Extending sampling approaches for great crested newt (*Triturus cristatus*) eDNA monitoring

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Abstract

1. Environmental DNA (eDNA) monitoring has been used for great crested newt (*Triturus cristatus*) survey in the UK since the publication of a Defra-funded trial in 2014. If eDNA results are to be used in support of a great crested newt licence, surveys must be performed during a 76-day survey window (15 April – 30 June) to coincide with peak great crested newt activity, and must follow the approved protocol for collection of 6 x 15 mL subsamples for ethanol precipitation. However, filtration may be a more effective and sustainable method of eDNA capture than ethanol precipitation, and detection may be possible outside of the current survey window.
2. We investigated whether the great crested newt eDNA survey season could be extended and filtration could be used for great crested newt eDNA capture by reviewing the available evidence and conducting a field study from April to October 2022. Paired water samples for ethanol precipitation (600 mL collected and 90 mL processed) and filtration (2.5 L collected and up to 2.5 L processed) were collected from ponds once a month, resulting in 124 ethanol precipitation kits and 124 filtration kits from 25 ponds. All samples were analysed with the approved quantitative PCR assay for the great crested newt.
3. Our results indicate that great crested newts can be reliably detected using both eDNA capture methods from April to August, with detection rates decreasing in September and October. Great crested newt eDNA detection was comparable or higher with filtration than ethanol precipitation, thus filtration could be a reliable and more sustainable means for assessing great crested newt presence.
4. *Practical implication.* Inclusion of filtration as an accepted eDNA capture method for great crested newt eDNA surveys would allow more water to be processed for robust and reliable estimates of great crested newt presence. Extending the great crested newt eDNA survey season to August would allow more waterbodies to be surveyed for great crested newt presence (but not absence), and identification of sites that provide important habitat for great crested newts outside of the breeding season. This would also remove logistical challenges and

costs associated with completing great crested newt surveys and laboratory analysis within 10 weeks, improving performance of surveyors and laboratories as well as quality of results. Furthermore, great crested newt eDNA surveys could be carried out alongside monitoring for other species, which would enable infrastructure projects to develop more effective mitigation measures as well as reduce time required from surveyors and survey costs.

Keywords: amphibian, environmental DNA, ethanol precipitation, filtration, freshwater, northern crested newt, ponds

1. Introduction

Great crested newts (*Triturus cristatus*) are a European protected species, meaning individuals, their eggs, their breeding sites, and their resting places are protected by law. In the UK, licences must be obtained for activities affecting great crested newts, including development and mitigation, from the appropriate statutory authority, e.g. Natural England, NatureScot or Natural Resources Wales. Where records suggest great crested newts may be present, there is suitable aquatic habitat, or suitable terrestrial habitat at or in the vicinity of a proposed development site, surveys are required to assess species distribution and abundance in order to take appropriate measures to avoid, mitigate or compensate for any negative effects on great crested newts. Traditionally, great crested newts were monitored using methods such as torchlight counts, bottle trapping, netting and egg searches to confirm presence or likely absence in waterbodies (Langton et al., 2001). Since 2015, environmental DNA (eDNA) analysis has offered a non-invasive, time-efficient and cost-effective alternative (Biggs et al., 2015; Rees et al., 2014), but within a regulated 76-day survey window (15 April – 30 June) to coincide with peak breeding activity and maximise chance of detection (Biggs et al., 2014).

Previous UK studies have shown that great crested newts can be successfully detected with eDNA analysis outside this regulated survey window (Buxton et al., 2017, 2018a; Gorman et al., 2020; Rees et al., 2017), possibly due to juveniles remaining beyond the breeding season, and juveniles and adults overwintering in waterbodies (Rees et al., 2023). Across Europe where the same regulations do not apply, eDNA surveys for great crested newts have also occurred over summer months (Knudsen et al., 2023; Strand et al., 2022). Extension of this survey window would allow more potential great crested newt waterbodies to be surveyed for robust and reliable estimates of great crested newt presence. It would also allow great crested newt eDNA surveys to be carried out alongside monitoring for other species, such as smooth newt (*Lissotriton vulgaris*), chytrid fungus (*Batrachochytrium dendrobaditis*; Taugbøl et al., 2021, 2025), and white-clawed crayfish (*Austropotamobius pallipes*; Troth et al., 2020), or amphibian assemblages (Charvoz et al., 2021; Davison et al., 2025; Dufresnes et al., 2019; Strand et al., 2022; Svenningsen et al., 2022) or whole vertebrate communities (L. R. Harper et al., 2018). This would enable infrastructure projects to develop more effective mitigation measures as well as reduce survey time and costs. However, additional evidence is needed to support extension of the great crested newt eDNA survey window and regulatory change (Rees et al., 2017, 2023).

Technical guidance published by Biggs et al. (2014) and approved by Natural England underpins the widely adopted protocol (WC1067) for great crested newt eDNA surveys in the UK. This guidance was based on a project conducted in 2013/14 that established the performance of and produced a technical advice note on eDNA techniques for the great crested newt. In accordance with this guidance, all great crested newt eDNA sampling kits produced by different commercial eDNA providers capture eDNA via ethanol precipitation (hereafter EP). This method purifies and concentrates DNA by adding salt and ethanol to water samples, which forces the precipitation of DNA out of the solution. The DNA can then be separated from the rest of the solution by centrifugation. EP is restricted to small water volumes (e.g. 90 mL), susceptible to contamination during laboratory processing, and expensive due to taxes, dangerous goods shipping and specialist waste disposal required for ethanol, especially for small

numbers of kits. EP kits also potentially present a risk to ecologists and any others who have to store and transport a large number of kits (Bruce et al., 2021; Rees et al., 2023).

Since 2014, eDNA applications have been continually evolving and multiple studies have identified that filtration may provide more efficient species detection than EP (e.g. Hinlo et al., 2017; Minamoto et al., 2015; Peixoto et al., 2020; Spens et al., 2016; Troth et al., 2020), although the optimal capture method can depend on environment sampled and inhibitor concentrations (Minamoto et al., 2015; Troth et al., 2020). Filtration separates DNA from water using a filter medium (e.g. paper, gauze) that allows the solution to pass through but not the DNA. Filters can process litres of water for higher detection rates, albeit less water may be processed and/or processing may take longer for turbid water samples (Andreou et al., 2023; Minamoto et al., 2015). Unlike EP samples, filters are self-contained and quicker to extract for lower contamination risk and reduced labour costs. There is minimal risk to ecologists and any others who have to store and transport them, meaning standard couriers can be used for shipping. They can also be stored at ambient temperature before and after (if a preservative solution is added to the filter) use, making storage of collected samples easier and less expensive (Bruce et al., 2021; Rees et al., 2023). In contrast, EP kits must be stored at 2-4°C if they cannot be delivered to a laboratory on the same day as sample collection (Biggs et al., 2014).

Here, we reviewed the evidence for extending the great crested newt eDNA survey season and using filtration for great crested newt eDNA capture. We performed a field comparison of in-season (April to June) versus out-of-season (July to October) eDNA survey, and EP versus filtration for great crested newt eDNA detection. We hypothesised that great crested newts would be detected beyond 30 June, and that filtration would outperform EP for great crested newt eDNA detection. We provide evidence-based recommendations for regulatory changes to evolve and streamline great crested newt eDNA surveys.

2. Materials and Methods

2.1. Literature review

A literature review was undertaken in June 2022 to synthesise published and grey literature on using filtration for great crested newt eDNA capture and great crested newt eDNA detection out-of-season. Published literature was searched with Google Scholar using the search terms “environmental DNA”, “eDNA”, “ethanol precipitation”, “filtration”, “qPCR”, “great crested newt”, “*Triturus cristatus*”, “GCN”, and “amphibians”. Literature was restricted to those referencing great crested newts as well as incorporating filtration and/or out-of-season eDNA surveys. Grey literature was sought by contacting a range of regulatory agencies, non-governmental organisations, and ecological consultancies. Nineteen organisations were contacted, including five regulatory agencies, three non-governmental organisations, and 11 ecological consultancies. Of these, 12 responded, including three which had relevant data but only two provided. Cited literature within studies returned by searches, including previous literature reviews and reports, was also accessed and assessed for relevance. Information extracted from each source was summarised in Microsoft Excel (Supporting Information: Appendix 1).

2.2. Field study

Ponds across three sites in Buckinghamshire, England, were selected based on results of previous surveys conducted on behalf of HS2 Ltd within the last four years but dating back to 2013 for some ponds (Table 1, Figure 1). Surveys had not been undertaken for two ponds included in the present study. Most ponds were created as part of ecological mitigation works and were receptor sites for great crested newt translocation. Ponds were located near Newton Purcell (Ponds 1-4), Great Missenden (Ponds 5-8), and Aylesbury (Ponds 9-25). The Newton Purcell and Great Missenden sites were over 35 km apart, with the Aylesbury site in between (over 20 km from Newton Purcell site and

over 15 km from Great Missenden site). Therefore, great crested newt dispersal between the three sites is highly unlikely. However, most ponds within each site were less than 500 m apart and within similar proximity to other ponds not included in this study, therefore great crested newts at each site could move between ponds in response to environmental and social cues (Unglaub, Cayuela et al., 2021).

