Analytical and methodological development for improved surveillance of the Great Crested Newt

WC1067

Final Report

Project contractors: Freshwater Habitats Trust, Spygen, Amphibian and Reptile Conservation and the Durrell Institute of Conservation and Ecology

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Non-technical summary

This report summarises work undertaken to support the development of surveillance monitoring for the Great Crested Newt. The work had two components: Part A was an evaluation of the use of environmental DNA (eDNA) to detect the presence and abundance of Great Crested Newts, particularly when used by volunteers. Part B comprised complementary work to develop statistically valid sampling strategies for detecting trends in pond occupancy by Great Crested Newts, the quality of their pond habitat and the numbers of ponds at national and Great Britain level.

Part A

Environmental DNA (eDNA) is nuclear or mitochondrial DNA that is released from an organism into the environment. Sources of eDNA include secreted faeces, mucous, gametes, shed skin, hair and carcasses. In aquatic environments, eDNA is diluted and distributed in the water where it persists for 7–21 days, depending on the conditions. Recent research has shown that the DNA of a range of aquatic organisms can be detected in water samples at very low concentrations using qPCR (quantitative Polymerase Chain Reaction) methods.

In this project we first developed and tested a primer – a length of artificial DNA which specifically binds to and amplifies the DNA of the target organism – which was able to detect Great Crested Newt eDNA successfully in water samples. We then undertook an intensive study at 35 ponds to compare the ability of eDNA and traditional survey methods (torch counting, bottle trapping and egg searches) to detect newts in the breeding season, from late April to late June. Volunteer surveyors also collected single eDNA samples from 239 ponds known to be used by Great Crested Newts across England, Wales and Scotland. We used the volunteer data to assess whether eDNA detection was affected by variations in pond physical and chemical environmental factors, and also to assess the practicality of the technique for use by volunteers. eDNA samples were collected at sites where newts were known to be present in order to determine how often false negatives occur i.e. when the survey method failed to detect animals that we knew to be present. At further sets of sites we also tested for false positives i.e. we wanted to check that the technique would not report that animals were present when they were not. For this analysis we visited 30 sites *outside* the known range of the Great Crested Newt in Great Britain and 30 sites *within the range* where we had good evidence that newts were absent.

In the detailed methodological study, eDNA detected Great Crested Newts 99.3% of the time i.e. out of 140 samples from ponds where we knew newts were present, eDNA detected newts 139 times. Of the traditional survey methods, bottle trapping and torching were similar in effectiveness, followed by egg searches, with the individual methods detecting newts respectively 76%, 75% and 44% of the time over the full survey period from April to June. When torch counting and bottle trapping were combined, as is normal practice in amphibian surveying, the traditional method was only slightly less effective than eDNA. At the volunteer survey sites, newts were successfully detected 91.2% of the time (218 out of 239 sites). There were no false positives: eDNA did not record newts where we believed them to be absent. We found that newt abundance was weakly correlated with the eDNA 'score': sites with low eDNA scores always had low newt counts but sites with higher eDNA scores did not always have more newts.

Overall, collecting eDNA appears to be a highly effective method for determining whether Great Crested Newts are present or absent during the breeding season. We do not know how effective the method is outside this period and, at the moment, eDNA provides only limited information on newt abundance. eDNA also seems to offer more certainty about zero values: traditionally it has been difficult to say that there are no Great Crested Newts at a pond because surveys might just have missed them. eDNA is also substantially quicker than traditional methods with a sample taking about 2 person/hours in the field (including travel to site) compared to about 48 person/hours for a four visit / multiple methods traditional survey to confirm absence with a similar level of certainty. Overall, the cost of an eDNA survey in England, with 50% of samples collected by volunteers, would be just over £400,000. In Wales and Scotland the cost would be about half this amount. Costs of analysing and reporting the results would be about £50,000.

Part B

A monitoring strategy for Great Crested Newts should provide information on stock (e.g. the total number of ponds supporting newts) and change in variables which are important for the conservation of the species. The UK statutory agencies have decided that for the Great Crested Newt monitoring should focus are pond numbers, Habitat Suitability Index, an indicator of pond habitat quality for newts, and the number of ponds where newts are found (pond occupancy).

There are already several schemes underway which collect data at Great Britain level on the number and condition of ponds and their biota, including the Countryside Survey, the National Amphibian and Reptile Recording Scheme and PondNet. However, the effectiveness of these schemes in generating statistically robust data to assess change for Great Crested Newts is uncertain. None of these surveys was designed to report solely on Great Crested Newt, and all have different approaches to survey design. In addition, Great Crested Newts, in particular, are difficult to monitor because they have a scattered distribution, can be hard to detect and fluctuate in population abundance and pond occupancy between years. As a result, real changes can be hidden in this noise if the number of sites being monitored is too small.

Power analysis can be used to determine the sample size required to detect changes between separate survey years. For example, power analysis can be used to decide whether it is better to assess pond occupancy using a survey conducted in 1996 followed by a second conducted in 2006; or by surveying annually over that time (e.g. 1996, 1997, 1998, 1999 and annually until 2006) to detect change. In both cases the output of power analysis is dependent on a good understanding of the variability of data within and between years. At present such data are surprisingly sparse for Great Crested Newts, despite the amount of information collected about the species, so that there is still considerable uncertainty in estimates of variability and whether this is different for the different regions.

We undertook a wide range of power analyses investigating potential scenarios for the design of Great Crested Newt surveys. Overall, results indicate that, in England, to monitor change in the number of ponds occupied by Great Crested Newts per 1 km grid square, about 550 1 km squares need to be visited, which is roughly 15 per county. This number of squares would also provide sufficient data for pond number assessments and could detect a c10% change in HSI score. The survey strategy requires all ponds in the 1 km square to be surveyed for newts. As the national average pond density is about 2 ponds / km^2 this indicates that, in England, data on Great Crested Newts would need to be collected at c1100 ponds, most likely by the collection of an eDNA sample. In Wales a total of about 300 1 km squares would need to be visited to assess the number of ponds with newts, about 30 per county within the Great Crested Newt's range. Again this is sufficient to estimate change in pond numbers and large changes in HSI score. Newt data would be needed from about 600 individual ponds. In Scotland a total of 290 1 km grid squares would need to be visited to assess change in the number of occupied newt ponds. This would be sufficient to detect at least 20% change in the number of ponds within the newt's range and large changes in HSI scores. Newt data would be needed from about 380 individual ponds. These numbers of squares and ponds would need to be surveyed each time the survey was undertaken.

Estimates for trend analysis were more speculative because of the lack of existing data on which to base models. Stand-alone surveys provide an immediate assessment of change, whereas trend analysis can only confidently report on whether change is occurring at the end of the survey period.

We think that it would be difficult to recruit sufficient volunteers to undertake a 4 visit / multiple method survey using traditional survey methods, or to fund a professional survey using this approach. However, an eDNA survey appears much more feasible, and could probably be undertaken by volunteers assisted by professional staff. However, it is also important to recognise that, for many volunteers, seeing newts is often an important factor motivating them to participate and they may be less interested in an eDNA survey which simply involves collecting water. For this reason, we also discuss the potential for a 'mixed method' survey which combines traditional survey methods at sites where newts can be recorded quickly and easily on a single visit (e.g. by egg searches) with an eDNA sample taken if newts are not immediately and quickly found on the first survey visit.

Executive Summary

Abbreviations of project partner names and surveys widely used in this report are:

ARC = Amphibian and Reptile Conservation

DICE = Durrell Institute of Conservation and Ecology

FHT = Freshwater Habitats Trust, formerly Pond Conservation

NARRS = National Amphibian and Reptile Recording Scheme.

Background

This report summarises work to support the development of a surveillance programme for the Great Crested Newt in Great Britain. It has two main components:

- an evaluation of the use of environmental DNA (eDNA) in detecting the presence of Great Crested Newts in ponds, particularly its use by volunteer surveyors, and its ability to detect newt abundance (Part A); and
- complementary statistical work to establish reliable survey strategies for detecting change in pond occupancy by Great Crested Newts, Habitat Suitability Index scores and pond numbers (Part B).

Methods

Part A: eDNA methods

We developed and tested a primer for the Great Crested Newt using a three stage protocol: *in silico*, *in vivo* and *in situ*. Markers were first tested *in silico* using ecoPCR software, followed by an *in vivo* check of primer specificity using tissue samples collected by swab sampling from 16 Great Crested Newts from south Hampshire, north-west England and north-east England. Finally we tested the primer *in situ* at three out-of-range ponds in Shetland, and six in-range locations in south Hampshire with known low/medium or high density populations. Out of range sites were all negative and in-range sites all positive. The quantities of eDNA detected were broadly, but not exactly, correlated with the low/medium and high densities.

To test the practical utility of the eDNA method we collected five main datasets.

(i) Out-of-range sites to test for false positive eDNA responses: eDNA samples were collected from a set of sites (n=30) just beyond the edge of the Great Crested Newt's known range, in Cornwall.

(ii) Sites for detailed comparison with 'traditional' survey methods: we surveyed 35 sites (20 in south Hampshire, 15 in north-east Wales) on four occasions between mid-April and late June, collecting eDNA on each sampling visit and at the same time recording newt occurrence and abundance by torch counting, bottle trapping, daytime visual searching and egg searching. In south Hampshire, surveys were undertaken by a professional survey team; in north-east Wales the work was conducted by a volunteer team of approximately 50 people, organised by Natural Resources Wales. All sites in the detailed methods study were known to support Great Crested Newts at varying densities, with peak torch counts varying from 1 to 47 individuals.

(iii) Volunteer survey sites: in order to assess the potential for volunteers to use the eDNA method, eDNA samples were collected on one occasion from 239 ponds across England, Wales and Scotland. Volunteers were either part of the PondNet[1] project, or had been

[1]PondNet is a Natural England/Defra funded project which is investigating whether it is possible to establish a new volunteer-based biodiversity surveillance network that will provide statistically valid stock and change data

involved in amphibian surveys previously, for example through the NARRS network. All sites were intended to be known Great Crested Newt ponds with evidence from the 2013 breeding season that newts were present. Just over 80 volunteers, plus six members of the project team, were involved in collecting the samples. Most volunteers (55%) collected samples from 1 or 2 sites; 1 volunteer, a highly experienced herpetologist with a special interest in the project, collected samples from 30 sites.

(iv) Test for within-range false positives: during the sampling programme we added a further subset of sites (n=30), which were not part of the originally planned work. These were ponds *within* the core range of the Great Crested Newt (in south Hampshire, Kent and London) where we were had good reason to believe Great Crested Newts were *absent* as assessed by local expert knowledge.

(v) Volunteer sampling quality assurance: professional members of the project team resurveyed 11% of sites (n=26) previously surveyed by volunteers to quality assure volunteer sampling.

The volunteer sites surveyed in the present project were representative of the sites occupied by Great Crested Newts across Great Britain in terms of their range, altitude, pond size, geology and associated land-use. However, it should be noted that the sites were not strictly statistically representative i.e. they were not a random stratified sample. Rather, the objective of the study was simply to collect eDNA samples from a good range of sites.

Part B: Statistical design of surveillance surveys for Great Crested Newt

We undertook a wide range of analyses, and associated tests of power, to determine the optimum sampling designs to detect change in three parameters: pond numbers, Habitat Suitability Index (HSI) and Great Crested Newt pond occupancy.

Unlike some other taxonomic groups, particularly birds and butterflies, stock and change data for Great Crested Newts and HSI score is poor, although data are available for pond numbers. This places some limits on the statistical design of survey strategies because we do not have reliable estimates of variability and it is not possible to fully validate the models against real data.

(i) Pond numbers: We used existing data on pond numbers from the Countryside Survey and Ordnance Survey MasterMap to explore sampling strategies and sample sizes needed to assess change in pond numbers.

Countryside Survey field data were originally collected as part of a Great Britain level survey comprising a stratified random sample of 1 km squares, undertaken by professional survey teams. Surveys were undertaken in 1998 and again in 2007 and data were available from 544 1 km squares. Field surveys are currently the only practical way of accurately estimating pond numbers and, in particular, overcoming problems associated with detecting small or temporary ponds, ponds beneath trees, ponds in generally wet environments, and determining which ponds no longer exist. Remotely surveyed data are unlikely to deal with such problems effectively (Biggs *et al.* 1996).

Ordnance Survey data are based on a mixture of ground survey and remote sensing. They are collected over a number of years and have several short-comings including variable dates of survey, irregular updating, inconsistent recording of waterbodies, an ill-defined lower size limit and uncertainties in recording of temporary ponds. Initial ground-truthing by Freshwater Habitats Trust staff of the difference between actually existing ponds and those shown on OS maps indicates that up to 30% of ponds that exist on the ground are not shown on OS maps. However, the Ordnance Survey data provide a much larger pool of samples on which to base pond number estimates than Countryside Survey, effectively all 243,000 1 km square that

for target species and habitats. The network, called PondNet, uses a habitat-centred monitoring approach with ponds used as the pilot habitat. The project is working with volunteers in three regions: south Hampshire, Cheshire and north-east Yorkshire.

make up Great Britain, compared to the 544 1 km squares available from Countryside Survey. Thus, despite the inaccuracies in Ordnance Survey data, the very large sample size provides better estimates of variability than the relatively small number of Countryside Survey samples.

(ii) HSI: We used data from the regional surveys undertaken by DICE / FHT in Kent and Wales, Countryside Survey and NARRS to provide data on HSI scores.

Recording HSI scores is important to both understand pond condition for Great Crested Newts at individual sites and also for Favourable Conservation Status (FSC) reporting at national and European levels. However, until recently HSI score has not been included in most monitoring schemes, with the exception of NARRS, so that data on levels of change and variability in the index between years is uncertain.

We re-surveyed 23 ponds in Kent and 25 ponds in Wales which had been surveyed previously by DICE in 2007 to better understand change over time. These data and existing results from analysis of Countryside Survey data (77 ponds) suggest that HSI scores may be increasing (i.e. pond quality is increasing for Great Crested Newts), but that rates of change are slow e.g. a 3% increase in HSI scores over 10 years. A much higher rate of change in is suggested by HSI scores collected during NARRS surveys (372 ponds).

Because of this difference in assessment of change we evaluated the different sampling strategies' abilities to detect an ecologically meaningful value (10%) in HSI scores with 95% confidence and 80% power, using the existing surveys to provide information about variability in HSI scores within and between years.

(iii) Great Crested Newt occupancy: We used two principal datasets to analyse the power of alternative sampling strategies for Great Crested Newts: (a) distribution data from the National Biodiversity Network Gateway (NBN), and other sources, for the period 1988 to 2012 localised by a grid reference but not associated with a specific waterbody and (b) National Amphibian and Reptile Recording Scheme data (NARRS) collected between 2007 and 2012 and based on surveys of ponds nearest to the south-west corner of the sampling square in 410 randomly selected 1 km squares, and so associated with a known waterbody.

We cleaned the NBN data and developed a simulated pond occupancy dataset by overlaying the NBN data on the MasterMap pond layer. We identified all ponds within 1 km of a newt record as potentially suitable for Great Crested Newts, giving a dataset of just over 57,000 ponds and a simulated pond occupancy of 10% in Great Britain, which is consistent with field data. This dataset was then used to explore a sampling strategy based on recording occupancy of *all* ponds in a 1 km square. This approach has a number of advantages both statistically and practically and is the approach currently being tested in the Defra and Natural England supported PondNet project. We evaluated the power of various sampling strategies based on this overall approach, testing different levels of power and different levels of change between sampling years.

We used the NARRS data to evaluate sampling strategies based on the pond occupancy with independence of sample units maintained through survey of only one pond in a 1 km grid square. An alternative strategy would be to survey all the ponds in a square to provide data on the number of occupied ponds per square. This strategy is being used in PondNet, but this network is still in a pilot phase and has not yet generated sufficient data for power analysis. Therefore, variability between squares was modelled using NBN data. Both strategies are valid and have benefits and drawbacks: importantly we are seeking ways to integrate the results to make the best use of all available data.

Results

Part A: eDNA survey

The results suggest that eDNA is highly effective at detecting the presence of Great Crested Newts in ponds. In the detailed methodological studies where newt occupancy was assessed on four occasions at 35 sites, newts were detected 99.3% of the time when known to be present. In volunteer surveys of single samples from 239 ponds newts were detected 91.2% of the time when known to be present using other methods. There was no evidence of false positives and no evidence of cross contamination between sites. There was no evidence that eDNA detectability varied during the sampling period (mid-April to early June), either in the detailed methodological study sites or in the broad volunteer survey. We do not yet know how the method performs outside the breeding season period.

In our detailed study, eDNA was more effective at detecting newts than individual 'traditional' survey methods (torch counts, bottle trapping, egg searches) over the course of the survey season. For traditional methods to achieve similar detection rates to eDNA, it was necessary to combine torch counts and bottle trapping, although later in the season eDNA was significantly more effective than even torch and bottle trapping combined. Torch counts and egg searches combined were not as effective as eDNA in England and only equal to eDNA in Wales early in the season.

Our laboratory technique detects the presence of eDNA below the level at which the amounts present can be reliably quantified. However, we believe that number of positive qPCR replicates that are amplified in each sample (the 'eDNA score') is related to the amount of eDNA present, so that the eDNA score is a surrogate of the amount of eDNA in the sample. Overall, there was a weak relationship between the eDNA score, reflecting the amount of DNA in the samples, and Great Crested Newt counts. Sites with low eDNA scores always had low numbers of newts. On average, sites with larger newt populations had higher eDNA scores (typically scoring 9 out of 12 or above), but it was possible for sites with high eDNA scores to have low newt counts. At present, the relationship between eDNA score and newt abundance is not strong enough to use eDNA as a reliable index of population size.

Quality assurance by professional surveyors obtained the same result as volunteers on 92% of occasions, suggesting that eDNA collection can be effectively deployed by volunteers with a low rate of error.

There were a small number of false negatives when newts were present but not detected by eDNA. Failure to detect newts when they were present appeared to be due mainly to (i) sites having very small Great Crested Newt populations, (ii) practical difficulties in obtaining water samples from areas newts were using e.g. in some ponds, which were apparently fully accessible, it was only possible to collect water from broad and very shallow marginal zones, with water 1-2 cm deep, when newts were in deeper water in the interior of the pond, (iii) access difficulties e.g. dense scrub preventing surveyors from collecting water samples from right around the pond. Results suggest that false negatives were most likely when more than one of these factors occurred together.

Spearman rank correlation analysis indicated that the detection of eDNA was related only to the overall HSI score. There was also a weak non-significant correlation with the absence of fish. This suggests that the main factor driving the ability to detect newt eDNA across Great Britain as a whole was the presence of Great Crested Newts and that, at the scale of a national survey, environmental factors had little, or perhaps no, influence on eDNA detection.

We conclude that theoretically it would be feasible for either volunteers or professionals to collect eDNA samples as part of a national survey. However, there are additional logistical issues that need to be addressed if eDNA surveys using volunteers are to be effective. First, attitude surveys of NARRS and PondNet volunteers show that volunteers prefer to go to sites which are close to home. Since most volunteers live in urban areas visiting more remote rural sites will inevitably require professional backup to ensure a properly structured set of sites is

visited. Secondly, volunteers are generally unwilling to visit sites where land ownership is not already known. Hence it is essential for a scheme organiser to obtain prior permission for survey. Thirdly, volunteers are time-limited and may not be willing to collect samples from all ponds in a 1 km square if more than two or three ponds are present. Finally, as many volunteers do pond surveys because they enjoy seeing amphibians, additional explanation and encouragement may be needed to ensure that volunteers find eDNA surveys - during which you do not need to see amphibians - sufficiently rewarding. Thus, as with other wildlife monitoring schemes, a significant element of professional volunteer support, survey work and other logistical backup is likely to be essential for eDNA surveys that involve volunteers.

Part B: Statistical design of surveillance surveys for Great Crested Newt

We evaluated survey strategies for detecting change in three parameters important to Great Crested Newt surveillance: pond numbers, HSI score and the number of ponds occupied by Great Crested Newts.

Compiling data on which to base power analyses
Overarching themes across the monitoring of the three parameters were:

1) The value of recording all ponds within a 1 km grid square to calculate pond numbers and pond occupancy. However, for Habitat Suitability Index scores, only one pond per 1 km grid square should be selected for survey, to maintain independence between sample units.
2) Although repeated measures surveys are optimal in terms of reducing sample size, this is outweighed by other limitations. Thus, 1 km grid squares should be selected at random each survey year.
3) The same number of 1 km grid squares should be visited each year to created balanced designs: if volunteers surveyors are used it is often difficult to get the same number of surveys completed every year, and special effort may be needed to achieve this (e.g. extra support for volunteer workers).
4) Data are currently incomplete for several parameters e.g. certain regions, trend data, assumptions that variability will remain the same in the future, etc. As new data are made available through surveillance the scope of the monitoring network can be refined.
5) The existing networks PondNet and NARRS will need to make some adjustments to their networks to better pool data. With these existing schemes the delivery of a statistically robust volunteer-led surveillance network for Great Crested Newt is a feasible option in theory.

Pond numbers

We have chosen to investigate pond numbers in terms of change in the average number of ponds per 1 km grid square. This unit is small enough to respond to changes in land use and can be scaled up to give an estimate of the number of ponds at Great Britain level.

Analysis of existing Countryside Survey data indicates that over the period 1998 to 2007 the power of the analysis at Great Britain level was 72.5% and detected an increase in pond density of from 1.86 to 2.10 ponds per km^2, a 17% change.

In terms of the design of future surveys, power analysis based on this design (matched pairs analysis) indicates that to detect a 20% change between two time periods would require a sample of 500 ponds at Great Britain level. To detect change in England alone would require a survey of 231 1 km squares, but nearly 10 times more squares would be required to detect change in Scotland or Wales alone, reflecting the greater heterogeneity in pond densities in these countries.

Ordnance Survey data are based on a mixture of ground survey and remote sensing. They are collected over a number of years and have several short-comings including variable dates of survey, irregular updating, inconsistent recording of waterbodies, an ill-defined lower size limit and uncertainties in recording of temporary ponds. Initial ground-truthing by Freshwater Habitats Trust staff of the difference between actually existing ponds and those shown on OS maps indicates that up to 30% of ponds that exist on the ground are not shown on the maps. However, the Ordnance Survey data provide a much larger pool of samples from which to understand spatial variability than Countryside Survey: effectively all 243,000 1 km squares that make up Great Britain, compared to the 544 1 km squares available from Countryside Survey.

Overall, considering both Countryside Survey and Ordnance Survey datasets, we conclude that the best strategy for estimating a 20% change in pond numbers with 80% power at Great Britain level is to use a balanced design, surveying different squares each survey year, but stratified to only include squares within the Great Crested Newt's range. All ponds within the grid square would need to be recorded to ensure that estimates were accurate. For Great Britain estimates this would require visiting 121 1 km squares. For England, Scotland and Wales separately, respectively 147, 135 and 208 1 km squares need to be surveyed.

Detecting smaller changes in the number of ponds per grid square would be more challenging and the results could not be used to describe changes in the number of ponds outside of Great Crested Newt's range. It is difficult to analyse the power of trend analysis for pond numbers because annual surveys have not been conducted and therefore it is unclear how variable they are over time.

Using the recommended Great Crested Newt sampling strategy proposed below, we would be able to detect at least a 20% change in pond numbers with 80% power in England (a survey of 549 1 km squares stratified to 50:50 known: unknown Great Crested Newt occupied squares), Scotland (a survey of 282 1 km grid squares stratified to 75:25 known: unknown Great Crested Newt occupied squares) and Wales (a survey of 294 1 km squares stratified to 90:10 known: unknown Great Crested Newt occupied squares).

HSI scores

Countryside Survey and DICE data suggest that at individual ponds HSI scores are likely to change as little as 3% over 10 years. However, for conservation purposes it is only necessary to detect larger changes of 10% or more. To detect this level of change there are three potential survey strategies.

The sampling strategy with the smallest sample size to detect this level of change in HSI scores is achieved using a repeated measures survey which involves revisiting the same ponds with discrete surveys at times t_1 and t_2. As part of a national monitoring network based on 1 km squares, this would involve surveying 1 pond in each 1 km grid square as a focal pond (as is currently occurring with PondNet). For a Great Britain-wide survey involving revisiting the same squares, only 32 1 km grid squares (with one pond per km square surveyed) would be required to detect a 10% change in HSI score with 80% power. However, repeat surveys have important limitations statistically in that they cannot easily be adapted to include new squares (the preferred option for assessing Great Crested Newt pond occupancy).

A second, and better, option for assessing change in HSI score is a random sampling strategy of different ponds in discrete surveys at t_1 and t_2. The number of squares for Great Britain surveys would be 109 1 km grid squares (with one pond per square surveyed) and for national surveys would be in England 215 1 km grid squares (one pond per square), in Scotland 118 1 km grid squares (one pond per square) and in Wales 159 1 km grid squares (one pond per square).

The third strategy, compiling data over several years, and comparing differences between survey periods, further increases the sample size required because:

- Analysis suggests that the total number of ponds needed to detect a given level of change is likely to be higher than in discrete surveys, to take account of variation between years (within sample periods) and between sample periods.
- Although the number of ponds required for a Great Britain-wide survey over 6 years to detect 20% change are feasible (c.500 1 km grid squares, with one pond per 1 km square), sample sizes for smaller changes are very much larger.
- The number of ponds required for a Great Britain-wide survey, to detect a 10% change in mean HSI score between sample periods (80% power), would be 396 1 km grid squares per year to overcome between-year variation over an 11 year survey.

Compilation of data over several years may become necessary if it is not possible to recruit and retain enough volunteers in the network. This highlights the need to co-ordinate survey networks to take advantage of the willing volunteer labour force and the need for coordination (resourcing) within organisations to support volunteer programmes.

Analysis of trend data over several years is more feasible. Detecting an annual rate of change of 2% in the Habitat Suitability Index at Great Britain level would require survey of 110 1 km grid squares per year. However, the data on which these estimates were based followed a slight but continual increase in HSI scores. As such they mirrored the sample size needed to detect a difference in pond number between the first and final year.

Great Crested Newt pond occupancy

To assess survey designs for pond occupancy by Great Crested Newts we evaluated the power of the existing NARRS survey design and modeled alternative scenarios using the NARRS data. We also modeled a range of scenarios using a simulated dataset derived from NBN / local records centre data. All analyses of Great Crested Newt pond occupancy assumed that the best available survey technique, or combination of techniques, would be used on each survey visit to have a high level of confidence that Great Crested Newts could be detected if they were present.

Power analyses of the currently available NARRS data suggest that the survey's strategy has relatively modest power to detect change in Great Crested Newt pond occupancy. Taking NARRS data and assuming that all 410 samples so far obtained were collected in year 1 (i.e. time t_1) and repeating the survey with a different set of 410 samples in a second year (time t_2), giving a fully random design, at Great Britain level there is only 12% power to detect a 20% change in pond occupancy, assuming an α (probability) level of 0.05. To detect a 30% change in pond occupancy with 80% power and an α level of 0.05, would require a survey of 1142 ponds. If analyses are conducted country by country in England, Scotland and Wales, power is also relatively limited.

Combining data over several years is a possibility, but the sample size increases to overcome both the variability within each year and the difference between years within the same period. As the number of samples within each year increases, the variability decreases - so it is optimal to have sufficient sample size within each year. If the difference between sample years is minimal then the variability between years is low and the number of years over which the survey data should be compiled does not have a significant effect on sample size. If, however, there is a large amount of variation between years, then to detect a difference between survey periods requires a large number of years to be sampled.

