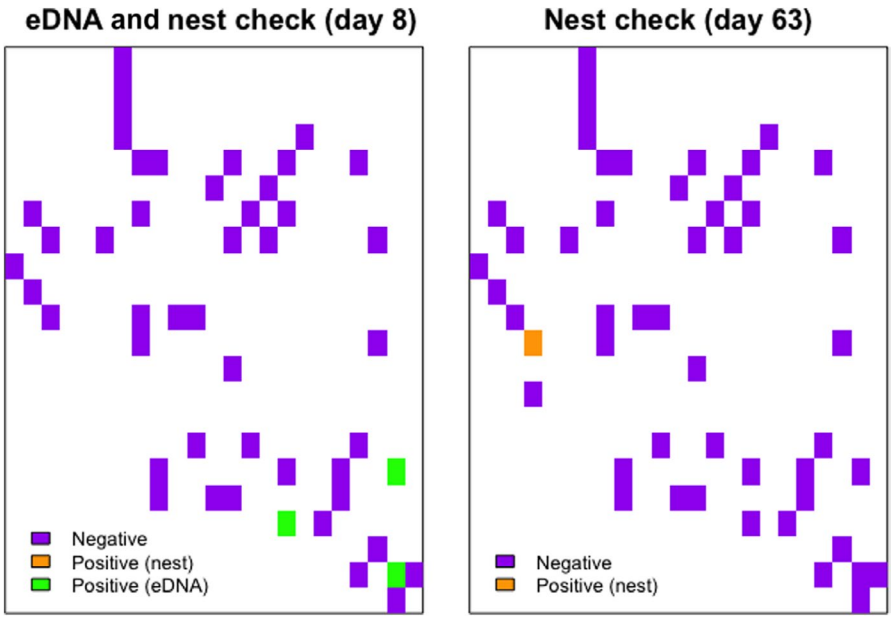


FIGURE 4 Relative (not to scale) position of nest tubes at the Surrey survey site, with three eDNA positive tubes on day 8 and one nest on day 63

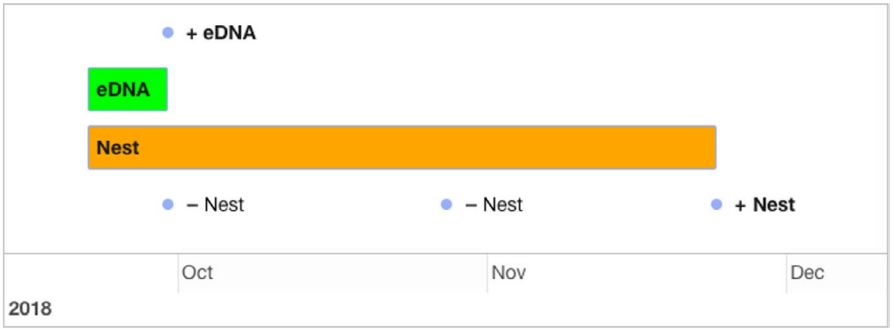


FIGURE 5 Timeline for the field survey. Nest tubes were set on 22 September 2018 and checked 8 days later. Three were positive for eDNA and all tubes were negative for nests. The tubes were checked again on days 36 and 63, with a nest found in one tube during this final check at the end of the survey season

construction could, therefore, have started at any point between days 36 and 63 (Figure 5).

Results from this study show a minimum 28-day saving and a maximum 55-day saving when sampling eDNA from survey nest tubes. They are also based on a field test during a low probability of detection month, October (Table 1).

By taking the maximum 8 days for eDNA detection and a minimum of 36 days for a nest, we can derive a conservative measure of survey efficiency. An ideal (100%) efficiency would be positive detection in all tubes on day 1. Based on Equation 2, survey efficiency with eDNA is a minimum of 0.75% and with nests, a maximum of 0.06%. The eDNA method improved survey efficiency at least 12-fold, in this study.

$$\text{Survey efficiency} = \frac{(\text{positive tubes} \times 100)/\text{survey days}}{\text{available tubes}}. \quad (2)$$

3.5 | Wider applicability: House mouse and wood mouse eDNA samples

Environmental DNA was extracted from standard paper left in wood mouse and house mouse enclosures, at a concentration of, 13 and 19 ng/μl respectively (NanoDrop™ 2000c Spectrophotometer). This eDNA was successfully amplified by universal mammal *cytb* primers and provenance confirmed by Sanger sequencing (S12, Supporting Information). There was considerably more urine visible on paper from these two species, than for captive hazel dormice.

4 | DISCUSSION

Results confirm that hazel dormice, wood mice and house mice urinate on paper and that this can be swabbed, amplified and used to confirm their presence. For the hazel dormouse, we have shown that this method can be quicker and increase the probability of detection (more positive tubes) than a traditional nest tube survey. This integrated approach combines eDNA with established survey methods and could therefore be applied with immediate effect (workflow at S13, Supporting Information).

