Needle in a haystack? A comparison of eDNA metabarcoding and targeted qPCR for detection of great crested newt (*Triturus cristatus*)

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Summary

1. Environmental DNA (eDNA) analysis is a rapid, cost-effective, non-invasive biodiversity monitoring tool which utilises DNA left behind in the environment by organisms for species detection. The method is used as a species-specific survey tool for rare or invasive species in a broad range of ecosystems. More recently, eDNA and 'metabarcoding' have been combined to describe whole communities as opposed to focusing on single target species. However, whether metabarcoding is as sensitive as targeted approaches for rare species detection remains to be evaluated.

2. The great crested newt *Triturus cristatus* is a flagship pond species of international conservation concern and the first in the UK to be routinely monitored using eDNA. We evaluate whether eDNA metabarcoding has comparable sensitivity to targeted real-time quantitative PCR (qPCR) for great crested newt detection, and examine the implications for the future of community eDNA monitoring. Extracted eDNA samples (N = 532) were screened for great crested newt by qPCR and analysed for all vertebrate species using High-Throughput Sequencing technology.

3. Of all ponds, 50% tested positive for great crested newt with qPCR when the detection threshold was set to 1/12 positive qPCR replicates. Detection rate decreased to 32% when the threshold was increased to 4/12 positive qPCR replicates. With metabarcoding, newts were detected in 34% of ponds when no detection threshold was applied, and in 28% of ponds when a threshold (0.028%) was applied. Therefore, qPCR generates a higher detection rate than metabarcoding, but the metabarcoding detection rate with no threshold is broadly equivalent to that of qPCR with the stringent detection threshold. The proportion of great crested newt sequences in each sample was positively associated with the number of positive qPCR replicates (qPCR score)

suggesting eDNA metabarcoding may be indicative of species abundance.

4. eDNA metabarcoding holds enormous potential for holistic biodiversity assessment and routine freshwater monitoring. We advocate this community approach to freshwater monitoring to guide management and conservation, whereby entire communities can be initially surveyed to best inform use of funding and time for species-specific surveys.

Key-words: environmental DNA (eDNA), great crested newt, High-Throughput Sequencing (HTS), metabarcoding, ponds, real-time quantitative PCR (qPCR), *Triturus cristatus*.

Introduction

Species monitoring has rapidly evolved with the advent of environmental DNA (eDNA) analysis (Lawson Handley 2015). eDNA analysis allows highly sensitive detection of rare and invasive species and is increasingly being used for surveys of aquatic species (Thomsen *et al.* 2012; Biggs *et al.* 2015; Davy, Kidd & Wilson 2015; Smart *et al.* 2015; Evans *et al.* 2017b). This non-invasive approach uses intra- and extracellular DNA (mucous, skin cells, faeces/urine, gametes, hair, deceased remains) released into the environment by organisms to survey for species and assess their distribution (Rees *et al.* 2014b; Lawson Handley 2015; Goldberg *et al.* 2016). Typically for eDNA analysis, DNA is extracted from environmental samples (water, soil, air) and analysed using a targeted or passive approach. The targeted approach uses species-specific primers with conventional PCR (PCR), real-time quantitative PCR (qPCR), or droplet digital PCR (ddPCR), to determine presence-absence and estimate abundance of single species (Shaw, Weyrich & Cooper 2016b; Goldberg *et al.* 2016). Conversely, the passive approach uses conserved primers and PCR to sequence whole communities with High Throughput Sequencing (HTS) (Shaw *et al.* 2016b; Valentini *et al.* 2016), commonly termed eDNA metabarcoding (Taberlet *et al.* 2012; Deiner *et al.* 2017). Passive eDNA monitoring is particularly attractive to ecologists for biodiversity assessment as a means to detect entire species assemblages alongside rare or invasive species (Lacoursière-Roussel *et al.* 2016; Blackman *et al.* 2017). However, this gain in community understanding may come at the cost of accuracy and sensitivity. Direct comparisons of these two approaches are essential to determine whether they have comparable power and yield similar results.

Although in its relative infancy, eDNA metabarcoding has proven effective for community biodiversity assessment across a range of taxa in varying environments, particularly freshwater

herpetofauna and fish (Valentini *et al.* 2016; Hänfling *et al.* 2016; Shaw *et al.* 2016a; Civade *et al.* 2016; Lacoursière-Roussel *et al.* 2016; Lopes *et al.* 2016; Evans *et al.* 2017a; Bálint *et al.* 2017). However, eDNA metabarcoding is confounded by potential amplification bias during PCR, preventing capture of all species present in a given area (Kelly *et al.* 2014). Species' DNA in community samples is also in competition to bind to metabarcoding primers during PCR, where more common templates are more likely to be amplified. High abundance species may thus prevent detection of low abundance species, whether by fewer individuals or less DNA shed, resulting in 'species masking' (Kelly *et al.* 2014; Brandon-Mong *et al.* 2015). eDNA metabarcoding may therefore be less capable of identifying eDNA of rare species DNA within a community than species-specific qPCR (Goldberg *et al.* 2016).

The sensitivity of eDNA metabarcoding has been evaluated against conventional biodiversity monitoring methods in freshwater ecosystems (Valentini *et al.* 2016; Hänfling *et al.* 2016; Shaw *et al.* 2016a; Civade *et al.* 2016; Lacoursière-Roussel *et al.* 2016; Lopes *et al.* 2016; Evans *et al.* 2017a; Bálint *et al.* 2017), yet specific investigations comparing the sensitivity of eDNA metabarcoding and targeted qPCR are sparse. Schnieder *et al.* (2016) achieved improved detection of Invasive Mosquito Species (IMS) with targeted qPCR and eDNA metabarcoding as opposed to conventional sampling. Although qPCR provided higher detection probability for two species, metabarcoding achieved comparable results for a third species, and allowed simultaneous detection of IMS and other taxa in a single sequencing run without development of multiple species-specific markers. In another study, eDNA metabarcoding failed to detect wood turtle *Glyptemys insculpta* (LeConte, 1830) in four rivers where targeted qPCR and conventional visual survey detected the species (Lacoursière-Roussel *et al.* 2016). Amplification of longer fragments during metabarcoding versus qPCR could account for difference in sensitivity of the two methods,

with the shorter qPCR assay being more capable of detecting heavily degraded DNA (Lacoursière-Roussel *et al.* 2016). Further research is clearly needed to determine whether these two approaches are comparable.

Great crested newt *Triturus cristatus* (Laurenti, 1768), hereafter crested newt, is a model organism for eDNA-based monitoring. Crested newt secrete mucous, breed in water, and produce aquatic eggs and larvae - all sources of DNA deposition in ponds. The species is rare in parts of the UK and Europe, and as such all life stages are protected by UK and European legislation (Rees *et al.* 2014a; Buxton *et al.* 2017). eDNA analysis using targeted qPCR has been repeatedly verified against conventional surveying (bottle trapping, torchlight counts, larval netting, egg searches) for crested newt and found to achieve comparable or improved species detection (Thomsen *et al.* 2012; Rees *et al.* 2014a; Biggs *et al.* 2015). eDNA sampling also requires less training, is cost-efficient (Biggs *et al.* 2014) and can be implemented in large-scale citizen science monitoring programmes without loss of species detection (Biggs *et al.* 2015). Crested newt is the first species to be routinely monitored using eDNA in the UK (Natural England 2015) and targeted eDNA assays are now offered as a commercial service by ecological consultancies. The targeted eDNA assay is highly effective for crested newt detection; however, should metabarcoding have comparable sensitivity, this approach would allow detection of crested newt alongside pond communities and potentially enable more cost-effective monitoring of entire ecosystems and ecological hypothesis testing.