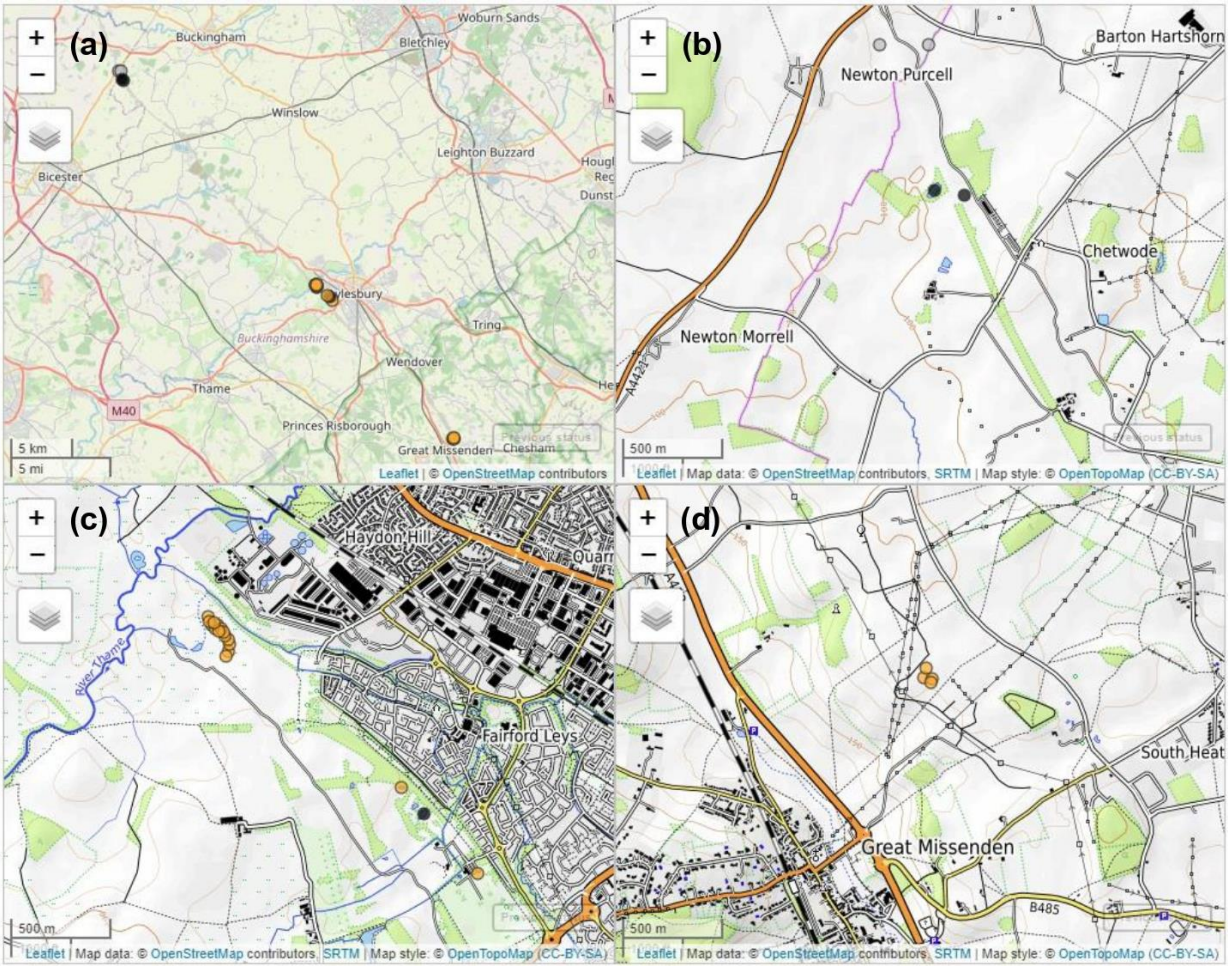


Figure 1. Maps of study ponds in Buckinghamshire. All ponds are shown relative to the wider landscape (a), then by clusters near Newton Purcell (b), Aylesbury (c), and Great Missenden (d). Points are coloured by previous great crested newt status, where orange is positive, black is negative, and grey is unknown.

199 **Table 1.** Characteristics of study ponds based on the ten suitability indices (SI) that are used to calculate the Habitat
200 Suitability Index (HSI) for great crested newts (Oldham et al., 2000), and define pond suitability (ARG-UK, 2010). SI1:
201 Geographic location (Zone A = optimal, Zone B = marginal, Zone C = unsuitable). SI2: Pond area
202 (rectangle/ellipse/irregular – m^2). SI3: Permanence (never dries/rarely dries/sometimes dries/dries annually). SI4: Water
203 quality (good/moderate/poor/bad). SI5: Shade (percentage of margin shaded 1 m from bank). SI6: Waterfowl
204 (absent/minor/major). SI7: Fish (absent/possible/minor/major). SI8: Pond count (number of ponds within 1 km). SI9:
205 Terrestrial habitat (good/moderate/poor/none). SI10: Macrophytes (percentage of the pond surface area occupied by
206 macrophyte cover).
207

Pond	Site	Great crested newt status prior to 2022	SI 1	SI2	SI3	SI4	SI 5	SI6	SI7	SI8	SI9	SI10	HSI score	Pond suitability	Population size assessment
1 *†	Newton Purcell	Unknown	A	Irregular – 400	Sometimes dries	Poor	0	Minor	Absent	13	Moderate	0	0.42	Average	None
2 *†	Newton Purcell	Unknown	A	Irregular – 300	Dries annually	Poor	0	Minor	Absent	13	Moderate	0	0.35	Below average	None
3 *	Newton Purcell	Negative	A	Irregular – 15	Dries annually	Moderate	0	Absent	Absent	13	Moderate	10	0.3	Poor	None
4 *	Newton Purcell	Negative	A	Irregular – 2770	Never dries	Moderate	70	Minor	Major	13	Moderate	15	0.46	Poor	None
5	Great Missenden	Positive	A	Irregular – 120	Rarely dries	Good	0	Minor	Absent	3 (within exclusion fencing)	Good	30	0.76	Good	Bottle trapping, torching, refuge checks
6	Great Missenden	Positive	A	Irregular – 230	Rarely dries	Good	0	Minor	Absent	3 (within exclusion fencing)	Good	60	0.84	Excellent	Bottle trapping, torching, refuge checks
7	Great Missenden	Positive	A	Irregular – 80	Rarely dries	Good	0	Minor	Absent	3 (within exclusion fencing)	Good	40	0.74	Good	Bottle trapping, torching, refuge checks
8 §	Great Missenden	Positive	A	Irregular – 20	Sometimes dries	Good	0	Minor	Absent	3 (within exclusion fencing)	Good	60	0.62	Average	Bottle trapping, torching, refuge checks

9 §	Aylesbury	Positive	A	Irregular – 35	Dries annually	Good	0	Minor	Absent	13	Good	30	0.56	Below average	Torching, refuge checks, netting
10	Aylesbury	Positive	A	Irregular – 180	Never dries	Good	0	Minor	Absent	13	Good	40	0.83	Excellent	Torching, refuge checks, netting
11	Aylesbury	Positive	A	Irregular – 120	Rarely dries	Good	0	Minor	Absent	13	Good	50	0.82	Excellent	Torching, refuge checks, netting
12	Aylesbury	Positive	A	Irregular - 200	Never dries	good	0	minor	absent	13	Good	50	0.85	Excellent	Torching, refuge checks, netting
13	Aylesbury	Positive	A	Irregular – 50	Sometimes dries	Good	0	Minor	Absent	13	Good	30	0.68	Average	Refuge checks, netting
14	Aylesbury	Positive	A	Irregular – 80	Rarely dries	Good	0	Minor	Absent	13	Good	40	0.77	Good	Torching, refuge checks, netting
15	Aylesbury	Positive	A	Irregular – 60	Rarely dries	Good	0	Minor	Absent	13	Good	30	0.74	Good	Torching, refuge checks, netting
16	Aylesbury	Positive	A	Irregular – 100	Rarely dries	Good	0	Minor	Absent	13	Good	25	0.77	Good	Refuge checks, netting
17 †§	Aylesbury	Positive	A	Irregular – 10	Dries annually	Good	0	Minor	Absent	13	Good	100	0.5	Below average	Torching, refuge checks, netting (assessed before pond dried in May)
18 †‖	Aylesbury	Positive	A	Irregular – 150	Sometimes dries	Poor	50	Minor	Possible	1 (only pond not separated by roads or fencing)	Good	60	0.62	Average	None
19 †‖	Aylesbury	Positive	A	Irregular – 2	Dries annually	Good	0	Minor	Absent	13	Good	0	0.39	Poor	None
20	Aylesbury	Positive	A	Irregular – 50	Rarely dries	Good	0	Minor	Absent	13	Good	50	0.75	Good	Torching, refuge checks, netting
21 †§	Aylesbury	Positive	A	Irregular – 20	Sometimes dries	Good	0	Minor	Absent	13	Good	50	0.64	Average	Torching, refuge

															checks, netting
22 §	Aylesbury	Positive	A	Irregular – 50	Rarely dries	Good	0	Minor	Absent	13	Good	25	0.72	Good	Torching, refuge checks, netting
23 †‖	Aylesbury	Positive	A	Irregular – 25	Sometimes dries	Good	0	Minor	Absent	13	Good	50	0.65	Average	None
24 *‡	Aylesbury	Negative	A	Irregular – 750	Never dries	Moderate	20	Minor	Possible	13	Good	30	0.83	Excellent	None
25 ‡¶	Aylesbury	Positive	A	Irregular – 150	Rarely dries	Good	0	Minor	Possible	13	Good	70	0.82	Excellent	Refuge checks, netting (out-of-season only)

* Control pond where great crested newt absence was confirmed or suspected and no population size assessment performed.

† Pond with in-season eDNA survey only.

‡ Pond with out-of-season eDNA survey only.

§ Pond subject to in-season population size assessment only as no eDNA survey able to be undertaken in October.

‖ Pond dried out before in-season population size assessment could be performed.

¶ Pond subject to out-of-season population size assessment only as no in-season eDNA survey.

Initially, 21 ponds were sampled in April 2022 to ensure 17 positive ponds and three negative ponds (i.e. control ponds) for great crested newts. From May onwards, 20 ponds were selected for repeat surveys. Ponds from the same three sites were added to replace any that dried and/or became unsafe to access, but ultimately the total number of ponds decreased each month (Supporting Information: Appendix 2).Each pond was sampled by AtkinsRéalis ecologists once each month using both NatureMetrics Great Crested Newt eDNA Kits (EP) and Pump Aquatic eDNA Kits (filtration; Figure 2).

Figure 2. AtkinsRéalis ecologists **(a)** collecting water for filtration and **(b)** processing water using an EP kit.

The latter included a sterile 0.8 µm pore size polyethersulfone filter and a 5 µm glass fibre pre-filter inside a 50 mm polypropylene capsule with luer lock fitting connections. Water was filtered and the filter air-dried using a Bürkle Vampire Sampler (a peristaltic pump with a flow rate of up to 2 L per minute) and a piece of silicone hose (1 m) with luer lock adapter. The hose was placed inside the sampling bag, filled with sample

236 water, then the filter capsule was attached to the adapter before continuing to run the
237 pump. Once all water had been filtered or the filter clogged, the hose was removed from
238 the sampling bag and pump run until no more water exited the filter. The filter capsule
239 was detached from the hose, following which the capsule was filled with 1.5 mL of
240 Longmire's solution for DNA preservation (Longmire et al., 1997). A new kit and a new
241 piece of silicone hose (1 m) with luer lock adapter were used for each pond. Field
242 blanks (one of each kit type using mineral water instead of pond water) were included
243 from May onwards to assess potential field contamination risk.
244
245 The water sampling protocol established by Biggs et al. (2014) was used for both eDNA
246 capture methods with minor modifications for filtration. For EP, 20 x 30 mL subsamples
247 were collected at equidistant intervals around the pond perimeter then pooled into a
248 single 900 mL sampling bag for homogenisation, following which 6 x 15 mL aliquots
249 were added to tubes containing 33 mL of absolute ethanol and 1.5 mL of sodium
250 acetate (3 M). For filtration, 20 x 125 mL subsamples were collected at equidistant
251 intervals around the pond perimeter and pooled into a single 3.5 L sampling bag for
252 homogenisation, following which as much water as possible was filtered. For EP, 600
253 mL of water was always collected, and 90 mL invariably processed. For filtration, 2.5 L
254 of water was always collected, with up to 2.5 L filtered but volumes varied for each pond
255 in each month (Supporting Information: Appendix 2, Figure S3). Water sampling for EP
256 or filtration took the same amount of time (20-30 minutes), but water processing was
257 typically faster with the EP kit (~10 minutes) than the filtration kit (~20 minutes) due to
258 the greater volumes of water being processed with filtration (Supporting Information:
259 Appendix 2, Figure S3). EP samples were stored at ambient temperature for same-day
260 courier collection or refrigerated until courier collection. Filtration samples were stored at
261 ambient temperature.
262
263 Where possible, population size class assessment surveys were performed for each
264 pond that was positive for great crested newt prior to 2022 (i.e. generally based on
265 surveys within the last four years for most ponds but occasionally based on surveys
266 dating back to 2013 for some ponds). These were based on six visits from May to June