The nest tube structure offers some protection from light and rain (Figure 1) but abiotic factors such as temperature, pH, humidity and ultraviolet light affect the rate of eDNA degradation (Harper, Buxton, et al., 2019). Hazel dormouse physiology, behaviour and differences between individuals (Sassoubre et al., 2016) may also affect the amount of DNA shed onto the paper. It would be unrealistic to factor all possible variables into decisions regarding sampling intervals. Practitioners would therefore need to balance resources versus the risk of eDNA degradation, but a cautious sampling interval would be <14 days, the time frame in which a decrease in amplification rate among technical replicates occurred for this study. Samples in this study were stored at −20°C within 3 hr of collection. Where that is not possible, the paper samples can be stored dry with silica beads or swabbed in the field. Swab tips can be ejected into a nuclease-free 2-ml flip-top plastic tube and stored dry with silica beads or in lysis buffer (Majaneva et al., 2018). We also recommend the use of a light adhesive to hold the paper inserts in place, as the paper fell from some of the tubes. This occurred after a positive result on day 8, so it did not affect the sample size here, but could for other studies. Practitioners should be mindful of non-target amplification of edible dormouse *Glis glis* DNA, specifically if just one technical replicate amplifies (1/12) at a high threshold cycle >45 and in locations where presence of this introduced species is suspected. In this instance, specificity can be validated by Sanger sequencing qPCR product. In the future, a synthetic gBlocks® Gene Fragment qPCR standard could be used (Conte et al., 2018) to establish the assay's LoD in DNA copies/μl. Fluorescence observed at a threshold cycle above 0 copies/μl would be discountable as non-target amplification.

New nest tubes were used for this study, so we do not know the effect of lining second-hand tubes on repeat deployments. Urine may not be the sole source of eDNA in the tubes and while swabbing urine directly from clean paper inserts is a pragmatic way to avoid cross-contamination, we have not tested this assumption. The use of field, extraction and non-template controls are therefore key (Goldberg et al., 2016). A UVA torch was used to highlight urine and target swabbing effort. While we consider the risk to be low (Mächler et al., 2018), we have not tested whether this brief exposure accelerated the rate of eDNA degradation.

Nest tubes were positioned alongside nest boxes which may have skewed results, as familiar housing (Chanin & Gubert, 2011) was available to the study species. That effect would be felt for both the nest and eDNA method so we can assume it did not affect their comparison but may have an impact on absolute values. In captivity and the field test, we found nests in paper-lined tubes, so do not believe the paper insert dissuades dormice from using them but

acknowledge that regular checks could be disruptive if conducted insensitively.

Footprint tunnel surveys also record hazel dormouse visits to survey apparatus, in as little as 2 days (Mills et al., 2016). The tubes are open at both ends (they are not intended for nests), paper-lined and bookended with ink pads. When a hazel dormouse walks through the ink and into the tube it leaves behind distinctive triangular prints on the paper. Fortnightly checks of 50 footprint tunnels at 12 sites (April–November) resulted in a 97.5% probability of detection at low density (Bullion et al., 2018). It would be interesting to compare the probability of detection for eDNA methods at a similar scale and check positive footprint paper for hazel dormouse eDNA. We do not know if every visit to a survey tube (footprint or nest) reliably results in an eDNA signal. Urinating in the tubes could represent hazel dormouse marking behaviour which while typical for rodents (Arakawa et al., 2008) is, to our knowledge, unknown for this species. Where footprint tunnels are used for surveys, results from crowded and ambiguous footprint sheets or a lack of data due to dry inkpads could be validated by swabbing for hazel dormouse eDNA.

The potential for eDNA methods to detect hazel dormouse presence, rewrite seasonal detection indices and reduce the number of survey tubes is clear and could, with minimal effort, be incorporated into nest tube, nest box or footprint tunnel surveys. The impact of these efficiency savings would be significant for large-scale spatial and temporal monitoring programmes, such as the National Dormouse Monitoring Programme (Great Britain).

While a method to confirm presence and infer absence is valuable to conservation practitioners, eDNA can offer additional insights. Amplification of larger fragments returns sex ratio, population genetics, behaviour and abundance estimates (Adams et al., 2019; Baker et al., 2018; Barnes & Turner, 2016; Parsons et al., 2018; Sigsgaard et al., 2019). These additional gains are more likely when swabbing defined spots of urine from clean paper, compared to eDNA extracted from a mixed, bulk environmental sample.

Beyond the hazel dormouse, capturing an eDNA sample on paper (or other clean substrate) could broaden the application and associated benefits to a range of terrestrial invasive (Browett et al., 2020; Valentin et al., 2020) and conservation priority species. The global authority on the conservation status of small mammals lists the top 20 most critically endangered and top 20 feared extinct species (IUCN Small Mammal Specialist Group, 2020a, 2020b). A total of 70% (14/20) of the former and 85% (17/20) of the latter list are, like the hazel dormouse, wood and house mouse, of the order Rodentia. Though a highly diverse order, it is likely that most, mark with urine (Ferkin, 2018) and are therefore suitable species for the eDNA method described in this study.

More broadly, where an eDNA survey of a terrestrial species is judged impractical, other clean eDNA samplers could be developed to 'catch' eDNA. Perhaps coarse paper under or on top of survey apparatus for reptiles (Rose et al., 2019 and Ratsch et al., 2020), manmade salt licks for tropical forest mammals (Ishige et al. 2017 sampled natural saltlicks) or conspicuous samplers deployed as lures for large mammal marking behaviour (Johnson, 1973).

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AUTHORS' CONTRIBUTIONS

V.P., R.A. and V.S. designed the research; V.P., R.A. and M.B. collected the data; V.P. analysed the data and wrote the first draft of the manuscript; all the authors then contributed to the final manuscript.

DATA AVAILABILITY STATEMENT

Data available from Zenodo Digital Repository https://doi.org/10.5281/zenodo.3514390 (Priestley et al., 2021).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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