Here, we perform a large-scale comparison (N = 532 ponds) of eDNA metabarcoding and targeted qPCR for crested newt detection to compare method sensitivity. A single primer pair that is vertebrate specific for mitochondrial DNA (mtDNA) and requires no *a priori* knowledge of species composition was employed for eDNA metabarcoding. The metabarcoding results were then compared to results obtained using the standard crested newt qPCR assay (Biggs *et al.* 2015).

Our hypotheses are: (1) eDNA metabarcoding will give equivalent results to qPCR for crested newt detection, (2) eDNA metabarcoding sequence read count for crested newt will increase as qPCR score (the number of positive qPCR replicates) increases, therefore eDNA metabarcoding may be indicative of species abundance, and (3) metabarcoding primers will amplify DNA from all taxa equally well and no bias towards amplification of crested newt will occur (bias would be indicated by a positive association between the proportion of crested newt sequence reads and PCR product concentration). We also examined the cost and investigator effort required by each approach to determine whether a trade-off between cost, time and amount of data generated exists.

Materials and methods

SAMPLING

Samples from 532 ponds distributed across three UK counties (Cheshire, Kent and Lincolnshire) were analysed for this project. Of these, 508 ponds were sampled as part of crested newt surveys through Natural England's Great Crested Newt Evidence Enhancement Programme. Crested newt egg searches were performed once during the daytime at 506/508 ponds. Any other life stages seen were also recorded. A further 24 ponds were sampled for eDNA by ecological consultants for private contracts but egg searches were not undertaken at these ponds. All water samples were collected using methodology outlined by Biggs *et al.* (2015). Briefly, twenty 30 mL water samples were collected at even intervals around the pond margin and pooled in a sterile 1 L Whirl-Pak®

stand-up bag, which was shaken to provide a single homogenised sample from each pond. Six 15 mL subsamples were taken from the mixed sample using a sterile plastic pipette (25 mL) and added to sample tubes containing 33.5 mL absolute ethanol and 1.5 mL sodium acetate 3 M (pH 5.2) for ethanol precipitation. Subsamples were then sent to Fera (Natural England), and ADAS (private contracts) for eDNA analysis according to laboratory protocols established by Biggs *et al.* (2015). Subsamples were centrifuged at 14,000 x g for 30 minutes at 6 ^{o}C, and the supernatant discarded. Subsamples were then pooled during the first step of DNA extraction with the Qiagen® DNeasy Blood & Tissue Kit, where 360 µL of ATL buffer was added to the first tube, vortexed, and the supernatant transferred to the second tube. This process was repeated for all six tubes. The supernatant in the sixth tube, containing concentrated DNA from all six subsamples, was transferred in a 2 mL tube and extraction continued following manufacturer's instructions to produce one eDNA sample per pond.

TARGETED qPCR FOR CRESTED NEWT

Prior to testing for crested newts, all extracted samples were tested for PCR inhibitors and sample degradation using methodology outlined by Biggs *et al.* (2015), where an Internal Positive Control was included in qPCR reactions of eDNA samples and a sample considered inhibited if replicates showed different C_q values (where samples move into the exponential phase of qPCR amplification). Targeted qPCR was carried out as part of the crested newt monitoring programmes mentioned above in the laboratories at Fera and ADAS during 2015 using a standardised protocol (Biggs *et al.* 2015). Extracted DNA was amplified by TaqMan probe qPCR using published

primers and probe to amplify an 81 bp fragment of the cytochrome b gene: TCCBL (5'-CGTAAACTACGGCTGACTAGTACGAA-3'), TCCBR (5-CCGATGTGTATGTAGATGCAAACA) and TCCB_Probe (5'-CCACGCTAACGGAGCCTCGC-3') (Thomsen *et al.* 2012). PCR reactions were set up in a total volume of 25 µL consisting of: 3 µL of extracted template DNA, 1 µL of each primer (0.4 µM), 1 µL of probe (0.1 µM), 1x TaqMan® Environmental Master Mix 2.0 (containing AmpliTaq GOLD DNA polymerase, Life Technologies) and ddH_2O. The PCR included an initial incubation for 5 min at 50 °C, then a 10 min denaturation step at 95 °C, followed by 55 cycles of denaturation at 95 °C for 30 s and annealing at 56.3 °C for 1 min. For each sample, 12 qPCR replicates were performed and a sample recorded as positive for crested newt if one or more qPCR replicates were positive. Positive (crested newt DNA: 1×10^{-1} ng/µL to 1×10^{-4} ng/µL) and negative controls (ddH_2O) were also included on each plate in quadruplicate. Following qPCR, the eDNA samples were placed in storage at -80 ^{o}C.

METABARCODING OF VERTEBRATE COMMUNITIES

eDNA samples were stored at -20 ^{o}C until PCR amplification. Metabarcoding was performed using published vertebrate-specific primers F1 (5'-ACTGGGATTAGATACCCC-3') and R1 (5'-TAGAACAGGCTCCTCTAG-3') (Riaz *et al.* 2011) which amplify a 73-110 bp fragment of 12S ribosomal RNA gene (rRNA). The assay was first validated *in silico* using ecoPCR software (Ficetola *et al.* 2010; Bellemain *et al.* 2010) against a custom, phylogenetically curated reference database for UK vertebrates. Full details of reference database construction are provided in

Supporting Information (Appendix 1). The complete reference database compiled in GenBank format has been deposited in the dedicated GitHub repository for this study: https://github.com/HullUni-bioinformatics/Harper_et_al_2017. Parameters set allowed a 50-250 bp fragment and maximum of three mismatches between the primer pair and each sequence in the reference database. Primers were then validated against tissue DNA extracted from UK amphibian species (Supporting Information: Appendix 2) having been previously validated *in vitro* for UK fish communities by Hänfling *et al.* (2016). After primer validation, a two-step PCR protocol was used to construct metabarcoding libraries from the eDNA samples. During the first PCR, the target region was amplified using metabarcoding primers, comprised of the aforementioned specific locus primer, random hexamers, sequencing primer and pre-adapter (Illumina 2011). DNA from the cichlid *Rhamphochromis esox* (Boulenger, 1908) was used for PCR positive controls (six per PCR plate; N = 114), whilst sterile molecular grade water (Fisher Scientific) substituted template DNA for No Template Controls, NTCs (six per PCR plate; N = 114). In the second PCR, Molecular Identification (MID) tags (unique 8-nucleotide sequences) and Illumina MiSeq adapter sequences were added to the amplified product. Two independent libraries were constructed, each containing 266 eDNA samples, 57 NTCs, and 57 positive controls. Sequencing was performed on an Illumina MiSeq using 2 x 300 bp V3 chemistry at Fera. The first sequencing run revealed human contamination across samples and in some PCR controls, therefore reactions prepared for the second sequencing run were sealed with mineral oil to minimise PCR contamination. Full details of the eDNA metabarcoding workflow are provided in Supporting Information (Appendix 2).