267 and one visit in October using different combinations of bottle trapping, torchlight
268 survey, refuge searching and netting. Bottle trapping could not be used where protected
269 mammal species were present and torchlight could not be used for ponds with
270 extensive vegetation cover (Table 1). Further details on each of these survey methods
271 can be found in Langton et al. (2001). Pond 25 was subject to an out-of-season
272 population survey only as this pond was included from July onwards as a replacement
273 for an eDNA positive pond which became dry. Ponds 8, 9, 17, 21 and 22 were subject
274 to an in-season population survey only as these ponds had dried out and/or become
275 unsafe to access by October. Ponds 1-4 and 24 were not assessed as these were
276 control ponds known or suspected to be negative for great crested newt, and Ponds 18,
277 19 and 23 were not assessed as they dried before population survey could occur.
278

279 ## 2.3. Laboratory analysis

280

281 At NatureMetrics, eDNA was precipitated via centrifugation at 14,000 x g for EP kits and
282 processed in accordance with Biggs et al. (2014). Briefly, resulting pellets from EP kits
283 and lysate from filtration kits were extracted using Qiagen DNeasy Blood and Tissue
284 kits following the manufacturer protocol, with elution in 200 µL of Buffer AE. All samples
285 were tested for degradation and inhibition using two commercial quantitative PCR
286 (qPCR) kits that target two separate synthetic Internal Positive Controls (IPC). One IPC
287 was included in the ethanol and sodium acetate of EP kits or the preservative solution of
288 filtration kits to test for degradation, and one IPC was added to DNA extracts to test for
289 inhibition. qPCR amplification for each IPC was carried out in duplicate on each sample.
290 qPCR amplification was then carried out in 12 technical replicates per sample using
291 species-specific primers and probe for the great crested newt, developed by Thomsen
292 et al. (2012) and adopted by Biggs et al. (2014), in the presence of extraction negative
293 controls, qPCR positive controls, and qPCR negative controls. The great crested newt
294 qPCR reagents (and their concentrations and volumes) and conditions were as
295 specified in the WC1067 protocol (Biggs et al., 2014), with 3 µL of template DNA used
296 in each reaction. A score is given for the number of positive technical replicates out of
297 12, i.e. eDNA score. A sample is deemed positive for great crested newts if the eDNA

score is 1 or higher. Results from the great crested newt qPCR assay are considered to have a high confidence rating according to the Confidence Assessment Tool for eDNA qPCR Results (K. J. Harper et al., 2021). Quality control methods included the use of field blanks, kit blanks, extraction negative and qPCR negative controls, and qPCR positive controls. The qPCR positive controls are standards of known great crested newt DNA concentration amplified in triplicate to determine correct amplifications.

2.4. Data analysis

Data were analysed in R v4.2.2 (R Core Team, 2022), and all plots were produced using the packages ggplot2 v3.5.1 (Wickham, 2016), ggpubr v0.6.0 (Kassambara, 2020), and patchwork v1.3.0 (Pedersen, 2024). Control ponds (ponds 1-4 and 24) and negative process controls were assessed for any evidence of contamination, then these were removed from the data set.

2.4.1. Generalised Linear Mixed-effect Models

Great crested newt detection success (both detection rate and eDNA scores) with EP and filtration in each month was statistically compared for Ponds 5-23 and 25. Next, for ponds with both in-season (April to June) and out-of-season (July to October) data (Ponds 5-16, 20 and 22), months were grouped as in-season or out-of-season to statistically compare detection success with EP and filtration between these two time periods. We examined the influence that September and October results had on in-season vs. out-of-season detection by repeating the statistical comparison without these data, i.e. only July and August results grouped as out-of-season (see Supporting Information for results of in-season vs. out-of-season comparisons).

Generalised Linear Mixed-effects Models (GLMMs) with binomial error family were used for these analyses. eDNA scores were converted to proportional eDNA scores (i.e. ranging from 0 to 1) for modelling. These GLMMs included an interaction term between eDNA capture method and month or time period, but the interaction term was dropped if

329 it was not significant. eDNA capture method and month or time period were retained as
330 fixed effects. We also examined the influence of volume of water filtered on proportional
331 eDNA scores using a GLMM with binomial error family (see Supporting Information).
332
333 In-season population sizes for Ponds 5-17 and 20-22 were available as quantitative
334 (peak adult counts) or qualitative (class) measurements. Classes were 'small' (up to 10
335 animals counted), 'medium' (11–100 animals counted) and 'high' (counts exceeding 100
336 animals) (English Nature, 2001). The population size and eDNA data for these ponds
337 were split by eDNA capture method. The EP and filtration subdatasets were each
338 analysed using three GLMMs with binomial error family, where both population size
339 measurements were included as fixed effects. The three GLMMs had different response
340 variables: (1) corresponding eDNA score, i.e. proportional eDNA score corresponding to
341 May when most in-season survey visits for population size assessment were made; (2)
342 average eDNA score, i.e. the average of proportional eDNA scores for in-season
343 months; and (3) peak eDNA score, i.e. the highest proportional eDNA score observed
344 across in-season months.
345
346 Pond number was included as a random effect in all GLMMs to account for repeat
347 visitation and unknown variation inherent to individual ponds. GLMMs, model
348 performance checks, extraction of model predictions, and post hoc analyses were
349 implemented using the packages glmmTMB v1.1.10 (Brooks et al., 2017), DHARMa
350 v0.4.7 (Hartig, 2022), easystats v0.7.3 (Lüdecke et al., 2022), arm v1.14-4 (Gelman &
351 Su, 2022), emmeans v1.10.5 (Lenth, 2022), and effects v4.2-2 (Fox & Weisberg, 2018;
352 Fox & Weisberg, 2019). If a significant effect was found for variables using the *drop1*
353 function from the package stats v4.2.2 (R Core Team, 2022), multiple pairwise
354 comparisons were performed using the *pairs* function from the package emmeans
355 v1.10.5. The full data set has been provided as Supporting Information: Appendix 2.
356

357 **2.4.2. Occupancy models**

358

359 A potential flaw with GLMMs is they do not account for imperfect detection via a
360 hierarchical dependence structure like multi-scale occupancy models do. Like other
361 monitoring methods, eDNA surveys do not always detect a species when it is present,
362 i.e. false negative. The difference with eDNA surveys is false negatives can occur at
363 different stages: species may not release enough DNA into the environment, eDNA may
364 not be captured during sample collection, and/or eDNA may not be amplified during
365 laboratory analysis (Dorazio & Erickson, 2018). Standard occupancy models assume no
366 false positives, i.e. species is absent but recorded as present. This can occur with other
367 survey methods where a surveyor mistakenly identifies a species based on visual or
368 audio cues, but may be more pronounced with eDNA surveys where a species' eDNA
369 can be transported to sites where the species is not present by surveyors or other
370 wildlife, or contamination can occur in the laboratory (Griffin et al., 2020).

371

372 There are now several multi-scale occupancy models available for eDNA data, which
373 account for false negative (and occasionally false positive) error in the field and the
374 laboratory. However, each has advantages and disadvantages, including the type of
375 error that can be estimated, the ability to specify different covariates at different levels,
376 and the ability to account for repeat visits to the same sites using multiple survey
377 methods. We applied three of these models to our data to examine false negative and
378 false positive error rates across months and with different eDNA capture methods.

379

380 The Griffin et al. (2020) model, implemented via the package eDNAShinyApp v1.0
381 (Diana et al., 2021), enables false positive and false negative error rates to be
382 estimated for eDNA surveys. This allows specification of covariates which may influence
383 site occupancy and detection probability, but different covariates cannot be specified at
384 different levels. In our case, we would expect eDNA capture method and month to
385 influence eDNA detection probability in water samples (Stage 1, θ), but we would only
386 expect eDNA capture method to influence eDNA detection probability in qPCR
387 replicates (Stage 2, p) due to differences in DNA extraction. The Dorazio and Erickson

(2018) model, implemented with a Gibbs sampler via the package msocc v1.1.0 for computational efficiency (Stratton et al., 2020), does allow different covariates to be specified at different levels, but only provides estimates of false negative error. Neither of these models account for repeat visits to the same site in the same way as a GLMM with a random effect does. Instead, each visit is treated as a unique site. Multi-method models (Nichols et al., 2008), implemented via the package RPresence v2.15.19 (MacKenzie & Hines, 2025), account for non-independence between sampling methods at shared, local scale sampling locations (Nichols et al., 2008). However, these implement a different hierarchical dependence structure (where Ψ is occupancy at landscape scale, θ is local-scale occupancy, and p is probability of detection) and only estimate false negative error rate.

Specification of each model is described in the Supporting Information. We present the results of these models but note that none are particularly well-suited to our experimental design, i.e. multiple eDNA sampling methods used on repeat visits to the same sites.

3. Results

3.1. Literature review

Seven studies were identified in the published (four studies) and grey literature (three studies) that used filtration for eDNA capture and/or out-of-season eDNA survey for great crested newt detection. Only two studies used filtration for eDNA capture with the remainder using EP, and up to 1 L of water was processed by those that used filtration. Across these two studies, filtration recovered more great crested newt eDNA than EP in a tank experiment but not in natural ponds where eDNA concentration was similar between methods (Buxton et al., 2017; 2018b). The remaining studies demonstrated that out-of-season detection was possible, including a 2019 data set supplied by

418 AtkinsRéalis where every pond surveyed was positive for great crested newt eDNA in
419 September (Gorman et al., 2020). There were differences in sampling methodology
420 across all studies, but the eDNA capture conditions for EP and filtration, the DNA
421 extraction method, and the amplification method were consistent (see Supporting
422 Information: Appendix 1).
423

424 3.2. Field study

425

426 3.2.1. Quality control

427

428 Results of quality control checks are summarised in Figure 3 and Supporting
429 Information: Appendix 2. Of five control ponds (Ponds 1-4 and 24) known or suspected
430 to be negative for great crested newts based on previous surveys, three ponds were
431 also negative in 2022, but two ponds produced positive results from filters in June (Pond
432 4, eDNA score = 1) and October (Pond 24, eDNA score = 2) respectively. All samples
433 passed tests for degradation and inhibition except four filters in May (Pond 22),
434 September (Ponds 3 and 4) and October (Pond 24) which were inhibited despite
435 troubleshooting undertaken to resolve inhibition. Samples are analysed for great crested
436 newt regardless of inhibition and degradation being identified. Results from the two
437 filters for Ponds 22 and 24 were returned as positive rather than inconclusive because
438 great crested newt DNA amplified successfully, and therefore these samples were
439 retained. However, results for the two filters from Ponds 3 and 4 were returned as
440 inconclusive rather than negative because great crested newt DNA did not amplify
441 which could be due to inhibition or absence of great crested newt DNA in the sample.
442 One field blank (EP kit) also produced an inconclusive result in October because it
443 failed the inhibition test. All other negative process controls were negative for great
444 crested newt DNA and passed quality control.
445

3.2.2. Sample size

In total, 25 ponds were sampled from April to October (Table 1, Figure 3). Ponds 1 and 2 were newly created drainage ponds and produced negative eDNA results in 2022. Pond 3 was not previously found to support great crested newts and also produced negative eDNA results in 2022. Ponds 4 and 24 were negative for great crested newts until 2022 when they produced positive eDNA results. The remaining 20 ponds that were previously found to support great crested newts also produced positive eDNA results in 2022 (Figure 3; Supporting Information: Appendix 2).

