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

Lynsey R. Harper[1,2], Kirsten J. Harper[1,3], Suzie Platts[4], Rebecca Irwin[1], Michael Bennett[1], Ben Jones[1], Oliver Taylor[1], Laura Plant[1], Danielle Eccleshall[5], Andrew Briscoe[1], Luke Gorman[6], Kat Stanhope[7,8], Bastian Egeter[1]

[1] NatureMetrics, 1 Occam Court, Surrey Research Park, Guildford, GU2 7HJ, UK
[2] Natural England, Electra Way, Crewe Business Park, Crewe, CW1 6GJ, UK
[3] Roslin Institute, The University of Edinburgh, Easter Bush Campus, Midlothian, EH25 9RG, UK
[4] Binnies, 60 High St, Redhill RH1 1SH, UK
[5] AtkinsRéalis, Woodcote Grove, Ashley Road, Epsom, Surrey, KT18 5BW
[6] AtkinsRéalis, Chadwick House, Warrington, Cheshire, WA3 6AE, UK
[7] High Speed Two (HS2) Limited , Two Snowhill, Snow Hill Queensway, Birmingham, B4 6GA, UK
[8] AtkinsRéalis, 2 Chamberlain Square, Birmingham, B3 3AX, UK

***Corresponding author**
Lynsey Harper, lynsey.harper2@gmail.com

Abstract

1. Environmental DNA (eDNA) monitoring has been used for great crested newt (*Triturus cristatus*, GCN) survey in the UK since the publication of a Defra-funded trial in 2014. Surveys can only be performed during a 76-day survey window (15 April – 30 June), and only if approved methods for collection of 6 x 15 mL subsamples for ethanol precipitation are used. 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 GCN eDNA survey season could be extended and filtration could be used for GCN eDNA capture by reviewing the available evidence and conducting a field study from April to October 2022. Paired water samples for ethanol precipitation and filtration 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 GCN.
3. Our results indicate that GCN can be reliably detected using both eDNA capture methods from April to August, with detection rates decreasing in September and October. GCN eDNA detection was comparable or higher with filtration than ethanol precipitation, thus filtration could be a reliable and more sustainable means for assessing GCN presence.
4. Inclusion of filtration as an accepted eDNA capture method for GCN eDNA surveys would allow more water to be processed for robust and reliable estimates of GCN presence. Extending the GCN eDNA survey season to August would allow more waterbodies to be surveyed and identification of sites that provide important habitat for GCN outside of the breeding season. This would also remove logistical challenges and costs associated with completing GCN surveys and laboratory analysis within 10 weeks, improving performance of surveyors and laboratories as well as quality of results. Furthermore, GCN 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*, hereafter GCN) are a European protected species. GCN, their eggs, their breeding sites, and their resting places are protected by law. Traditionally, GCN were monitored using methods such as torchlight counts, bottle trapping, netting and egg searches to confirm presence or likely absence in waterbodies in support of development and mitigation licences (Langton et al., 2001). Since 2015, environmental DNA (eDNA) analysis has offered a non-invasive, time-efficient and cost-effective alternative (Rees et al., 2014; Biggs et al., 2015), but within a regulated 76-day survey window (15 April – 30 June) (Biggs et al., 2014).

Previous studies have shown that GCN can be successfully detected with eDNA analysis outside this regulated survey window (Buxton et al., 2017, 2018a; Rees et al., 2017), possibly due to juveniles remaining beyond the breeding season, and juveniles and adults overwintering in waterbodies (Rees et al., 2023). Extension of this survey window would allow more potential GCN waterbodies to be surveyed for robust and reliable estimates of GCN presence. It would also allow GCN eDNA surveys to be carried out alongside monitoring for other species, enabling 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 GCN 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 for GCN eDNA surveys. 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 GCN. In accordance with this guidance, all GCN eDNA sampling kits produced by different commercial eDNA providers capture eDNA via ethanol precipitation (hereafter EP). This method 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, 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). Filters can process litres of water for higher detection rates. They 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 (Bigg et al., 2014).