If, as is currently the case, NARRS surveys are conducted over several years and combined as a time period, p_1, and compared to another time period, p_2, there is added variability due to between year differences. Based on the unbalanced design, with different numbers of sites visited each year, and the level of variability based on visiting 410 ponds over 6 years,

the analysis suggests that 6 years would not be sufficient to detect anything other than very large changes in Great Crested Newt occupancy (e.g. a 50% change at only 50% power).

Using NBN data we evaluated several approaches to detecting change in Great Crested Newt occupancy. These were: (i) changes in the proportion of occupied 1 km **squares**, using either a paired sample approach, in which the same squares are revisited, or independent random samples, both compared at times t_1 and t_2, (ii) changes in the proportion of occupied **squares** but stratified to include only squares **with ponds**, and then surveyed either using a paired design (same sites revisited) or a design in which a new set of random sites is visited at time t_2 (iii) change in the number of occupied ***ponds*** rather than the number of occupied squares with several alternative strategies, visiting randomly selected ponds or ponds stratified to include different numbers of sites known to support Great Crested Newts, varying from 50% to 90% known Great Crested Newt squares.

As a general principle we recommend that surveys focus on occupied ***ponds*** (or ponds within 1 km squares) rather than occupied squares alone. This is because there is a substantial danger that in squares with a number of occupied ponds (a common phenomenon with Great Crested Newts) substantial loss of occupied ponds would only be recorded as a 'loss' when all the ponds in that square lost their newts. Thus a substantial decline in newts could go unrecorded as long as there was one occupied pond remaining in the square.

With no stratification i.e. when ponds are simply chosen at random, 3000-7000 1 km squares are needed at Great Britain level to achieve 80% power to detect a 30% change at $\alpha = 0.05$. On average there are about 2 ponds in each 1 km square in Great Britain, so roughly double this number of ponds would need to be surveyed. Sample sizes would also be large on an individual country basis.

Much more realistic sample sizes can be achieved by choosing a proportion of squares known to support Great Crested Newts. The smallest samples are needed when ***only*** Great Crested Newt squares are visited but this constrains the survey's ability to assess the expansion of newt populations: i.e. only expansion into unoccupied ponds in the known newt squares could be assessed, and not expansion into new squares. Therefore, strategies which involve surveying both known Great Crested Newt squares, and squares where their status is unknown, are preferable.

Given the substantial differences in the heterogeneity of Great Crested Newt populations in England, Wales and Scotland, we recommend that the mixed known/unknown squares approach is used but with the proportions of squares tailored to each country. Taken together the three countries survey would then provide a view for Great Britain as a whole but would not be analyzed as a single dataset. A single Great Britain-wide strategy is not ideal since such a strategy provides good estimates for England but does not provide reliable data for Scotland and Wales when broken down to country level.

In England we suggest a survey based on 50:50 stratification where half the squares surveyed are known to support Great Crested Newts, and half are squares where status is unknown. This would require a survey of 549 squares to detect a 30% change in occupancy with 80% power, involving an estimated 1100 ponds. In Scotland and Wales we recommend strategies much more focused on known sites. In Scotland we propose that 75% of squares in the network are known to support Great Crested Newts, with 25% selected from the 5 km buffer zone around the known distribution to detect expansion. To detect a 30% change in the percentage of occupied ponds per 1 km grid square with 80% power, a total of 282 1 km grid squares would need to be surveyed. In Wales, 90% of squares should be known to support Great Crested Newts, with 10% selected from the 5 km buffer zone around the known distribution. This would require a survey of 294 1 km squares to achieve 80% power to detect 30% change. These numbers of squares and ponds would need to be surveyed on each occasion the survey was undertaken.

Estimates for trend analysis were more speculative because of the lack of existing data on which to base models, but a survey of 50 squares per year, over 10 years, should be sufficient to detect a 30% change in the average number of occupied ponds per 1 km square. We recommend that this approach is adopted only after stand-alone surveys are undertaken for c. 6 years - one survey per year in order to provide sufficient data on which to refine the model. This would allow for immediate assessment and reporting on status change with a view to undertaking longer term trend analysis in the future.

For all parameters we recommend that sample sizes are based on those calculated for detecting change between two sample years. These are robust estimates which will allow detection of change with sufficient power (80%). Once data have been generated by the network they can be analysed to determine whether smaller sample sizes will be sufficient to detect longer term rates of change.

There are potentially three alternative methodological approaches to obtaining data on Great Crested Newt pond occupancy: using traditional survey methods (torch counting and bottle trapping) alone, eDNA survey alone or a combination of these approaches. Although it is theoretically possible that traditional surveys could be undertaken by volunteer surveyors, the level of recruitment, organizational challenge and cost would be substantial and project success could not be guaranteed. eDNA surveys by professional surveyors are eminently feasible, and completion of at least part of the survey by volunteers is also a viable option. However, it is unlikely to be possible to recruit enough volunteers to visit all the sites needed, particularly in areas away from population centres. An additional, and currently unknown consideration, in an eDNA-only study design is the motivation of volunteers. Seeing amphibians is often an important motivation for volunteers and undertaking a survey which only involves collecting water samples has the potential to alienate volunteers and undermine existing volunteer survey work. We currently have no data on the magnitude of this effect. If an eDNA only survey is adopted, we recommend that it is accompanied by an investigation of this potential problem.

Once at a site, professionals and trained volunteers can sometimes rapidly establish the presence of amphibians by direct observation of adults or eggs, particularly where the survey timing is optimal and there are good newt populations. For this reason we also suggest that a ‘mixed method’ survey could be undertaken in which professional or volunteer surveyors, when visiting sites, briefly first searched for amphibians. If a positive sighting was made, this would be sufficient to prove presence. If newt traces are not evident, an eDNA sample would be collected. This approach has advantages and disadvantages: although cost savings are likely to be small, because most of the cost of the survey is in obtaining permission to visit sites, the approach does offer greater potential for volunteers, in particular, to see Great Crested Newts.

Costs of eDNA sampling

The cost of collecting eDNA samples is made up of three components: obtaining permission to visit sites (about 2 days / kilometre square), collecting the samples and analysis of the eDNA. Obtaining permission to visit is the main cost being more than half of the total. Costs of collecting an eDNA dataset are about 6 - 10 times less than an equivalent standard conventional survey using torch counting and bottle trapping.

In England the cost of collecting an eDNA dataset is about £410,000, assuming that 50% of samples are collected by volunteers. In both Scotland and Wales the costs are similar, being about £180,000 for each country. In all cases the largest part of the cost is obtaining the permissions to visit sites. This cost would be incurred in each year the survey was undertaken.

Costs of analysing and reporting results would be additional to this, but are the same whether eDNA or traditional methods are used.

Conclusions and recommendations

The project has shown that eDNA is a highly effective technique for detecting the presence of Great Crested Newts during the breeding season. Previously, the relatively limited pool of skilled surveyors available to undertake survey work voluntarily, the substantial cost of full multi-method, multi-visit professional surveys and the practical challenges of Great Crested Newt surveying, have prevented the full roll-out of a statistically robust surveillance programme for Great Crested Newts.

eDNA seems to have overcome many of these problems. eDNA surveys have a better detection rate than traditional survey methods and can be used by a larger pool of surveyors because they need less skill and time commitment than the equivalent traditional survey methods. A national surveillance survey could be feasibly achieved either by a combination of volunteers and professional biologists, or a fully professional team.

To cover England, where most Great Crested Newt sites occur, about 550 1 km squares need to be visited, which is roughly 15 per county. This number of squares would also provide sufficient data for pond number assessments and could detect a c10% change in HSI score. In Wales a total of about 300 1 km squares would need to be visited, about 30 per county within the Great Crested Newt's range; again this is sufficient to estimate change in pond numbers and large changes in HSI score. In Scotland about 290 1 km grid squares would need to be surveyed within Great Crested Newt range to have confidence that we could detect changes in pond occupancy, HSI scores and pond numbers. This also equates to about 30 ponds per county. These numbers of squares and ponds would need to be surveyed on each occasion the survey was undertaken.

In total, in England, Scotland and Wales eDNA samples would be needed from about 2000 ponds, approximately 2 ponds per 1 km square, each time the survey was carried out. Overall, the cost of an eDNA survey in England, with 50% of samples collected by volunteers, would be just over £400,000. In Wales and Scotland the cost is about half this amount. Costs of analysing and reporting the results would be in the region of £50,000. These costs would be incurred in each year the survey was undertaken.

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Contents

1. Background and project aims

This report describes work to evaluate the use of environmental DNA to monitor the Great Crested Newt and to develop improved statistical designs for sampling methodologies for the species. The work has been supported primarily by Defra and Natural England, with additional financial and technical support from JNCC, Natural Resources Wales and Scottish Natural Heritage.

The report is divided into two main sections:

Part A deals with the results of surveys to test the effectiveness of eDNA as a survey methodology for assessing occupancy and abundance of Great Crested Newts, particularly for use by volunteers.

Part B is concerned with developing statistical sampling designs for surveys to assess change at Great Britain and country level (England, Scotland, Wales) in three metrics: pond numbers, HSI score and pond occupancy by Great Crested Newts.

1.1 Part A: Developing and testing the eDNA concept

The use of environmental DNA for the detection of cryptic or difficult to survey freshwater organisms is a rapidly developing area of research and practice which offers considerable potential benefits for nature conservation (Lodge *et al*. 2012; Sutherland *et al*. 2013). Environmental DNA (eDNA) is nuclear or mitochondrial DNA that is released from an organism into the environment. Sources of eDNA include secreted faeces, mucous, and gametes; shed skin and hair; and carcasses. eDNA can be detected in cellular or extracellular (dissolved DNA) form. DNA obtained directly from environmental samples as a method to assess the diversity of macro-organism communities was first applied to ancient sediments, revealing the past of extinct and extant mammals, birds and plants (Willerslev *et al*. 2003). Subsequently, the approach has been successfully used on several different modern and ancient environmental samples including terrestrial sediments, lake and ice cores, and freshwater lakes and rivers (Hofreiter *et al*. 2003; Haile *et al*. 2007, 2009; Willerslev *et al*. 2007; Ficetola *et al*. 2008; Matisoo-Smith *et al*. 2008; Jerde *et al*. 2011).

Ficetola et al. (2008) demonstrated the first use of eDNA to detect a cryptic aquatic animal, using the technique to detect the presence of Bullfrog (*Lithobates catesbeiana*). Since 2008, the method has so far been demonstrated to be effective at the 'proof of concept' level for a range of species groups, including amphibians (Ficetola *et al*. 2008; Goldberg *et al*. 2011; Dejean *et al*. 2011, 2012; Olson *et al*. 2012; Pilliod *et al*. 2013), fish (Takahara *et al*. 2012,), aquatic invertebrates (Thomsen *et al*. 2012) and aquatic mammals (also Thomsen *et al*. 2012) (Table 1.1). For Great Crested Newts specifically, Thomsen *et al*. (2012) were able to detect the species successfully in a sample of 11 Danish ponds, and Schmidt (pers. comm.) in a sample of 30 Swiss ponds. Thomsen *et al*. (2012) achieved a 91% detection rate (i.e. 10 out 11 sites positive) and Schmidt (pers. comm.) about a 60% detection rate.

The present project is the first to assess the use of the eDNA technique throughout the national range of a species, the Great Crested Newt, and to relate this to the statistical design of a monitoring programme.

The main aims of Part A of the project were to:

- Develop and test a primer for the Great Crested Newt
- Develop and put in place a programme of field testing to evaluate factors that might affect eDNA detectability of Great Crested Newts, including both presence/absence and abundance
- Carry out a statistical analysis comparing eDNA and traditional sampling approaches
- Produce practical guidance on the eDNA sampling methodology (note that this guidance will be produced in a short separate manual for practitioners).

Table 1.1 eDNA studies of the distribution of freshwater aquatic organisms (modified from Pilliod *et al*. 2012)

Species	Source
American bullfrog (*Lithobates catesbeianus*)	Dejean *et al*. 2012b; Ficetola *et al*. 2008
Big headed carp (*Hypophthichthys nobilis*) Silver carp (*H. molitrix*)	Jerde *et al*. 2011
Idaho giant salamander (*Dicamptodon aterrimus*) Rocky Mountain tailed frog (*Ascaphus montanus*)	Goldberg *et al.* 2011
Common spadefoot toad (*Pelobates fuscus*) Great crested newt (*Triturus cristatus*) European weather loach (*Misgurnus fossilis*) Eurasian otter (*Lutra lutra*) White-faced darter (*Leucorrhinia pectoralis*) Tadpole shrimp (*Lepidurus apus*)	Thomsen *et al*. 2012a
Sturgeon (*Acipenser baerii*)	Dejean *et al*. 2011
Common carp (*Cyprinus carpio*)	Takahara *et al*. 2012
Eastern hellbenders (*Cryptobranchus a. alleganiensis*)	Olson *et al*. 2012
Great crested newt (*Triturus cristatus*)	Benedikt Schmidt *pers. comm.*

1.1.1 Short overview of the use of eDNA methods in the detection of aquatic organisms

Environmental DNA (eDNA) is composed of intracellular DNA, present in living or freshly dead cells, and extracellular DNA, released after cell lysis (Levy-Booth *et al*. 2007). Because of this, freshwater environments (and also the soils and oceans), constitute a substantial reservoir of such environmental DNA (Pote *et al*. 2009).

Once released from organisms, DNA in the environment may persist or be adsorbed onto organic or inorganic particles. It may also be transformed by competent microorganisms or be degraded (see Levy-Booth *et al*. 2007 for a comprehensive review). Several factors operate in DNA degradation. Endogenous nucleases, water, UV radiation and the action of bacteria and fungi in the environment all contribute to DNA decay (Shapiro, 2008). A number of studies have demonstrated that medium length DNA fragments of 300 - 400 bp could be detected in water samples for up to one week in controlled conditions (Alvarez *et al.* 1996; Matsui *et al*. 2001; Romanowski *et al.* 1992). In contrast, short DNA fragments are usually very slowly degraded and can be more easily recovered from environmental samples (Deagle *et al*. 2006).

As a new technique there is still relatively limited information about some key aspects of the methodology including sensitivity of the technique, persistence of DNA in the water, the types of waterbody where the method can be most effectively used and the impact of environmental factors on degradation of DNA.

Persistence of eDNA in the water is a critical issue for eDNA detection. Thomsen *et al.* (2012) working on various freshwater species, including Great Crested Newt, demonstrated that short fragments of DNA (i.e. < 100 bp) were detectable c.a. 2 weeks after the removal of the DNA source (Figure 1.1).

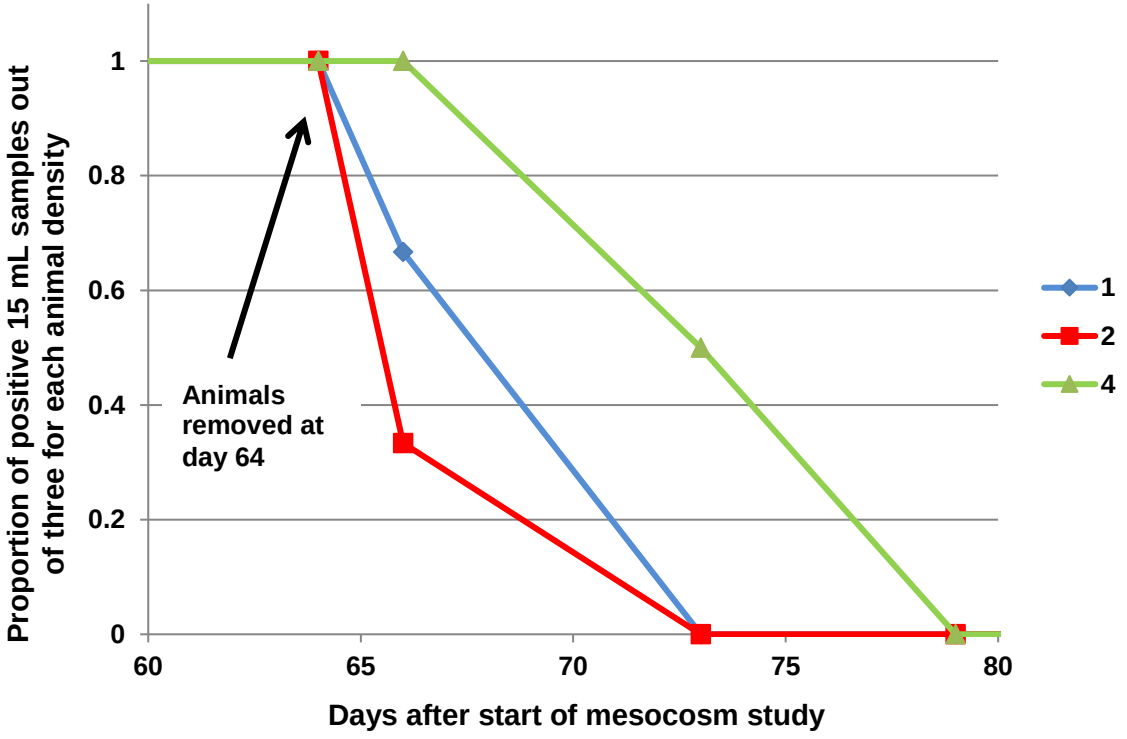


Figure 1.1. Decay of Great Crested Newt eDNA in a mesocosm situation. Newts were removed from mesocosms on day 64. Decay of eDNA to undetectable was 9, 9 and 14 days in mesocosms with 1, 2 and 4 newts, respectively. Data derived from supplementary data supplied with Thomsen *et al.* (2012).

Slightly longer persistence was noted for Bullfrog *(Lithobates catesbeianus)* tadpoles and the Siberian Sturgeon (*Acipenser baerii*) by Dejean *et al.* (2011), who found that in laboratory situations and natural waters eDNA could be detected for about a month

Equally important is the impact of environmental factors on eDNA degradation. As noted above, a range of factors are known to influence eDNA breakdown. However, much less is known about the effect of specific factors in freshwater e.g. temperature, light, and other chemicals dissolved in the water.

One of the few studies in freshwater is that of Pilliod *et al*. (2013a) who found that eDNA was no longer detectable in full-sun samples after 8 days, whereas it was still detectable in 20% of shaded samples after 11 days. Cooling also slowed breakdown: eDNA could still be detected in 100% of refrigerated control samples after 18 days.

1.2 Part B: Developing statistical sampling frameworks to assist with the development of an effective surveillance strategy for the Great Crested Newt

The aim of Part B of the project was to design statistically robust sampling strategies for three parameters of importance in reporting the conservation status of the Great Crested Newt: (i) pond turnover, (ii) habitat suitability and (iii) pond occupancy.

Specifically, the aims were to:

- Explore the power to detect change at individual country, joint England+Wales and Great Britain levels, in pond turnover, Great Crested Newt habitat suitability and pond occupancy by Great Crested Newt between two time points, using different sampling strategies.
- Explore how power changes in the three different parameters with additional repeat surveys.
- Assess the power of existing Great Crested Newt and amphibian sampling strategies.
- Present options for future sampling strategies.

The results provide an evaluation of the level of confidence in current survey and monitoring programmes, and form the basis for recommendation on new cost-effective sampling strategies.

We used existing datasets collected by the Durrell Institute of Conservation and Ecology (DICE), Amphibian and Reptile Conservation (particularly the NARRS survey), the National Biodiversity Network, the Countryside Survey and those generated by the current project, to provide data for power analysis of existing sampling strategies. We used these datasets as the basis for evaluating the strengths and weaknesses of a range of alternative sampling strategies to assess pond turnover, habitat suitability and pond occupancy by Great Crested Newts.

2. Methods

2.1 Part A methods: eDNA study

2.1.1 Approach and overview of method development

The overall aim of the eDNA work was to the evaluate the method as a monitoring technique, particularly for use by volunteers. Specifically, the eDNA work had two main stages: initial development and testing of the primer, followed by field application to evaluate its effectiveness in a wide range of ponds typifying those used by Great Crested Newts.

We **developed and tested a primer** *in silico, in vivo* and *in situ*. Suitable sequences were identified using the EMBL-Bank database, and databases held by Spygen, and used to develop a primer specifically for the Great Crested Newt. We then tested this primer against tissue swabs collected from UK Great Crested Newts and then against field water samples from three locations with no Great Crested Newts, three with low density Great Crested Newt populations and three with medium/high density Great Crested Newt populations (Figure 2.7 i).

Given the above tests showed that the method specifically detected Great Crested Newts in the laboratory and field, we then collected specific sets of eDNA samples to test its effectiveness in five different situations:

1. Out of range sites to test for false positives. We collected eDNA samples from ponds just beyond the known range of the Great Crested Newt. All samples were collected professionally from a region to the west of the known species range in Cornwall (n=30) (Figure 2.7 ii).

2. Sites for detailed comparison with 'traditional' survey techniques. We surveyed 20 ponds in the New Forest and 15 ponds in north-east Wales on four occasions from mid-April to the end of June 2013 (Figure 2.7 iii). Fifteen of the sites in the New Forest were surveyed by a professional team from Hampshire Ecological Services Ltd who are highly experienced in Great Crested Newt field survey work. The remaining sites were surveyed by volunteers supervised directly by the project team. In north-east Wales, all ponds were surveyed by a volunteer team led Natural Resources Wales. This team typified Great Crested Newt 'volunteer' surveyors in that it included both highly experienced professionals herpetologists working in their own time, and much less experienced environment sector workers who, as well as giving their own time to the project, also aimed to gain experience of working with Great Crested Newts.

3. Volunteer sites: single samples collected from 239 ponds widely distributed through the Great Crested Newt's range in England, Scotland and Wales (Figure 2.7 iv). The sampling programme was intended to assess the effectiveness of the technique for use by volunteers and to evaluate environmental factors influencing the eDNA method. An eDNA sample was collected from each site once during the peak period of Great Crested Newt breeding activity from mid-May to June 2013. All locations were selected on the basis that there was evidence this year that the pond supported Great Crested Newts, normally as a result of torch counts or other standard survey work undertaken by the volunteers. All sites were confirmed as Great Crested Newt sites before eDNA test kits were sent to volunteers. Volunteers in the three PondNet trial regions collected samples in Cheshire, Hampshire and Yorkshire. Outside these areas samples were collected mainly by volunteers with an interest in amphibian recording. These sites provide a test of the occurrence of ***false negatives*** in volunteer collected samples i.e. sites where newts are present but an eDNA survey or analysis does not detect them.

4. Test for within-range false positives. Although not originally part of the planned work, because the results of the eDNA work were looking so positive, project staff and volunteers surveyed a further 30 sites ***within*** the range of the newt - mostly in Hampshire, Oxfordshire and Greater London - to assess the more realistic risk of ***within range false positives***. All sites were locations where there was local knowledge that it was ***very unlikely*** newts would

be present, although we did not survey the sites specifically to assess this (Figure 2.7 iii). For example, in Hampshire sites included field study centre ponds which are regularly used for teaching, where Great Crested Newts had not been seen over a number of years, large fish ponds where local surveyors had never seen Great Crested Newts, and garden ponds belonging to members of the survey team where Great Crested Newts had never been seen.

5. Volunteer sampling quality assurance. We planned to resurvey 30 volunteer sites using a professional member of our team to assess variability amongst volunteer surveyors. In practice, 26 sites were resurveyed because at 3 sites the volunteer did not send back a water sample, and at one site the volunteer's sample leaked in the post.

2.1.2 Developing and testing the primer

Great Crested Newt (*Triturus cristatus*) DNA was amplified using primers and probes designed by Thomsen *et al*. (2012) that amplify a fragment of the cytb gene. Those markers were first tested *in silico* PCR using the ecoPCR software (Taberlet *et al.* 2007, available at http://www.grenoble.prabi.fr/trac/ecoPCR) on the EMBL-Bank release 114 (released in December 2012) and SPYGEN's reference database. When analysing only the primer pairs, without taking into account the probe, the primers amplified 63 species present in GenBank. When the bioinformatic analysis was done using the primers and the probe they were found to bind perfectly with *T. cristatus* DNA, but also, with some mismatches, to *Melanotaenia splendida, Taricha torosa, Triturus carnifex* and *Triturus karelinii. Melanotaenia splendida* is a warm water fish native to Australia and *Taricha torosa* is the California newt. The two *Triturus* species are, respectively, the Italian Crested Newt and the Southern Crested Newt.

Because of the number of mismatches and their position on the primer binding sites, the chance of amplifying these species is very low, but we cannot exclude their amplification. All are absent from the UK, except for the Italian Crested Newt which is known from the Newdigate area in Surrey, and perhaps elsewhere, although these populations are not believed to be spreading (Jehle *et al.* 2011). None of the sites at which eDNA samples were collected were suspected to be supporting this species.

Primers and probes were tested *in vitro* against 16 swabs collected in three different populations of *T. cristatus* in Great Britain. DNA was extracted using the DNA Blood and Tissue kit (Qiagen®) following the manufacturer's instructions. The quantitative PCR was performed in a final volume of 25 µL, which included 3 µL of template DNA, 12.5 µL of TaqMan® Environmental Master Mix 2.0 (Life Technologies ®), 6.5 µL of ddH2O, 1 µL of each primer (10 µM, TCCBL and TCCBR) and 1 µL of probe (2.5 µM TCCB Probe) all under thermal cycling at 50 °C for 5 minutes and 95 °C for 10 minutes, followed by 55 cycles of 95 °C for 30 seconds and 56.3 °C for 1 minute. Samples were run on a BIO-RAD® CFX96 Touch real time PCR detection system. The DNA extracted was quantified using a Qubit (Life Technologies ®). A dilution series of *T. cristatus* DNA, ranging from 10^{-1} ng μL^{-1} to 10^{-4} ng μL^{-1}, was used as qPCR standard. Additionally, primers and probes were also tested on tissue samples of *Triturus marmoratus* and *Triturus carnifex* and none of these samples were amplified, showing the suitability of the primer pair and probe.

The limit of detection (LOD, i.e. the minimum amount of target DNA sequence that can be detected in the sample) and the limit of quantification (LOQ, i.e. the lowest amount of target DNA that yields an acceptable level of precision and accuracy) were calculated by running a dilution series of a known amount of *T. cristatus* DNA, ranging from 10^{-1} ng μL^{-1} to 10^{-10} ng μL^{-1} (10^{9} and 1 molecules, respectively) with 12 replicates per concentration. These tests demonstrated that the LOQ in this study was 10^{-3} ng μL^{-1} and that *T. cristatus* DNA can still be detected at a concentration of 10^{-9} ng μL^{-1}, with at least one qPCR replicate in twelve showing a positive result. This concentration was set as the LOD.

Following *in vitro* testing, primers and probe were tested *in situ.* Nine samples were collected between April and May in ponds where *T. cristatus* density was known. Three samples were collected from ponds with low density Great Crested Newt populations, three with

medium/high density populations and three from ponds where the species was absent. DNA was extracted following the protocol proposed by Ficetola *et al.* (2008) after slight modifications. At each of the nine sites a standard eDNA sample was collected comprising 6 replicates of 15 mL of pond water preserved with 35 mL of ethanol (see Section 2.1.3 below describing the field sampling method). The six subsamples per site were centrifuged at 14000 x g, for 30 minute, at 6 °C and the supernatant discarded. After this step, 360 µL of ATL Buffer of the DNeasy Blood & Tissue Extraction Kit (Qiagen) were added in the first tube, the tube was vortexed and the supernatant was transferred to the second tube. This operation was repeated for all the six tubes. Finally, the supernatant in the 6th tube, containing the DNA concentrated from all 6 sub-samples, was transferred in a 2 mL tube and the DNA extraction was performed following the manufacturer's instructions. DNA extraction was performed in a room dedicated for degraded DNA samples. An extraction control was performed to monitor possible contaminations.