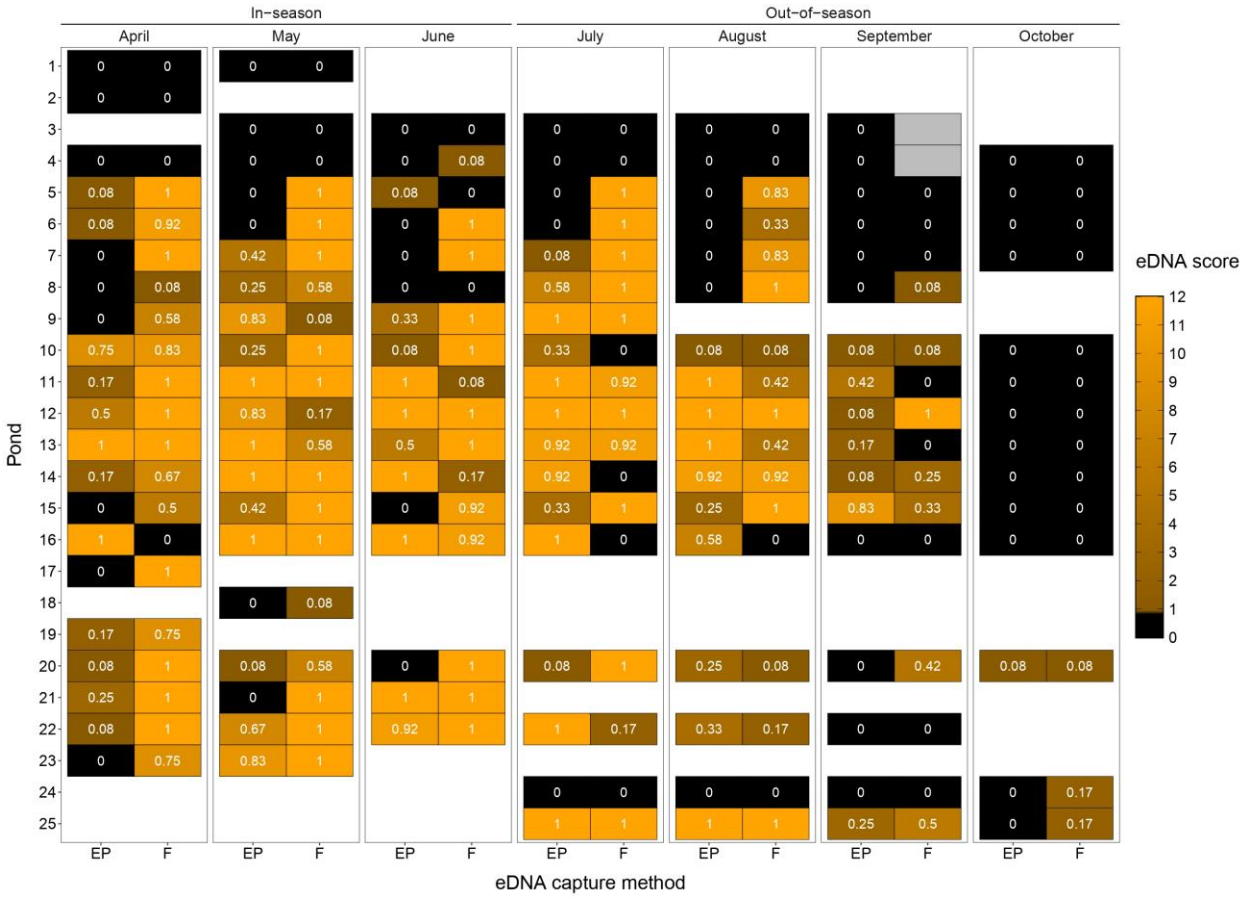
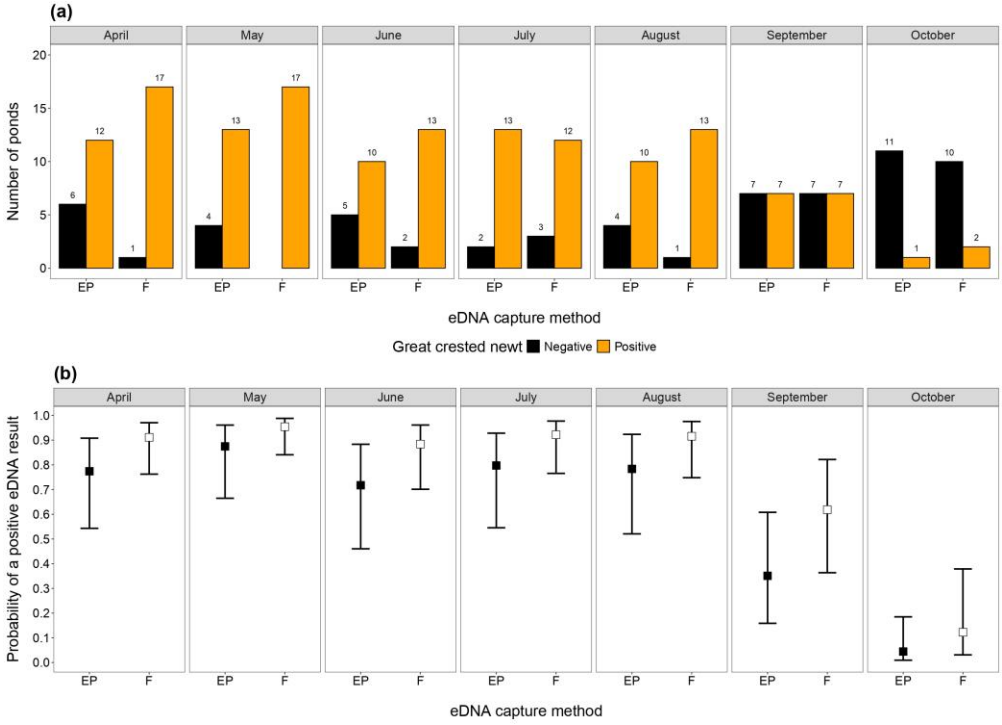


Figure 3. Great crested newt status of ponds surveyed from April to October using ethanol precipitation (EP) kits and filtration (F) kits. A pond is classed as positive for positive for great crested newts if one or more of the qPCR replicates performed on the

461 eDNA sample are positive, i.e. eDNA score >= 1. Cells are shaded according to kit
462 result (black = negative, orange = positive, grey = inconclusive), with positive results on
463 a colour gradient according to eDNA score. White cells represent months where a pond
464 was not surveyed due to drying out and/or health and safety concerns. Numbers in cells
465 indicate proportional eDNA scores used for modelling.
466
467 Of the 25 ponds sampled that produced positive and negative results over the course of
468 the study, 16 had in-season and out-of-season eDNA data, while seven had in-season
469 eDNA data only (typically spanning 2 months) and two had out-of-season eDNA data
470 only. Two ponds with both in-season and out-of-season eDNA data were control ponds.
471 This left 14 ponds that were previously positive for great crested newts using
472 conventional and eDNA surveys that also produced a positive eDNA result for at least
473 one in-season month in 2022. All 14 ponds were surveyed in July, decreasing to 13
474 ponds in August and September, and 11 ponds in October due to ponds drying out
475 and/or health and safety issues (Figure 3).
476
477 All control ponds and laboratory controls were excluded from downstream analysis. All
478 remaining ponds (Ponds 5-23 and 25, n = 20) were all used in statistical comparisons of
479 eDNA detection success in each month (sections 3.2.3 and 3.2.5). Great crested newt
480 ponds with both in-season and out-of-season eDNA data (Ponds 5-16, 20 and 22, n =
481 14) were used in statistical comparisons of eDNA detection success in-season vs. out-
482 of-season (Supporting Information). Great crested newt ponds with in-season eDNA
483 and population data (Ponds 5-17 and 20-22, n = 16) were used in statistical analysis of
484 eDNA score in relation to population size (section 3.2.4).
485
486 **3.2.3. eDNA capture method and month**
487
488 The number of positive ponds generated by each eDNA capture method in each month
489 ranged from 1 to 17 (Figure 4a). The effect of eDNA capture method did not depend on
490 month and vice versa as the interaction term was not significant (X^2_6 = 9.101, P =
491 0.168), but the individual effects of eDNA capture method (X^2_1 = 8.030, P = 0.005) and

492 month (X^2_6 = 61.959, P < 0.001) were significant (Table 2). Filtration (mean probability
493 of a positive eDNA result ± SE = 0.823 ± 0.057) consistently produced more positive
494 ponds than EP (0.608 ± 0.083) overall (z = -2.727, P = 0.006). May (0.924 ± 0.045) had
495 the highest number of positive ponds and significantly differed to September (0.484 ±
496 0.124, z = 3.382, P = 0.013) and October (0.075 ± 0.052, z = 5.039, P < 0.001) but not
497 other months (April and June to August: z = -0.969-1.353, P > 0.05). October had the
498 fewest positive ponds and significantly differed to all other months (April to August: z =
499 4.628-5.039, P < 0.001), although the difference between September and October was
500 on the cusp of significance (z = 2.981, P = 0.045; Figure 4b).
501



502
503

504 **Figure 4.** The effects of eDNA capture method and month on GCN detection. In **(a)**,
505 bars show the number of ponds that were positive (orange) and negative (black) for
506 great crested newt each month with EP and filtration (F) kits. The number of ponds
507 corresponding to each category are displayed above bars. In **(b)**, square points
508 represent the model-predicted positive eDNA result probabilities with EP (black) and F
509 (white) in each month, and error bars show the 95% confidence intervals for these
510 predictions.