Here, we reviewed the evidence for extending the GCN eDNA survey season and using filtration for GCN 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 GCN eDNA detection. We hypothesised that GCN would be detected beyond 30 June, and that filtration would outperform EP for GCN eDNA detection. We provide evidence-based recommendations for regulatory changes to evolve and streamline GCN 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 GCN eDNA capture and GCN 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 GCN 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. 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. Surveys had not been undertaken for two ponds included in the present study. Initially, 21 ponds were sampled in April 2022 to ensure 17 GCN positive ponds and three GCN negative ponds (i.e. control ponds). 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 GCN eDNA Kits (EP) and Pump Aquatic eDNA Kits (filtration). The latter included a sterile encapsulated 0.8 µm pore size polyethersulfone filter and a 5 µm glass

fibre pre-filter. Water was filtered and the filter air-dried using a Bürkle Vampire Sampler, following which the capsule was filled with 1.5 mL of preservative solution. Field blanks (one of each kit type using mineral water instead of pond water) were included from May onwards to assess potential field contamination risk.

The water sampling protocol established by Biggs et al. (2014) was used for both eDNA capture methods with minor modifications for filtration, where 20 x 125 mL subsamples were collected at equidistant intervals around the pond perimeter and pooled into a single 3.5 L sampling bag for homogenisation, following which as much water as possible was filtered. Water sampling for EP or filtration took the same amount of time (20-30 minutes), but water processing was typically faster with the EP kit (~10 minutes) than the filtration kit (~20 minutes) due to the greater volumes of water being processed with filtration (Figure 1). EP samples were stored at ambient temperature for same-day courier collection or refrigerated until courier collection. Filtration samples were stored at ambient temperature.

Figure 1. AtkinsRéalis ecologists **a)** collecting water for filtration and **b)** processing water using an EP kit.

Population size class assessment surveys were performed for each pond that was positive for GCN prior to 2022 based on six visits from May to June and one visit in October (Supporting Information: Appendix 3). Pond 25 was subject to an out-of-season population survey only as this pond was included from July onwards as a replacement for an eDNA positive pond which became dry. Ponds 8, 9, 17, 18, 19, 21, 22 and 23 were subject to an in-season population survey only as these ponds had dried out and/or become unsafe to access by October. Habitat Suitability Index (HSI) assessments (Oldham et al., 2000) were performed for all ponds regardless of GCN status (Supporting Information: Appendix 4).

2.3. Laboratory analysis

At NatureMetrics, eDNA was precipitated via centrifugation at 14,000 x g for EP kits and processed in accordance with Biggs *et al*. (2014). Briefly, resulting pellets from EP kits and lysate from filtration kits were extracted using Qiagen DNeasy Blood and Tissue kits following the manufacturer protocol. All samples were tested for degradation and inhibition using two quantitative PCR (qPCR) tests that target separate Internal Positive Controls (IPC). One IPC was included in the ethanol and sodium acetate of EP kits or the preservative solution of filtration kits to test for degradation, and one IPC was added to DNA extracts to test for inhibition. qPCR amplification for each IPC was carried out in duplicate on each sample. qPCR amplification was then carried out in 12 technical replicates per sample using GCN-specific primers and probe, developed by Thomsen et al. (2012) and adopted by Biggs et al. (2014), in the presence of extraction negative controls, qPCR positive controls, and qPCR negative controls. A score is given for the number of positive technical replicates out of 12, i.e. eDNA score. Results from the GCN 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, including the use of field blanks, kit blanks, additional extraction negative and qPCR negative controls, and qPCR positive controls, exceeded the requirements outlined

in Biggs et al. (2014). The qPCR positive controls are standards of known GCN 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) and ggpubr v0.6.0 (Kassambara, 2020). Control ponds and negative process controls were assessed for any evidence of contamination, then these were removed from the data set.

GCN detection success with EP and filtration in each month was statistically compared as well as in-season versus out-of-season, both including and excluding the later months of September and October (see Supporting Information for results of the latter analysis). Generalised Linear Mixed-effects Models (GLMMs) with binomial error family were used. 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 it was not significant. We also examined the influence of volume of water filtered on proportional eDNA scores using a GLMM with binomial error family (see Supporting Information).

Population sizes for each pond were available as quantitative (peak adult counts) or qualitative (class) measurements. The data were split by eDNA capture method and both measurements were included in GLMMs with binomial error family testing for a relationship with corresponding eDNA score (i.e. proportional eDNA score corresponding to May when most in-season survey visits for population size class assessment were made), average eDNA score (i.e. the average of proportional eDNA scores for in-season months), and peak eDNA score (i.e. the highest proportional eDNA score observed across in-season months).