BIOINFORMATIC PROCESSING

Illumina data was converted from raw sequences to taxonomic assignment using a custom pipeline for reproducible analysis of metabarcoding data: metaBEAT (metaBarcoding and eDNA Analysis Tool) v0.8 (https://github.com/HullUni-bioinformatics/metaBEAT). Bioinformatic data processing/analysis largely followed the workflow outlined by Hänfling *et al.* (2016), with minor modifications. In brief, raw reads were quality trimmed using Trimmomatic v0.32 (Bolger, Lohse & Usadel 2014), both from the read ends (minimum per base phred score Q30), as well as across sliding windows (window size 5bp; minimum average phred score Q30). Reads were clipped to a maximum length of 110 bp and reads shorter than 90 bp after quality trimming were discarded. To reliably exclude adapters and PCR primers, the first 25 bp of all remaining reads were also removed. Sequence pairs were merged into single high quality reads using FLASH v1.2.11 (Magoč & Salzberg 2011), if a minimum of 10 bp overlap with a maximum of 10% mismatch was detected between pairs. For reads that were not successfully merged, only forward reads were kept. To reflect our expectations with respect to fragment size, a final length filter was applied and only sequences of length 80-120 bp were retained. These were screened for chimeric sequences against our custom reference database using the uchime algorithm (Edgar *et al.* 2011), as implemented in vsearch v1.1 (Rognes *et al.* 2016). Redundant sequences were removed by clustering at 97% identity ('--cluster_fast' option) in vsearch v1.1 (Rognes *et al.* 2016). Clusters represented by less than five sequences were considered sequencing error and omitted from further analyses. Non-redundant sets of query sequences were then compared against our custom reference database using BLAST (Zhang *et al.* 2000). For any query matching with at least 98% identity to a reference sequence across more than 80% of its length, putative taxonomic identity was assigned using a

lowest common ancestor (LCA) approach based on the top 10% BLAST matches. Sequences that could not be assigned (non-target sequences) were subjected to a separate BLAST search against the complete NCBI nucleotide (nt) database at 98% identity to determine the source via LCA as described above. To ensure reproducibility of analyses, the described workflow has been deposited in the GitHub repository.

DATA ANALYSIS

All downstream analyses were performed in the statistical programming environment R v.3.3.2. (R Core Team 2016). Data and R scripts have been deposited in the GitHub repository. Manipulation of the dataset produced by metaBEAT is described in Supporting Information: Appendix 3.

Detection thresholds and contamination

At present, there are no standard guidelines for eDNA analysis to indicate minimum number of positive eDNA samples or replicates required to class sites as species positive (Goldberg *et al.* 2016). Samples analysed by qPCR in this study were previously considered crested newt positive if one or more qPCR replicates gave a positive result (Biggs *et al.* 2015). We term this analysis qPCR NT (No Threshold). This inference of species presence is employed across many studies but may not be reliable or reproducible (Goldberg *et al.* 2016). More stringent qPCR thresholds

reduced detection sensitivity for palmate newt *Lissotriton vulgaris* (Smart *et al.* 2015) but may be necessary to ensure consistency and prevent false positives (Rees *et al.* 2014b). To facilitate comparison with current qPCR scoring (our NT interpretation) and eDNA metabarcoding, we also applied a stringent qPCR threshold of four or more positive qPCR replicates (out of 12) to infer species presence, and termed the new analysis qPCR TA (Threshold Applied).

The raw eDNA metabarcoding dataset with no detection thresholds applied was termed metabarcoding NT (No Threshold). A second dataset was constructed to reduce the potential for false positives by application of a species-specific threshold: a species was only classed as present at a given site if its sequence frequency exceeded a species-specific threshold. Thresholds for each species were defined by analysing sequence data from PCR positive controls (N = 114) and identifying the maximum sequence frequency for a given species across all PCR positive controls (Supporting Information: Table S2). For example, the species-specific false positive sequence threshold for great crested newt was 0.028% to omit all false detections in the PCR positive controls. The resultant dataset was termed metabarcoding TA (Threshold Applied).

We tested whether mineral oil reduced contamination by analysing the distribution of positive control sequences (*R. esox*) and human DNA in eDNA samples, and any DNA in NTCs, across both sequencing runs using binomial generalised linear mixed effects models (GLMMs) within the R package 'lme4' v1.1-12 (Bates *et al.* 2015). The response variable was presence-absence of contamination and explanatory variables were PCR plate (random effect) and sequencing run i.e. mineral oil sealed versus non-sealed (fixed effect). Human DNA may be present in eDNA samples as a real environmental signal or contaminant prior to PCR and thus may not be a true PCR contaminant. Consequently, contamination in eDNA samples was examined using several model permutations, where contamination comprised both cichlid and human DNA,

cichlid DNA alone and human DNA alone. An information-theoretic approach using Akaike's Information Criteria (AIC) to evaluate model fit was employed, where low AIC models are more parsimonious than high AIC models (Akaike 1973). Significance of the fixed effect in the model was tested by a Likelihood Ratio Test (LRT).

Comparison of eDNA methods for crested newt detection

We next tested the null hypothesis of no significant difference in sensitivity of qPCR and metabarcoding. Overall agreement between eDNA metabarcoding and qPCR for crested newt detection was measured using Cohen's kappa coefficient (Cohen 1960), following which Pearson's Chi-squared Test for Independence was used to test equality of crested newt detection between eDNA approaches.

The relationship between read count and qPCR score has not been examined previously, and whether read production is indicative of DNA concentration remains unknown. qPCR score is positively correlated with crested newt abundance and can be proxy for amount of DNA present (Biggs *et al.* 2015). We hypothesised samples with higher qPCR score would have increased crested newt read count. First, the average number of crested newt reads produced by eDNA metabarcoding per qPCR score (1-12 out of 12) was calculated. A Spearman Rank Correlation was then used to test for a relationship between average read count and qPCR score.

Following data exploration (see Supporting Information: Appendix 3), a negative binomial GLMM was used to counter overdispersion and improve model fit. The GLMM examined read count in relation to qPCR score, accounting for other variables that may affect metabarcoding

signal strength. Variation in crested newt read count was examined using the proportion of crested newt reads within the total number of reads produced for each eDNA sample as the response variable. Sequencing run and PCR plate were considered random effects and all other explanatory variables as fixed effects (qPCR score, sample degradation, sample inhibition, post-PCR eDNA concentration). Presence-absence of sample degradation and inhibition was determined by qPCR in 2015 using methodology outlined by Biggs *et al.* (2015). Model fit was again evaluated using AIC and significance of fixed effects in the model was tested with stepwise backward deletion of terms from the model informed by LRTs. All values were bound in a new data frame and model results plotted for evaluation using the R package 'ggplot2' v 2.1.0 (Wickham, 2009).

Results

TARGETED qPCR AND EGG SEARCHES

Targeted qPCR detected crested newt in 253 (49.80%) samples analysed by Fera (N = 508). Of 255 (50.20%) samples that were crested newt negative, one was inhibited and nine were degraded. qPCR and egg searches produced consistent results for 297 (58.47%) ponds, with 51 (10.04%) positive and 246 (48.43%) negative ponds by both methods. Of the 211 ponds where there was disagreement between methods, 202 (39.76%) were qPCR positive but negative by egg searches, and 7 (1.38%) were positive with egg searches but qPCR negative. Of 24 samples analysed by

ADAS, 12 (50.00%) were qPCR negative and 12 (50.00%) were qPCR positive for crested newt. No egg search data were available for these ponds.