The samples were amplified using the protocol described above. In the *in situ* tests, Great Crested Newts were detected at all sites where they were present and in none of the sites where they were absent (Table 2.1).

Table 2.1 In situ sites where the primer was tested

Code[1]	Location name	Density	eDNA qPCR positive replicates[2]
WM00854	Shetland Site 1	Absent	0/12
WM00855	Shetland Site 2	Absent	0/12
WM00848	Shetland Site 3	Absent	0/12
WM00826	Hatchet Triangle	Low	4/12
WM00823	Windyeats Pond	Low	3/12
WM00822	Standing Hat	Low	9/12
WM00845	Valley Gardens	Medium/High	12/12
WM00830	Woods Corner	Medium/High	3/12
WM00837	Balmer Lawn	Medium/High	12/12

Key: 1 = the unique code for all eDNA samples collected in the project; 2 = the eDNA qPCR positive replicates (0/12 to 12/12) refers to the number of qPCR replicates in which DNA was detected.

2.1.3 Field method for collecting eDNA sample

To collect eDNA we followed the sampling procedure developed by Spygen, described here. Samples were collected following this procedure in all components of the study: the detailed methodological study, the volunteer survey and the tests for false positives. Water samples were collected using the sampling kit supplied for this work by Spygen. The sampling kit has 5 components (Figure 2.1):

- A sterile 30 mL ladle
- A sterile self-supporting plastic bag with 1 litre capacity
- A sterile 10 mL pipette to resample the pond water
- Six sterile 50 mL centrifuge tubes containing preservative (Absolute Ethanol (200 Proof), Molecular Biology Grade, Fisher BioReagents™ and other markers)
- 2 pairs of sterile gloves.

Sample kits were supplied to the Freshwater Habitats Trust in Oxford by Spygen in several batches. Kits were stored in a cold room for up to c.1 month before they were dispatched to volunteers or used in the field by professional staff. We advised volunteers or professional surveyors working away from Oxford to store kits prior to use in a domestic fridge although we did not check whether all did so. We do not now believe this is technically necessary and will modify sampling instructions accordingly.

To collect an eDNA sample, surveyors collected a 30 mL water sample at 20 locations around the pond margin using the sterile ladle supplied in the sampling kit (total approximately 600 mL). Samples were collected whilst the surveyor stood only on the pond bank or muddy pond edges, but without entering the water. The 20 separate samples were pooled into a single sample in the sterile, self-supporting, plastic bag. The water samples were homogenised by gently shaking the bag to ensure that eDNA was evenly mixed through the sample. Six subsamples of 15 mL of pond water were then pipetted from the bag into sterile tubes containing 35 mL of ethanol to preserve the eDNA sample. Samples were then returned at ambient temperature to Oxford where they were stored in a cold room before being transported to the Spygen laboratories in France for analysis. We advised volunteers who were posting samples back to Oxford to store collected samples in a domestic refrigerator temporarily. We adopted this approach because, although alcohol is a good preservative for DNA at room temperature, breakdown is slower at lower temperatures. As we were retaining samples in the UK for up to 1 month in England before transport to Spygen labs in France we adopted a precautionary approach of keeping samples refrigerated. However, the short periods at ambient temperature (e.g. during the transportation of samples) do not affect the quality of the results. For future stages of the programme we believe it will be sufficient to keep sampling kits at cool ambient temperatures before use, and refrigerated once collected. As the samples contain an artificial DNA marker to check for unexpected decay of DNA it remains possible to assess whether kits have degraded unacceptably before analysis. There was no evidence of DNA decay during the study.

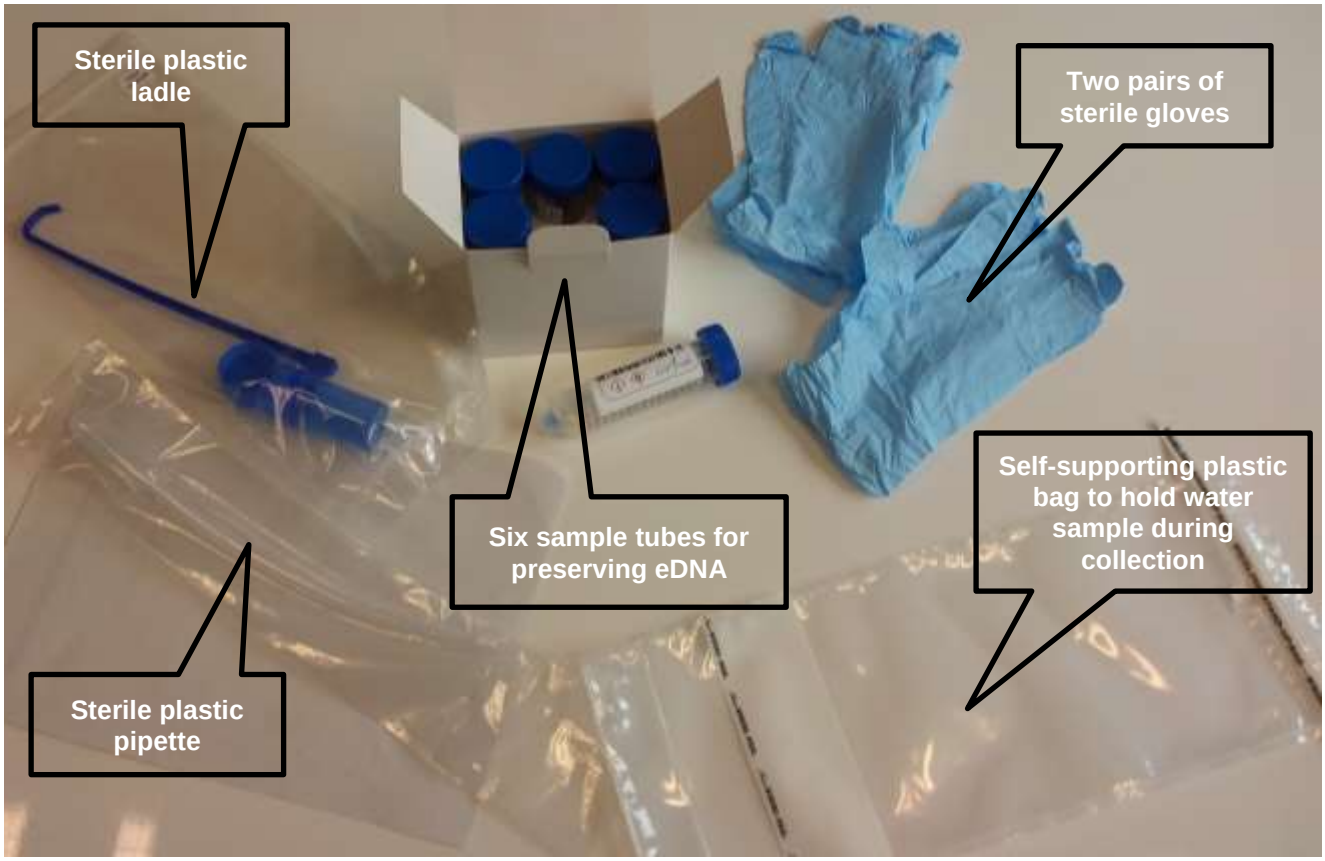


Figure 2.1 The sampling equipment used to collect the eDNA sample

A joint field meeting with Spygen, FHT staff and Hampshire Ecological Services Ltd was held before beginning sampling to finalise water sampling methods. Following this, written instructions were provided for both professionals and volunteers using the method (see Appendix 1). We did not specifically train either professionals or volunteers face to face, relying solely on the written instruction.

In total, 89 volunteers requested eDNA sampling kits with most returning one or more samples (6 project team members also collected a small number of samples but are excluded from this total). 26 volunteers came from the three PondNet pilot regions, with the remainder reached mainly through amphibian networks, particularly NARRS, the National Amphibian and Reptile Recording Scheme (Table 2.2). Sampling kit distribution was co-ordinated from the Freshwater Habitats Trust. Generally we posted samples in small batches to volunteer surveyors, most collecting one or two samples, although one volunteer surveyor organised collection from 30 sites (Figure 2.2). A small number of volunteers were unable to return kits to us having previously indicated that they would: in total we distributed 256 kits to volunteers of which 239 were used and returned to us, a 7% wastage rate. The total number of eDNA samples collected in different sections of the project is summarised in Table 2.5.

Table 2.2 Number of PondNet and other volunteers who collected the volunteer eDNA samples

Total number of volunteers	Number of PondNet volunteers	Number of other volunteers
86	26	60
Note that the total number of volunteers excludes 6 paid members of the project team who also collected a small number of volunteer samples		

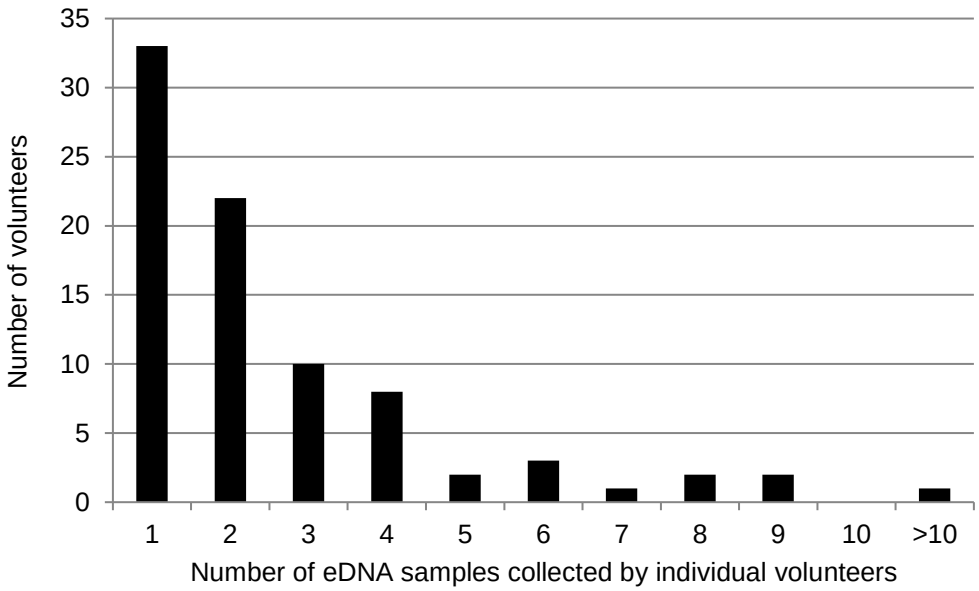


Figure 2.2 Number of eDNA samples collected by individual volunteers. Most volunteers collected one or two samples. One volunteer collected 30 samples.

Our objective was to collect samples during the peak of the Great Crested Newt breeding season and this was broadly achieved. Samples were collected by volunteers between 6 May 2013 and 6 July 2013 (Figure 2.3). 84% of samples were collected between 15 May and 17 June 2013.

Once samples had been collected, kits were returned to Oxford and held in the cold room for up to c 3 weeks, and then returned to Spygen at Le Bourget du Lac, France in three batches. Sample delivery by road took about 12 hours and was undertaken at ambient temperature.

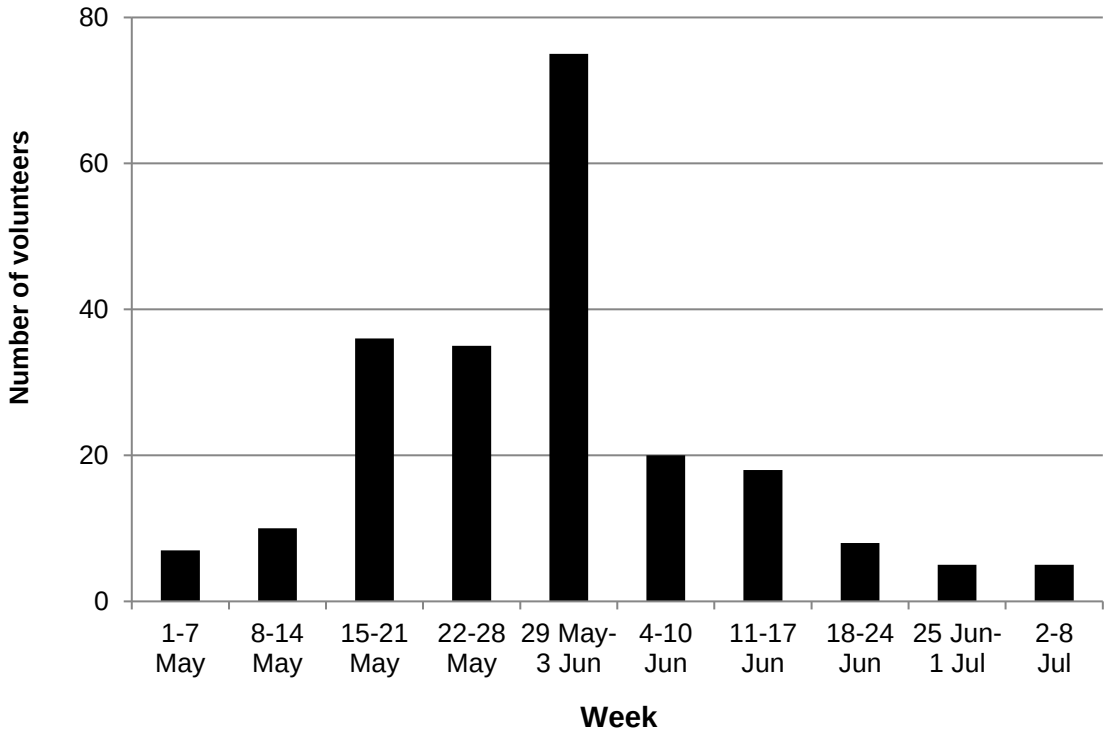


Figure 2.3 Time of volunteer eDNA sample collection.

2.1.4 eDNA laboratory analytical methods

All the samples were extracted following the protocol described in Section 2.1.2. DNA extraction was performed in a room dedicated to degraded DNA sample analysis. Extraction controls were systematically performed to monitor possible contamination.

After the DNA extraction the samples were tested for inhibition by qPCR. The quantitative PCR was performed in a final volume of 25 µL, using 3 µL of template DNA, 3 µL of 10^{-3} ng/µL of DNA of a synthetic gene, 12.5 µL of TaqMan® Environmental Master Mix 2.0 (Life Technologies ®), 3.5 µL of ddH2O, 1 µL of each specific primer for the synthetic gene (10 µM) and 1 µL of probe (2.5 µM) under thermal cycling 50 °C for 5 minutes and 95 °C for 10 minutes, followed by 55 cycles of 95 °C for 30 seconds and 52 °C for 1 minute. All the samples were analysed in duplicate. If at least one of the replicates showed a different Ct than expected, the sample was considered inhibited and diluted twice before the amplification with *T. cristatus* primer and probes. Fifty-two samples (c.a. 11.3% of the total) were found to be inhibited and diluted twice before amplification with *T. cristatus* primer and probes.

The samples were amplified using the protocol described in the Section 2.1.2. Each sample was run in 12 replicates. A dilution series of *T. cristatus* DNA, ranging from 10^{-1} ng μL^{-1} to 10^{-4}

ng μL^{-1}, was used as a qPCR standard. Four negative (UHQ water) controls were systematically added during the qPCR step.

eDNA results are reported as the proportion of the 12 replicate qPCR samples from each water sample that were successfully amplified ('positive'). In the qPCR analysis the 12 replicates are arranged in wells along one side of a PCR well plate (Figure 2.4) where each PCR reaction takes place. Throughout the report, for simplicity, we have called the number of qPCR positive replicates the 'eDNA score'. In all cases, the estimated concentrations of DNA were below the limit of quantification (LOQ, i.e. less than 10^{-3} ng/l^{-1}.), meaning that eDNA quantification was not possible.

Despite this we have been able to use eDNA score as a proxy for the amount of eDNA in the water. Our working assumption is that the more eDNA there is in the sample, the greater the number of positive qPCR replicates: thus we believe that a sample with 1/12 positive replicates has less eDNA than a sample with 12/12 positive replicates, although at present we cannot formally test this assumption.

In reporting the results of PCR replicates we have either described the proportion of the replicates in which DNA was successfully amplified (e.g. 1/12, 12/12) or converted the proportion to a decimal fraction to facilitate analysis. (i.e. 1/12 becomes 0.08 and 12/12 becomes 1.0).

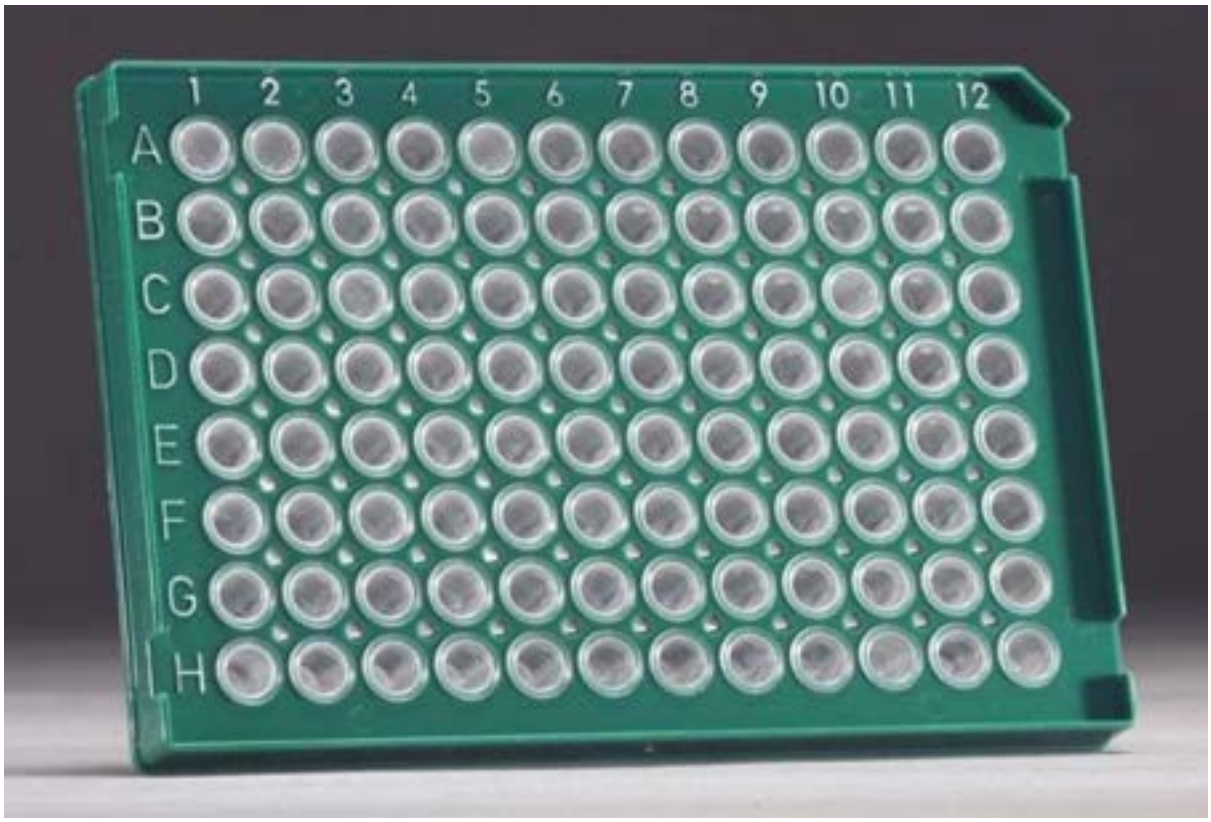


Figure 2.4. A PCR well plate. Replicates from a sample are arranged along a line of 12 wells (numbered 1-12). Samples are arranged in lines from A – H.

2.1.5 Methods used in the detailed methodological study to compare eDNA and traditional Great Crested Newt survey methods

(i) Field methods

Detailed comparisons of traditional survey methods and eDNA survey methods were undertaken. Ponds were visited on four occasions at 2-3 week intervals, from mid-April to late June 2013 (Figure 2.5 a,b; Table 2.3). 20 ponds were surveyed in the New Forest and 15 ponds in north-east Wales. In the New Forest, each group of visits (Visit 1, 2, 3, 4) was spread over a period of a week or more because ponds were further apart and could not all be visited on the same night. In Wales, apart from Visit 1, at each visit all ponds were visited during the same 24 hour period.

In the New Forest, surveys of 15 sites were undertaken by a professional survey team from Hampshire Ecological Consultants. 5 further sites were surveyed by volunteers co-ordinated by Freshwater Habitats Trust. In north-east Wales all sites were surveyed by a volunteer team co-ordinated by Natural Resources Wales.

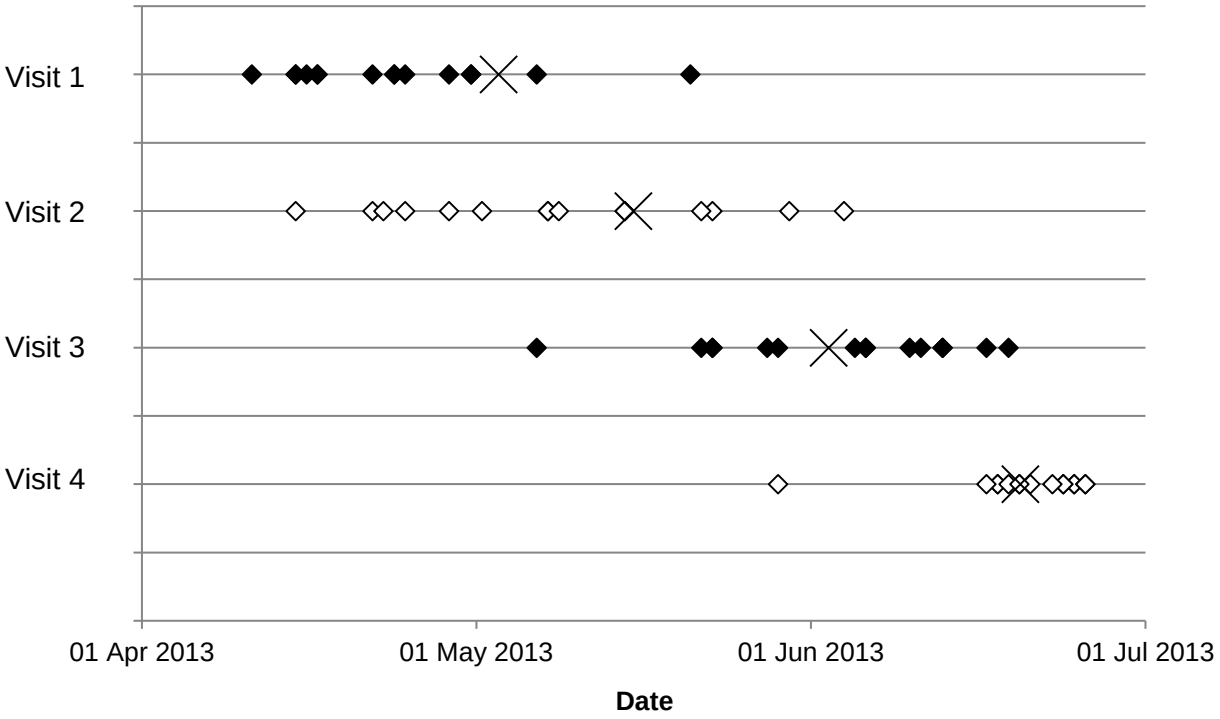
Four traditional methods were compared: torch counting, bottle trapping, egg searches and daylight visual detection of adults, immatures or larvae. In practice, daylight detection did not prove a practically useful technique as very low numbers of animals were detected by this method and it was not considered further in the analysis. We did not use netting as a technique because it may damage vegetation which is used by Great Crested Newts and because experienced surveyors now consider this technique is likely to lead to unacceptable levels of injury to the larval stages of Great Crested Newts.

Surveys were designed to provide independent data on each of the survey methods. At all sites evening torch counts were undertaken first, followed by collection of eDNA and then setting of bottle traps. Torch counts used a 1 million candlepower Cluelight CB2 torch. Torch counts were undertaken in the New Forest mainly between 21:30 and 23:30 hrs and took on average 24 minutes. On average 84% of the ponds‘ shorelines was accessed. In north-east Wales two-thirds of torch counts were carried out between 21.50 and 22.50 hrs and most of the remaining third between 22.50 and 23.50 hrs. On average counts took 24 minutes in north-east Wales and 97% of the ponds’ shorelines was accessed.

Bottle traps were set at approximately 2 m intervals around the shoreline and numbers of traps deployed varied from 17 to 62 per pond (Table 2.4).Traps were left overnight and collected in the morning. If eggs had not been seen at any other time during the survey, a search lasting up to 20 minutes was undertaken for newt eggs in the morning when traps were retrieved. Bottle traps followed the same design as used by Sewell *et al*. (2010).

The sequence of survey work (which required collecting eDNA samples after dark) was designed to (a) avoid going in the water or disturbing sediments before the eDNA sample was collected and (b) to ensure that methods were, as far as practically feasible, independent of each other and did not influence the subsequent observation. For true independence all surveys would need to be undertaken on randomly selected dates but this was not feasible within the practical constraints of the project. Thus we delayed the setting of traps and approaching the pond to collect eDNA until *after* torch counts were competed to avoid unnecessarily disturbing animals which might then seek refuge amongst vegetation or in deeper water areas, potentially reducing numbers encountered when torching.

(a) Timing of sites visits in New Forest



(b) Timing of sites visits in north-east Wales

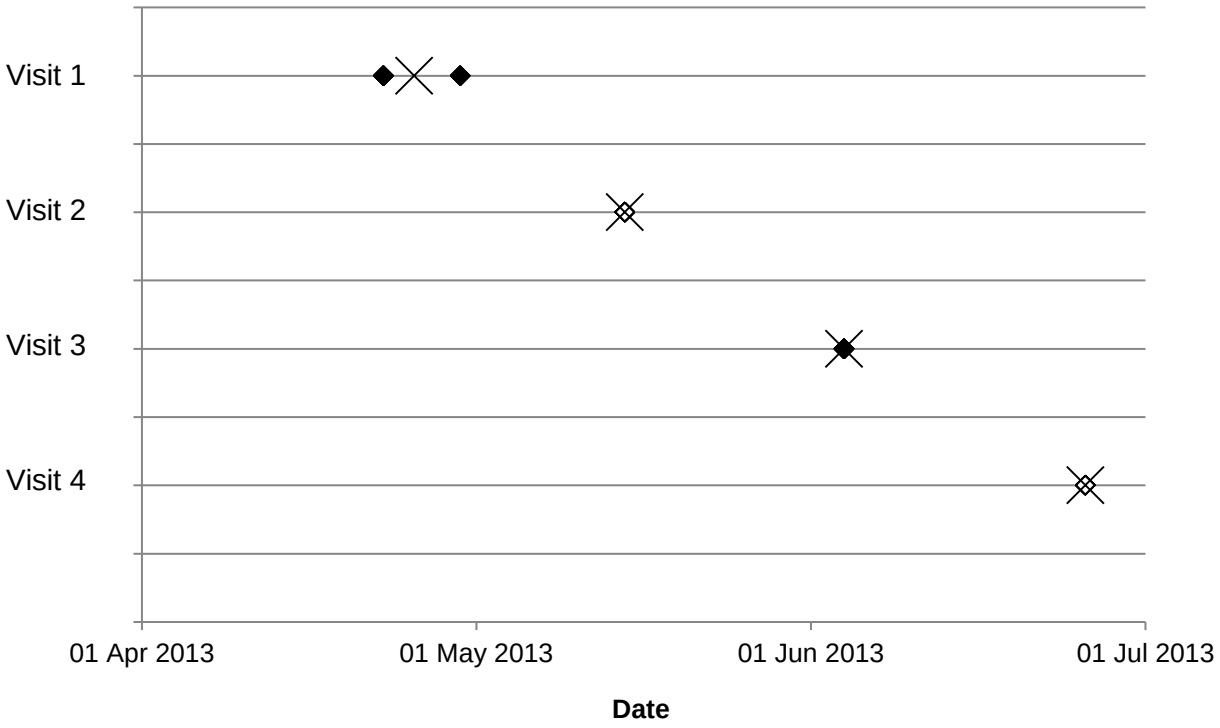


Figure 2.5 Timing of surveys in detailed methodological studies: (a) New Forest site visits (b) north-east Wales site visits.
The large X marks the mean date of visits. In the New Forest the average time between Visits 1-2, 2-3 and 3-4 were, respectively, 2.1, 3.6 and 2.5 weeks. In north-east Wales, where all ponds were on the same site, visits were all usually made on the same day. Time between visits in Wales was, respectively, 2.7, 2.9 and 3.1 weeks.