Pond number was included as a random effect in each GLMM to account for unknown variation inherent to individual ponds. GLMMs, model performance checks, extraction of

model predictions, and post hoc analyses were implemented using the packages glmmTMB v1.1.10 (Brooks *et al*., 2017), DHARMa v0.4.7 (Hartig, 2022), easystats v0.7.3 (Lüdecke *et al*., 2022), arm v1.14-4 (Gelman & Su, 2022), emmeans v1.10.5 (Lenth, 2022), and effects v4.2-2 (Fox & Weisberg, 2018; Fox & Weisberg, 2019). If a significant effect was found for variables using the *drop1* function from the package stats v4.2.2 (R Core Team, 2022), multiple pairwise comparisons were performed using the *pairs* function from the package emmeans v1.10.5. The full data set has been provided as Supporting Information: Appendix 2.

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 GCN 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 outperformed ethanol precipitation in a tank experiment but not in natural ponds (Buxton et al., 2017; 2018b). The remaining studies demonstrated that out-of-season detection was possible, including a 2019 data set supplied by AtkinsRéalis where every pond surveyed was positive for GCN eDNA in September (Gorman et al., 2020). There were differences in sampling methodology across all studies, but the eDNA capture conditions for ethanol precipitation and filtration, the DNA extraction method, and the amplification method were consistent (see Supporting Information).

3.2. Field study

3.2.1. Quality control

Of five control ponds (Ponds 1, 2, 3, 4 and 24) known or suspected to be negative for GCN based on previous surveys, three ponds were also negative in 2022, but two ponds produced positive results from filters in June (Pond 4, eDNA score = 1) and October (Pond 24, eDNA score = 2) respectively. All samples passed tests for degradation and inhibition except four filters in May (Pond 22), September (Ponds 3 and 4) and October (Pond 24) which were inhibited despite troubleshooting undertaken to resolve inhibition. Samples are analysed regardless of inhibition and degradation being identified. Results from Ponds 22 and 24 were returned as positive rather than inconclusive because GCN DNA amplified successfully. However, results for Ponds 3 and 4 were returned as inconclusive rather than negative because GCN DNA did not amplify which could be due to inhibition or absence of GCN DNA in the sample. One field blank (EP kit) also produced an inconclusive result in October because it failed the inhibition test. All other negative process controls were negative for GCN and passed quality control.

3.2.2. Sample size

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

Of the 25 ponds, 16 had in-season and out-of-season eDNA data, while seven had in-season eDNA data only (typically spanning 2 months) and two had out-of-season eDNA data only. Two ponds with both in-season and out-of-season eDNA data were control ponds. This left 14 ponds that were previously positive for GCN using conventional and

eDNA surveys that also produced a positive eDNA result for at least one in-season month in 2022. All 14 ponds were surveyed in July, decreasing to 13 ponds in August and September, and 11 ponds in October due to ponds drying out and/or health and safety issues (Table 1).

3.2.3. eDNA capture method and month

The number of positive ponds generated by each eDNA capture method in each month ranged from 1 to 17 (Figure 2a). The effect of eDNA capture method did not depend on month and vice versa as the interaction term was not significant, but the individual effects of eDNA capture method and month were significant (Table 2). Filtration consistently produced more positive ponds than EP overall. May had the highest number of positive ponds and significantly differed to September and October but not other months. October had the fewest positive ponds and significantly differed to all other months, although the difference between September and October was on the cusp of significance (Figure 2b).

Table 1. GCN status of ponds surveyed from April to October using ethanol precipitation (EP) kits and filtration (F) kits. Cells are shaded according to kit result (navy = negative, turquoise = positive, grey = inconclusive), and white cells represent months where a pond was not surveyed due to drying out and/or health and safety concerns. Numbers in turquoise cells indicate eDNA scores for positive kits.