VERTEBRATE METABARCODING

The *in silico* and *in vitro* primer validation confirmed that crested newt, and other native UK amphibians tested, can be reliably amplified and identified with the chosen assay (Supporting Information: Appendix 4, Fig. S1). Furthermore, the *in silico* approach showed that the majority of all UK vertebrates can be amplified (see Appendix 4 for details). Both sequencing runs had comparable yield and sequencing quality score; summary statistics for each sequencing run and read counts for taxonomic assignment levels are provided in Supporting Information: Appendix 4 (Tables S3, S4). A full summary of sequence read count data is also given in Supporting Information: Appendix 5. eDNA metabarcoding identified a combined total of 60 species (Supporting Information: Appendix 4, Fig. S2) across both sequencing libraries, with 375,954 and 508,879 sequences assigned to crested newt from each library. Analyses of overall pond species compositions inferred by eDNA metabarcoding (Supporting Information: Appendix 4, Fig. S3, Table S5) are reported separately (Harper *et al.* in preparation).

All samples (N=532) were sequenced and of 57 samples that did not produce visible PCR bands, nine generated sequence reads. Notably, the 57 samples were not inhibited or degraded at time of qPCR. Weak PCR bands were observed in some NTCs therefore all PCR controls were sequenced (Supporting Information: Appendix 4, Fig. S4). Six NTCs contained crested newt DNA but only one exceeded 100 crested newt reads (307/330 reads). Twelve other sources occurred in

NTCs (Supporting Information: Appendix 4, Table S6); seven occurred in more than one NTC and eight had high maximum read counts (> 100 reads).

Contamination of NTCs (any DNA) and environmental samples (cichlid/human DNA) was observed (Supporting Information: Appendix 4, Figs. S4, S5, S6). Read counts of NTC contaminants were reduced between sequencing runs with the addition of mineral oil to PCR reactions included on the second sequencing run (Supporting Information: Appendix 4, Figs. S4, S5, S6) but this reduction was not statistically significant (GLMM: $\chi^2_1 = 2.083$, $F_1 = 1.941$, $P > 0.05$). Mineral oil did not reduce human DNA signal in environmental samples between sequencing runs either (GLMM: $\chi^2_1 = 3.608$, $F_1 = 3.591$, $P > 0.05$); however, it did reduce human DNA in combination with cichlid DNA (GLMM: $\chi^2_1 = 10.348$, $F_1 = 21.143$, $P < 0.01$), and cichlid DNA contamination alone (GLMM: $\chi^2_1 = 5.053$, $F_1 = 6.978$, $P < 0.05$) of environmental samples.

eDNA METABARCODING VS qPCR FOR CRESTED NEWT DETECTION

Crested newt detection by metabarcoding NT (34.21%) was less sensitive than qPCR NT (49.81%) but marginally higher than qPCR TA (32.71%) (N = 532 ponds, Fig. 1). Metabarcoding TA had lower detection efficiency (28.01%) and failed to detect crested newt in 116 and 25 ponds where the species was detected by qPCR NT and qPCR TA respectively. Nonetheless, both molecular approaches attained higher crested newt detection than daytime egg searches (11.46%) in 506 ponds where all three approaches were implemented.

Overlap between survey methods for positive crested newt ponds (N = 277), and unique detections by each method is summarised in Fig. 2. Negative crested newt ponds (N = 229) are

examined in combination with species positive ponds in Supporting Information: Appendix 4 (Table S7). Each survey method detected the species in ponds where other methods failed. Despite lower crested newt detection efficiency, egg searches detected the species in six ponds where it went undetected by qPCR and metabarcoding. Metabarcoding NT and metabarcoding TA revealed crested newt in seven ponds which other methods did not, whilst qPCR NT and qPCR TA detected crested newt in 33 ponds unique to other methods. All methods detected crested newt in 32 ponds, and both metabarcoding and qPCR identified crested newt in 86 ponds. Disagreement between molecular methods was more likely when samples were positive rather than negative by qPCR. Without thresholds, 39.25% of qPCR positive ponds (N = 265) were negative by metabarcoding, but 7.87% of qPCR negative ponds (N = 267) were positive by metabarcoding. With thresholds, 29.31% of qPCR positive ponds (N = 174) were negative by metabarcoding, whereas 7.26% of qPCR negative ponds (N = 358) were positive by metabarcoding. Agreement between eDNA approaches is summarised in Table 1. Agreement was strongest between eDNA approaches when the qPCR detection threshold was applied, irrespective of whether the metabarcoding detection threshold was applied. Metabarcoding (NT or TA) and qPCR TA did not significantly differ in their detection of crested newt (Table 1).

An identical positive correlation was observed between qPCR score and the average number of crested newt reads obtained for samples belonging to each qPCR score (r_s = 0.648, df = 11, P < 0.05), regardless of threshold application to the metabarcoding data. Despite some inconsistency across qPCR scores, samples with a higher qPCR score generally had more crested newt reads, supportive of a relationship between metabarcoding and abundance of eDNA from single species. Notably, metabarcoding produced crested newt reads for qPCR NT and qPCR TA crested newt negative samples, but the crested newt metabarcoding signal of these (qPCR $NT_{negative}$

= 2639 reads max., qPCR $TA_{negative}$ = 3075 reads max.) was much lower than samples with higher qPCR score (max. 65,325 reads; Supporting Information: Appendix 5). Further examination of the relationship between qPCR score and metabarcoding TA revealed qPCR score and post-PCR eDNA concentration of samples also influenced the proportion of crested newt reads i.e. relative crested newt sequence read production (Table 2). A significant positive relationship was observed between qPCR score and the proportion of crested newt reads within total reads per sample ($P < 0.001$) (Fig. 3a). Conversely, post-PCR eDNA concentration had a significant negative influence on the proportion of crested newt reads ($P < 0.001$), where read proportion decreased as post-PCR eDNA concentration increased (Fig. 3b).

COMPARISON OF METHOD COST AND INVESTIGATOR EFFORT

A full breakdown of expenditure per pond is given in Supporting Information (Table S8) and summarised in Fig. 4. Cost of materials, and investigator effort and salary (assuming hourly rate of £21.20) were calculated for eDNA samples but estimates do not include procedural controls, qPCR standards, or consumables and reagents required for assay optimisation. Time required to perform PCR for metabarcoding and qPCR was estimated assuming available machinery to run four PCR plates in parallel and one qPCR plate.

Discussion

We have demonstrated eDNA metabarcoding is a highly sensitive tool for monitoring crested newt alongside the wider biological community, corroborating other comparisons of eDNA metabarcoding and qPCR for single-species monitoring (Lacoursière-Roussel *et al.* 2016; Schneider *et al.* 2016). Despite marginal reduction in single-species detection, eDNA metabarcoding revealed a wealth of biodiversity information and could enable more effective freshwater monitoring networks and better understanding of community structure and ecosystem function alongside crested newt monitoring (Biggs, von Fumetti & Kelly-Quinn 2016). However, both eDNA approaches have advantages and drawbacks which must be considered for design and implementation of biodiversity monitoring programmes.

SINGLE-SPECIES DETECTION BY qPCR AND METABARCODING

A direct comparison of sensitivity between qPCR and metabarcoding is not straightforward: stochasticity in qPCR largely occurs during amplification (volume of template DNA and technical replication), whereas stochastic variation during metabarcoding arises through PCR amplification and sequencing (depth and replication) (Kelly *et al.* 2014; Thomsen *et al.* 2016; Deiner *et al.* 2017).