Ponds in the New Forest were located at a variety of sites over a roughly 50 km^2 area; ponds in north-east Wales were all on the Brookhill Great Crested Newt mitigation site at Buckley. The Brookhill ponds were specially designed for Great Crested Newts and are located close together (Figure 2.6) and may therefore present an especially good situation for Great Crested Newt surveys. We explored obtaining additional sites by working with consultants who were already undertaking 4 visit type standard surveys in the course of Great Crested Newt mitigation projects. In practice, this proved too hard to organise and we did not obtain further survey data by this route.

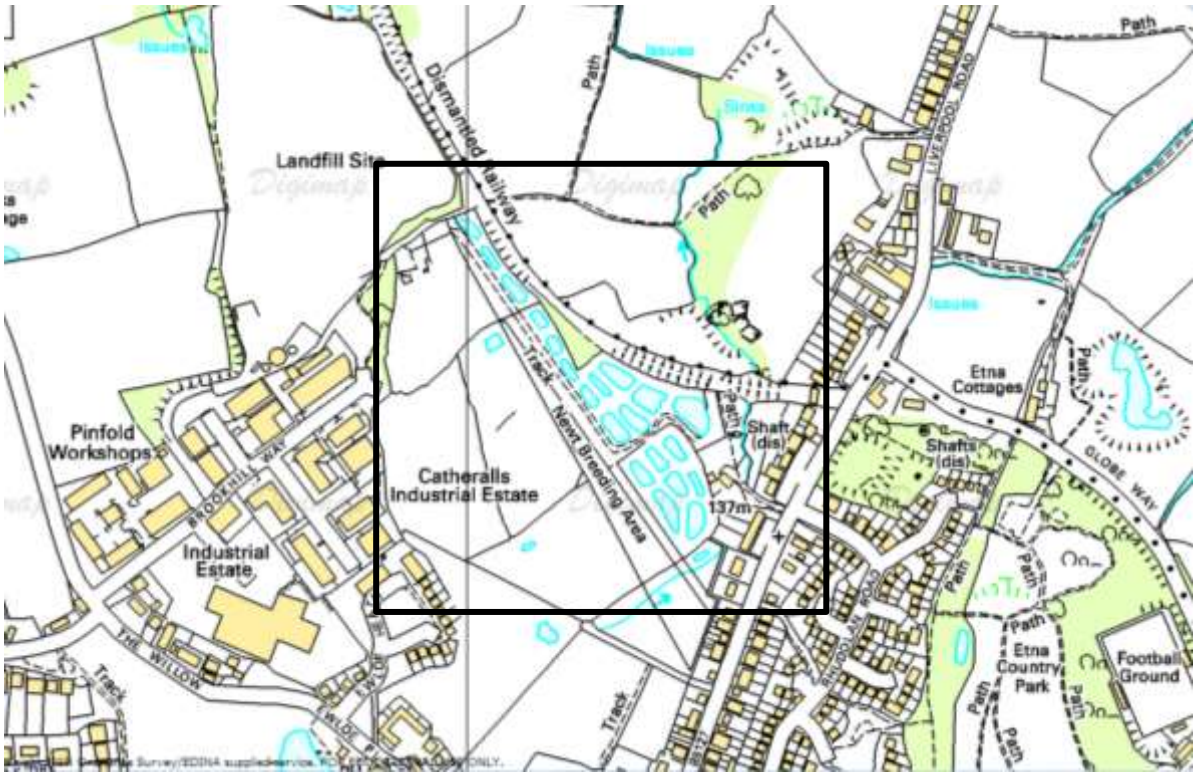


Figure 2.6 Ponds on the Brookhill Great Crested Newt mitigation site at Buckley / Bwcle in north-east Wales (Flintshire). 15 ponds on this site were used as part of the detailed methodological study.

At each visit an eDNA sample was collected using the standard methods outlined in Section 2.1.3 and newt presence and abundance assessed by the traditional methods. This gave a total of 140 sampling occasions when eDNA could be compared to 'traditional' methods. Pond survey dates are shown in Table 2.3.

Table 2.3 Dates of survey visits in the detailed methodological study

Note that in Wales, pond numbering follows a pre-existing system already applied to the Brookhill site, where all the ponds are located, to assist future survey work. Thus ponds 1, 7, 16 and 17 were not included in this survey.

	Date of survey visit			
New Forest	**Visit 1**	**Visit 2**	**Visit 3**	**Visit 4**
Pond 1	17-18 April	22-23 April	23-24 May	25-26 June
Pond 2	16-17 April	25-26 April	22-23 May	20-21 June
Pond 3	15-16 April	30 Apr-1 May	29-30 May	18-19 June
Pond 4	15-16 April	23-24 April	28-29 May	18-19 June
Pond 5	24-25 April	8-9 May	10-11 June	25-26 June
Pond 6	11-12 April	15-16 April	7-8 May	29-30 May
Pond 7	17-18 April	8-9 May	6-7 June	24-25 June
Pond 8	22-23 April	29-30 April	23-24 May	17-18 June
Pond 9	29 April	23-24 May	17-18 June	19-20 June
Pond 10	21-22 May	4-5 June	19-20 June	24-25 June
Pond 11	1-2 May	15-16 May	13-14 June	26-27 June
Pond 12	1-2 May	15-16 May	13-14 June	26-27 June
Pond 13	24-25 April	2-3 May	6-7 June	23-24 June
Pond 14	25-26 April	9-10 May	11-12 June	21-22 June
Pond 15	7-8 May	22-23 May	5-6 June	20-21 June
Pond 16	5-6 May	18-19 May	1-2 June	22-23 June
Pond 17	24-25 April	15-16 May	5-6 June	19-20 June
Pond 18	24-25 April	15-16 May	5-6 June	19-20 June
Pond 19	22-23 April	9-10 May	29-30 May	20-21 June
Pond 20	25-26 April	13-14 May	28-29 May	18-19 June
North-east Wales	**Visit 1**	**Visit 2**	**Visit 3**	**Visit 4**
Pond 2	23-24 April	15-16 May	4-5 June	26-27 June
Pond 3	23-24 April	15-16 May	4-5 June	26-27 June
Pond 4	23-24 April	15-16 May	4-5 June	26-27 June
Pond 5	23-24 April	15-16 May	4-5 June	26-27 June
Pond 6	23-24 April	15-16 May	4-5 June	26-27 June
Pond 8	23-24 April	15-16 May	4-5 June	26-27 June
Pond 9	23-24 April	15-16 May	4-5 June	26-27 June
Pond 10	23-24 April	15-16 May	4-5 June	26-27 June
Pond 11	23-24 April	15-16 May	4-5 June	26-27 June
Pond 12	30 Apr-1 May	15-16 May	4-5 June	26-27 June
Pond 13	30 Apr-1 May	15-16 May	4-5 June	26-27 June
Pond 14	30 Apr-1 May	15-16 May	4-5 June	26-27 June
Pond 15	30 Apr-1 May	15-16 May	4-5 June	26-27 June
Pond 18	30 Apr-1 May	15-16 May	4-5 June	26-27 June
Pond 19	30 Apr-1 May	15-16 May	4-5 June	26-27 June

Table 2.4 Number of bottle traps used on each sampling occasion in the detailed methodological study.

0 values indicate visits when ponds were too shallow to use traps without risk of injury to newts. Note that in Wales, pond numbering follows a pre-existing system already applied to the Brookhill site, where all the ponds are located, to assist future survey work. Thus ponds 1, 7, 16 and 17 were not included in this survey.

	Number of bottle traps deployed			
New Forest ponds	**Visit 1**	**Visit 2**	**Visit 3**	**Visit 4**
1	30	28	28	25
2	40	40	45	25
3	30	30	30	15
4	30	44	45	45
5	45	45	45	45
6	15	15	15	15
7	45	45	44	45
8	45	45	43	44
9	21	21	21	21
10	30	29	29	29
11	20	20	20	20
12	29	30	30	30
13	31	31	30	28
14	60	59	60	60
15	35	45	46	45
16	35	45	46	45
17	15	15	10	0
18	15	15	15	15
19	15	10	0	0
20	20	20	20	20
North-east Wales ponds	**Visit 1**	**Visit 2**	**Visit 3**	**Visit 4**
2	48	39	48	30
3	57	52	49	39
4	32	34	30	29
5	57	62	61	49
6	31	31	25	26
8	33	41	37	26
9	47	40	38	34
10	43	37	40	45
11	30	27	41	26
12	26	27	25	23
13	25	28	24	24
14	37	41	35	35
15	21	21	21	21
18	31	31	31	30
19	17	14	12	24

Table 2.5 Total number of eDNA samples collected

Section of the study	Number of sites
Development of the primer	**9**
Out of range zero sites	**30**
Zero sites within range	**30**
Detailed methodological study	**142**
Volunteer Survey	**239**
- England	***163***
Cheshire	27
Hampshire	40
Yorkshire	31
Other areas of England	63
- Scotland	***39***
- Wales	***39***
Quality assurance	**25**
Additional autumn samples	**9**
All samples	**484**

In the project proposal we indicated that the data collected in the detailed methodological study would be used to extend the work of Sewell *et al.* (2010). In practice this was not possible, mainly because of unexpected methodological difference between the present work and the Sewell *et al.* (2010) study. In that work, four survey techniques were used: daytime visual encounter surveys searching for all life stages, but particularly for eggs; night counts using a 500,000 candlepower torch; netting using a standard dip net with a 2 mm mesh; and bottle trapping using a simple trap constructed from 2 litre plastic bottles. However, in practice the total number of animals recorded in daylight visual encounters, torch counts and netting were summed as a single value so it was not possible to assess independently the effectiveness of these methods. Data from bottle traps ***were*** recorded separately so the Sewell study assesses, effectively, the benefit of adding bottle trapping to the three other methods. The study therefore did not allow the comparative effectiveness of all individual methods to be tested, as was the case here. There were other small but notable methodological differences: in the Sewell *et al.* (2010) study the maximum number of bottle traps deployed did not exceed 25 suggesting that there was probably greater trapping effort used in the present work. This has implications for the interpretation of the effectiveness of bottle trapping as a technique.

(ii) Analytical methods

Detailed methodological study. We evaluated differences in rates of detection of Great Crested Newts by different 'traditional' survey methods and eDNA in the detailed methodological study. All sites were known to support newts so we were able simply to compare the success rate of different methods at detecting newts. The statistical significance of differences was tested using McNemar's test. McNemar's test is the appropriate test for binary paired data. It assesses whether the difference in the numbers of discordant pairs is greater than you would expect by chance. In this context, discordant pairs are the number of times when eDNA detected Great Crested Newts when other methods (e.g. torching, bottle trapping) did not.

Newt abundance and eDNA. eDNA scores were non-normal. We examined the relationship between eDNA score and newt counts using Spearman's rank correlation. eDNA scores show substantial variability (see for example the approach adopted by Pilliod *et al*. 2013). To

explore the data further, therefore, we also grouped low, medium and high eDNA scores and compared groups with the Kruskal Wallis H statistic.

2.1.6 The Tom Langton dataset: a volunteer collected set of eDNA samples from 30 sites around the Dew's Farm Special Area of Conservation

Over the last 20 years Tom Langton (a highly experienced herpetologist) has been managing and creating ponds around the Dew's Farm Special Area of Conservation, created for Great Crested Newts, in Suffolk. Annual torch counts of Great Crested Newts are available from 30 ponds in a cluster extending over an area of about 5 square kilometres. Monitoring of these sites has been taking place for up to 22 years; new ponds, or recently renovated ponds have shorter time series' of records. Torch count and eDNA data were also collected by Tom and colleagues in 2013. 28 of the ponds were known to have Great Crested Newts present; at 2 ponds Great Crested Newts had been seen very rarely or never.

We compared eDNA scores with the annual count data for 2013, and with the peak count for previous years (up to 22 years).

2.1.7 Volunteer site survey methods

We organised the collection by volunteers of a single eDNA sample from a wide variety of ponds in England, Wales and Scotland. Samples were taken in the three PondNet pilot regions (Cheshire, South Hampshire and north-east Yorkshire), and more widely in England, Wales and Scotland, by 86 volunteers (Appendix 2). In a few locations we paid for the time and travel expenses of professional ecologists to collect the samples. The distribution of sampling locations is shown in Figure 2.7.

In addition to testing the ability of volunteers to collect eDNA samples effectively, the survey was specifically intended to assess the frequency of false negatives. For this reason, all sampling locations were initially selected as sites with recent evidence of the occurrence of Great Crested Newts, as far as possible from observations made in the current 2013 field season.

Sites in the PondNet regions were chosen by a combination of volunteers local knowledge and Freshwater Habitats Trust staff regional co-ordinators. Sites spread more widely outside the PondNet regions were selected by volunteers on the basis of their local knowledge, advice from local specialists and in discussion with other project team members. In practice, a small proportion of sites (less than 3%, a total of 7 sites) were locations where there was good evidence that newts were ***absent***. These sites were retained in the analysis because they provided useful information on the likely occurrence of false positive results.

Sampling locations were broadly representative of ponds in the range of the Great Crested Newt (see Section 3.5). However, as the sampling locations were selected by the volunteers, and did not follow a stratified random design, we evaluated relationships between environmental factors and eDNA detection using non-parametric analytic methods (see Section 3.5).

At the volunteer locations a standard HSI score sheet was completed and the eDNA sample collected. Most volunteers collected between 1 and 6 samples, although one volunteer with a detailed long-term knowledge of newt populations around a Suffolk Great Crested Newt SAC collected eDNA from 30 sites.

2.1.8 Within-range false positives

We did not originally plan to test within-range false positives. However as the project progressed and most sites with newts had positive eDNA results (i.e. had a low rate of false negatives) we decided it would be valuable to assess the more realistic risk of ***within range false positives***. Within range sites which do not have newts are difficult to confirm conclusively as there is always the potential for one or two newts to briefly visit a site. In this section of the study all sites were locations where there was local knowledge that it was ***very***

unlikely newts would be present, although we did not survey the sites specifically to confirm newt absence. 30 sites very unlikely to support newts were sampled by project staff and volunteers ***within*** the range of the newt - mostly in Hampshire, Oxfordshire and Greater London. In each case a standard eDNA sample was collected and an HSI survey completed. The ponds surveyed comprised closely watched garden ponds belonging to project team members where Great Crested Newts had either never been seen, or only seen very rarely; large fish-inhabited ponds with recent evidence that newts were absent or extremely infrequently observed; and sites at local nature reserves known to lack Great Crested Newts. Several sites were located in Fryent Country Park in the north London suburbs where a regular programme of amphibian monitoring over the last 20 years has never recorded Great Crested Newts. In fact, at this site reintroduction of Great Crested Newts is actively under consideration.

2.1.9 Volunteer sampling quality assurance

We planned to resurvey 30 volunteer sites using a professional member of our team to assess variability amongst volunteer surveyors. In practice only 26 sites were resurveyed (11%) as at three sites volunteers did not collect a sample as planned, and the fourth sample leaked in the post and was lost.

2.1.10 Analysis of environmental factors which may influence eDNA detection

(i) Were the ponds surveyed representative of those with a closely associated Great Crested Newt record throughout Great Britain?

To evaluate the extent to which the volunteer eDNA survey ponds reflected the heterogeneity of ponds which are likely to be used by Great Crested Newts nationally we examined their representativeness in four ways:

- Did the volunteer ponds cover a substantial proportion of the ponds likely to be used by the Great Crested Newt throughout its range in the UK?
- Did the volunteer ponds represent the full range of pond sizes likely to be used by Great Crested Newts?
- Did the volunteer ponds match the altitudinal distribution of ponds likely to be used by newts?
- Did volunteer ponds occur in the same proportions in Defra land classes as the ponds nationally which are likely to be used by Great Crested Newts?

We then compared the characteristics of the volunteer survey ponds to the characteristics of ponds close to (i.e. within 1 km) known Great Crested Newt records. We simulated the distribution of Great Crested Newt ponds by relating newt distribution data from GB records centres and the NBN to mapped ponds shown on the OS Mastermap water layer. Record centre data do not normally identify the waterbody with which a record is associated. To do this we related the record, through its grid reference, to the nearest pond waterbodies within 1000 m of the newt record. For further explanation of this approach see also section 4.4.2. This gave a dataset of just over 57,000 ponds which, because of their proximity to a Great Crested Newt record, could support the species if the ponds were of suitable quality. We then characterised these ponds in terms of their area, altitude and Defra Land Class from mapped data for the national dataset and from a combination of field and mapped data for the volunteer ponds.

For the national dataset, pond area was described from the polygon size of each pond shown on MasterMap. Altitude was derived by finding the nearest contour to the pond, using 50 m interval contours provided in Ordnance Survey SRTM data. We overlaid the pond sites with the Defra land classes shown in Figure 3.12 to describe the land class.

(ii) Environmental factors influencing the detection of eDNA

We assessed the relationship of different environmental factors with eDNA scores in the volunteer survey sites. We evaluated the relationships of the following factors, mainly derived from the HSI assessment, with the addition of altitude:

- Pond numbers
- Shade
- Pond area
- Likelihood of drying out
- Water quality
- Occurrence of waterfowl
- Occurrence of fish
- Terrestrial habitat quality
- Vegetation cover in the pond
- Overall HSI score
- Altitude

Data were highly non-normal and could not be corrected by transformation (Anderson-Darling Normality test). To analyse relationships we ran a series of Spearman's rank order correlations with a Bonferroni correction to counteract the problem of multiple comparisons.

Although we could not take account of correlation between the variables with this approach, the lack of correlation between most variables and eDNA score suggests that this is not a serious problem. With the Bonferroni correction applied, the minimum significance level for tests was $p < 0.004$.

Figure 2.7 Distribution of eDNA study sites

2.2 Part B methods: statistical support for producing GB trends for the Great Crested Newt

2.2.1 Datasets

(i) Pond numbers datasets

To assess changes in pond turnover we used two datasets: the sample-based Countryside Survey and the Ordnance Survey MasterMap water layer which shows ponds mapped by the Ordnance Survey.

Countryside Survey data currently provide the most reliable information in Great Britain on changes in pond numbers. In the present project we used this dataset to describe statistical characteristics of change in pond numbers between 1998 and 2007. Countryside Survey data from 1998 are derived from a field survey of 569 1 km squares and in 2007 from 591 1 km squares, with a stratified random distribution throughout Great Britain (Williams *et al*. 2010). The stratified random sample of sites was the same in both years but extra sites were added in 2007. The distribution of sample squares is shown in Figure 2.8. The size of water bodies counted in the Countryside Survey ranges from 25 m^2 to 2 ha. The survey excludes very small ponds (1 m^2 - 24 m^2) which are hard for field surveyors to count effectively. Such small ponds are relatively infrequently used by Great Crested Newts but can be important for other biotic groups (see Figure 2.9, for example).

Figure 2.8 Distribution of 1 km sample square in Countryside Survey. Black dots show squares surveyed in both 1998 and 2007. Red dots show squares only surveyed in 2007. Reproduced from Carey *et al*. (2008).

Figure 2.9 This small pond, the site of the rediscovery in Scotland in 2004 of the Tadpole Shrimp *Triops cancriformis*, is about 5 m^2 in area.
It would not be recorded as a pond in the Countryside Survey despite being biologically one of the most important waterbodies in Great Britain. Photo: Larry Griffin

Ordnance Survey MasterMap data are a compilation of many years mapping of waterbodies by Ordnance Survey. The data are provided as a combined 'water layer', including all freshwater habitats.

At first sight the OS MasterMap data appears to provide a census of pond numbers. However, there are a range of inconsistencies in the data owing to the way in which OS maps are updated: substantial and long established ponds may not be recorded; many ponds which are long gone are still shown on OS maps, and recently created ponds are recorded rather haphazardly. Some examples of these inconsistencies are shown in Figures 2.10-2.12.

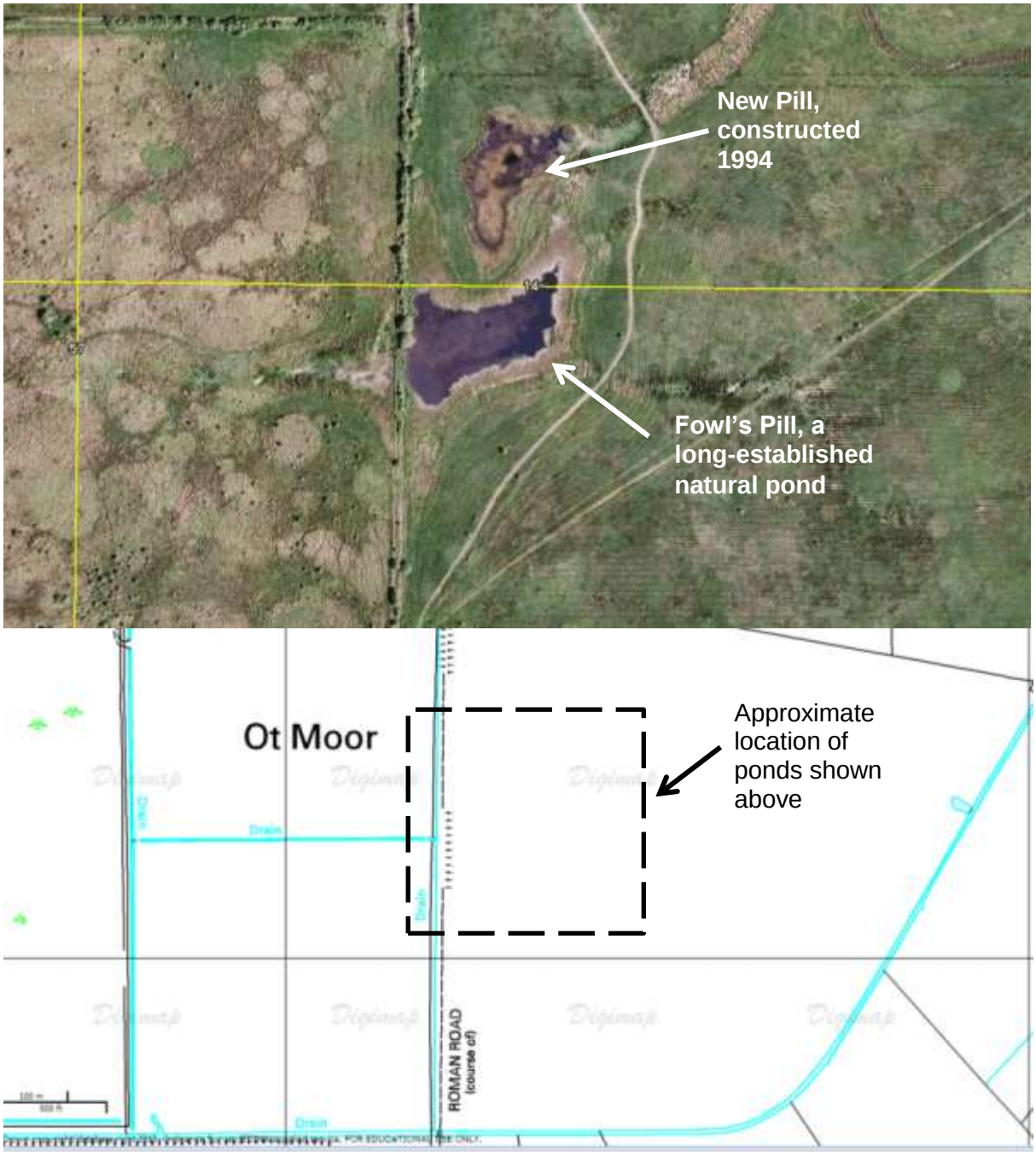


Figure 2.10 Examples of large ponds not shown on Ordnance Survey maps: the Fowl's Pill and the New Pill on Otmoor, Oxfordshire.
Both ponds have a surface area of about 1 ha. The Fowl's Pill is a long established (greater than 100 years old) natural feature. The New Pill was constructed by the Freshwater Habitats Trust and the Environment Agency in 1994. Both are priority ponds and amongst the most important sites for their freshwater biota in southern England. Although both ponds are clearly visible on current aerial images, neither waterbody is shown on Ordnance Survey maps. Interestingly, new features created on an adjacent RSPB nature reserve are shown on recent OS maps.

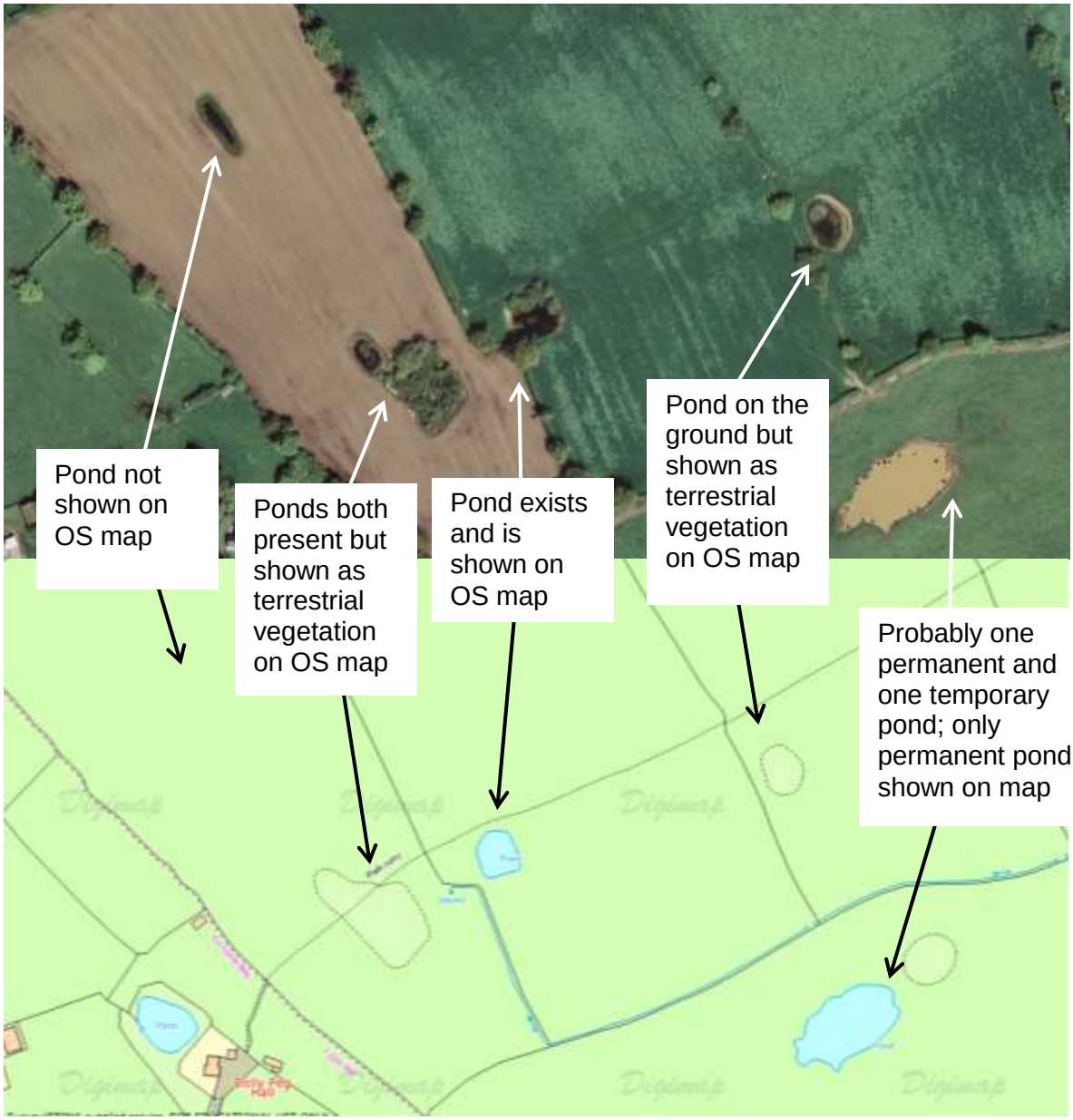


Figure 2.11 Examples of long-established ponds which are shown incorrectly on Ordnance Survey maps: Brown Heath, Cheshire.
The map has two types of error: ponds which are present and almost certainly long established, but not shown on the map and ponds which are present but shown as terrestrial habitat on the map.