Pond	Month and eDNA capture method													
	Apr		May		Jun		Jul		Aug		Sep		Oct	
	EP	F	EP	F	EP	F	EP	F	EP	F	EP	F	EP	F
1														
2														
3														
4						1								
5	1	12		12	1			12		10				
6	1	11		12		12		12		4				
7		12	5	12		12	1	12		10				
8		1	3	7			7	12		12		1		
9		7	10	1	4	12	12	12						
10	9	10	3	12	1	12	4		1	1	1	1		
11	2	12	12	12	12	1	12	11	12	5	5			
12	6	12	10	2	12	12	12	12	12	12	1	12		
13	12	12	12	7	6	12	11	11	12	5	2			
14	2	8	12	12	12	2	11		11	11	1	3		

15		6	5	12		11	4	12	3	12	10	4		
16	12		12	12	12	11	12		7					
17		12												
18				1										
19	2	9												
20	1	12	1	7		12	1	12	3	1		5	1	1
21	3	12		12	12	12								
22	1	12	8	12	11	12	12	2	4	2				
23		9	10	12										
24														2
25							12	12	12	12	3	6		2

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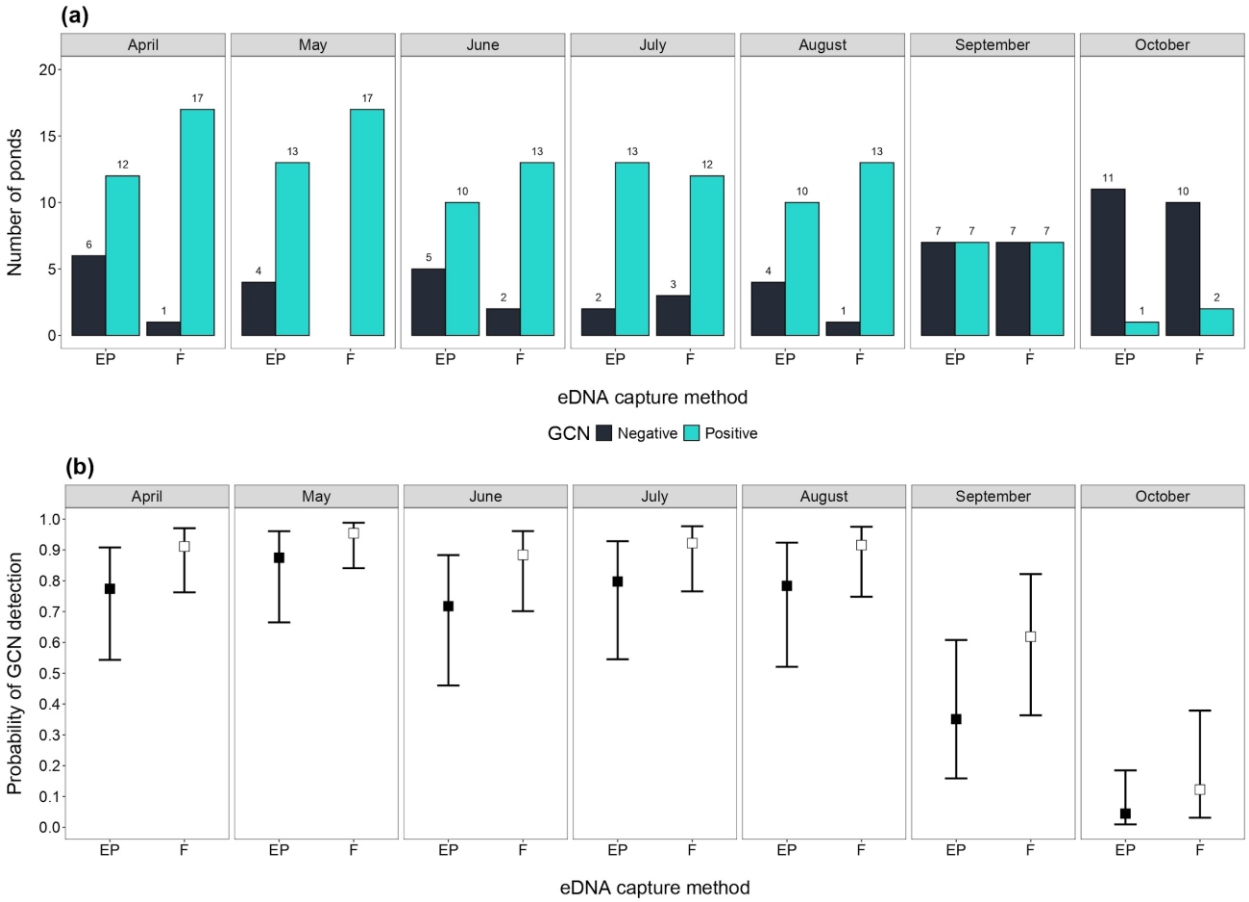