In our study, 12 independent qPCR replicates were performed for each sample but due to limited resources, metabarcoding was based on three pooled PCR replicates which were sequenced once only. Therefore, to enable a fair comparison between methods in terms of PCR effort, a threshold of >= 4/12 positive replicates (qPCR TA) was applied to the qPCR data. Indeed,

detection sensitivity was most similar between methods with the qPCR threshold and without the metabarcoding threshold. Both eDNA metabarcoding and qPCR displayed reduced crested newt detection when thresholds were applied; although, this may reflect reduced false positive detections rather than decreased sensitivity. Lower sensitivity of the eDNA metabarcoding approach used here may also stem from sample degradation during long-term storage. The samples used were stored for more than 12 months at -80 ℃ before metabarcoding. However, long-term storage and continual freeze-thawing of samples may allow aggregation of inhibitory substances which impair PCR amplification and cause false negatives (Takahara, Minamoto & Doi 2015).

Despite lower sensitivity, strength of eDNA signal produced by metabarcoding was correlated with that of qPCR, where both crested newt average read count and read proportion broadly increased with qPCR score of eDNA samples. The correlation was inconsistent though, where high average or proportional crested newt read count did not always correspond to high qPCR score. Biggs *et al.* (2015) also found a variable positive association between qPCR and visual counts of crested newt, where high qPCR score did not always correlate with high counts. Our results indicate sequence reads may allow relative abundance to be estimated but this must be statistically verified against established population census methods.

The comparison between qPCR and metabarcoding must also be examined in context of the sequencing effort. Here, we sequenced a large number of samples (380 including positive and negative controls) per run to provide a realistic cost scenario for routine monitoring. Yet, metabarcoding detection sensitivity would likely improve with an increase in read depth per sample (Kelly *et al.* 2014). In order to directly compare eDNA signal production by these approaches, it may be necessary to perform sequencing replicates to verify true positives where rare species are expected and generate an “eDNA metabarcoding score” system similar to qPCR

(Brandon-Mong *et al.* 2015; Port *et al.* 2016; Civade *et al.* 2016; Thomsen *et al.* 2016). PCR and sequencing replication in metabarcoding may enhance species detection probability through improved amplification of low abundance or highly degraded DNA (Ficetola *et al.* 2015; Port *et al.* 2016) that is readily amplified by qPCR (Lacoursière-Roussel *et al.* 2016). Similarly, sequencing of independent biological replicates, opposed to pseudoreplicates from a single water sample (established crested newt eDNA sampling strategy), may improve detection and minimise false negatives produced by eDNA metabarcoding (Andruszkiewicz *et al.* 2017; Bálint *et al.* 2017).

Metabarcoding assays are also susceptible to problems from taxon bias, DNA swamping and bioinformatics related problems (Taberlet *et al.* 2012; Kelly *et al.* 2014; Shaw *et al.* 2016b). Potential reduction in sensitivity of passive community sequencing versus targeted qPCR approaches may relate to the performance of metabarcoding primers for target species. During metabarcoding, DNA from rare species may be masked by highly abundant species (Schneider *et al.* 2016), or under-represented due to disproportionate eDNA shedding rates across species and preferential amplification of other species (Kelly *et al.* 2014). We found crested newt read proportion was negatively associated with post-PCR concentration of eDNA samples. As a positive relationship was not observed, this would suggest PCR amplification with our selected marker and primers was not biased toward our focal species. However, we cannot conclude that our metabarcoding assay was free of primer bias as post-PCR concentration of eDNA samples can be influenced by PCR stochasticity.

FALSE NEGATIVES

The aim of this study was not to evaluate sensitivity of molecular methods against standard crested newt survey methodologies. Egg searches were used to detect false negatives produced by qPCR and metabarcoding and in doing so, revealed some interesting results. Biggs *et al.* (2015) previously demonstrated that qPCR had higher detection rate than egg searches (as well as torchlight, netting, and bottle trapping), but here we show this also holds true for metabarcoding. Importantly, absence of eggs does not infer absence of adults, and this method is highly dependent on weather conditions and water clarity (Rees *et al.* 2014a; Biggs *et al.* 2015). Despite considerably higher detection rate of both eDNA approaches, eggs were recorded in a small number of ponds that were eDNA negative. eDNA analysis can incorrectly infer absence or low abundance of species if inhibition or interference from non-target DNA has occurred (Goldberg *et al.* 2016). Alternatively, eDNA false negatives may have been a by-product of sampling strategy and effort for crested newt. Larger water volumes and/or more biological replication instead of pseudoreplication (6 x 15 mL sampled from 600 mL for crested newt) may improve detection (Lopes *et al.* 2016; Andruszkiewicz *et al.* 2017; Bálint *et al.* 2017). All methods revealed crested newt in ponds where other approaches failed, emphasising that these species monitoring tools are complementary and should be used in combination to achieve maximum detection probability. However, integrative strategies combining molecular and conventional tools are often not cost-efficient for most applications.

FALSE POSITIVES

False positives may arise from field contamination and eDNA transport in the environment - particularly by waterfowl (Shaw *et al.* 2016a). eDNA is retained by predators, discarded in faeces, and transported by anthropogenic activity, combined with natural water currents and flow (Hänfling *et al.* 2016). In the laboratory, PCR-accumulated and sequencing error, including primer mismatch (Andersen *et al.* 2012) and 'tag jumps' (Schnell, Bohmann & Gilbert 2015), can induce misassignment leading to false positives, cross-contamination between samples, or laboratory contamination (Andruszkiewicz *et al.* 2017).

False positives can be identified using Site Occupancy Modelling of metabarcoding data (Ficetola *et al.* 2015) or risk of false positives minimised using a sequencing threshold i.e. the number of sequence reads required for a sample to be species positive (Hänfling *et al.* 2016; Civade *et al.* 2016; Evans *et al.* 2017a). However, such thresholds can reduce detection of rare species, a primary goal of this study, and may fail where false and true positives occur at similar frequency (Hänfling *et al.* 2016). Instead, we calculated species-specific sequence thresholds to more accurately control for false positives in our dataset without compromising crested newt detection.

In our study, human DNA occurred at high frequency and abundance; this may have been a true environmental signal from pond water, or real contaminant as encountered in other metabarcoding research (Port *et al.* 2016; Valentini *et al.* 2016; Thomsen *et al.* 2016). Blocking primers can prevent amplification of abundant non-target DNA like human (Valentini *et al.* 2016) but may fail (Thomsen *et al.* 2016) or prevent amplification of target taxa (Port *et al.* 2016). Alongside human, other aquatic and terrestrial vertebrate DNA occurred at high frequency in NTCs (Supporting Information: Appendix 4), although these were not removed by addition of

mineral oil.

Our results also highlight the importance and impact of qPCR thresholds when inferring species presence-absence. Similar to Smart *et al.* (2015), we found a stringent qPCR threshold reduced detection sensitivity. As yet, there is no guidance to indicate how many samples or replicates must be positive to class a site as species-positive (Goldberg *et al.* 2016; Smart *et al.* 2016) but this clearly needs to be addressed to improve standardisation and reproducibility of eDNA research. Importantly, less stringent thresholds (and false positives inherent to these) are somewhat precautionary and may enable better protection of crested newt by preventing development. Therefore, whilst reduction or removal of false positives is desirable, detection thresholds must not compromise protection of threatened species either. Until a suitable threshold can be established, it may be more appropriate to re-analyse samples which yield one positive qPCR replicate to prevent false positives (Rees *et al.* 2014b; Goldberg *et al.* 2016).