511 **Table 2.** Summary of GLMM and post hoc analysis performed to examine the influence of eDNA capture method and
512 month (as individual and interacting effects) on the GCN status of ponds. Significant p-values (<0.05) are in bold and df
513 denotes degrees of freedom.
514

GLMM					Post hoc analysis				
Formula	**Variable**	**Effect**			**Levels**	**Mean probability ± SE**	**Pairwise comparison**		
		X^2	**df**	**P**			**Pair**	**z**	**P**
GCN status ~ eDNA capture method + month + eDNA capture method : month	eDNA capture method : month	9.101	6	0.168	NA	NA	NA	NA	NA
	eDNA capture method	8.030	1	**0.005**	EP	0.608 ± 0.083	EP : F	-2.727	**0.006**
					F	0.823 ± 0.057			
	month	61.959	6	**<0.001**	Apr	0.856 ± 0.066	Apr : May	-0.969	0.961
							Apr : Jun	0.449	0.999
							Apr : Jul	-0.197	1.000
							Apr : Aug	-0.079	1.000
							Apr : Sep	2.783	0.079
							Apr : Oct	4.710	**<0.001**

GLMM					Post hoc analysis				
Formula	**Variable**	**Effect**			**Levels**	**Mean probability ± SE**	**Pairwise comparison**		
		X^2	**df**	**P**			**Pair**	**z**	**P**
					May	0.924 ± 0.045	May : Jun	1.353	0.826
							May : Jul	0.729	0.991
							May : Aug	0.829	0.982
							May : Sep	3.382	**0.013**
							May : Oct	5.039	**<0.001**
					Jun	0.815 ± 0.082	Jun : Jul	-0.615	0.996
							Jun : Aug	-0.493	0.999
							Jun : Sep	2.343	0.224
							Jun : Oct	4.427	**<0.001**
					Jul	0.872 ± 0.067	Jul : Aug	0.110	1.000
							Jul : Sep	2.821	0.071

GLMM					Post hoc analysis				
Formula	**Variable**	**Effect**			**Levels**	**Mean probability ± SE**	**Pairwise comparison**		
		X^2	**df**	**P**			**Pair**	**z**	**P**
							Jul : Oct	4.727	**<0.001**
					Aug	0.863 ± 0.072	Aug : Sep	2.691	0.101
							Aug : Oct	4.628	**<0.001**
					Sep	0.484 ± 0.124	Sep : Oct	2.981	**0.045**
					Oct	0.075 ± 0.052			

515

516 The eDNA score produced by each eDNA capture method in each month ranged from 0
517 to 12 (proportional eDNA score = 0-1; Figure 5a). The effect of eDNA capture method
518 did not depend on month and vice versa as the interaction term was not significant (X^2_6
519 = 4.888, P = 0.558), but the individual effects of eDNA capture method (X^2_1 = 12.903, P
520 < 0.001) and month (X^2_6 = 52.380, P < 0.001) were significant (Table 3). Filtration
521 (mean proportional eDNA score ± SE = 0.492 ± 0.085) produced higher eDNA scores
522 than EP (0.233 ± 0.063) overall (z = -3.474, P < 0.001; Figure 5b). The highest eDNA
523 scores were observed in July (0.691 ± 0.090) and lowest eDNA scores observed in
524 September (0.143 ± 0.067) and October (0.011 ± 0.019). Scores from April to August
525 did not significantly differ (April to August: z = -1.384-1.277, P > 0.05). Scores in May
526 (0.654 ± 0.088), June (0.613 ± 0.096) and July significantly differed to scores in
527 September (May: z = 3.638, P = 0.005; June: z = 3.343, P = 0.015; July: z = 3.801, P =
528 0.003), but not October (Table 3; Figure 5c).

529 **Table 3.** Summary of GLMM and post hoc analysis performed to examine the influence of eDNA capture methods and
530 month (as individual and interacting effects) on the proportional eDNA score of ponds. Significant p-values (<0.05) are in
531 bold and df denotes degrees of freedom.
532

GLMM					Post hoc analysis				
Formula	**Variable**	**Effect**			**Levels**	**Mean proportional eDNA score ± SE**	**Pairwise comparison**		
		X^2	**df**	**P**			**Pair**	**z**	**P**
Proportional eDNA score ~ eDNA capture method + month + eDNA capture method : month	eDNA capture method : month	4.888	6	0.558	NA	NA	NA	NA	NA
	eDNA capture method	12.903	1	**<0.001**	EP	0.233 ± 0.063	EP : F	-3.474	**<0.001**
					F	0.492 ± 0.085			
	month	52.380	6	**<0.001**	Apr	0.514 ± 0.090	Apr : May	-1.122	0.922
							Apr : Jun	-0.760	0.989
							Apr : Jul	-1.384	0.811
							Apr : Aug	-0.034	1.000
							Apr : Sep	2.861	0.064

GLMM					Post hoc analysis				
Formula	**Variable**	**Effect**			**Levels**	**Mean proportional eDNA score ± SE**	**Pairwise comparison**		
		X^2	**df**	**P**			**Pair**	**z**	**P**
							Apr : Oct	2.535	0.147
					May	0.654 ± 0.088	May : Jun	0.328	1.000
							May : Jul	-0.307	1.000
							May : Aug	1.017	0.950
							May : Sep	3.638	**0.005**
							May : Oct	2.841	0.068
					Jun	0.613 ± 0.096	Jun : Jul	-0.615	0.996
							Jun : Aug	0.682	0.994
							Jun : Sep	3.343	**0.015**
							Jun : Oct	2.741	0.088
					Jul	0.691 ± 0.090	Jul : Aug	1.277	0.863

GLMM					Post hoc analysis				
Formula	**Variable**	**Effect**			**Levels**	**Mean proportional eDNA score ± SE**	**Pairwise comparison**		
		X^2	**df**	**P**			**Pair**	**z**	**P**
							Jul : Sep	3.801	**0.003**
							Jul : Oct	2.926	0.053
					Aug	0.519 ± 0.102	Aug : Sep	2.788	0.078
							Aug : Oct	2.533	0.147
					Sep	0.143 ± 0.067	Sep : Oct	1.483	0.755
					Oct	0.011 ± 0.019			

533

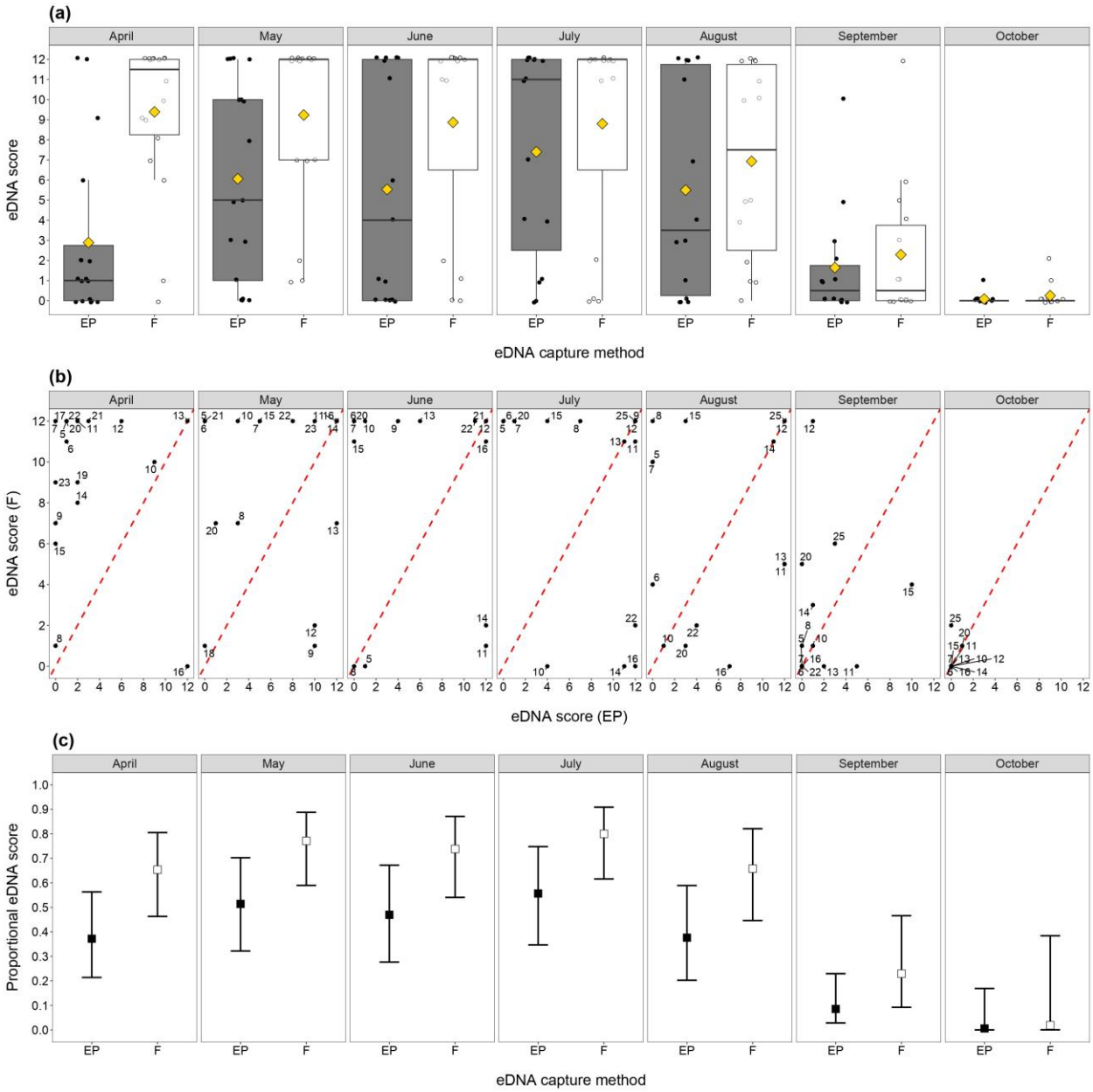


Figure 5. The effects of eDNA capture method and month on eDNA score. In **(a)**, the spread of eDNA scores with EP and filtration (F) kits in each month is represented by boxes which show the 25th, 50th and 75th percentiles, whiskers which show the 5th and 95th percentiles, and thick horizontal lines which show the median. Circular points (EP = black, F = white) represent the eDNA score for individual ponds, and yellow diamonds represent the mean eDNA score for EP and F. In **(b)**, eDNA scores with EP are plotted against eDNA scores with F for each pond in each month. The red dashed line