Figure 2. The effects of eDNA capture method and month on GCN detection. In **(a)**, bars show the number of ponds that were positive (turquoise) and negative (navy) for GCN each month with EP and filtration (F) kits. The number of ponds corresponding to each category are displayed above bars. In **(b)**, square points represent the model-predicted GCN eDNA detection probabilities with EP (black) and F (white) in each month, and error bars show the 95% confidence intervals for these predictions.

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

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**
					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
							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			

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The eDNA score produced by each eDNA capture method in each month ranged from 0 to 12 (Figure 3a). The effect of eDNA capture method did not depend on month and vice versa as the interaction term was not significant, but the individual effects of eDNA capture method and month were significant (Table 3). Filtration produced higher eDNA scores than EP overall (Figure 3b). The highest eDNA scores were observed in July and lowest eDNA scores observed in September and October. Scores from April to August did not significantly differ (Figure 3c).

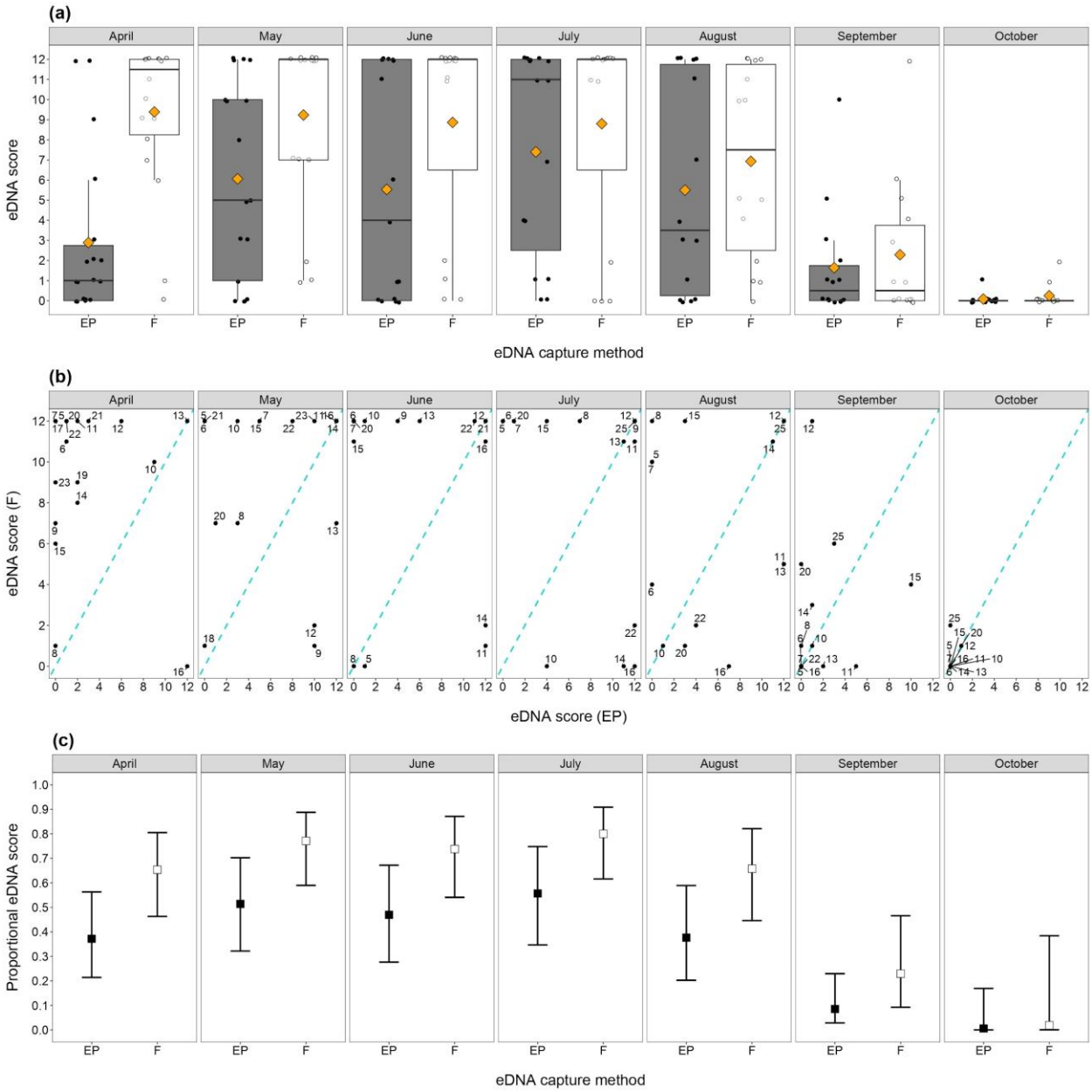

Figure 3. The effects of eDNA capture method and month on eDNA score. In **(a)**, the spread of eDNA scores with EP and filtration (F) kits in each month is represented by boxes which show the 25th, 50th and 75th percentiles, whiskers which show the 5th and 95th percentiles, and thick horizontal lines which show the median. Circular points (EP = black, F = white) represent the eDNA score for individual ponds, and orange 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 turquoise dashed line represents the same eDNA score being produced by both eDNA capture methods; points to the left of this line are ponds where F produced a higher eDNA eDNA score and points to the right of this line are ponds where EP produced a higher eDNA score. Numbers represent the number allocated to each pond for the present study (see Table 1). In **(c)**, the square points represent the model-predicted proportional eDNA scores with EP (black) and F (white) in each month, and error bars show the 95% confidence intervals for these predictions.

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

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
							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
							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			

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3.2.4. Population size