Figure 2.12 Relatively recently created ponds, dug in 1990-91, which are depicted rather haphazardly by Ordnance Survey mapping.
The site is Pinkhill Meadow, Oxfordshire, a demonstration site created by Freshwater Habitats Trust, Environment Agency and Thames Water. The site has 15 separate ponds on the ground but these are shown as two waterbodies on the Ordnance Survey MasterMap layer.

We extracted pond water bodies from the OS water layer by filtering on size and shape (to remove linear waterbodies that were not ponds). In the main inland water file there were 1,426,413 water features. Those with an area >2 ha and with area or length/width ratio of less than 3.5 were removed. This left 708,862 water features. An OS river shapefile (available from http://bit.ly/9krdUU) was intersected with the pond layer to remove further 'non-pond' features. This left 676,021 ponds up to 2 ha in area.

(ii) HSI score datasets

We used 3 datasets to analyse differences in HSI scores between years to determine the power of these surveys and the sample sizes required to detect change between years or sampling periods:

1. DICE/Freshwater Habitats Trust HSI data
2. Countryside Survey data
3. Amphibian and Reptile Conservation NARRS data and SNH data.

The DICE (Durrell Institute of Conservation and Ecology) HSI data collected in 2007 comprised 25 ponds in Wales and 23 ponds in Kent. Countryside Survey for 1996 and 2007 comprised 77 ponds which had been surveyed in both years, of which 83% were in England, 9% in Scotland and 8% in Wales. In 2007, as part of NARRS, Amphibian and Reptile Conservation obtained HSI data from 108 ponds in Great Britain with 67% from England, 29% from Scotland and 5% from Wales. Results from the 2012 SNH survey are excluded from this analysis because data were all collected in 2012, were from a restricted geographical area and were collected professionally, factors which were likely to introduce biases into the dataset when compared to the main body of the NARRS dataset. All data sets show normal distribution.

1. DICE / Freshwater Habitats Trust HSI data. There is very little information available on change in HSI scores over time. To provide further information on such changes we resurveyed sites in Kent and Wales which had previously been surveyed by Sewell and colleagues in 2007 and 2008 (Sewell *et al*. 2010). The distribution of these sites is shown in Figure 2.13. In 2007 sites in both Kent and Wales were surveyed by David Sewell.

Figure 2.13 Sites in Wales and Kent used by Sewell *et al*. (2010) and resurveyed in the present study to assess change in HSI score with time

Sites surveyed by Sewell were revisited in 2013 by the DICE team in Kent and in Wales by Freshwater Habitats Trust to collect HSI data. In June and July 2013 at each of the ponds 13 simple variables were measured as follows: pond temperature, elevation, turbidity and the 10 parameters used to assess HSI.

These data provide an indication of the variability of HSI scores between ponds within year (which is critical for determining power) and the real levels of change in HSI score which may be expected over a c.10 year period, covering a core Great Crested Newt area (Kent) and a peripheral area of the range (Wales).

2. Countryside Survey data. We approximated HSI scores using Countryside Survey data from 1996 and 2007. Environmental variables collected in the Countryside Survey closely match, but are not exactly the same as, the ten variables collected in a standard HSI survey (Table 2.6). Variables in the table highlighted in white are measured in a slightly different way in the Countryside Survey compared to a standard HSI score. The reason for this difference is that the Countryside Survey methodology is based on the Freshwater Habitats Trust National Pond Survey methodology which was developed in the early 1990s before HSI scores were developed (Biggs *et al.* 1998).

Countryside Survey was based on a random survey of 1 km grid squares and the ponds therein. The same ponds (n=77) were visited in the Lowland Pond Survey 1996, a sub-project of Countryside Survey, and the full Countryside Survey 2007. For each occasion the ponds were given an approximate HSI score calculated using the environmental variable that most closely match the HSI variable. Further details are given in Williams and Biggs (2012).

The scores cannot be related to Great Crested Newt presence because this was not recorded in Countryside Survey and the location of ponds is confidential, but the majority of ponds (83%) were located in England. These data can provide an indication of the variability of HSI scores within year and the levels of change in HSI score which may be expected over a c.10 year period at the scale of Great Britain.

Analysis by Williams and Biggs (2012) of change in Countryside Survey approximate HSI scores found a small (2.6%) non-significant increase in HSI values between 1996 and 2007.

3. Amphibian and Reptile Conservation NARRS data and SNH data.
Data from the NARRS study provide a large dataset (n=c500 sites) with HSI scores and information on Great Crested Newt occupancy (about 14% of sites had Great Crested Newts). The distribution of these sites in the 6 survey years (2007-2012 so far) is shown in Figure 4.9. Sites are selected using a stratified random sampling protocol but the take up of samples is dependent on volunteer engagement so may still contain some distributional biases.

Table 2.6 Comparison of the variables comprising the Great Crested Newt Habitat Suitability Index (HSI) and the approximate equivalent derived from the Countryside Survey. Variables highlighted in white are measured in a slightly different way in the Countryside Survey compared to a standard HSI score

HSI variable	How measured for HSI	How derived from Countryside Survey data
1 Geographic location	Based on UK location within three map zones	Based on UK location within three map zones
2 Pond area	Surface area of the pond when water is at its highest	Surface area of the pond when water is at its highest
3 Permanence Ranked in 4 categories: 1=Never dries, 4=always dries	Deduced using local knowledge and personal judgement	Based on a range of data including water depth, drawdown height, and whether the pond dried out in the drought year of 1996, but not in the wetter year of 2007
4 Water quality Ranked in 4 categories: 1=good, 4=bad	Subjective assessment based on factors including invertebrate diversity, presence of submerged water plants, water source and agricultural inputs	Based on a range of factors including measured nutrient levels, water source, land use, submerged plant abundance, and plant biotic assessment using PSYM
5 Shade % overhang by trees and buildings	% of the pond margin overhung to at least 1 m from the shore	% of the total pond area overhung
6 Waterfowl Ranked in 3 categories: 1=absent, 3=major	Based on a 3 category ranked score	Based on a 5 category ranked score of waterfowl impact or text box information
7 Fish Ranked in 4 categories: 1=good, 4=bad	Based on a 4 category ranked score	Based on a 5 point ranked score of fish impact or text box information
8 Pond count	Number of ponds occurring within 1 km radius around pond	Number of ponds in the 1 km survey square
9 Terrestrial habitat Ranked in 4 categories: 1=good, 4=bad	Based on availability of suitable habitat within 250 m of the pond	Based on surrounding land use type within 100 m of the pond
10 Macrophytes % abundance of wetland plants.	% of the pond surface area occupied by emergent, submerged and floating plants excluding duckweed	% of the pond surface area occupied by emergent, submerged and floating plants excluding duckweed

(iii) Great Crested Newt occupancy dataset

To obtain a baseline dataset to model the distribution of occupied Great Crested Newt ponds in Great Britain we collated data held on the NBN and also contacted all GB records centres to ask them for all Great Crested Newt records from 1988 onwards. Data were received from 37 out of 46 English counties with remaining data coming from the NBN. We believe this to be a dataset which is as good as is currently available, although there are certainly further data held in other sources which we have not been able to access (e.g. in confidential consultancy reports, in the private records of surveyors who do not wish to make their data publicly available) (see also Section 4.4.2(i) for further discussion of this point).

Normally NBN and records centre data do not identify the waterbody with which the record is associated. To relate records to specific waterbodies we mapped the newt data over the OS MasterMap pond layer and related the records to the nearest pond waterbodies that were within 1000 m of the newt record. This provided a dataset of just over 57,000 ponds with an associated record of a Great Crested Newt. The distribution of these ponds is shown in Figure 2.14.

We originally proposed creating three map layers:

(i) a cleaned data set of all records

(ii) a second dataset with only records that coincided with a pond (using the OS MasterMap derived pond layer).

(iii) a third dataset using only ponds in the south west corner of each 1 km grid square to replicate the NARRS sampling strategy, to remove surveyor bias and ensure independence between sample units.

In practice we decided to use only datasets (ii) and (iii) to model the distribution of Great Crested Newts as these were the only practical scenarios (i.e. ponds which would be surveyed for newts) that would be used in a real world surveillance strategy. In addition, the estimate of pond numbers potentially supporting Great Crested Newts derived by these methods broadly agrees with existing estimates.

It is important to note that these datasets provide virtually no information on how Great Crested Newt occupancy changes from year to year.

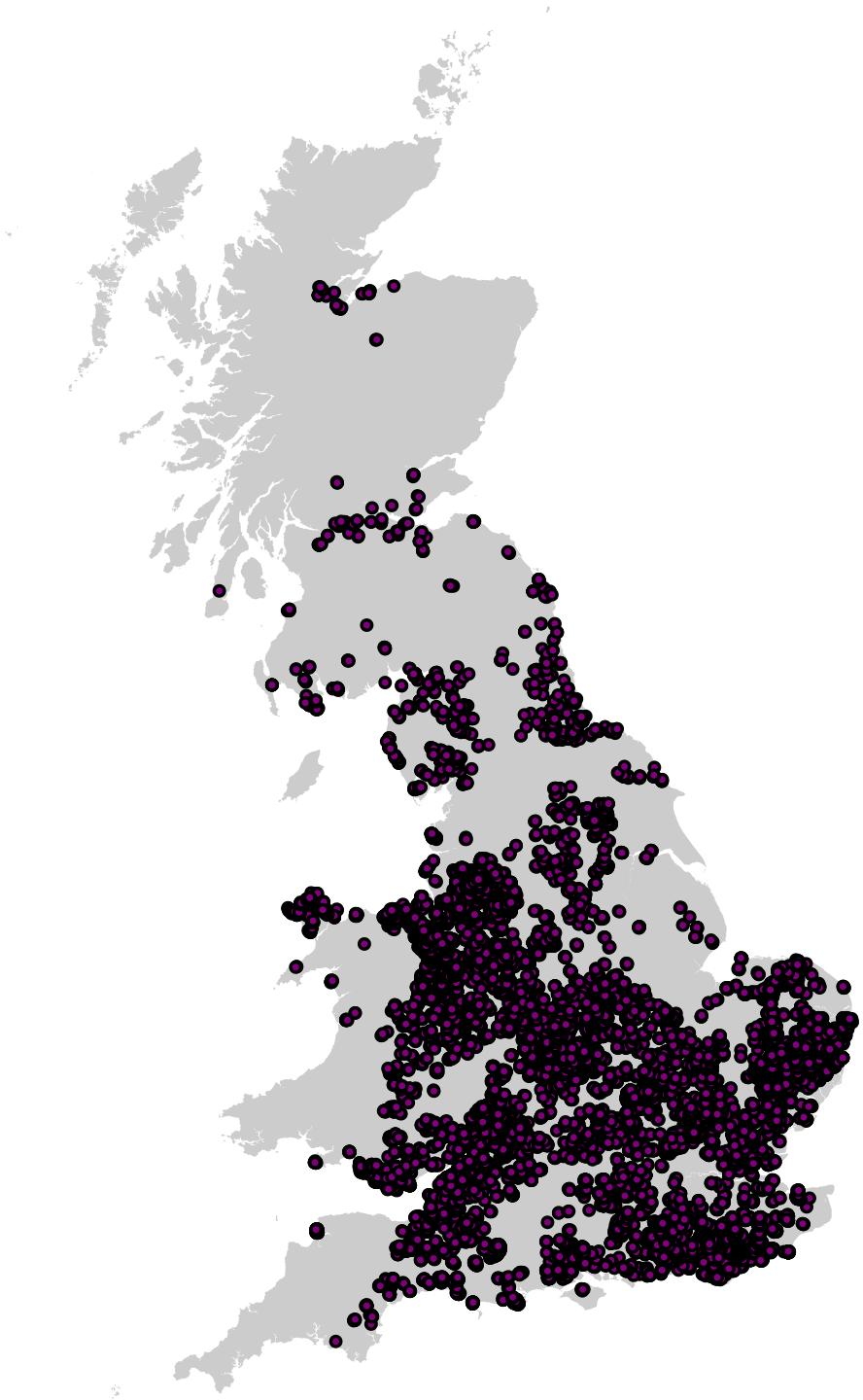


Figure 2.14 Distribution of ponds (n=57,000) with a closely associated record for Great Crested Newt. The map was created by overlaying post-1988 Great Crested Newt records on the OS MasterMap pond layer and identifying all ponds within 1 km of a Great Crested Newt record. The distribution of ponds was then used (a) to provide realistic simulations of the proportion of all ponds, the proportion of 1 km grid squares and the number of ponds per 1 km grid square occupied by Great Crested Newts for power analyses and (b) to assess whether ponds from which eDNA samples were collected by volunteers in the study were broadly representative of ponds in the range of, and likely to be used by, Great Crested Newts.

2.2.2 Analytical approach

Statistical methods

Power analysis was used to determine the sample size needed to detect changes in pond numbers within the Great Crest Newt's range, Habitat Suitability Index scores and pond occupancy. Power (1-β) is the probability of detecting an effect if one exists in the population, and is largely dependent on sample size N, effect size and levels of variance in sample groups σ^2. Type II errors (β) may occur if there is a failure to reject the null hypothesis, when in fact the alternative hypothesis is true. Confidence that the observed results are statistically different from the random variation seen in the environment is controlled by alpha. As the size of alpha increases, so does the risk of detecting a significant result when one does not exist, a Type I error. Robust experimental designs reduce the risk of Type I and Type II errors occurring, but at the same time should minimise the cost of analysing too many samples unnecessarily.

Analysis of power was undertaken in R[2] using the pwr package[3] and G*Power[4].

Change between sampling years for each parameter was specified as 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40% and 50%. There has not yet been agreement amongst the Agencies as to which levels of change will be acceptable for reporting purposes. For the summary reporting we have chosen to compare 10% change at 80% power for pond numbers and HSI scores and 20% and 30% change in Great Crested Newt occupancy at 80% power. The sample sizes required to achieve 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% and 95% power at each of these levels of change was calculated at two different levels of alpha (α=0.05, α=0.10).

The sample sizes required for different approaches (e.g. matched pairs and independent samples) and different units of measurement (e.g. number of occupied ponds per 1 km grid square, proportion of occupied ponds and proportion of occupied 1 km grid square) were then compared at Great Britain, England, Scotland, Wales and England+Wales levels. If random selection of sample units was found to require very large sample sizes, selection was stratified to increase representation of 1 km grid squares known to support Great Crested Newts.

Estimates of variance for each parameter were based on previous surveys (e.g. Countryside Survey, DICE, NARRS), OS MasterMap and collation of data from the NBN gateway.

Data were analysed using MINITAB© 14 Statistical software. Data were tested for normality using the Anderson-Darling test for normality. If levels of α were found to be greater than 0.05, the data were assumed to be normal. Data were also tested for equal variance using Levene's test for equal variance. If levels of α were greater than 0.05 the data were assumed to have equal variance.

Tests of difference between group means for parametric data were 2-sample t-tests and paired t-tests. For non-parametric data, the difference between the median value of ranked data was tested using the Mann-Whitney U-test (independent samples) and Wilcoxon's matched pairs analysis. Nested two-way Analysis of Variance (ANOVA) was used to test for differences between group means within and between sample periods. Non-parametric data were tested using nested Kruskal-Wallis (Oron and Hoff 2006). Change in proportional data

[2] R Core Team (2012). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.

[3] Champely, S (2009) R Package 'pwr': Basic functions for power analysis. V 1.1.1. Published 2012-10-29 08:59:31, URL http://cran.r-project.org/web/packages/pwr/pwr.pdf.

[4] Faul F (1992 – 2012) G*Power 3.1.5. http://www.psycho.uni-duesseldorf.de/abteilungen/aap/gpower3/download-and-register

were analysed using Fisher's exact test for independent samples and McNemar's test for matched pairs analysis.

The statistical analyses applied, data used, survey stratification type and overall analysis design are summarised for each analysis at the head of individual results tables which summarise the results of power, and other, analyses. Detailed tables of results are given in the Appendix 3 (separate volume).

Approach to analysis of pond numbers datasets

(i) Theoretical design of pond number sampling strategy.

We used existing data on pond numbers to explore the sampling strategies and sample sizes needed to achieve different levels of statistical power for assessing changes in pond numbers. We assessed:

- Change in pond numbers assuming a random survey design between two time points
- Change in pond numbers assuming the survey combines randomly reselected ponds and a proportion of ponds which are revisited on each occasion (which would be expected to reduce between survey variation, and therefore reduce sample size)
- The effect of collecting survey data over several years - typically this would be expected to increase survey variation (by adding between year variability) which is traded off against increased sample size
- Whether complete sample surveys, undertaken in one year, of pond numbers are necessary or cost effective compared to surveys spaced several years apart. We also assessed whether partial surveys in which the dataset was generated over several years would be effective.

We derived theoretical pond number datasets from the cleaned OS MasterMap water layer of c580,000 ponds in Great Britain. To assess the power of different sampling strategies we resampled at random 1 km grid squares, measuring pond numbers per grid square. We resampled the pool of c225,000 1 km squares present in GB using the computer package Resampling Stats™ (Resampling Stats, 2006).

(ii) Power of existing sampling strategies to detect change in pond numbers.

In the light of the above theoretical analysis we also evaluated current sampling strategies to assess their power to detect change in pond numbers, specifically:

(i) the Countryside Survey, based on a stratified random sample of c590 1 km squares throughout Great Britain allocated amongst 32 land classes. In each square all ponds are counted

(ii) the PondNet approach based on counting pond numbers per 1 km square. All ponds are counted

(iii) the NARRS approach, based on estimating pond numbers (from OS data) within 1 km of a single focal pond located closest to the south-west corner of the selected 1 km square (Figure 2.15).

Power analysis was used to determine the sample size needed to accurately estimate the number of ponds nationally, within acceptable 95% confidence limits, and to detect change in total pond numbers. Alpha in this analysis was 0.1. The results show the number of 1 km grid squares to be surveyed in each survey period.

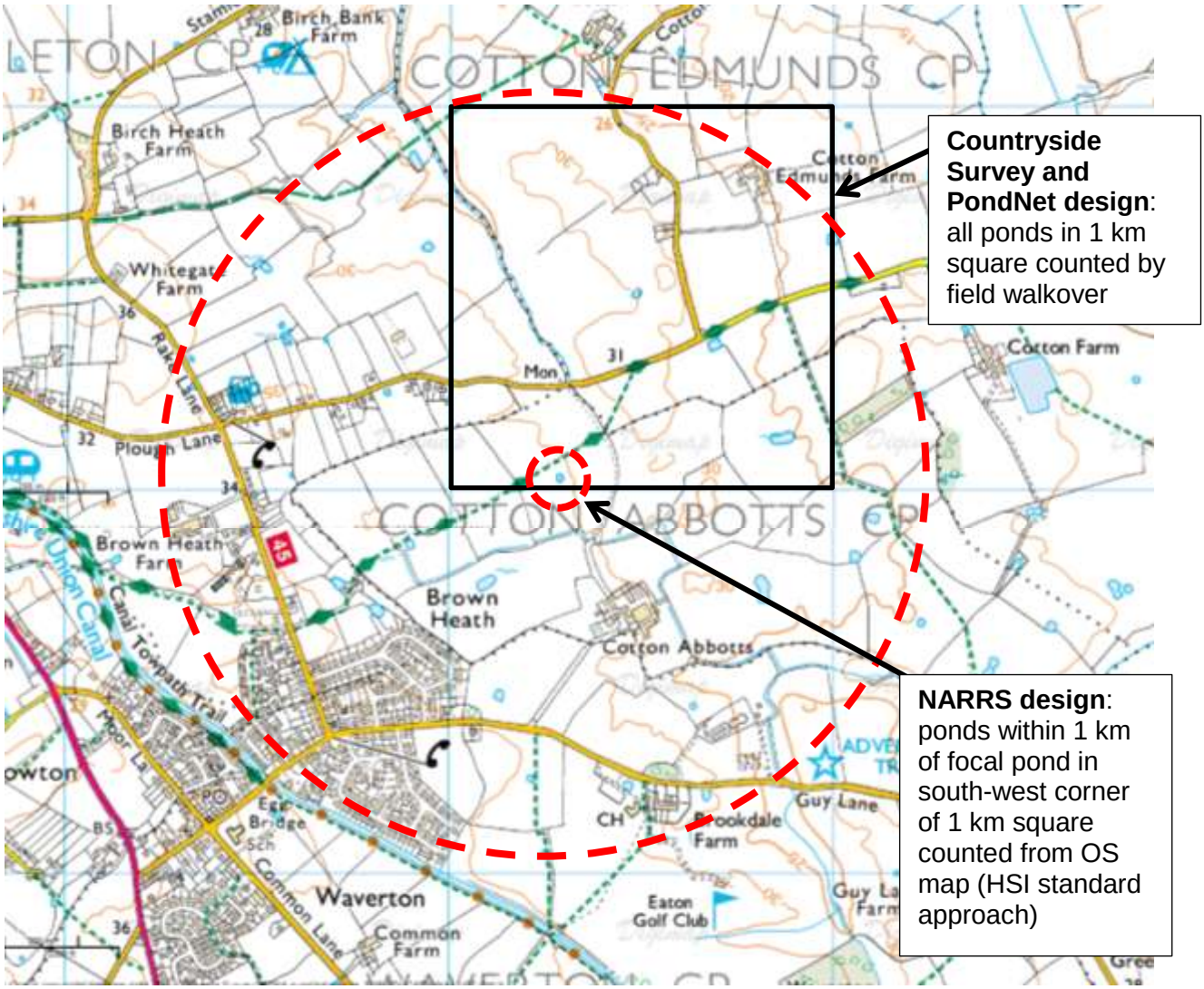


Figure 2.15 The alternative approaches to assessing pond numbers in three different surveys that describe pond numbers: (i) Countryside Survey (ii) PondNet and (iii) NARRS. Countryside Survey and PondNet assess pond numbers by counting all ponds in the focal 1 km square (black box). NARRS assesses pond numbers by estimating pond numbers from OS map in 1 km circumference of focal pond.

Approach to the analysis of HSI datasets

Presence of Great Crested Newts has been shown to correlate well with Habitat Suitability Index scores (Oldham *et al*. 2000). However, previous work has not looked at levels of change in HSI scores between years or the sample size needed to detect these changes.

We used the three existing datasets described above (Section 2.2.1 ii), and theoretical data, on HSI scores to explore: (a) the degree of change seen in HSI scores over differing time periods and (b) the sample sizes required to achieve different levels of power to detect expected changes in HSI values at different special scales, using different sampling strategies.

The analyses we undertook followed three conventions:

(i) A sampling strategy for HSI scores would operate at the sample unit "pond".

(ii) To maintain sampling independence each pond should be a minimum distance from other ponds in the sample - for ease and to remain in line with previous surveys we ensured that ponds were in different 1 km grid squares.

(iii) In the results, surveys comparing results between years are referred to as survey t_1 and t_2; surveys which compile data over several years and then compare survey periods are referred to as p_1 and p_2.

(i) Degree of change in HSI scores.

There is little currently available data on changes over time in HSI scores. Reanalysis of the Countryside Survey (1996 to 2007) has already indicated fairly small changes occur in HSI scores over relatively long times (Williams and Biggs 2012) with differences found in this dataset not statistically significant. However, only a relatively small sample of ponds surveyed in both years was available (n=77). In the present study we theoretically increased the levels of change in HSI score over the 11 year time period to observe the effect on sample size needed to detect change at different levels of power.

We also further examined changes in HSI score over time by repeating surveys undertaken in 2007 at 23 ponds in Kent and 25 ponds in Wales by Sewell and colleagues to assess actual levels of change in HSI scores (Sewell *et al*. 2010).

(ii) What is the optimum sampling strategy for detecting change in HSI scores in terms of sample size, sample type and repetition of surveys?

We mapped ARC HSI data and used repeat sampling statistics to understand the extent to which randomised or stratified surveys could detect the full range of HSI pond types. Of particular importance in this analysis was the observation that the number of ponds with high HSI scores is low and not evenly distributed. This provides information about how many sampling sites and which strategies are needed to provide a good assessment of the range of pond quality. We also simulated different levels of change in mapped HSI scores and used standard power analysis to calculate the power of different sampling strategies and the power of different spatial sampling scales (country, England + Wales and Great Britain levels).

We also investigated whether there was any geographical correlation between ponds that have good HSI scores which may influence sampling strategies. We created theoretical HSI pond layers based on ARC results at Great Britain level, to allow analysis of a larger dataset and provide a baseline against which we could model change.

We combined the DICE/FHT HSI data collected in 2007 and 2013, and the theoretical layers of HSI scores for ponds at country and Great Britain levels, to generate model datasets to test two questions:

- *What is the effect on power if data are collected over several years, combining data to look at several years as a single time point.*
- *Is there any benefit in annual surveys to determine HSI scores or are periodic surveys sufficient to detect change and what is the impact of this on power?*

A third question:

- *What power can be achieved if some of the sample squares are repeats and others are different squares each year?*

presents a fundamental challenge to pond survey design, although solved for other habitats (Scott 2008), and cannot readily be answered. The question is discussed further in section 4.3.3 (i) of the results.

Approach to analysis of Great Crested Newt occupancy datasets

The detailed methodological field study indicated that we could expect to achieve c.95% efficiency of detection if Great Crested Newts were present using either a single eDNA sample, or combined torch counting and bottle trapping undertaken over four visits (see Figures 3.1 - 3.3). Using the national dataset on Great Crested Newt pond occupancy that was created (see Figure 2.14) we then tested alternative sampling strategy/power combinations to design the optimum sampling strategy for Great Crested Newt.