543 represents the same eDNA score being produced by both eDNA capture methods;
544 points to the left of this line are ponds where F produced a higher eDNA eDNA score
545 and points to the right of this line are ponds where EP produced a higher eDNA score.
546 Numbers represent the number allocated to each pond for the present study (see Table
547 1). In **(c)**, the square points represent the model-predicted proportional eDNA scores
548 with EP (black) and F (white) in each month, and error bars show the 95% confidence
549 intervals for these predictions.
550
551 **3.2.4. Population size**
552
553 No great crested newts were found during population size class assessment surveys
554 conducted in October, but three ponds produced a positive eDNA result. Pond 20 was
555 positive using both filtration and EP kits, whereas Ponds 24 and 25 were only positive
556 using filtration kits. Consequently, no analysis of population size in relation to eDNA
557 score out-of-season was undertaken.
558
559 Sixteen ponds (Ponds 5-17 and 20-22) had in-season population size data (peak adult
560 counts and classes) that could be used to analyse population size in relation to eDNA
561 score. The remaining nine ponds were unsuitable because: a) they were control ponds,
562 b) population size class assessment surveys were not undertaken as ponds were
563 removed from the study due to drying out and/or health and safety reasons, or c) they
564 were replacements added to the study from July onwards and only had out-of-season
565 data.
566
567 There was no influence of peak adult counts or population class on corresponding
568 eDNA score, average eDNA score, or peak eDNA score with EP or filtration (see
569 Supporting Information: Table S3, Figure S4). Therefore, adult population size did not
570 appear to influence the amount of great crested newt eDNA captured with either eDNA
571 capture method.
572

573 **3.2.5. Occupancy modelling**

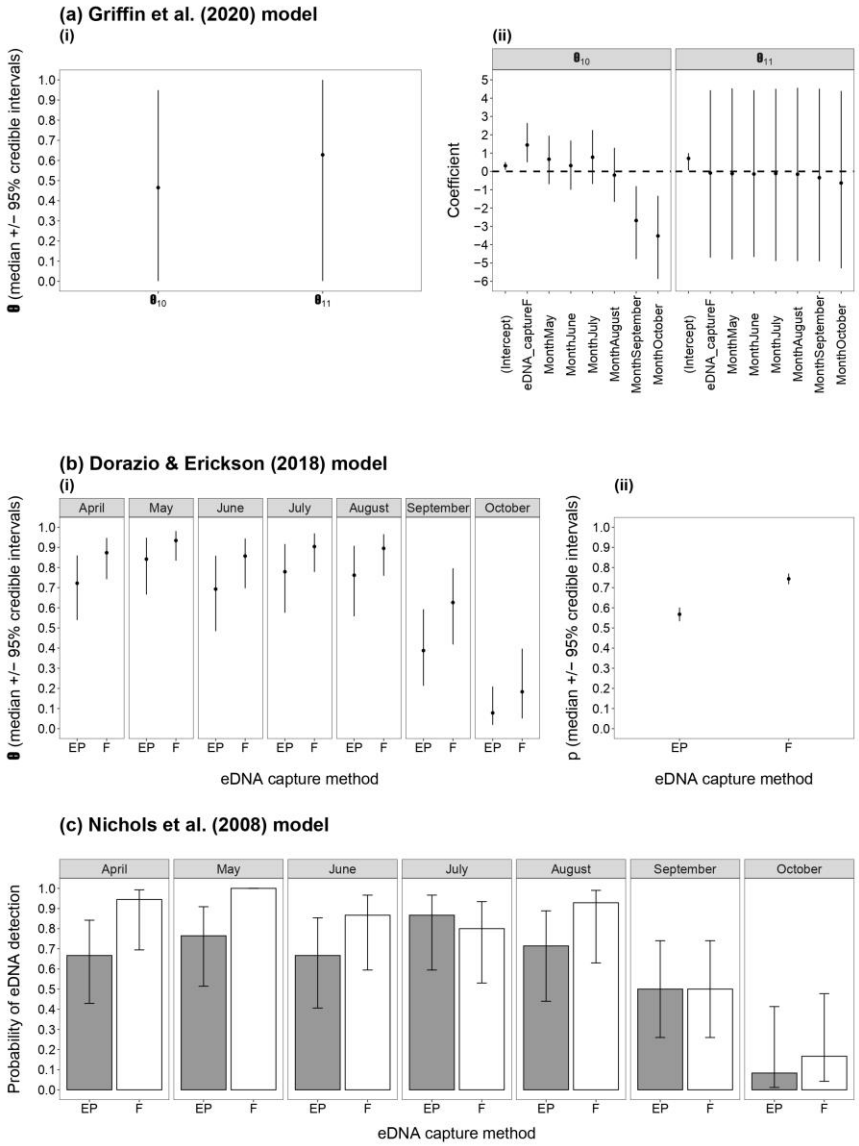
574

575 With the Griffin et al. (2020) model implemented in the eDNAShinyApp package, the
576 posterior mean occupancy for all sites was 0.078 with posterior credible intervals (PCI)
577 ranging from 0.002 to 0.763. At Stage 1 (sampling), the true positive rate (mean
578 posterior probability of θ_{11}) was high, ranging from 0.556-0.711 (mean = 0.637; median
579 = 0.628; PCI = 0.002-0.999954). The false positive rate (mean posterior probability of
580 θ_{10}) was moderate, ranging from 0.022-0.781 (mean = 0.426; median = 0.465; PCI =
581 0.001-0.949) (Figure 6ai). At Stage 2 (laboratory analysis), the true positive rate (mean
582 posterior probability of p_{11}) was very high at 0.936 (PCI = 0.918-0.952). The false
583 positive rate (mean posterior probability of p_{11}) was very low at 0.097 (PCI = 0.082-
584 0.118). False negative probability is calculated as 1—true positive: this was lower than
585 the false positive rate at both Stage 1 (1 – 0.637 = 0.363), and Stage 2 (1 – 0.936 =
586 0.064). Both eDNA capture method and month were found to have Posterior Inclusion
587 Probability (PIP) scores of 1 for Stage 1 true positive and false positive rates,
588 suggesting an influence on probability of great crested newt eDNA capture in water
589 samples. The probability of true positives at Stage 1 was highest in July, followed by
590 May then June. Filtration also produced a higher probability of true positives. However,
591 these patterns were the same for false positives (Figure 6aii).

592

593 With the Dorazio & Erickson (2018) model implemented in the msocc package, the
594 model with eDNA capture method and month as sample-level covariates (but no
595 interaction between these), and eDNA capture method as a replicate-level covariate
596 performed best. The probability of eDNA capture in a water sample (θ) was higher with
597 filtration each month (median values: April = 0.874, May = 0.934, June = 0.856, July =
598 0.902, August = 0.895, September = 0.627, October = 0.182) than with EP (median
599 values: April = 0.723, May = 0.845, June = 0.695, July = 0.778, August = 0.762,
600 September = 0.390, October = 0.078), albeit with overlapping CI. The probability of
601 eDNA capture in a water sample was highest in May then July and August with either
602 EP or filtration, and lowest in October (Figure 6bi). Detection probability in a qPCR

603 replicate (*p*) was higher with filtration (0.744) than EP (0.568), with no overlapping CI
604 (Figure 6bii)
605
606 With the Nichols et al. (2008) model implemented in the RPresence package, the model
607 included eDNA capture method and month as covariates for detection (*p*). Filtration had
608 a higher predicted probability of detection than EP in most months except July.
609 Probability of detection was highest in May, followed by April then August with filtration,
610 or July followed by May then August with EP. Probability of detection was lowest in
611 October with either eDNA capture method (Figure 6c).
612



613

614

615 **Figure 6.** Outputs of three different multi-scale occupancy models for levels at which
616 covariates were considered. For the Griffin et al. (2020) model **(a)**, the θ_{11} (true positive
617 error rate) and θ_{10} (false positive error rate) estimates are shown with posterior credible
618 intervals **(i)** alongside the Stage 1 coefficients and posterior credible intervals for each
619 level of covariates where PIP values were greater than 0.5 **(ii)**. For the Dorazio &
620 Erickson (2018) model **(b)**, the MCMC estimates of the parameters θ as a function of
621 eDNA capture method (EP or filtration – F) and month **(i)**, and *p* as a function of eDNA
622 capture method **(ii)** are shown, summarised as the median and 95% credible intervals
623 of estimated values. For the Nichols et al. (2018) model **(c)**, the parameter estimates for
624 *p* with EP and filtration (F) in each month are shown with 95% credible intervals.

625

626 The results of all three multi-scale occupancy models are consistent with the GLMM
627 analysis, where filtration improved the probability of eDNA capture and subsequently
628 great crested newt detection, with similar detection probabilities from April to August.
629 However, the Griffin et al. (2020) model does indicate some potential for false positives.

630

631

632 # 4. Discussion

633

634 ## 4.1. Quality control

635

636 Filtration for two control ponds produced unexpected positive eDNA results. Pond 24 is
637 adjacent to a pond (~50 m apart) not sampled in this study where great crested newt
638 presence was confirmed in 2020, thus this positive detection may have resulted from
639 great crested newts migrating from the adjacent pond in combination with higher
640 sensitivity of filtration due to larger volumes of water processed (Rees et al., 2023).
641 Pond 4 is located on a site where all ponds were likely or suspected to be negative for
642 great crested newts and produced negative results either side of the positive result in
643 June. Field and laboratory contamination is unlikely as all negative process controls

644 were negative for great crested newt DNA. Possible explanations for sporadic eDNA
645 detection include great crested newt adults or sub-adults temporarily using ponds for
646 foraging or aquatic refuge (Priol et al., 2023), or eDNA transport by humans, domestic
647 animals or other wildlife, e.g. great crested newt eDNA in water trapped in waterfowl
648 feathers or predated great crested newts in waterfowl faeces (Beng & Corlett, 2020). At
649 each of the three sites, it is not possible to rule out great crested newt dispersal from
650 unsampled ponds in the wider landscape. It is also not possible to eliminate hydrological
651 movement at the Aylesbury site where ponds are in closer proximity to each other and a
652 stream is present (Figure 1c).
653
654 One field blank (EP kit) failed the inhibition test. Reasons for this are unknown but could
655 include chemicals or ions present in the mineral water or contamination with an inhibitor
656 present in the field (Schrader et al., 2012). However, given that all other negative
657 process controls were negative for great crested newt DNA, great crested newt DNA
658 was unlikely to be present in this field blank either.
659

660 ## 4.2. Great crested newt eDNA survey season

661
662 Both the desk and field studies indicated that great crested newts can be detected in
663 spring as well as summer, likely due to the presence of adults and larvae (Buxton et al.,
664 2017; Rees et al., 2017), with reduced detection from September onwards. Therefore,
665 the survey window could potentially be doubled (c. 15 April – 31 August instead of 15
666 April – 30 June), which would facilitate land access and relieve logistical pressures for
667 ecological consultants, commercial eDNA providers, and developers (Rees et al., 2017).
668
669 The great crested newt eDNA assay has undergone substantial validation, thus a
670 positive eDNA result means that great crested newts are very likely present and a
671 negative eDNA result means great crested newts are likely absent assuming
672 *appropriate timing* and replication in sampling (Thalinger et al., 2021). Ponds that were
673 positive with either EP or filtration in July and August in the present study are likely to be
674 true positives as most eDNA scores were high (Figure 3). Extending the great crested

newt eDNA survey season could enable identification of ponds used for breeding and occupied by larvae as well as ponds only used for foraging or refuge that are critical to great crested newt survival but may be missed by the current eDNA survey window (Buxton et al., 2021b; Priol et al., 2023). Out-of-season detections could be used to begin planning conservation or mitigation activities (Gorman et al., 2020), including which waterbodies should be subject to in-season survey the following year (Rees et al., 2023). However, out-of-season non-detections require more scrutiny and cautious interpretation (Buxton et al., 2021b).