No GCN were found during population size class assessment surveys conducted in October, but three ponds produced a positive eDNA result. Pond 20 was positive using both filtration and EP kits, whereas Ponds 24 and 25 were only positive using filtration kits. Consequently, no analysis of population size in relation to eDNA score out-of-season was undertaken.

Sixteen ponds had in-season population size data that could be used to analyse population size in relation to eDNA score. The remaining nine ponds were unsuitable because: a) they were control ponds, b) population size data could not be obtained from HS2 Ltd subcontractors, c) population size class assessment surveys were not undertaken as ponds were removed from the study due to drying out and/or health and safety reasons, or d) they were replacements added to the study from July onwards and only had out-of-season data.

There was no influence of peak adult counts or population class on corresponding eDNA score, average eDNA score, or peak eDNA score with EP or filtration (see Table S3 and Figure S4 in Supporting Information). Therefore, population size did not appear to influence the amount of GCN eDNA captured with either eDNA capture method.

3.2.5. HSI score

HSI scores for ponds ranged from 0.3 to 0.85, i.e. Poor to Excellent (Supporting Information: Appendix 4). Ponds that were positive for GCN tended to be Average, Good or Excellent, except Ponds 9 and 17 which were both positive for GCN but Below Average. Ponds that were negative for GCN tended to be Average, Below Average or Poor, except Pond 24 which was negative for GCN but Excellent.

4. Discussion

4.1. Quality control

Filtration for two control ponds produced unexpected positive eDNA results. Pond 24 is adjacent to a pond where GCN presence was confirmed in 2020, thus this positive detection may have resulted from GCN migrating from the adjacent pond in combination with higher sensitivity of filtration due to larger volumes of water processed (Rees et al., 2023). Pond 4 is located on a site where all ponds were likely or suspected to be negative for GCN and produced negative results either side of the positive result in June. Field and laboratory contamination is unlikely as all negative process controls were negative for GCN. Possible explanations for sporadic eDNA detection include GCN adults or sub-adults temporarily using ponds for foraging or aquatic refuge (Priol et al., 2022), or eDNA transport by humans, domestic animals or other wildlife, e.g. GCN eDNA in water trapped in waterfowl feathers or predated GCN in waterfowl faeces (Beng & Corlett, 2020). One field blank (EP kit) failed the inhibition test. Reasons for this are unknown but could include chemicals or ions present in the mineral water or contamination with an inhibitor present in the field (Schrader et al., 2012). However, given that all other negative process controls were negative for GCN, GCN DNA was unlikely to be present in this field blank either.