We used existing and theoretical data on Great Crested Newt occupancy to explore the sample sizes required to achieve different levels of power to detect change at different special scales using different sampling strategies.

We evaluated the five following sampling strategies:

Strategy 1: Sample as many ponds in a 1 km squares as possible. The sample of 1 km squares comprised 50% fully random and 50% random from 1 km squares known to be occupied by Great Crested Newt (i.e. the recommended option from PondNet project).

In PondNet this enabled us to look at the change in occupancy at a national level in the number of occupied 1 km grid squares (376 1 km grid squares for Great Crested Newt in England to detect 30% change at 70% power) and change in the number of occupied ponds per 1 km grid square (121 1 km grid squares needed in England to detect 30% change at 70% power). In the present study we have mainly reported pond occupancy rather than square occupancy because it is a more sensitive measure of change in the distribution of Great Crested Newts.

Strategy 2: Sample one pond in each 1 km square. Use a random selection of 1 km squares. (i.e. NARRS approach). PondNet has looked at the number of 1 km squares needed in a random survey of ponds in England (414 1km grid squares to detect 30% change at 70% power). Similarly, at the pond level it was possible to show that a random survey of ponds in England would require a survey of 2005 ponds. Neither of these analyses precisely matched the NARRS approach, so we used OS MasterMap data and GIS tools to simulate the NARRS methodology and then analysed power and levels of change using a random selection strategy.

Strategy 3: Use a combination of the above to investigate varying the proportion of squares where only one pond is sampled, and the differences from sampling (i) fully random squares and (ii) random within known distribution regions for Great Crested Newt. To do this we modelled a range of scenarios varying the proportion of known and unknown squares from sampling strategy 1, and the proportion of ponds surveyed from each square according to this strategy. We also compared sample size and power at different levels of change between Strategy 1 and Strategy 2.

Strategy 4: Addition of non-random squares from designated sites or those under environmental stewardship schemes. We determined the proportion of squares using the

sampling strategies (1-3) above, that overlap with (a) protected sites, (b) land under environmental stewardship schemes and whether additional non-random squares would need to be added to a survey network to provide adequate data to analyse trends within these site.

In the project proposal we originally suggested a fifth strategy to assess the sampling needed to monitor other amphibian species. This work was not undertaken as part of the present project.

We used the modelled data to determine:

(i) the effect on power if data are collected over several years - combining data to look at several years as a single time point.

(ii) whether there is benefit in annual surveys to determine trends in Great Crested Newt occupancy or whether periodic surveys are sufficient to detect change and the impact of this on power.

(iii) the ecological robustness of some annual and some periodic surveys to allow for natural fluctuations in Great Crested Newt populations.

We originally proposed assessing what power can be achieved if some of the sample squares are repeats and others are different squares each year. However, as noted above it is not statistically feasible to use this option. This point is further discussed in Section 4.3.3 (ii).

We have evaluated these questions assuming that detectability is close to 95% - either by using eDNA as a survey method or combining torch counts with bottle trapping.

We used the Random Statistics™ programme to randomly select 50 1 km grid squares from the pool of all grid squares and calculated the mean number of occupied newt ponds in those 50 1 km grid squares. We then resampled the total pool of 1 km squares 1000 times to generate a sampling distribution. We did this to avoid issues with pseudo-replication.

3. Results Part A: eDNA Study

3.1 Detailed methodological study

3.1.1. Presence or absence of newts

In the detailed methodological study, at ponds where newts were known to be present, eDNA successfully detected the presence of newts 99.3% of the time. Specifically, newts were detected with eDNA on 139 out of 140 sampling visits, spread throughout the survey season from mid-April to late June. eDNA failed to detect newts in one sample (Figure 3.1).

The eDNA method was significantly better than any individual traditional method at detecting the presence of Great Crested Newts. Bottle trapping and torch counting were similar in effectiveness, followed by egg searching, the methods detecting newts respectively 76%, 75% and 44% of the time over the full survey period from April to June. Differences between methods were significant at $p<0.0005$ (McNemar's test).

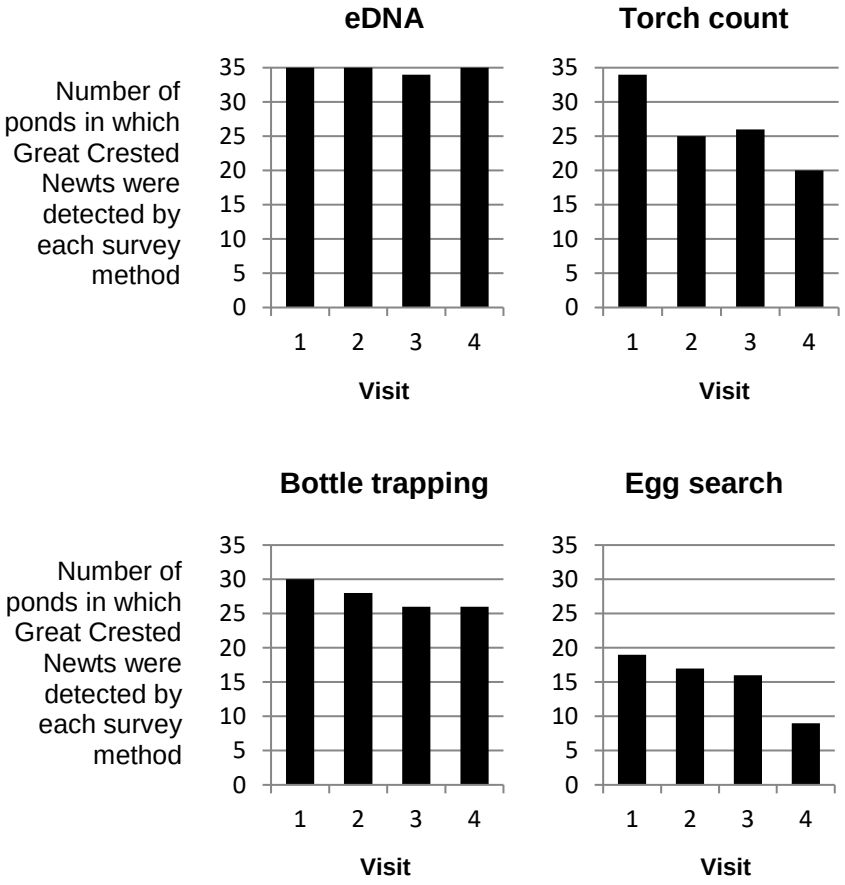


Figure 3.1 The ability of different survey methods to detect Great Crested Newts at four times during the survey season from mid-April to late June.
All sites combined: south Hampshire and north-east Wales. Visits were made at roughly three week intervals through the survey period.

To achieve detection rates with traditional methods that equalled those achieved by eDNA it was necessary to combine methods, as is normal practice in amphibian surveying. When torch counting and bottle trapping were combined newts were detected 95% of the time, a figure only slightly (but significantly) lower than for eDNA (Figure 3.2).

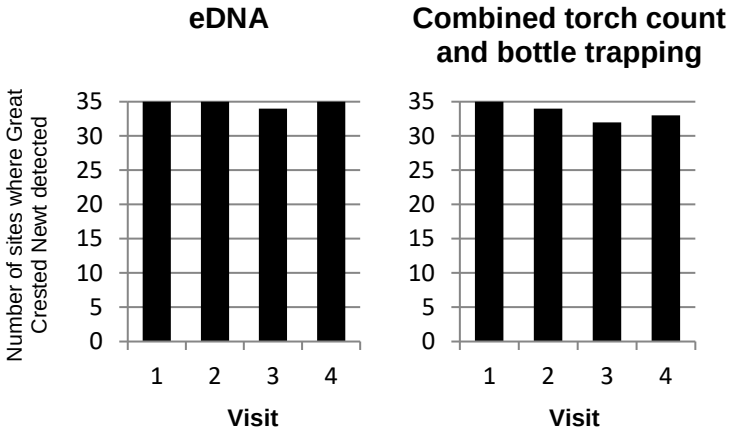


Figure 3.2 Comparison of the effectiveness of eDNA with combined torch counting and bottle trapping in detecting Great Crested Newt presence. N = 35 (15 sites in north-east Wales, 20 in south Hampshire).
Visits were made at roughly three week intervals during the survey period from mid-April to late June. The difference between the two detection approaches is statistically significantly (McNemar's test; $p<0.05$).

Although in the detailed methodological study the combined traditional methods were only slightly less effective than eDNA, it is unlikely that the use of the combined methods could be replicated at a national scale except in a professionally undertaken survey (see Section 5 below).

Figure 3.1 shows all sites combined (south Hampshire and north-east Wales). The results from the two regions are shown separately in Figure 3.3.

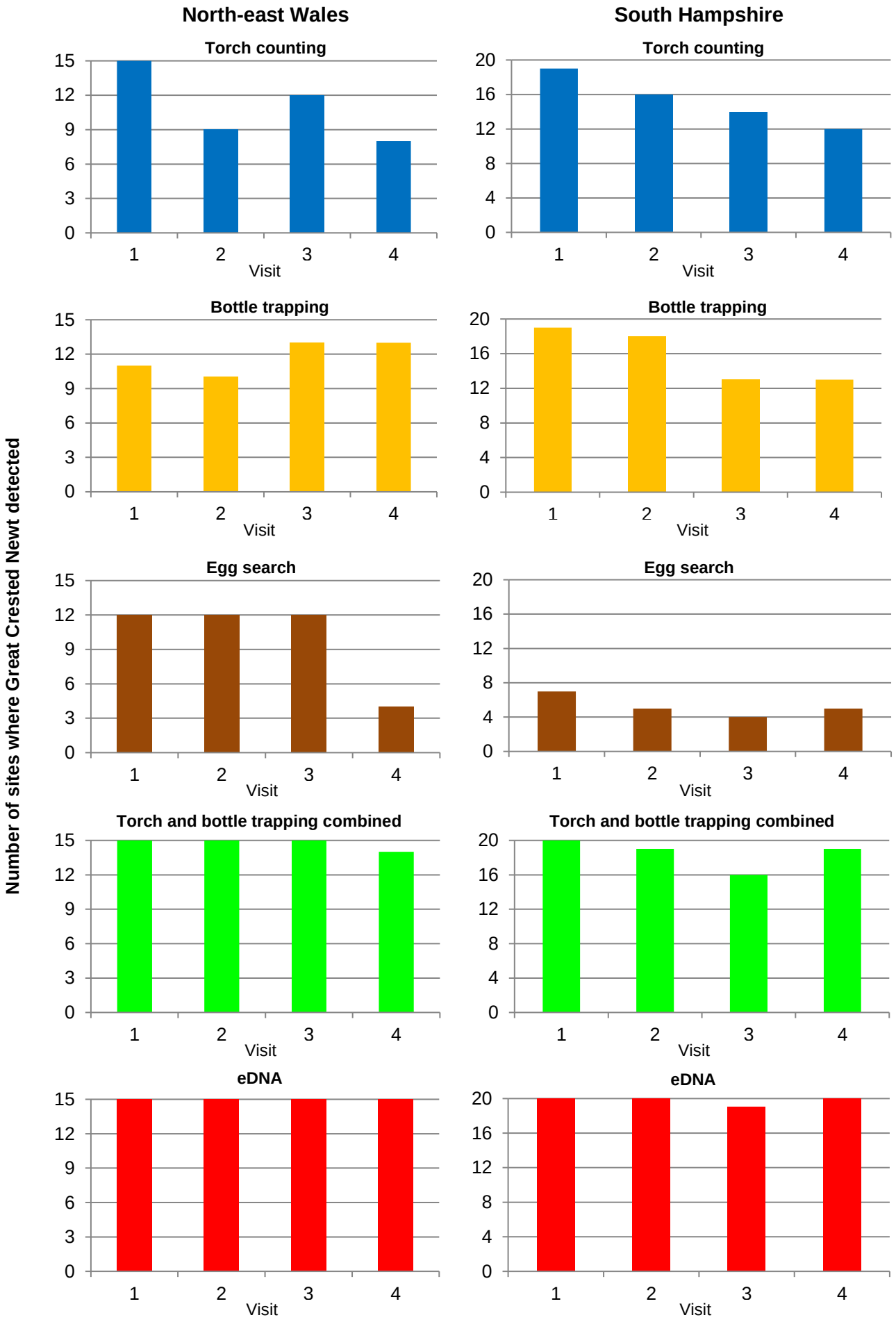


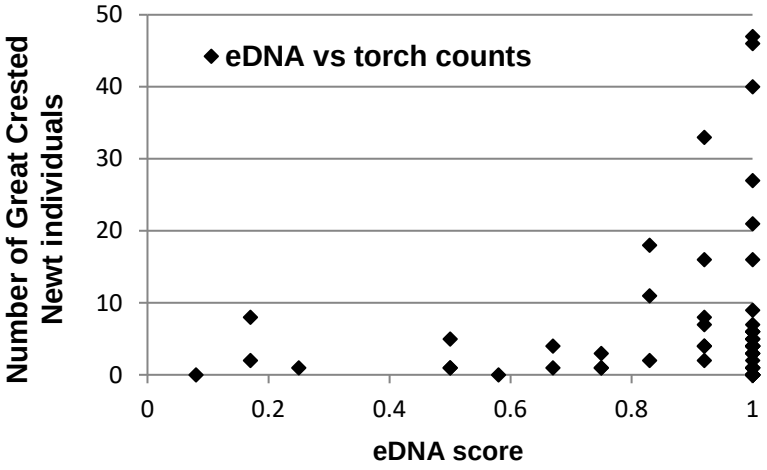
Figure 3.3 The ability of different survey methods to detect Great Crested Newts at four times during the survey season from mid-April to late June in Hampshire and north-east Wales. Visits were made at roughly three week intervals through the survey period.

3.1.2 Relationship between newt abundance and eDNA

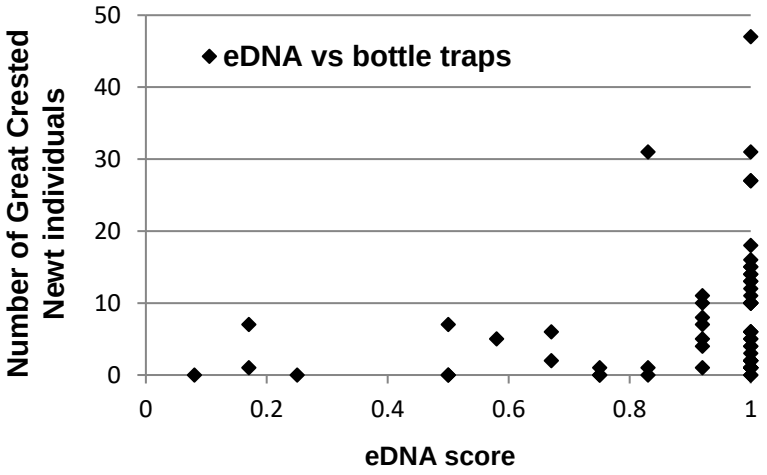
Results from the detailed methodological study suggest that eDNA score was broadly related to the abundance of newts, as measured by torch counting or bottle trapping (see also discussion section 5.1.4 for comparison with results of Thomsen *et al*. 2012 and Pilliod *et al.* 2013 who also evaluated the potential for eDNA to assess amphibian abundance). Although our method cannot quantify the amount of eDNA reliably (see Section 2.1.4) we believe that the number of positive qPCR replicates is related to the amount of eDNA in the sample i.e. the higher the eDNA score, the more eDNA is present. As we expect the amount of eDNA in the water to increase with newt abundance, this allows us to assess whether eDNA can provide information on newt abundance as well as presence.

In both south Hampshire and north-east Wales there was a suggestion that eDNA scores were higher where Great Crested Newts counts were higher (Figure 3.4 a,b). In south Hampshire the trend was statistically significant only for bottle trap data (p=0.002). Note that although graphs in Figure 3.4a look very similar, the median newt count for bottle trapping was significantly higher than for torch counting (Mann-Whitney U test: Z = -2.33, p<0.05). This difference was the main reason for the difference in the Spearman r value. In north-east Wales neither torch counts or bottle traps were significantly correlated with eDNA scores (p > 0.05). However, it should be noted that there was very little variation in the Welsh sites with all eDNA scores relatively high, with no values less than 0.75, the decimal equivalent of a 9/12 score.

(i) South Hampshire



n=62, Spearman r=0.04, p=0.790



n=62, Spearman r=0.39, p=0.002

Figure 3.4a The relationship between eDNA score and Great Crested Newt abundance. The eDNA score is the decimal fraction of the original values which range from 0 out of 12 to 12 out of 12 PCR replicates where eDNA was successfully amplified.

(ii) North-east Wales

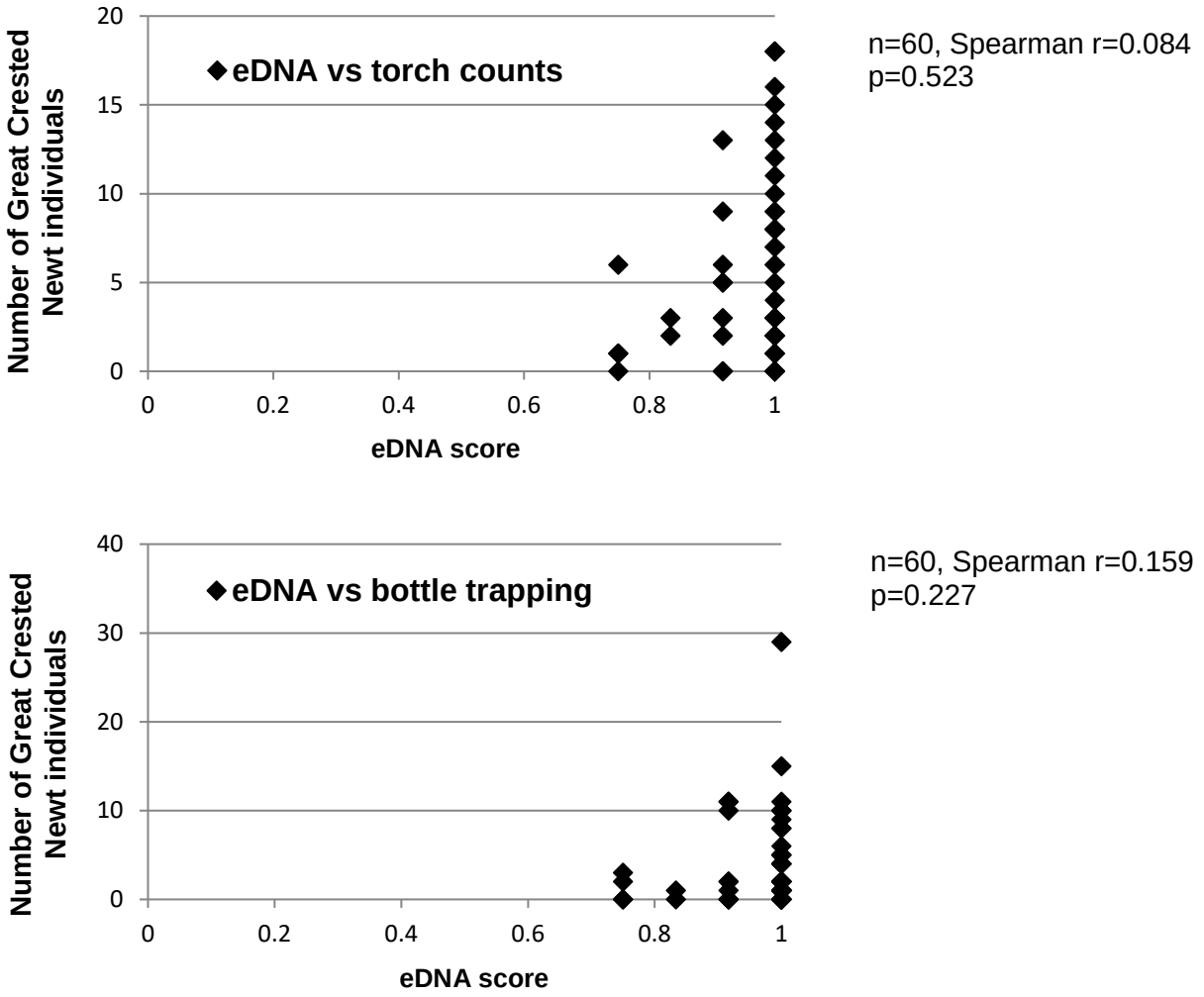


Figure 3.4b The relationship between eDNA score and Great Crested Newt abundance in north-east Wales ponds. The eDNA score is the decimal fraction of the original values which range from 0 out of 12 to 12 out of 12 PCR replicates where eDNA was successfully amplified.

There is also a clear indication from the south Hampshire data that, in terms of individual site values, low eDNA scores are always associated with low newt abundance (Figure 3.4a), with a suggestion of a similar trend in Wales (Figure 3.4b). In contrast, abundance may be *either* high or low when eDNA scores are high.

In an effort to reduce the noise in the analysis, the eDNA scores were grouped into three categories: 'low': 0/12 to 4/12, 'medium': 5/12 to 8/12 and 'high': 9/12 to 12/12. This analysis also suggests that eDNA scores were higher at sites with higher Great Crested Newt counts (Figures 3.5a,b; 3.6a,b; 3.7a,b) although differences in newt counts at sites with low, medium or high eDNA scores were not statistically significant. However, the highest newt counts, which are indicated in the box plots as the outliers, were all restricted to the high eDNA score groups. Practically, this means that protection of sites with high eDNA scores (i.e. 9/12 and above) would ensure that all sites with high newt counts were protected, even though this group of sites would also include some locations with low newt counts.

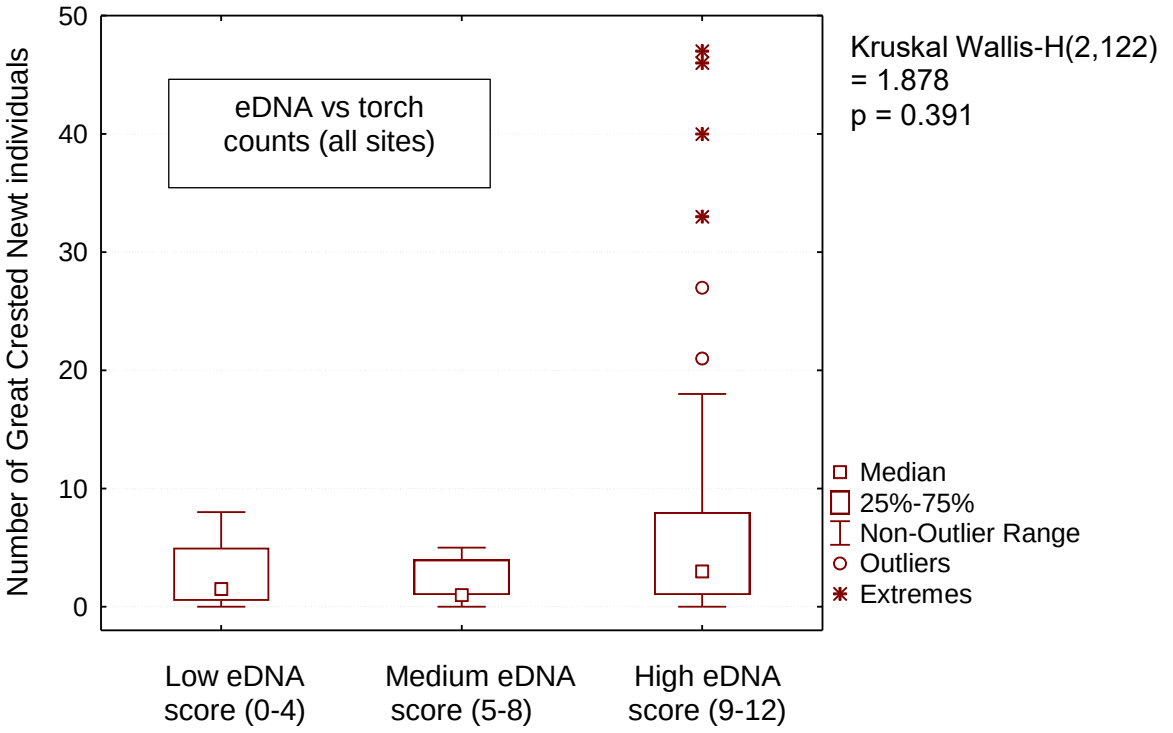


Figure 3.5a All sites: eDNA vs mean number of Great Crested Newts recorded by torch counts.

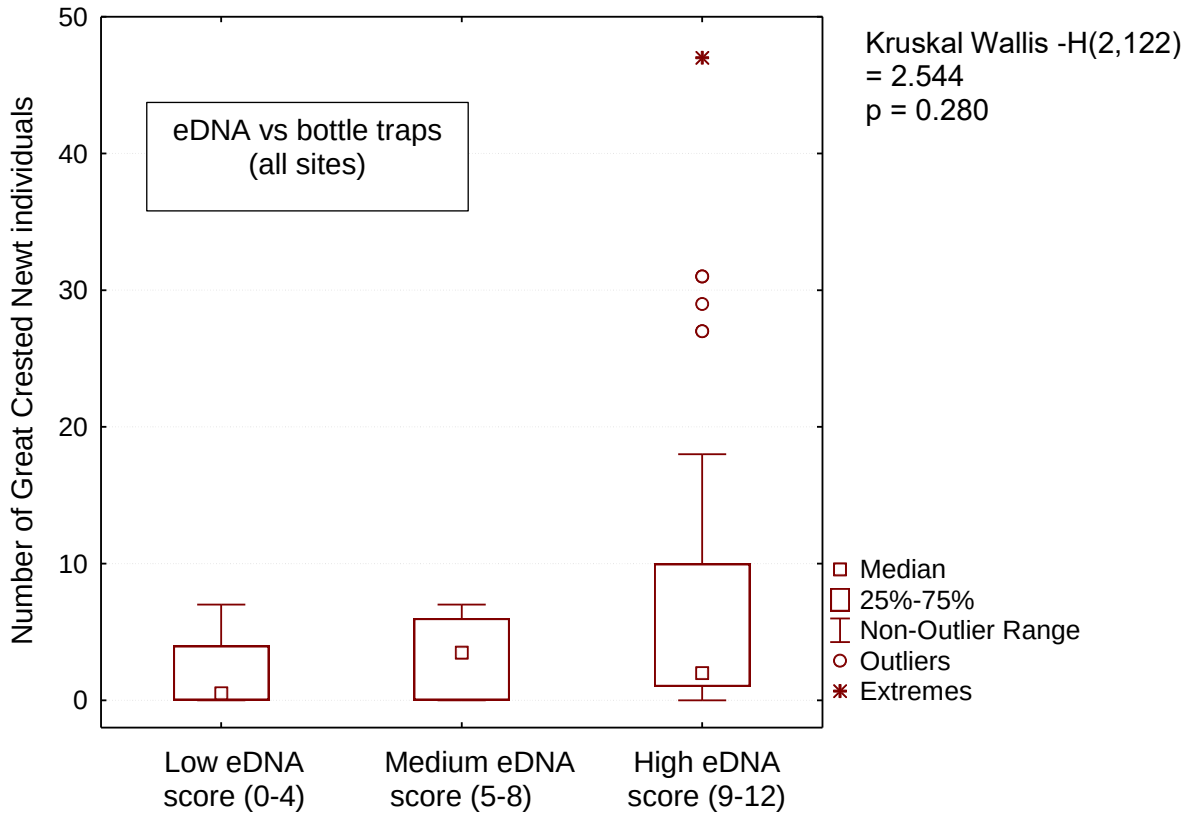


Figure 3.5b All sites: eDNA vs median number of Great Crested Newts captured with bottle trapping.