Although positive eDNA results outside the sampling timeframe originally used in assay validation are plausibly reliable, negative eDNA results are not as the survey timing is no longer optimal for DNA release from the target species (Thalinger et al., 2021; Troth et al., 2021). False negatives were observed for three ponds out-of-season where great crested newts were detected in other months that preceded or followed a month of non-detection with either filtration or EP. Yet, this anomaly was also observed across in-season months where great crested newts went undetected in 10 ponds in one month but were detected in preceding or following months with either EP or filtration. This highlights the influence of eDNA capture method and population dynamics on great crested newt eDNA detection. Great crested newts exist as spatially structured populations with many interconnected sub-populations within the wider landscape. Individuals are capable of frequent short-distance (<400 m) dispersal and occasionally long-distance (>1 km) dispersal between ponds. Some ponds are ‘sources’ (i.e. core breeding ponds) whereas others ‘sinks’ (i.e. occupied but not primarily used for breeding), resulting in a patchy population or metapopulation structure depending on the direction of dispersal. Sources and sinks can change over time due to environmental conditions (Griffiths et al., 2010; Unglaub, Cayuela et al., 2021).

As the highest number of positive ponds with either eDNA capture method was observed in May, the peak month of the core breeding season, regulators need to consider what acceptable levels of uncertainty and error are if eDNA surveys are undertaken in different months of the breeding season or outside the breeding season.

706 Waterbodies occupied by breeding great crested newt adults, eggs or larvae may
707 produce false negatives if they support fewer individuals or were not sampled when
708 breeding activity peaked (both of which increase the rate of eDNA release and amount
709 of eDNA present), they were not utilised by great crested newts in a given year, all eggs
710 and larvae were predated, or the pond temporarily dried then refilled. In our study, two
711 ponds that dried by July or August were occupied by great crested newts from April to
712 June or July, and four ponds were occupied in April and/or May before drying. More
713 ponds are likely to experience temporary drying before refilling in winter with declines in
714 summer precipitation and increases in summer temperature and winter precipitation
715 (Griffiths et al., 2010). Therefore, variable use of ponds is a key consideration for out-of-
716 season survey.
717
718 Waterbodies occupied by non-breeding great crested newt adults in spring only may not
719 be identified by eDNA surveys in July and August, resulting in false negatives (Buxton
720 et al., 2017, 2021b). Similarly, waterbodies without larvae and increasingly fewer great
721 crested newt adults as they disperse during out-of-season months, or waterbodies that
722 are sporadically used by great crested newt adults and sub-adults for foraging and
723 refuge in out-of-season months, may produce false negatives if great crested newts are
724 not present at densities that would produce sufficient amounts of eDNA for detection
725 (Buxton et al., 2021b; Priol et al., 2023). Cruickshank et al. (2021) found the number of
726 non-breeding amphibian populations in Switzerland was substantially higher than
727 breeding populations, and non-breeding populations had lower detection probabilities
728 than breeding populations due to lower abundance. Extinction risk of non-breeding
729 populations may be lowered through habitat management thereby increasing breeding
730 probability. Therefore, identification of non-breeding populations is key in promoting the
731 favourable conservation status of a species.
732
733 Out-of-season great crested newt survey is unlikely to be appropriate for determining
734 species absence and is an important caveat attached to regulatory acceptance of out-
735 of-season eDNA survey. Interpretation of non-detections out-of-season as great crested
736 newts being absent poses a potential risk to conservation or mitigation under UK and

European legislation (Buxton et al., 2021a, b). To account for this risk, waterbodies that produce a negative result out-of-season should also be resurveyed the following spring to confirm whether great crested newts are indeed present or absent.

Nonetheless, false negatives can be encountered with any monitoring tool (Buxton et al., 2021a, 2022) as evidenced by non-detection with torching, bottle trapping or refuge searches at three ponds in October which produced positive eDNA results albeit with low eDNA scores. These detections highlight the detection sensitivity of eDNA analysis for detecting ponds with low numbers of great crested newts, possibly facilitated by processing more water (Rees et al., 2023). Other studies have also shown eDNA detection via single species or metabarcoding assays outperformed conventional survey methods for amphibians (Moss et al., 2022; Wikston et al., 2023), including great crested newts (Biggs et al., 2014, 2015; L. R. Harper et al., 2018; Priol et al., 2023; Rees et al., 2014).

Ideally, multiple survey methods and repeated sampling events should be used to minimise false negatives, including eDNA and conventional surveys where time and resources allow (Moss et al., 2022; Rees et al., 2014; Wikston et al., 2023). False negatives could also be minimised by increasing biological or field sample replication (Buxton et al., 2021a, 2022; L. R. Harper et al., 2018). False positive detections, as potentially indicated by a low eDNA score (Buxton et al., 2022), could also be verified by reanalysing samples (Rees et al., 2014), increasing the eDNA score threshold for classing a sample as positive (Buxton et al., 2021a), or applying statistical models that account for false negative and false positive error rate (Buxton et al., 2021a, 2021b, 2022; Griffin et al., 2020). The Griffin et al. (2020) model indicated a moderate portion of detections in our study could be false positives originating from field sample collection, whether due to contamination from surveyors, other wildlife or hydrological connectivity, yet no field negative controls returned unexpected results (see section 4.1). The eDNAShinyApp package was not well-equipped to handle our experimental design (i.e. repeat visits to the same ponds), and we had a small number of sites, so it is possible that the false positive rate observed is inflated. The development of a multi-scale

768 occupancy model with a hierarchical dependence structure that can handle repeat visits
769 using multiple methods for eDNA data would enable more accurate estimation of false
770 positive and false negative error rates. In the interim, applying the Griffin et al. (2020)
771 model to similar data sets involving unique sites may improve understanding of false
772 positive and false negative error rates for different eDNA capture methods and survey
773 dates. However, all of the measures described to handle false positives would require
774 further modification to the approved great crested newt eDNA protocol (Biggs et al.,
775 2014).
776
777 Critically, surveys could not be conducted in February and March 2022 due to the
778 timeframe for funding proposal submission, review, and acceptance. However, great
779 crested newt adults are known to start returning to waterbodies in these months and
780 even begin breeding in February and March (Buxton et al., 2017; Rees et al., 2017),
781 especially in colder climates (L. R. Harper et al., 2019). Compared to in-season months,
782 Rees et al. (2017) observed lower eDNA scores in February but similar eDNA scores in
783 March. Additional work is needed to confirm whether survey in these months produces
784 detection rates comparable with April to August or lower detection rates like those seen
785 in September and October in the present study and previous studies (Buxton et al.,
786 2018a, b; Rees et al., 2017). Ideally, this would be contracted by regulators in a large-
787 scale, multi-year evaluation of great crested newt eDNA detection in-season versus out-
788 of-season (February to October), encompassing waterbodies of known great crested
789 newt status and variable eDNA scores throughout the UK which span the full spectrum
790 of physicochemical variability seen in these systems (Rees et al., 2017, 2023).
791 However, local-scale evaluations (either independent or as part of regulatory
792 monitoring) will also generate vital evidence that will further improve understanding of
793 out-of-season great crested newt eDNA detection and allow regulators to make
794 informed decisions.
795

796 ## 4.3. eDNA capture method

797

798 EP and filtration were comparable in terms of eDNA yield and eDNA score in the
799 published studies that have compared both eDNA capture methods for great crested
800 newt DNA to date (Buxton et al., 2017, 2018b; Rees et al., 2024). In the present field
801 study, we found that filtration outperformed EP, both in terms of number of positive
802 ponds and eDNA score, echoing other published work where filtration outperformed EP
803 for targeted eDNA analysis (e.g. Hinlo et al., 2017; Minamoto et al., 2015; Peixoto et al.,
804 2020; Spens et al., 2016; Troth et al., 2020).

805

806 Importantly, there were key differences between our study and previous works. Buxton
807 et al. (2017, 2018b) surveyed eight small ponds (600 L, 1 m by 2 m and up to 0.6 m
808 deep) within a small plot of land owned by the University of Kent. A single 1 L surface
809 water sample was taken from the centre of each pond, thus the field protocol differed to
810 Biggs et al. (2014). Glass microfibre (0.7 µm) filters were used and frozen until DNA
811 extraction. More recently, Rees et al. (2024) analysed polyvinylidene difluoride (0.45
812 µm) filters from 88 natural ponds across Cheshire, Yorkshire and Norfolk, with filtered
813 volumes ranging from 25 mL to a maximum of 500 mL. An ethanol-based preservative
814 solution was added to filters for storage at ambient temperature for up to 8 weeks prior
815 to DNA extraction. In our study, ponds ranged in surface area from 2 to 2,770 m^2 (mean
816 = 238.68 m^2, median = 80 m^2; Table 1), and the field protocol from Biggs et al. (2014)
817 was adapted to accommodate larger subsamples and greater total sample volume.
818 Polyethersulfone (0.8 µm) filters with glass fibre pre-filters (5 µm) were used and
819 Longmire's solution added for storage at ambient temperature for up to 1 week prior to
820 DNA extraction. Therefore, successful filtration and greater detection in the present field
821 study may be due to pond characteristics, sampling strategy, volume of water collected
822 and filtered, filter material and pore size, inclusion of a pre-filter, use of a peristaltic
823 pump, and/or preservation strategy.