4.2. GCN eDNA survey season

Both the desk and field studies indicated that GCN can be detected in spring as well as summer, likely due to the presence of adults and larvae respectively (Buxton et al., 2017; Rees et al., 2017), with reduced detection from September onwards. Therefore, the survey window could potentially be doubled (c. 15 April – 31 August instead of 15 April – 30 June), which would facilitate land access and relieve logistical pressures for ecological consultants, commercial eDNA providers, and developers (Rees et al., 2017).

The GCN eDNA assay has undergone substantial validation, thus detection means that GCN are very likely present and non-detection means GCN are likely absent assuming appropriate timing and replication in sampling (Thalinger et al., 2021). Ponds that were positive with either EP or filtration in July and August in the present study are likely to be true positives. Extending the GCN eDNA survey season could enable identification of breeding ponds occupied by larvae as well as vital non-breeding ponds that are used for foraging or refuge and are critical to GCN survival but that may be missed by the current eDNA survey window (Priol et al., 2022). 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).

Although false negatives were observed for three ponds out-of-season where GCN were detected in other months that preceded or followed a month of non-detection with either filtration or EP, this anomaly was also observed across in-season months where GCN went undetected in 10 ponds in one month but were detected in preceding or following months with either EP or filtration. This would suggest that the choice of eDNA capture method is more likely to influence GCN eDNA detection than the timing of eDNA survey. However, non-detection with either eDNA capture method in out-of-season months introduces some uncertainty.

Importantly, waterbodies occupied by non-breeding GCN adults in spring only may not be identified by eDNA surveys in July and August, resulting in false negatives (Buxton et al., 2017). Similarly, non-breeding waterbodies without larvae and increasingly fewer GCN adults as they disperse during out-of-season months, or non-breeding waterbodies that are sporadically used by GCN adults and sub-adults for foraging and refuge in out-of-season months, may produce false negatives if GCN are not present at densities that would produce sufficient amounts of eDNA for detection (Priol et al., 2022). This is an important caveat attached to regulatory acceptance of out-of-season GCN eDNA survey. Whether non-detections out-of-season are interpreted as GCN being absent will depend on the level of uncertainty around eDNA detection, and potential risk that false negatives

pose to conservation or mitigation under UK and European legislation, that regulators are prepared to accept.

Nonetheless, false negatives can be encountered with any monitoring tool (Buxton et al., 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 GCN, 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 GCN (Biggs et al., 2014, 2015; L. R. Harper et al., 2018; Priol et al., 2022; 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., 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, or applying statistical models that account for false negative and false positive error rate (Buxton et al., 2022). However, all of these measures would require further modification to the approved GCN protocol (Biggs et al., 2014).

Critically, surveys could not be conducted in February and March 2022 due to the timeframe for funding proposal submission, review, and acceptance. However, GCN adults are known to start returning to waterbodies in these months and even begin breeding in February and March (Buxton et al., 2017; Rees et al., 2017), especially in colder climates (L. R. Harper et al., 2019). Compared to in-season months, Rees et al. (2017) observed lower eDNA scores in February but similar eDNA scores in March. Additional work is needed to confirm whether survey in these months produces detection

rates comparable with April to August or lower detection rates like those seen in September and October in the present study and previous studies (Buxton et al., 2018a, b; Rees et al., 2017). Ideally, this would be contracted by regulators in a large-scale, multi-year evaluation of GCN eDNA detection in-season versus out-of-season (February to October), encompassing waterbodies of known GCN status and variable eDNA scores throughout the UK which span the full spectrum of physicochemical variability seen in these systems (Rees et al., 2017, 2023). However, local-scale evaluations (either independent or as part of regulatory monitoring) will also generate vital evidence that will further improve understanding of out-of-season GCN eDNA detection and allow regulators to make informed decisions.