COST AND INVESTIGATOR EFFORT

Comparisons of qPCR and conventional survey for species monitoring have included cost projections (Biggs *et al.* 2014; Davy *et al.* 2015; Smart *et al.* 2016; Evans *et al.* 2017b) but cost has not been thoroughly assessed in qPCR and eDNA metabarcoding comparisons (Lacoursière-Roussel *et al.* 2016; Schneider *et al.* 2016). We found eDNA metabarcoding was more costly than qPCR but both approaches required similar investigator effort. qPCR scales to the number of samples being processed (Schneider *et al.* 2016) whereas metabarcoding has fixed costs including reagent kit for HTS platform (Bálint *et al.* 2017). eDNA metabarcoding becomes more cost-

efficient as more samples are processed (Bálint *et al.* 2017) but fewer replicates would reduce qPCR cost (Davy *et al.* 2015; Smart *et al.* 2016). Travel was excluded from our cost estimate but inclusion of this expense would further reduce cost efficiency of both approaches. Cost of eDNA monitoring is influenced by sample size, methods, replication, laboratory, statistical power and occupancy modelling (Davy *et al.* 2015; Evans *et al.* 2017b). Consequently, cost is proportional to project requirements (Davy *et al.* 2015) and will vary depending on choice of qPCR or metabarcoding workflow. Nevertheless, cost efficiency combined with the overarching aim of a monitoring or conservation programme should always be considered.

Conclusion

eDNA metabarcoding holds promise for holistic biodiversity monitoring of freshwater ponds opposed to targeted qPCR for flagship or indicator species such as crested newt. Metabarcoding can reveal entire species assemblages from environmental samples without prior ecosystem information, and provide broad-scale distribution data for multiple species simultaneously. This diagnostic power over conventional monitoring has been repeatedly demonstrated for freshwater vertebrates (Valentini *et al.* 2016; Hänfling *et al.* 2016; Shaw *et al.* 2016a; Civade *et al.* 2016; Lacoursière-Roussel *et al.* 2016). Nonetheless, the method at present appears to be less sensitive than qPCR for single-species monitoring, and species detection by molecular and conventional methods was incongruent.

Comprehensive study of the influence of water volume, eDNA capture and extraction

method, and sample storage on single-species and community detection in lentic and lotic systems is required. Currently, 90 mL water is sampled during crested newt eDNA survey, followed by ethanol precipitation and Qiagen® DNeasy Blood and Tissue kit extraction (Biggs *et al.* 2015). While this may be appropriate for highly-sensitive targeted qPCR, larger water volumes and filtration may be required to capture eDNA from less abundant vertebrates and characterise community diversity (Shaw *et al.* 2016b). Additionally, eDNA from different species can be unevenly distributed throughout water bodies and may be concentrated in particular areas (Hänfling *et al.* 2016; Evans *et al.* 2017a), thus sampling strategies must be carefully designed to ensure eDNA samples are representative of biodiversity present. Indeed, how individuals within a species are distributed in the habitat sampled can also influence detection (Biggs *et al.* 2015).

Minimising the risk of false positives and contamination remains a pressing issue in metabarcoding, and standard contamination measures (Goldberg *et al.* 2016) may be insufficient for analysis of vertebrate assemblages. An even stricter forensic laboratory set-up, such as that employed for ancient DNA (aDNA) should be adopted to ensure robustness of the data. Positive and negative controls should be included at each stage of metabarcoding workflows to monitor contamination (Deiner *et al.* 2017). However, preventive measures inevitably increase research cost and some degree of contamination is unavoidable in metabarcoding (Kelly *et al.* 2014; Brandon-Mong *et al.* 2015; Port *et al.* 2016; Thomsen *et al.* 2016).

Currently, cost and investigator effort required for metabarcoding and qPCR are broadly equivalent. Yet, where qPCR is an established technology and has reached its price ceiling, HTS is relatively new technology and prices will continue to drop. This reduction in cost means higher sample throughput and more technical replication will be possible. We therefore argue that metabarcoding will become more cost-efficient in the long-term, providing more data at lower cost

and comparable sensitivity to qPCR. However, where samples cannot be processed in large batches, qPCR may retain cost-efficiency.

We conclude that eDNA metabarcoding is not yet a replacement for targeted qPCR and conventional survey but rather another tool in the ecologist toolbox. Ultimately, choice of monitoring tool(s) is specific to aims of each conservation project. At present, qPCR retains sensitivity for crested newt populations of all sizes, regardless of sample number processed. Under a realistic conservation monitoring scenario, where funding is limited and samples must be processed in large batches, metabarcoding may suffer from false negatives due to reduced sequencing depth and replication. However, in many cases, the biodiversity information generated by this approach, and its implications for community ecology and conservation, will eclipse lower sensitivity. This passive screening approach would be most effective for initial survey of water bodies to generate broad-scale multi-species distribution data. This holistic data can then inform best use of funding and time for targeted species-specific survey.

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Author contributions

B.H., L.R.H., L.L.H and N.B. conceived and designed the study. H.C.R., K.C.G., and N.B. contributed samples for processing. H.C.R. performed extractions under licence from Natural England and qPCR for eDNA samples from private contracts at The University of Nottingham. N.B. performed qPCR for eDNA samples from Natural England's Great Crested Newt Evidence Enhancement Program. L.R.H. performed remaining laboratory work and analysed the data. I.P.A. and E.L. offered advice on and supervised sequencing. C.H. assisted with bioinformatics analysis. P.B. and S.P. contributed datasets for analysis. L.R.H. wrote the manuscript, which all authors revised.

Data accessibility

R scripts and corresponding data are deposited in the dedicated GitHub repository: https://github.com/HullUni-bioinformatics/Harper_et_al_2017.

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Table 1. Summary of analyses testing for agreement between eDNA approaches, with and without thresholds, for crested newt detection. Cohen's kappa coefficient (k) represents strength of agreement between methods (1 = 100%). Pearson's Chi-squared Test for Independence tested whether methods significantly differed for crested newt detection.

Comparison	**Probability of observed agreement**	**Probability of expected agreement**	**k**	**Overall agreement**	χ^2	**d.f.**	***P***
Metabarcoding NT qPCR NT	0.77	0.50	0.53	Moderate	25.94	1	< 0.001
Metabarcoding TA qPCR NT	0.74	0.50	0.48	Moderate	52.291	1	< 0.001
Metabarcoding NT qPCR TA	0.84	0.56	0.63	Good	0.207	1	> 0.05
Metabarcoding TA qPCR TA	0.86	0.58	0.66	Good	2.561	1	> 0.05

Table 2. Summary of analyses testing for variation in proportion of crested newt sequence reads in a sample produced by eDNA metabarcoding, attributable to qPCR score or post-PCR eDNA concentration. Test statistic is for LRT used.