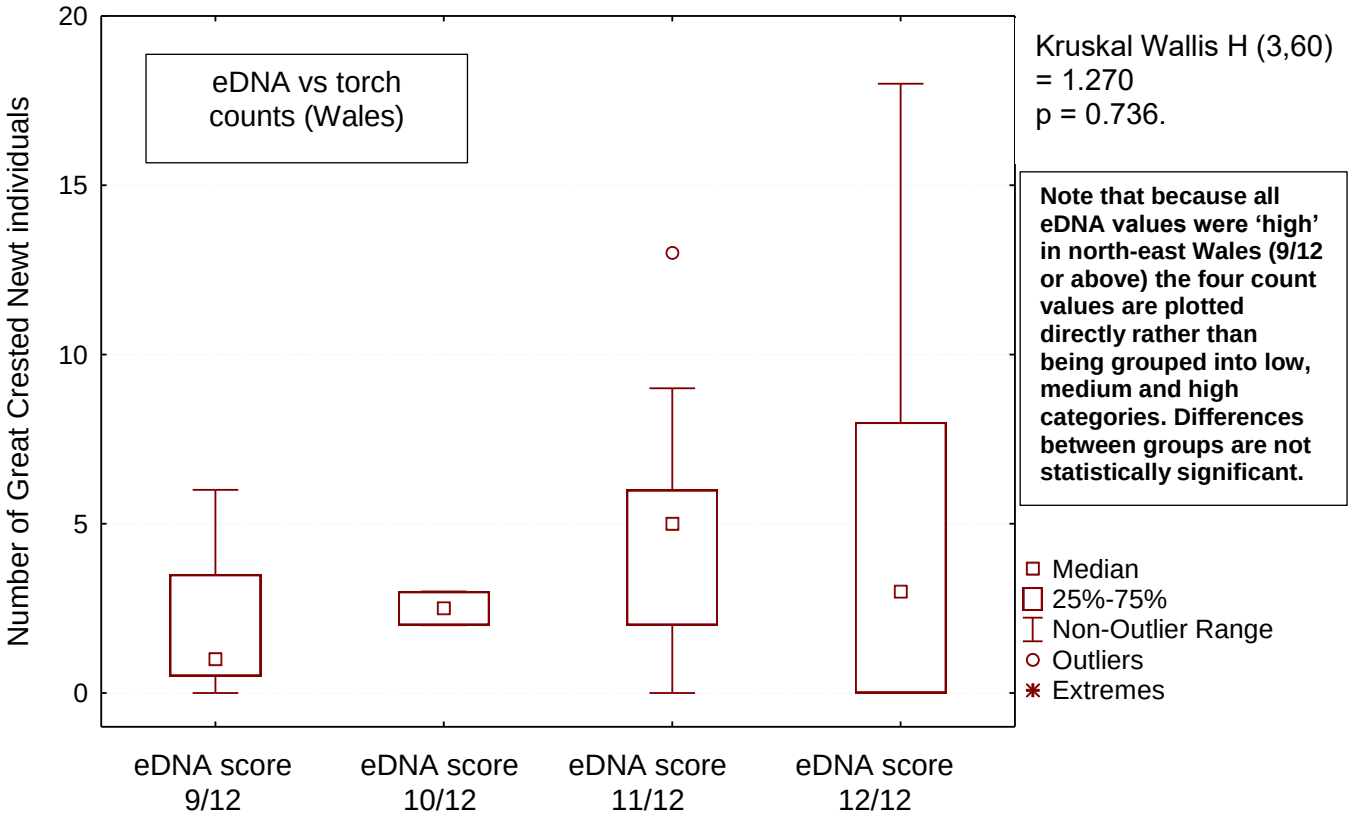


Figure 3.6a North-east Wales: eDNA vs median number of Great Crested Newts detected by torch counts.

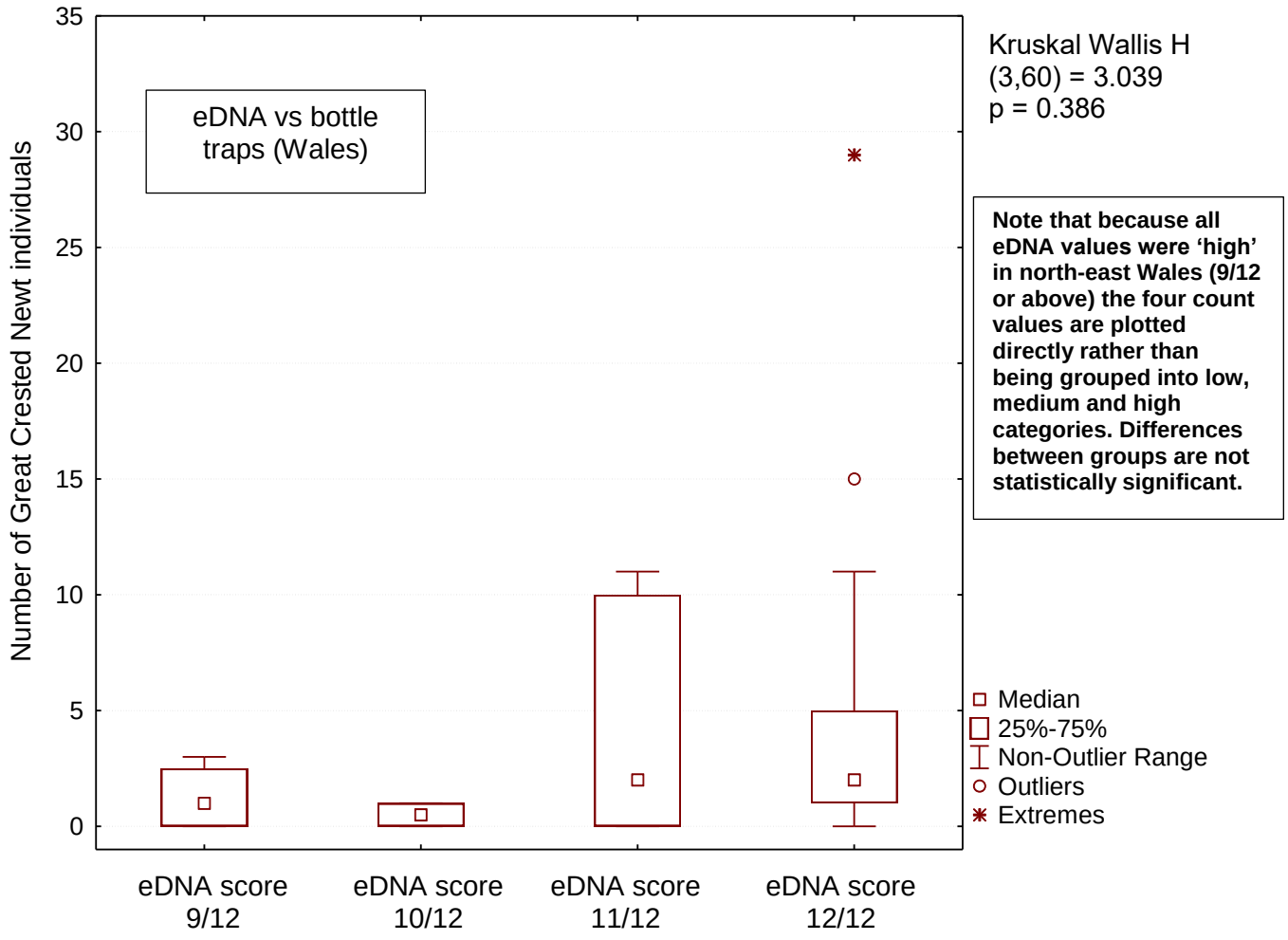


Figure 3.6b North-east Wales: eDNA vs median number of Great Crested Newts detected by bottle trapping.

A third analysis was undertaken using data collected over the last 20 years by Tom Langton. We compared Langton's torch counts of Great Crested Newts in 2013, and the peak torch counts over a period of up to 22 years, with the eDNA scores collected in 2013.

Inspection of the data suggests that, as in south Hampshire and north-east Wales, higher eDNA scores were associated with higher Great Crested Newt counts. In this case the eDNA scores are highly correlated with Great Crested Newt torch counts, both in 2013 (Figure 3.7a; $p < 0.0001$) and for peak count data over several years (Figure 3.7b; $p < 0.0001$). As in south Hampshire there is a clear indication that low eDNA scores are always indicative of low newt abundance (see Figure 3.7a,b) whereas abundance may be either low or high when eDNA scores are high.

Interestingly there was a much clearer relationship in Langton's data when eDNA was grouped into three categories (low, medium and high). In this case there were significant differences between median newt counts at different levels of eDNA, both in 2013 (Figure 3.8a) and when the peak count over several years (mean = 9 years) was used (Figure 3.8b)

It is not immediately clear why the relationship between eDNA and Great Crested Newt counts was so much stronger at these sites than in the south Hampshire and Welsh sites. As noted in the discussion, understanding of the dynamics of eDNA in the water, and the relationship between eDNA abundance and animal abundance, is still an area where further research is needed.

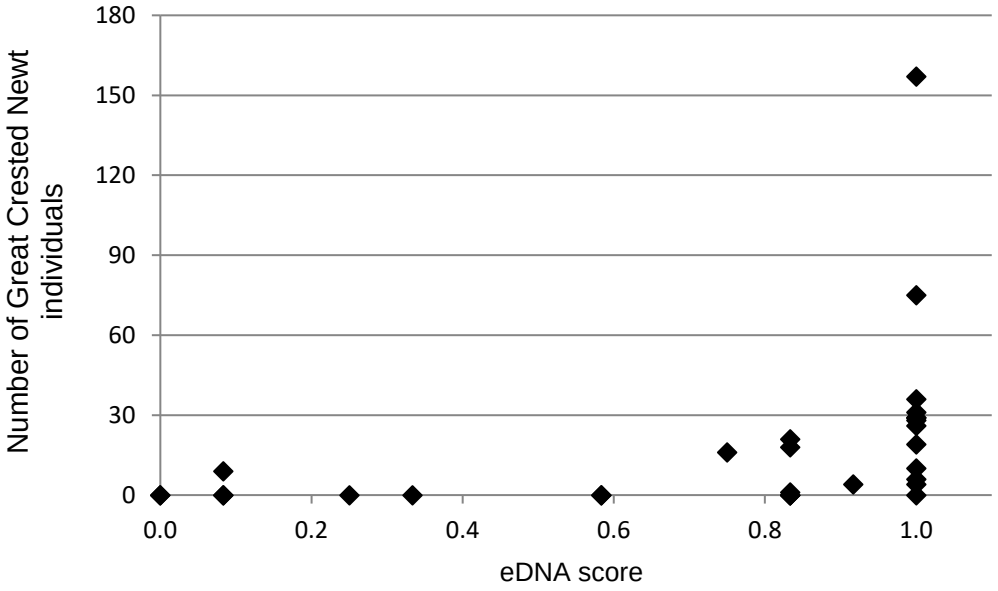


Figure 3.7a The relationship between eDNA score and Great Crested Newt abundance around Dew's Farm, Suffolk, in 2013, as measured by torch counting.

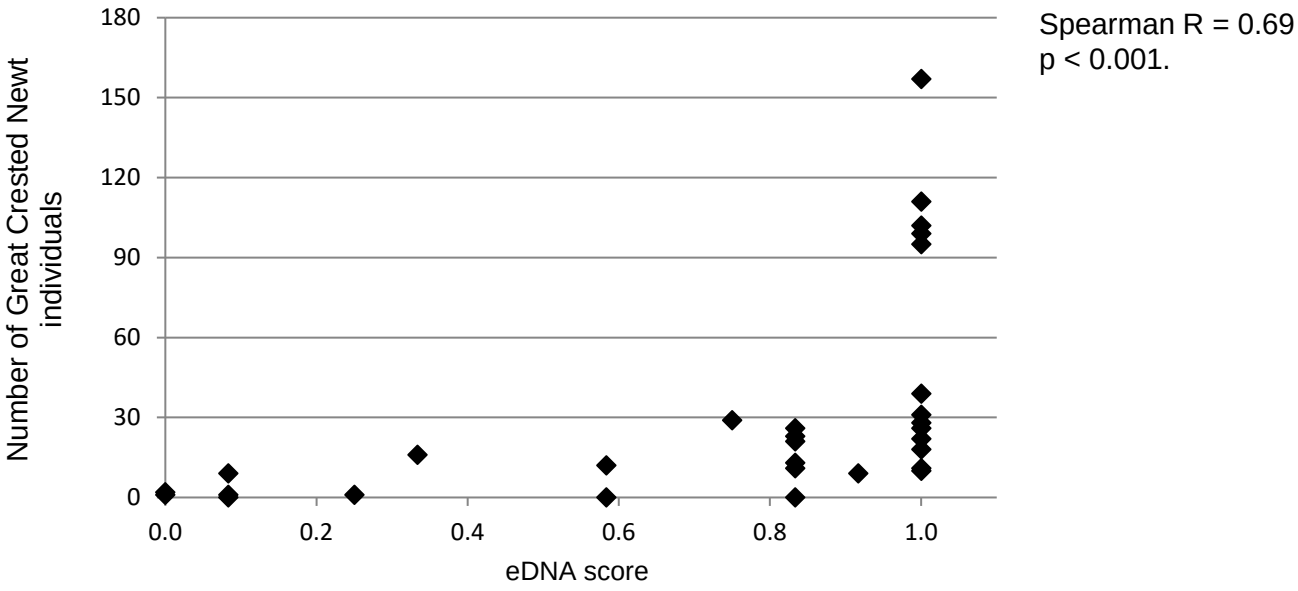


Figure 3.7b The relationship between eDNA score and Great Crested Newt counts in ponds around Dew's Farm, Suffolk: peak counts over several years, as measured by torch counting.

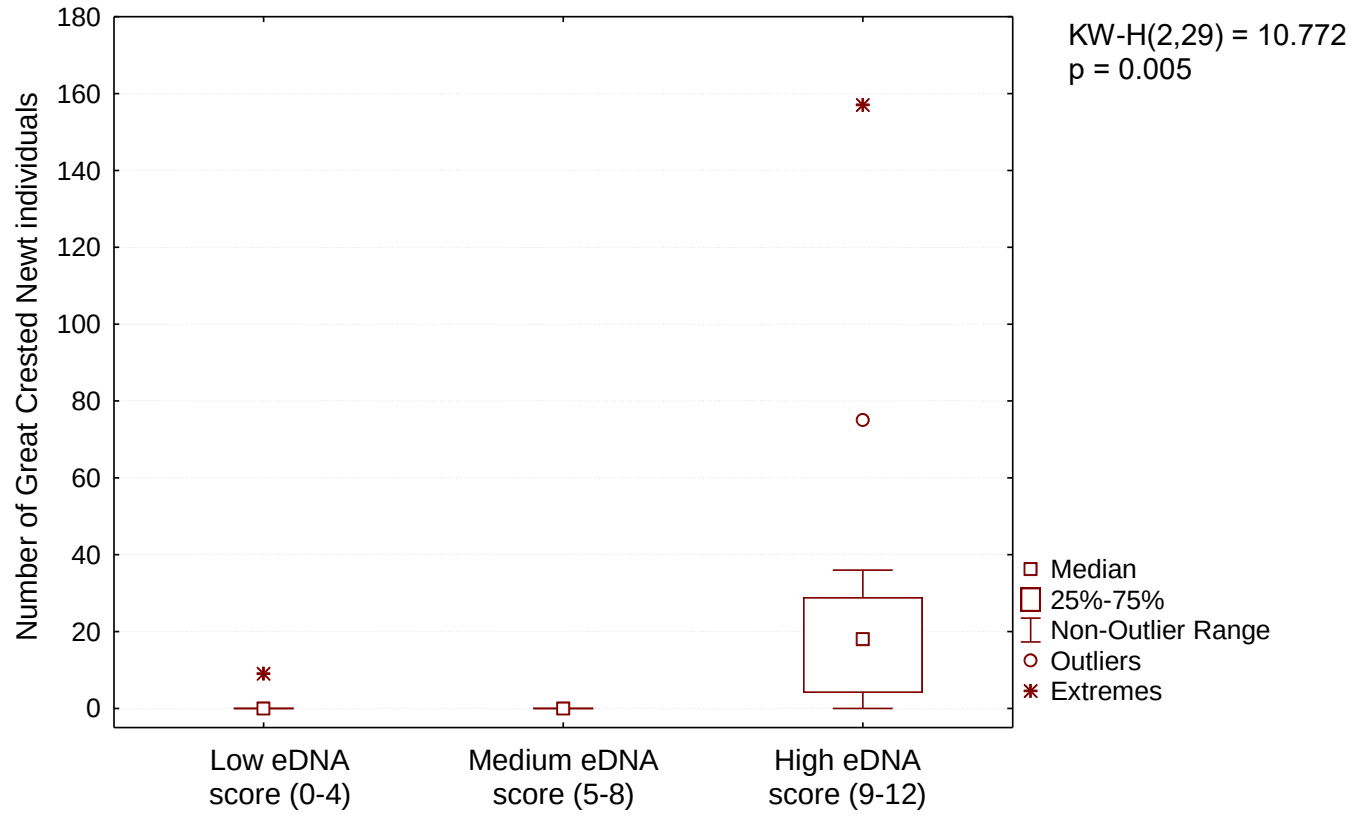


Figure 3.8a Tom Langton sites: eDNA scores vs peak number of Great Crested Newts found in 2013 (peak of three counts)

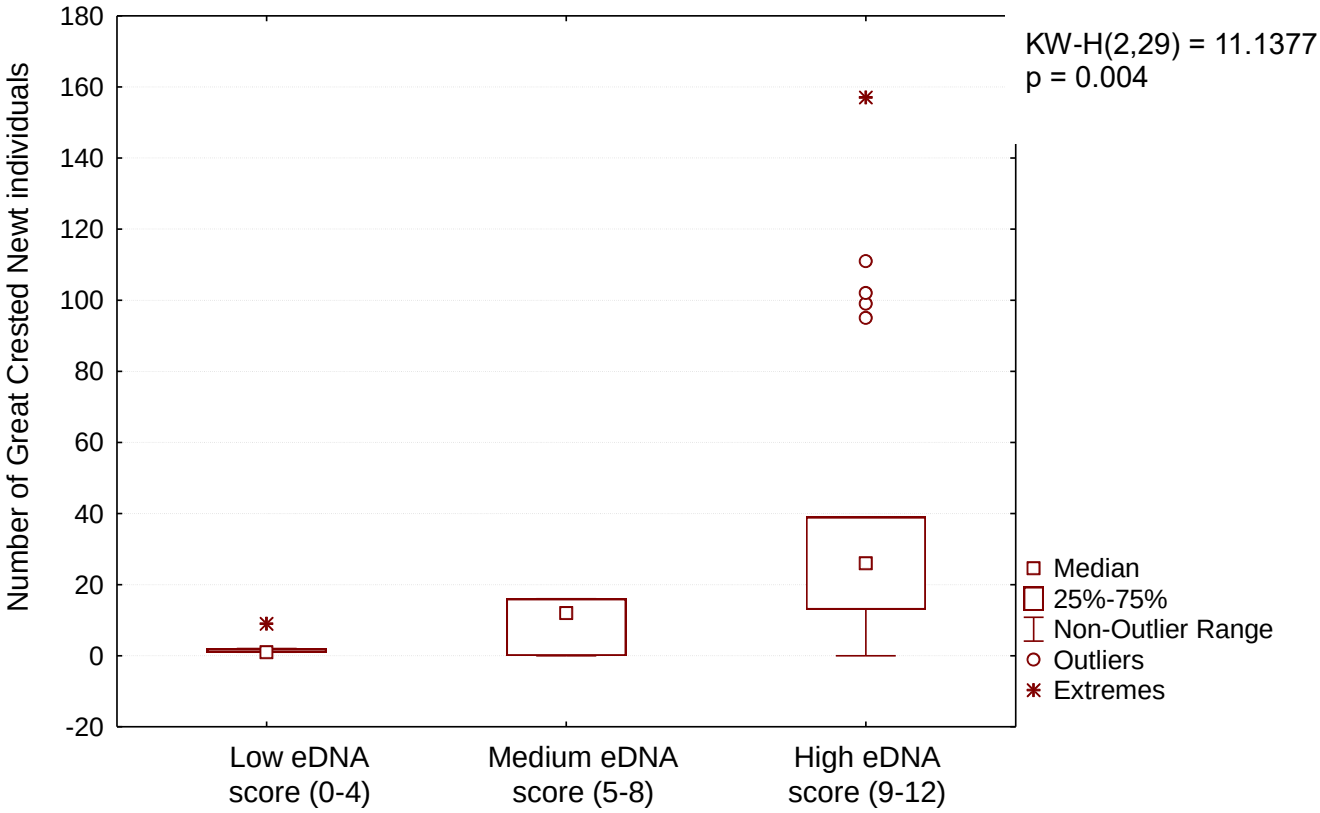


Figure 3.8b Tom Langton sites: eDNA vs peak number of Great Crested Newts found in torch counts over several years (maximum 22 years).

3.2 Volunteer survey

In the volunteer-collected eDNA survey of 239 ponds across England, Scotland and Wales, eDNA analysis correctly detected newts at 218 (91.2%) of sites. Thus in the volunteer survey there were a small number of **false negatives (**8.8% of sites) with good evidence that newts were present during the 2013 field season where they were not detected by eDNA.

Although relatively few in number, the false negatives provide a valuable indication of the factors that influence the effectiveness of the eDNA technique. They are discussed further in Section 3.4.

There was no evidence that **false positives** were generated. eDNA did not detect Great Crested Newts in the 30 out of range sites in Cornwall or 3 out of range sites in Shetland.

Within the range of the newt, there were also **no false positives** i.e. at the 30 in-range sites where, as far as we knew, there were no newts, eDNA records were all negative.

3.3. Quality assurance of volunteer samples

The same eDNA results were obtained at 24 of the 26 sites (92%) at which a second eDNA sample was collected professionally to quality assure the volunteer sample. 22 samples were positive on both occasions, and two sites were negative on both occasions. At two sites the volunteer and quality assurance samples differed: both were ponds where very low amounts of DNA was recorded (initial samples scored 1 out of 12 for eDNA, with the maximum possible being 12 out of 12).

3.4 Factors leading to false negatives

Three main factors appear to lead to false negative eDNA results:

(i) *Ponds with very low numbers of newts*. About a quarter of the false negatives are locations where there appear to be small populations of newts. For example, at the Blackmuir Wood site in Scotland one local surveyor had looked twice this year before the sample was collected and had seen no adult Great Crested Newts or eggs and the site was also known to have a diminishing newt population over the last few years, possibly due to fish introduction. Despite this, later in the year and after the eDNA sample was collected, a Great Crested Newt larva was photographed proving presence. No eDNA was detected at the site, i.e. it was a false negative. Subsequently the surveyor commented: 'The main pond currently has a low water table and maybe where water samples were collected around the pond edge there weren't any Great Crested Newts'.

(ii) *Ponds with wide shallow edges*. In several locations, very shallow water over a wide drawdown zone and/or margins dominated by dense vegetation reduced the volunteer's ability to collect a positive eDNA reading. This may have been either because these areas were less favoured by newts, or because the dense vegetation prevented mixing of the water. Volunteers were also specifically instructed not to enter the pond or disturb the sediment when taking the sample which, in ponds with very broad shallow margins, limited their ability to collect water from areas more likely to coincide with newt activity in the pond. At Madeley, in Cheshire, we tested this specifically by sampling amongst a dense marginal floating mat of grasses over a shallow drawdown zone and compared this to the pond's centre which had deeper water on the edge of the marginal vegetation. This could only be reached by attaching the sampler to a long pole. eDNA was not found amongst the floating mat but was detected in the open water.

(iii) *Ponds where sampling was restricted to a small part of the pond*. There was clear support for the suggestion that it was necessary to sample right round the pond, as specified in the survey method. For example, at Bowldish in Dorset, where a false negative result was obtained, the volunteer commented that '…I was only able to sample a small section in one area as the pond was surrounded by blackthorn bushes growing right down to the edge…'. Similarly in Scotland, at Dunmore Swamp Pond there were access difficulties due to thick vegetation and steep banks, so that the sampling area was limited to about 10% of the pond perimeter. No eDNA was detected at this site although newts were known to be present.

The occurrence of false negatives is likely to be greater when low newt density and access difficulties occur in combination.

There are two main actions which may help reduce false negatives:

(i) to provide surveyors with face to face training. In the present survey, most surveyors were only given written instructions on how to collect the eDNA sample. It is possible that hands-on demonstrations would improve the results by reinforcing the importance of surveying, as far as possible, all around the pond.

(ii) following the experience of the first year survey we would reiterate some aspects of the survey, and suggest some small modifications to the survey method. Specifically:

- Ensuring that samples are collected from as much of the pond as possible
- Avoiding broad pond marginal areas with very shallow water (e.g. less than 5 cm deep), where newts are unlikely to be found
- Avoiding very densely vegetated areas (for example dense floating mats of vegetation, which newts may not be able to penetrate); conversely it is probably desirable to collect water samples as close as possible to areas which are likely to be used by newts for egg-laying.

3.5 Environmental factors influencing the eDNA approach

3.5.1 Were the sites representative of the range of the Great Crested Newt?

To evaluate the extent to which the volunteer eDNA survey ponds reflected the heterogeneity of ponds occupied by Great Crested Newts nationally we examined the volunteer pond's representativeness in four ways:

- Did the volunteer ponds cover a substantial proportion of the ponds likely to be used by the Great Crested Newt throughout its range in the UK?
- Did the volunteer ponds represent the full range of pond sizes likely to be used by Great Crested Newts?
- Did the volunteer ponds match the altitudinal distribution of ponds likely to be used by newts?
- Did volunteer ponds occur in the same proportions in Defra land classes as the ponds nationally which are likely to be used by Great Crested Newts?

In each case we compared the volunteer ponds with the simulated set of 57,000 ponds identified as potential Great Crested Newt sites owing to their proximity to existing records for Great Crested Newt (see Sections 2.1.10 and 4.4.2 for description of methods used to generate the simulated Great Crested Newt pond dataset).