4.3. eDNA capture method

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

Importantly, there were key differences between our study and previous works. Buxton et al. (2017, 2018b) surveyed eight small ponds (600 L, 1 m by 2 m and up to 0.6 m deep) within a small plot of land owned by the University of Kent. A single 1 L surface water sample was taken from the centre of each pond, thus the field protocol differed to Biggs et al. (2014). Glass microfibre (0.7 µm) filters were used and frozen until DNA extraction. More recently, Rees et al. (2024) analysed polyvinylidene difluoride (0.45 µm) filters from 88 natural ponds across Cheshire, Yorkshire and Norfolk, with filtered volumes ranging from 25 mL to a maximum of 500 mL. An ethanol-based preservative solution was added to filters for storage at ambient temperature for up to 8 weeks prior to DNA extraction. In our study, ponds ranged in surface area from 2 to 2,770 m^2 (mean = 238.68 m^2, median

80 m^2; Supporting Information: Appendix 1), and the field protocol from Biggs et al. (2014) was adapted to accommodate larger subsamples and greater total sample volume. Polyethersulfone (0.8 µm) filters with glass fibre pre-filters (5 µm) were used and a preservative solution added for storage at ambient temperature for up to 1 week prior to DNA extraction. Therefore, successful filtration and greater detection in the present field study may be due to pond characteristics, sampling strategy, volume of water collected and filtered, filter material and pore size, inclusion of a pre-filter, use of a peristaltic pump, and/or preservation strategy.

Nonetheless, EP and filtration were inconsistent as standalone methods across all months and produced incongruent results to each other for individual months. Often, GCN were detected with one eDNA capture method but not the other across several months, or GCN were detected in one month but not the next month with a single eDNA capture method. Each eDNA capture method produced different results to the other method within a given month for 18 ponds. Filtration produced incongruent results in eight ponds across several months. EP produced incompatible results in 10 ponds across several months. During in-season months, 12 ponds that were positive for GCN with filtration tested negative with EP, representing 12 potential failures to protect GCN under current legislation due to filtration not being accepted by regulators in line with best practice for eDNA analysis. During out-of-season months, four ponds that were positive with filtration tested negative with EP, and one pond that was positive with EP tested negative with filtration. Inconsistent eDNA results using EP and filtration at the same ponds were also observed by Rees et al. (2024). Similar to in-season versus out-of-season detection, inconsistent detection between eDNA capture methods introduces some uncertainty and risk of false negatives with either eDNA capture method. This could be mitigated by using both eDNA capture methods at ponds being surveyed, but this would inherently increase survey time and cost.

The advantages of filtration for field surveyors and laboratories as well as evidenced efficiency indicate that regulators should consider acceptance of filtration methods for non-regulated application at local scales. However, persistent barriers to uptake may be

the high variability in filters (e.g. material, diameter, pore size, surface area) available to buy from scientific suppliers (Rees et al., 2023) as well as the strategies to preserve captured DNA (e.g. freezing, silica beads, preservation buffers) and storage conditions (e.g. temperature, duration), which can all influence the quality and quantity of eDNA extracted from samples and subsequently species detection (Hinlo et al., 2017; Jeunen et al., 2019; Minamoto et al., 2015; Peixoto et al. 2020; Spens et al., 2016). Concerns have also been expressed over effort required for manual filtration using syringes, filter clogging, negative eDNA results obtained with EP in previous surveys being undermined, a mismatch in detection sensitivity between filtration and ecologists performing population surveys, value of monitoring small, non-breeding populations, and implications for downstream analysis including proficiency and degradation testing (Rees et al., 2023, 2024).

Additional evidence is needed to overcome some of these 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 GCN 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 GCN 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.

5. Conclusions

We have provided strong evidence that GCN eDNA detection is comparable or higher with filtration compared to EP. Therefore, we recommend that this approach be included as an accepted eDNA capture method for GCN eDNA surveys. This will allow more water to be processed for robust and reliable estimates of GCN presence. Extending the GCN eDNA survey season to August is recommended to allow more potential GCN sites to be surveyed and identification of ponds that provide important habitat for GCN outside of the breeding season. This will also remove the logistical challenges and costs associated with surveying all potential GCN 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 GCN 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 GCN 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 GCN eDNA survey season and use filtration for GCN eDNA capture are: 1) further consultation with regulators (i.e. Nature England, NatureScot, Natural Resources Wales) 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 GCN eDNA capture at local, regional and national scale to enable regulatory acceptance at the corresponding spatial scale.

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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.

Data availability

Scripts and corresponding data have been deposited in a dedicated Zenodo repository (https://doi.org/10.5281/zenodo.13960689).

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. 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, Luke Gorman and Kat Stanhope are employed by AtkinsRéalis, for profit environmental consultancies.

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