Model variables	***N* (ponds)**	**d.f.**	**AIC**	**Effect size**	**Standard error**	χ^2	***F***	***P***
qPCR score	532	1	1578.3	0.373	0.032	150.682	147.117	*< 0.001*
post-PCR eDNA concentration	532	1	1441.9	-0.056	0.015	14.272	12.457	*< 0.001*

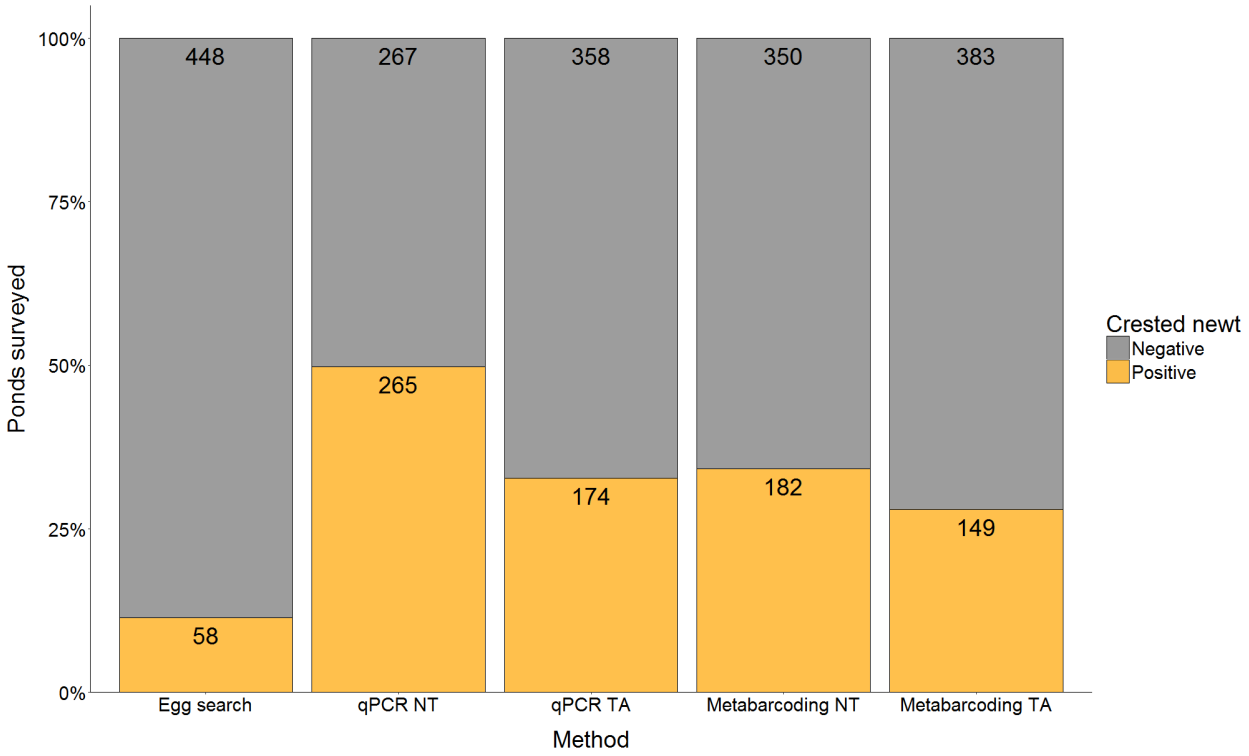

Figure 1. Comparison of survey methodology for crested newt detection in freshwater ponds across the UK. Bars represent proportion of positive and negative crested newt ponds by each method with frequency displayed on bars.

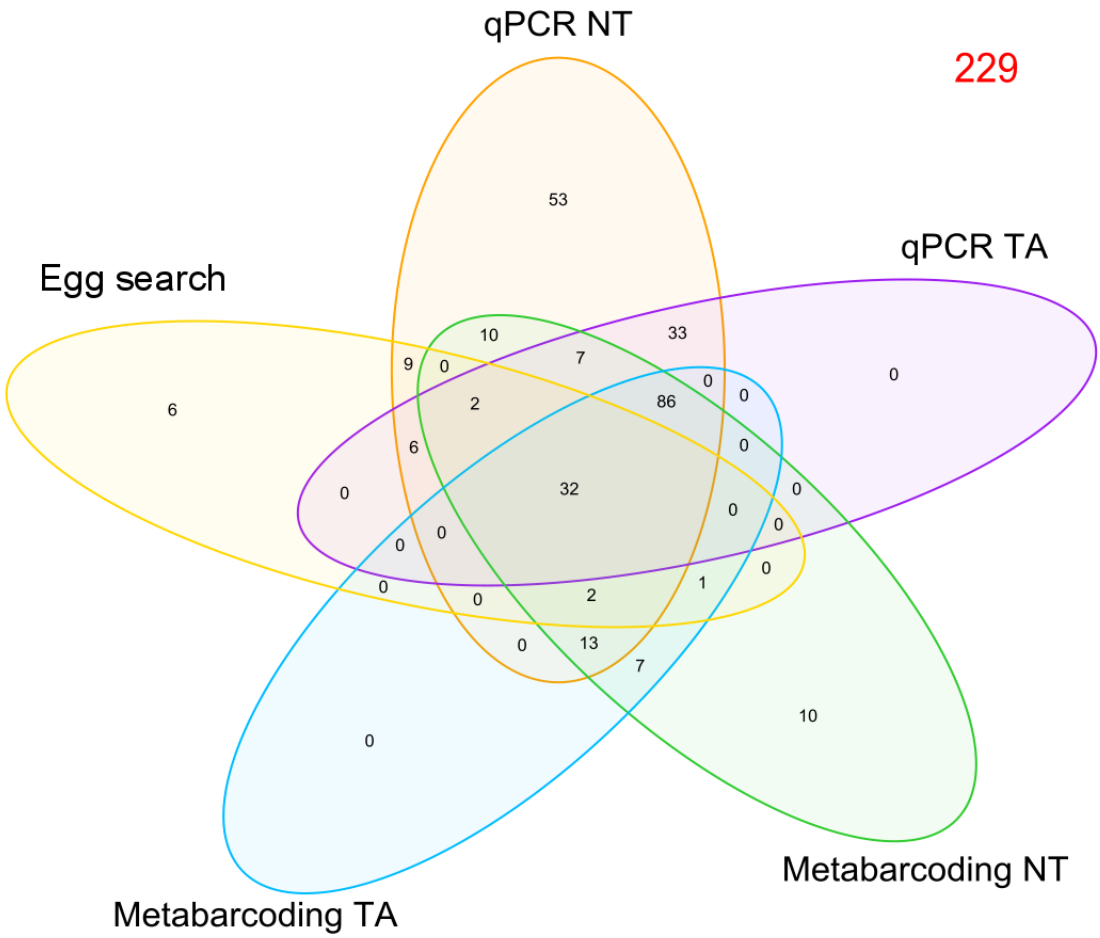

Figure 2. Venn diagram which summarises the number of positive crested newt detections (N = 277) by each method (egg search, qPCR NT, qPCR TA, metabarcoding NT, and metabarcoding TA), and overlap in crested newt detection between methods, for 506 ponds where all methods were applied. Negative crested newt detections (N = 229) are highlighted in red.