824

825 Nonetheless, EP and filtration were inconsistent as standalone methods across all
826 months and produced incongruent results to each other for individual months. Often,

827 great crested newts were detected with one eDNA capture method but not the other
828 across several months, or great crested newts were detected in one month but not the
829 next month with a single eDNA capture method. Each eDNA capture method produced
830 different results to the other method within a given month for 18 ponds. Filtration
831 produced incongruent results in eight ponds across several months. EP produced
832 incompatible results in 10 ponds across several months. During in-season months, 12
833 ponds that were positive for great crested newt DNA with filtration tested negative with
834 EP, representing 12 potential failures to protect great crested newts under current
835 legislation due to filtration not being accepted by regulators in line with best practice for
836 eDNA analysis. This contrasts with two ponds that tested positive with EP and negative
837 with filtration. During out-of-season months, four ponds that were positive with filtration
838 tested negative with EP, and one pond that was positive with EP tested negative with
839 filtration. Inconsistent eDNA results using EP and filtration at the same ponds were also
840 observed by Rees et al. (2024). Similar to in-season versus out-of-season detection,
841 inconsistent detection between eDNA capture methods introduces some uncertainty
842 and risk of false negatives with either eDNA capture method. This could be mitigated by
843 using both eDNA capture methods at ponds being surveyed, but this would inherently
844 increase survey time and cost. A compromise may be to resurvey ponds that are
845 negative with EP kits using filtration kits.
846
847 The advantages of filtration for field surveyors and laboratories as well as evidenced
848 efficiency indicate that regulators should consider acceptance of filtration methods for
849 non-regulated application at local scales. However, persistent barriers to uptake may be
850 the high variability in filters (e.g. material, diameter, pore size, surface area) available to
851 buy from scientific suppliers (Rees et al., 2023) as well as the strategies to preserve
852 captured DNA (e.g. freezing, silica beads, preservation buffers) and storage conditions
853 (e.g. temperature, duration), which can all influence the quality and quantity of eDNA
854 extracted from samples and subsequently species detection (Hinlo et al., 2017; Jeunen
855 et al., 2019; Minamoto et al., 2015; Peixoto et al. 2020; Spens et al., 2016). Concerns
856 have also been expressed over effort required for manual filtration using syringes, filter
857 clogging, negative eDNA results obtained with EP in previous surveys being

858 undermined, a mismatch in detection sensitivity between filtration and ecologists
859 performing population surveys, value of monitoring small, non-breeding populations,
860 and implications for downstream analysis including proficiency and degradation testing
861 (Rees et al., 2023, 2024). In particular, increased time and physical effort required for
862 manual filtration using syringes (Andreou et al., 2023) may reduce the number of ponds
863 (or sites) that can be surveyed in a single day and subsequently increase survey costs.
864 In the present study, filtration time was ~20 minutes compared to ~10 minutes for EP,
865 but using a peristaltic pump which may not always be logistically or financially feasible
866 (Buxton et al., 2018b). Syringe filtration assisted by a silicone gun may be a suitable
867 compromise to remove the need for a pump and increase volume filtered but reduce
868 surveyor time and discomfort (Andreou et al., 2023). A standardised volume of water to
869 process with filtration will also be necessary to ensure consistency and comparability for
870 regulatory monitoring whilst maintaining the same level of detection sensitivity (Andreou
871 et al., 2023; Buxton et al., 2018b).
872
873 More broadly, changing methods could have implications for the national great crested
874 newt eDNA data set. Changing protocols in long-term monitoring schemes, particularly
875 statutory or regulatory monitoring, is challenging due to the need for consistency and
876 comparability of resulting data and potential for introducing bias. Indeed, this is a
877 challenge frequently encountered when end users decide to incorporate eDNA survey
878 into a monitoring scheme that has traditionally used other methods. Calibration is
879 needed to assess and remove variability, such as that undertaken for acceptance of
880 eDNA survey for great crested newt (Biggs et al., 2014, 2015) and determining the
881 European Union Water Framework Directive for the lake fish biological element using
882 eDNA metabarcoding (Sellers et al., 2024) in the UK. Alternatively, modelling is needed
883 to assess changes in detection sensitivity resulting from methodological change, as
884 applied to bighead carp and Asian carp eDNA monitoring in the United States (Song et
885 al., 2019). Similar considerations are required for any changes to laboratory processing
886 of great crested newt eDNA samples (Rees et al., 2023).
887

Additional evidence and a cost-benefit analysis of different filtration approaches (e.g. manual, pump-driven, on-site, off-site) versus EP is needed to overcome some of the aforementioned barriers and to determine the optimal filtration method for regulated application at national scale (Rees et al., 2023, 2024). To generate that evidence, two approaches may be considered. First, experiments, similar to the present study, are carried out that encompass waterbodies of known great crested newt status throughout the UK which span the full spectrum of physicochemical variability seen in these systems. The results would provide definitive answers for nationwide application. Alternatively, regulatory acceptance of results from projects similar to this study would allow local/regional great crested newt eDNA surveys to be conducted using filtration. This would require these projects to survey 10-20 ponds that capture the spectrum of physicochemical variability seen in the locality or region. With either approach, the filtration conditions (e.g. on-site versus off-site, manual versus pump, pore size, material etc.) should be taken into account. It would be beneficial for any future validation experiments to incorporate filter types and preservatives that have already been validated in other local contexts (Buxton et al., 2018b; Rees et al., 2024). Allowing various filter types and preservatives to be used nationally would not be robust and likely increase the rate of false negatives relative to that of current EP kits (Biggs et al., 2014, 2015) unless a study comparing filter types and preservatives at national scale was also undertaken.

4.4. Population size

Links between population size and amount of eDNA present continue to be inconsistent in the eDNA literature, with some studies finding a positive relationship and others finding no relationship. In natural systems, biotic and abiotic factors can influence the release, persistence, and degradation of eDNA, and these effects may be exacerbated in ponds which exhibit greater physical and chemical heterogeneity (L. R. Harper et al. 2019). Buxton et al. (2017) found that adult numbers influenced eDNA concentration during the breeding season, but number of larvae and their size were the main drivers of eDNA concentration outside the breeding season. In our study, adult population size

919 in the breeding season did not influence any permutation of eDNA score tested.
920 However, different ponds were surveyed with different methods, including bottle,
921 torchlight, netting and refuge searches (Table 1). Newt counts via torchlight can be
922 compromised by water turbidity, vegetation cover, or weather conditions which affect
923 visibility, and temperature which affects newt activity. Counts via refuge searching are
924 also less reliable than other survey methods as even if newts are present, they may not
925 be using refuges (Langton et al., 2001). Furthermore, methods for counting adults may
926 only produce a minimum estimate of population size (Griffiths & Inns, 1998), and high
927 adult counts may not correspond to breeding adults who are more likely to shed DNA.
928 These factors as well as environmental conditions may be why no relationship was
929 found here.
930

931 **4.5. Change brings opportunity**

932

933 In mainland Europe, where great crested newt eDNA monitoring is not subject to the
934 same regulations as the UK, out-of-season sampling and filtration are frequently used.
935 In Denmark, citizen scientists were recruited to collect filters from waterbodies between
936 May and September for qPCR analysis of 14 species, including great crested newt
937 (Knudsen et al., 2023). In Norway, water samples were collected in May and July then
938 filtered off-site for qPCR analysis to examine presence of amphibians, including great
939 crested newt, and chytrid fungus (*B. dendrobatidis*) in acidic ponds (Strand et al., 2022).
940 Also in Norway, filters were analysed with droplet digital PCR assays for great crested
941 newt, smooth newt (*Lissotriton vulgaris*) and chytrid fungus (*B. dendrobatidis*) to
942 examine infection and potential declines (Taugbøl et al., 2021), and optimise field
943 methods (Taugbøl et al., 2025). In France, qPCR analysis of filters collected in spring
944 was used to investigate great crested newt occupancy alongside other methods (Priol et
945 al., 2023).
946

947 The literature grows when studies with a whole community focus using eDNA
948 metabarcoding are included. In Denmark, eDNA metabarcoding of filters collected in
949 July was compared to conventional surveys for amphibians including great crested newt

(Svenningsen et al., 2022). In Switzerland, eDNA metabarcoding of filters was used to examine distribution of great crested newt in relation to the invasive Italian crested newt (*Triturus carnifex*) and potential for hybridisation (Dufresnes et al., 2019). Also in Swtizerland, water samples were collected and filtered off-site for eDNA metabarcoding to examine amphibian distribution in urban areas (Charvoz et al., 2021). In the Netherlands, filters collected in April were analysed with qPCR analysis for chytrid fungus (*B. salamandrivorans*) and eDNA metabarcoding for amphibians to investigate possible transmission and infection (Davison et al., 2025).

These studies demonstrate the broader applicability of eDNA analysis for understanding great crested newt ecology, including distribution, occupancy, species interactions and disease susceptibility. Some studies examined the false positive and false negative error rate associated with their methods, but sampling was typically not repeated to examine the influence of survey timing and filtration was not compared to EP. Therefore, the future investigations described in sections 4.2 and 4.3 are still needed to ground truth and calibrate out-of-season survey and filtration, but this investment will allow for greater insights from great crested newt eDNA survey.

5. Conclusions

We have provided strong evidence that great crested newt eDNA detection is comparable or higher with filtration compared to EP, echoing many studies demonstrating this for other species and supporting a small number evidencing this for the great crested newt. Therefore, we recommend that this approach be included as an accepted eDNA capture method for great crested newt eDNA surveys. This will allow more water to be processed for robust and reliable estimates of great crested newt presence. Extending the great crested newt eDNA survey season to August could allow more potential great crested newt sites to be surveyed to assess species presence (but not absence for reasons discussed) and identification of ponds that provide important habitat for great crested newts outside of the breeding season. This will also remove the

logistical challenges and costs associated with surveying all potential great crested newt sites and sending eDNA samples to a laboratory within 10 weeks, improving performance of surveyors and laboratories as well as quality of results. Furthermore, it will allow eDNA surveys for great crested newts to be carried out alongside ecological surveying and monitoring for other species. This will enable infrastructure projects to develop more effective mitigation measures as well as reduce time required from surveyors and survey costs.

We have highlighted future investigations that should be conducted to optimise great crested newt eDNA surveys and implement the changes desired by developers planning projects, consultants working in the field, and technicians working in the laboratory. However, change will only be possible with regulatory acceptance and incorporation of new evidence into established frameworks. Actions needed to extend the great crested newt eDNA survey season and use filtration for great crested newt eDNA capture are: 1) further consultation with regulators (i.e. Nature England, NatureScot, Natural Resources Wales), the Chartered Institute of Ecology and Environmental Management which oversees the ecological consultancy industry, and amphibian conservation organisations regarding the results of the present study and their interpretation, 2) consideration of the results from the present study together with other published evidence, 3) undertaking of a similar study to that reported here but including eDNA survey in February and March, and 4) validation experiments on filtration as a method of great crested newt eDNA capture at local, regional and national scale to enable regulatory acceptance at the corresponding spatial scale.

Author contributions

Lynsey Harper and Bastian Egeter designed the study; Kat Stanhope managed the project on behalf of HS2 Ltd; Kirsten Harper performed the literature review; Suzie Platts, Laura Plant, Danielle Eccleshall, Luke Gorman and Kat Stanhope facilitated sample collection and provided supporting data; Rebecca Irwin and Michael Bennett

performed the laboratory work; Ben Jones, Oliver Taylor and Lynsey Harper analysed the data; Lynsey Harper wrote the manuscript, which all authors revised and gave final approval for publication.

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Conflict of interest

Lynsey Harper is employed by Natural England but they do not endorse this work. Some of this data has been published on the HS2 Learning Legacy website: https://learninglegacy.hs2.org.uk/document/environmental-dna-surveys-for-great-crested-newts-time-for-regulatory-changes/. Rebecca Irwin, Andrew Briscoe and Bastian Egeter are employed by NatureMetrics Ltd, a for profit company dedicated to the analysis of environmental DNA. Suzie Platts is employed by Binnies, and Danielle Eccleshall and Luke Gorman are employed by AtkinsRéalis, for profit environmental consultancies. Kat Stanhope is employed by National Grid, a for profit electricity, natural gas and clean energy delivery company.

Data availability

Scripts and corresponding data have been deposited in a dedicated Zenodo repository (https://doi.org/10.5281/zenodo.16544635).

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