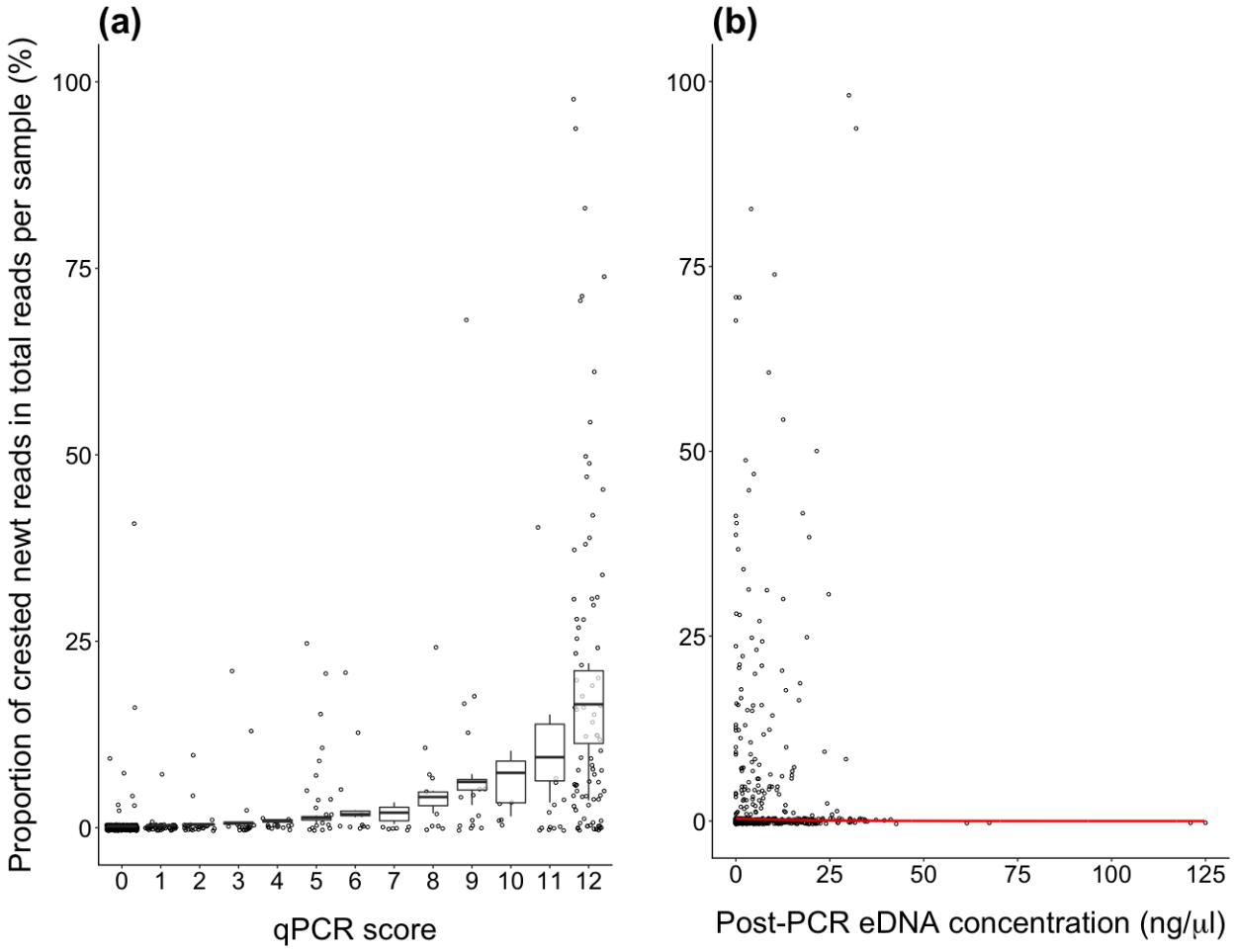

Figure 3. Relationship between fixed effects (qPCR score, post-PCR eDNA concentration) and response variable (proportion of crested newt reads) in eDNA samples, as predicted by the negative binomial GLMM. The 95% CIs, as calculated using the predicted proportions, and standard error for these predictions are given for each relationship. The observed data (points) are also displayed against the predicted relationships (boxes, line). The proportion of crested newt reads within eDNA samples increased as qPCR score increased **(a)**. Conversely, the proportion of crested newt reads decreased as post-PCR eDNA concentration increased **(b)**.

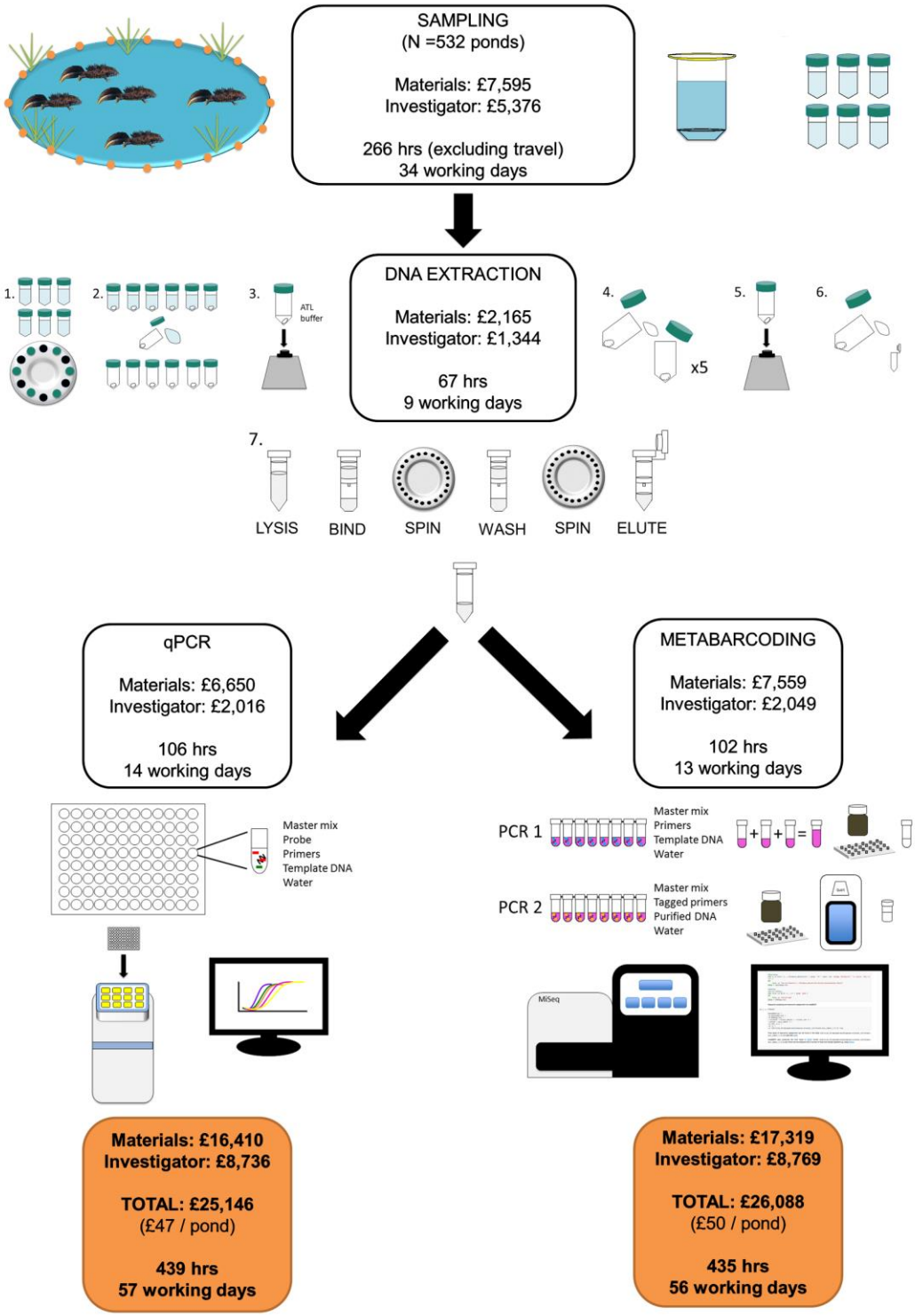

Figure 4. Cost and investigator effort required for targeted qPCR of crested newt and eDNA metabarcoding of vertebrate communities from pond water samples.