(i) Species range

The ponds with a closely associated record for Great Crested Newt are shown in Figure 3.9 (black spots). The volunteer survey sites in the current project are shown by green spots in

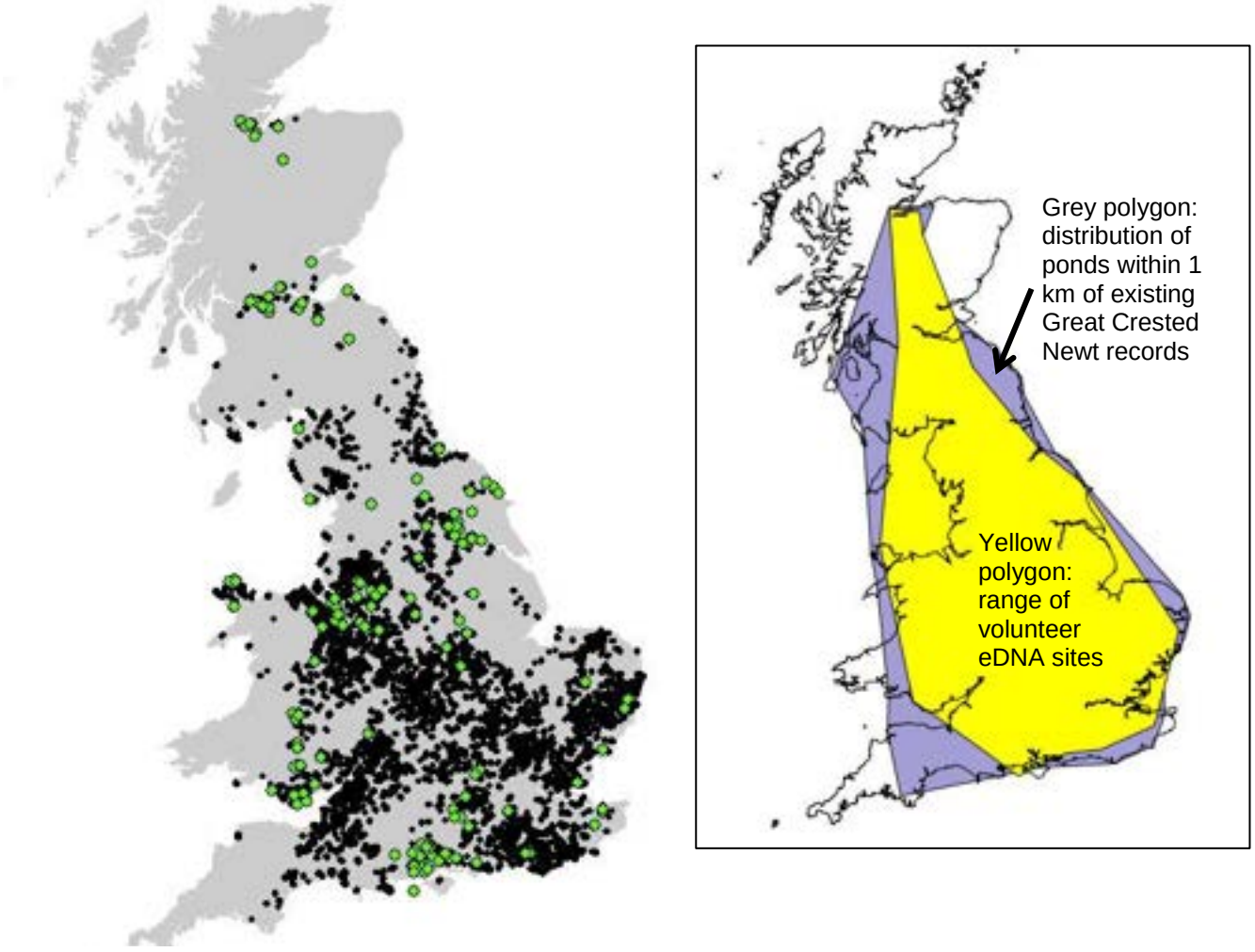


Figure 3.9 Distribution of Great Crested Newt ponds (black spots) and eDNA volunteer surveyed ponds (green spots). The inset shows the broad range encompassed by known Great Crested Newt sites (grey polygon) and the range encompassed by the volunteer sampled eDNA sites (yellow polygon).

this figure. The simulated range over which Great Crested Newts occur in Great Britain is encompassed by a polygon extending over about 217,000 km^2 (grey area in the inset within Figure 3.9). The volunteer sites broadly reflected this range and are encompassed by a yellow polygon covering about 75% of this area (c. 166,000 km^2) (Figure 3.9).

(ii) Area of Great Crested Newt ponds in the study

The area of ponds which could be used by Great Crested Newts (i.e. within 1 km of known records) was derived from the Ordnance Survey water layer pond outlines.

The area of ponds survey for eDNA by volunteers closely matched the national pattern (Figure 3.10).

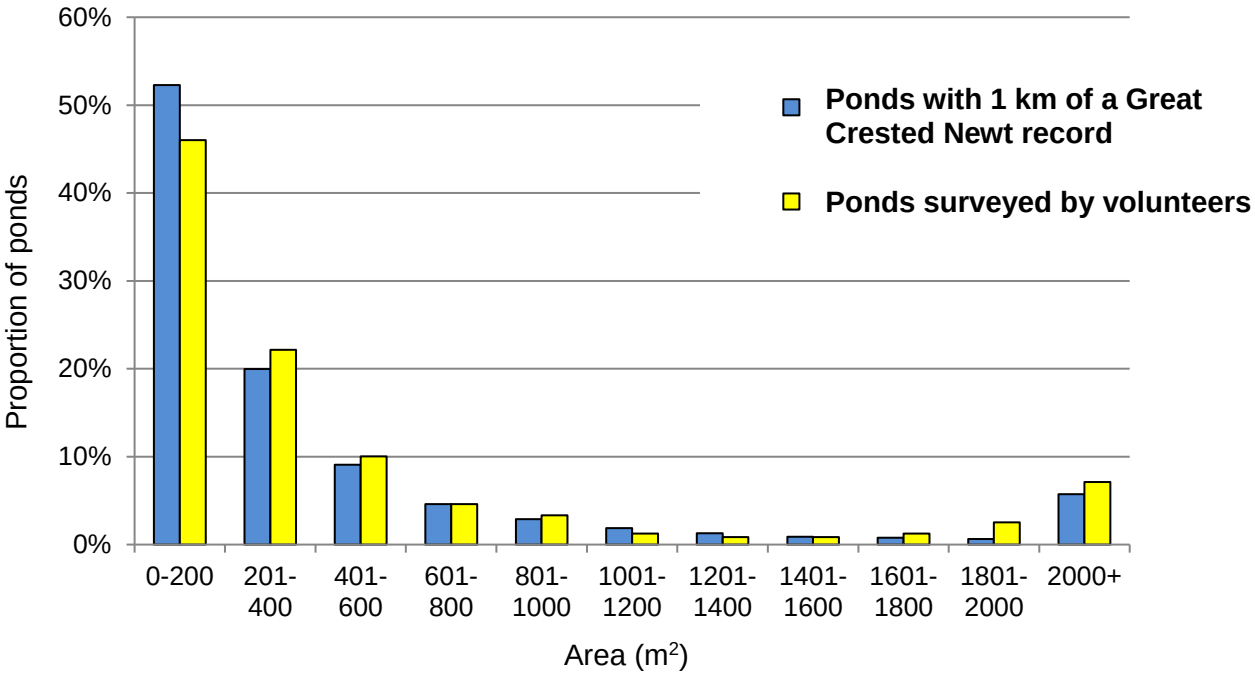


Figure 3.10 Comparison of the area of all ponds within 1 km of post-1988 Great Crested Newt records (n=57,021) and ponds in the volunteer eDNA survey (n=239)

(iii) Altitudinal range of Great Crested Newt ponds in the study

The volunteer sampling locations broadly matched the national altitudinal range of ponds within 1 km of a known Great Crested Newt record (Figure 3.11).

Nearly 90% of the ponds which could be used by Great Crested Newt nationally are found in the 0-100 m altitude range. A small number are found above this altitude, up to 450m.

The volunteer sites closely matched this altitudinal range, with slightly more sites in the higher altitudinal ranges than the national proportions.

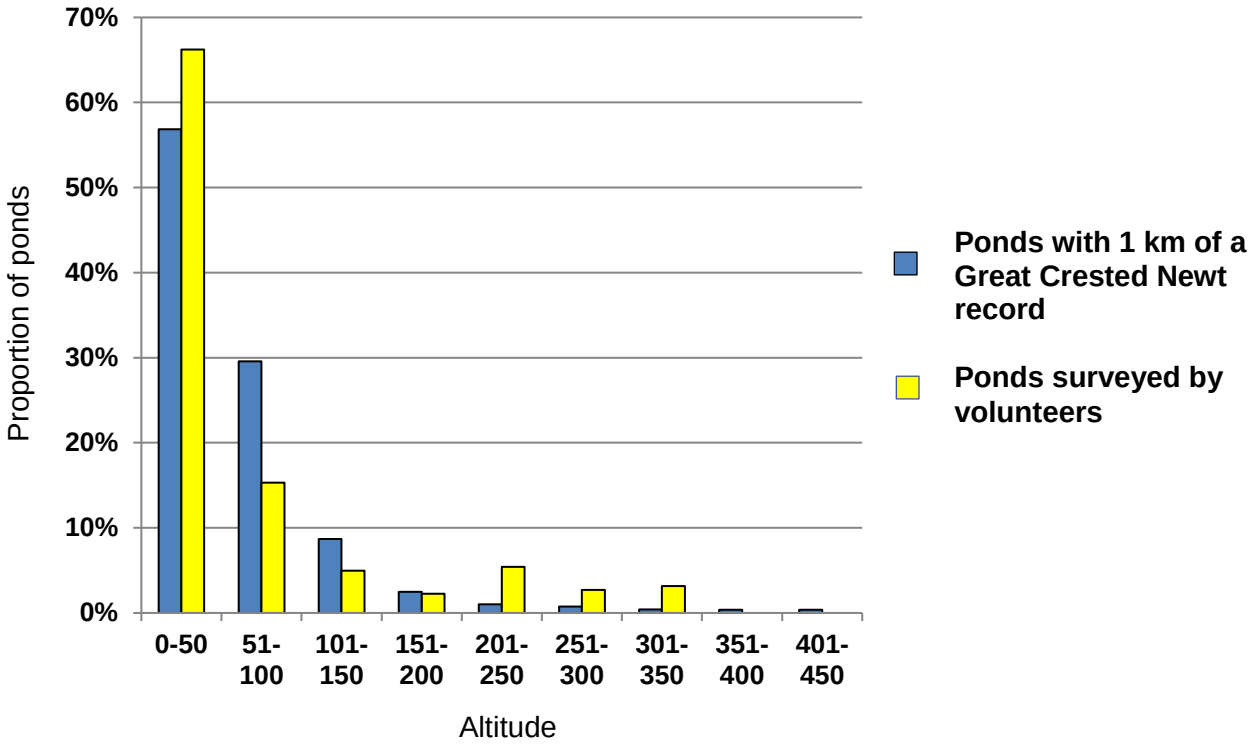


Figure 3.11 Comparison of altitudinal range of all ponds within 1 km of post-1988 Great Crested Newt records in Great Britain (n=57,021) and ponds in the volunteer eDNA survey (n=239)

(iv) Land classes of Great Britain

The agricultural landscape classes defined in the Defra 'Aquatic ecosystems in the UK agricultural landscape' project are shown in Figure 3.12. Land classes were defined in terms of soil, hydrology, land use and cropping characteristics likely to have a significant impact on aquatic ecosystems.

Ponds which could support Great Crested Newts are predominantly found in eutrophic till landscapes (Land Class 4) and pre-Quaternary clay landscapes (Land Class 6) (Figures 3.12, 3.13 and 3.14). Volunteer sample sites were also predominantly located in these landscapes, although a relatively large proportion of the volunteer sites were located in non-agricultural areas. Nationally, ponds which could be used by Great Crested Newt are found in all land classes, although only in very small numbers within Land Classes 11 and 12, which are restricted to a small area in Scotland. Volunteer sites reflected this pattern (Figure 3.13).

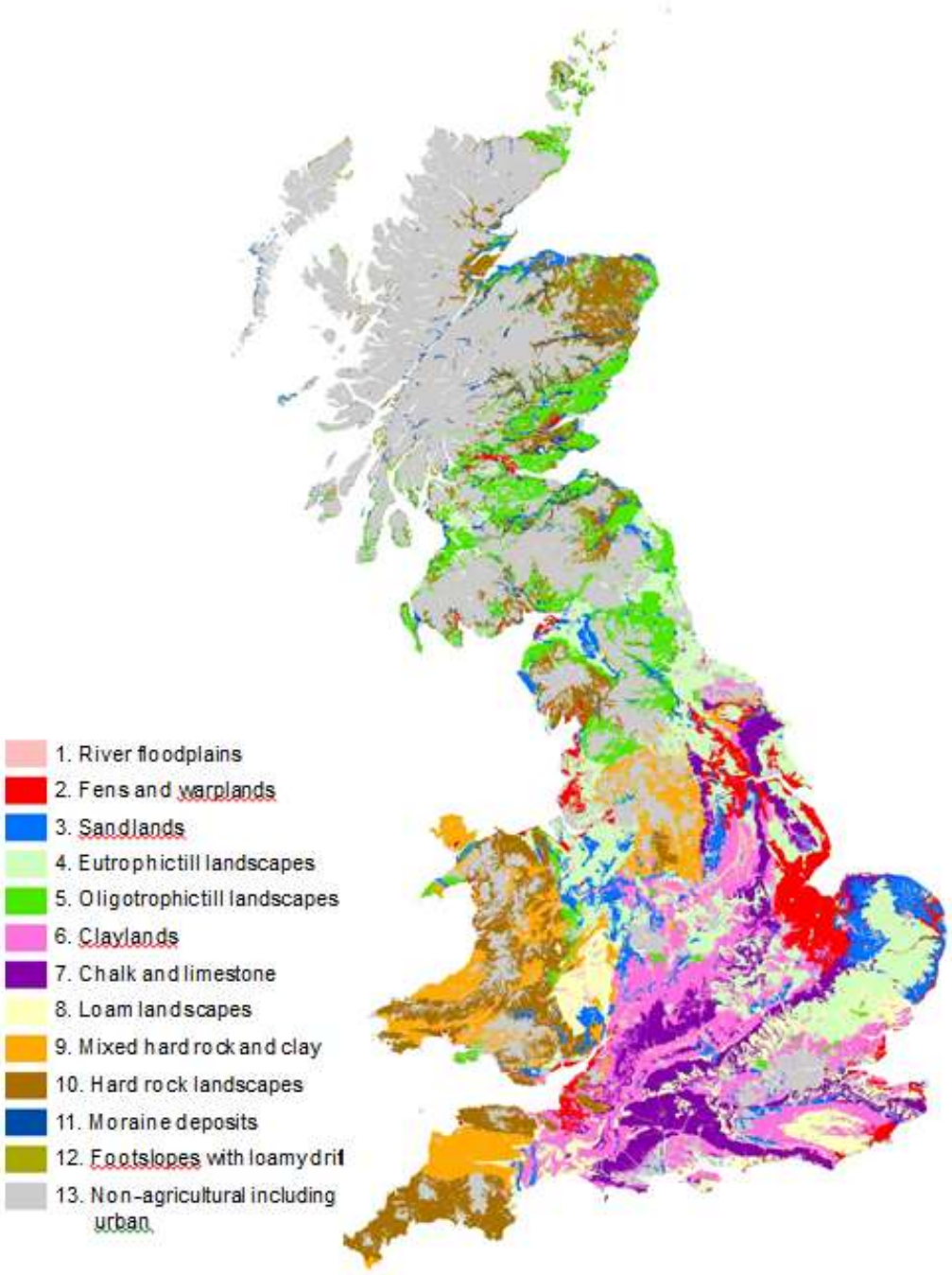


Figure 3.12 The distribution of agricultural land classes in Great Britain.
Land classes were developed during Defra project 'PN0931 Aquatic ecosystems in the UK agricultural landscape' (Brown *et al.* 2006). Characteristics of land classes are summarised in Table 3.1.

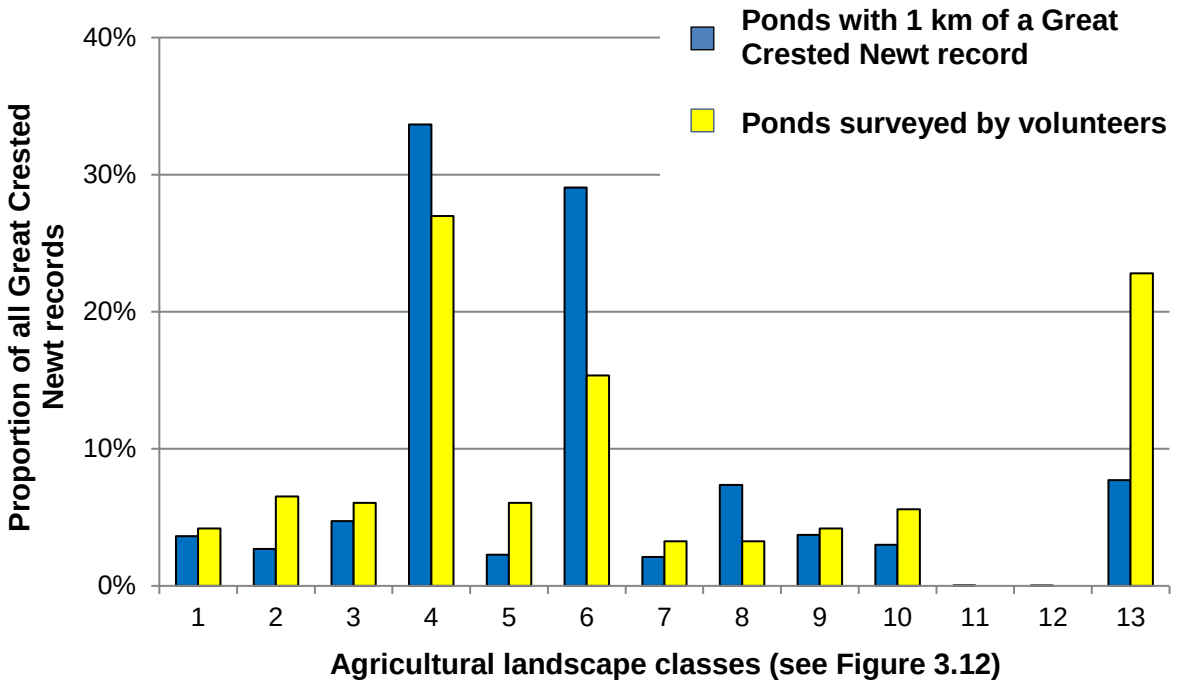


Figure 3.13 Distribution of Great Crested Newt ponds in Defra agricultural landscape classes.
Blue bars show distribution of all ponds within 1 km of a known Great Crested Newt record (n=57,021); yellow bars show distribution of volunteer survey sites in the present project (n=239).

Table 3.1 Description of the Defra landscape classes

No.	Landscape	Description	Total area (km^2)	Crops (minor crops in parentheses)
1	River floodplains and low terraces	Level to very gently sloping river floodplains and low terraces	7,781	Permanent grass, some cereals and oil-seed rape, probably more intensive on terraces
2	Warplands, fenlands and associated low terraces	Level, broad 'flats' with alluvial very fine sands, silts, clays and peats	9,017	Cereals (oil-seed rape, beans), sugar beet, potatoes, peas, vegetables, top fruit
3	Sandlands	Level to moderately sloping, rolling hills and broad terraces. Sands and light loams	10,871	Cereals (oil-seed rape, beans and peas), sugar beet, potatoes (peas in East Anglia)
4	Till landscapes	Level to gently sloping glacial till plains. Medium loams, clays and chalky clays, with high base status (eutrophic). Some lighter textured soils on outwash	22,151	Cereals, oil-seed rape and beans (peas in E. Yorks.), permanent and rotational grass (mainly in west)
5	Till landscapes	Level to gently sloping glacial till plains. Medium loams and clays with low base status (oligotrophic). Some lighter textured soils on outwash	15,449	Permanent and rotational grass with some cereals and oil-seed rape
6	Pre-quaternary clay landscapes	Level to gently sloping vales. Slowly permeable, clays (often calcareous) and heavy loams. High base status (Eutrophic)	19,706	Permanent grass, cereals (>10-15%), leys, oil-seed rape, maize (not in NE or Weald) and beans
7	Chalk and limestone plateaux and coombe valleys	Rolling 'Wolds' & plateaux with 'dry' valleys; shallow to moderately deep loams over chalk & limestone	14,197	Cereals (and oil-seed rape, beans), sugar beet, potatoes, peas
8	Pre-quaternary loam landscapes	Gently to moderately sloping ridges, vales and plateaux. Deep, free-draining & moderately permeable silts & loams	10,072	Permanent & rotational grass, cereals and oil-seed rape with some beans, grass, hops and fruit
9	Mixed, hard, fissured rock and clay landscapes	Gently to moderately sloping hills, ridges and vales. Moderately deep free draining loams mixed with heavy loams and clays in vales	12,259	Permanent grass, rotational grass and some cereals (<10-15%)
10	Hard rock landscapes	Gently to moderately sloping hills and valleys. Moderately deep free draining loams over hard rocks. Some slowly permeable heavy loams on lower slopes and valleys	23,342	Permanent grass, rotational grass and some cereals (<10-15%)
11	Moundy morainic and fluvioglacial deposits	Gently and moderately sloping mounds, some terraces. Free draining moraines, gravels & sands on mounds, poorly draining gleys in hollows	2,270	Permanent and rotational grass, some cereals
12	Footslopes with loamy drift	Concave slopes or depressional sites often with springlines	1,081	Permanent and rotational grass
13	Non-agricultural	All areas not cultivated with arable (including orchards, soft fruit and horticultural) or maintained grassland	79,690	No crops
Total			**227,886**	

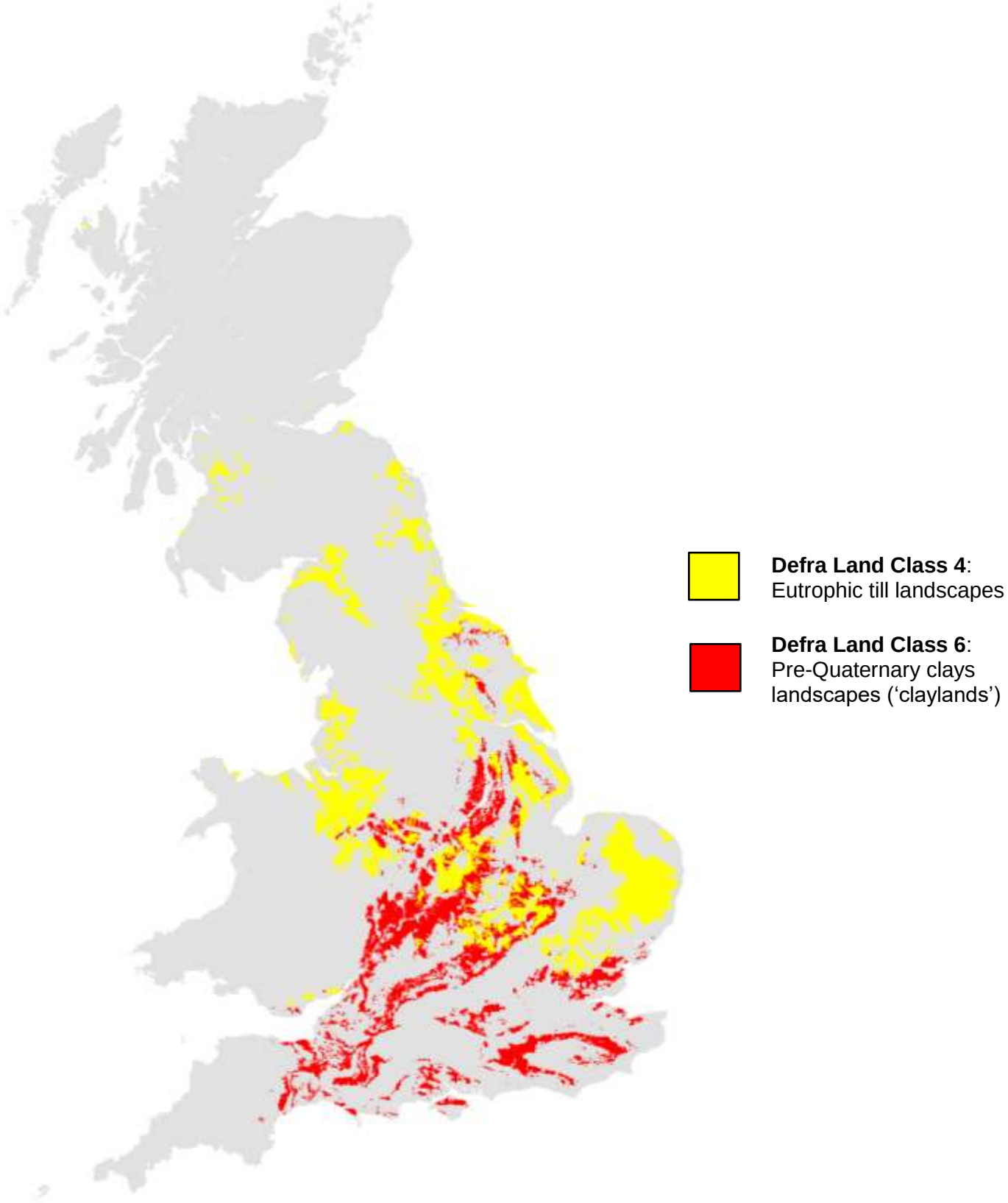


Figure 3.14 Land class 4 (eutrophic till landscapes) and land class 6 (pre-Quaternary clay landscapes) support c63% of all ponds within 1 km of a known post-1988 Great Crested Newt record

3.5.2 Environmental factors influencing the detection of eDNA

(i) The relationship between eDNA detection and HSI score in the volunteer survey sites

Overall, there was a weak positive correlation between the amount of eDNA detected and HSI score in the volunteer survey sites (Figure 3.15; Spearman rank correlation, r = 0.33, p<0.001). Grouping sites into low, medium and high eDNA scores suggests that, as in the detailed methodological study, the high eDNA score sites are distinct from the low and medium score sites in terms of HSI. HSI scores above 0.7 are generally regarded as good (Figure 3.16).

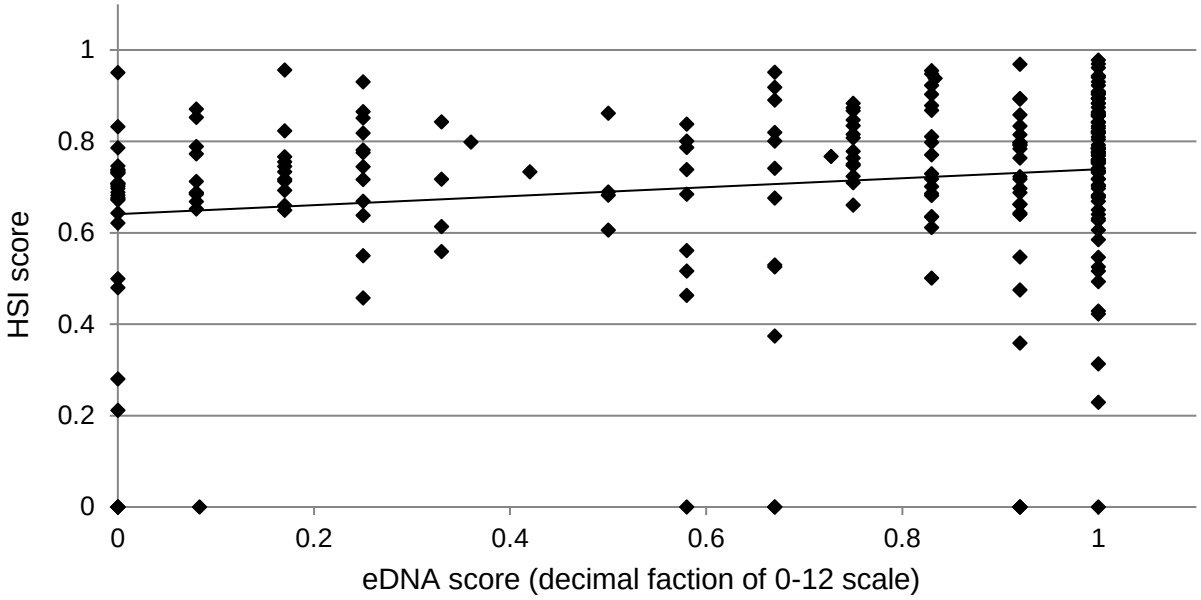


Fig 3.15 Relationship between eDNA value and HSI score

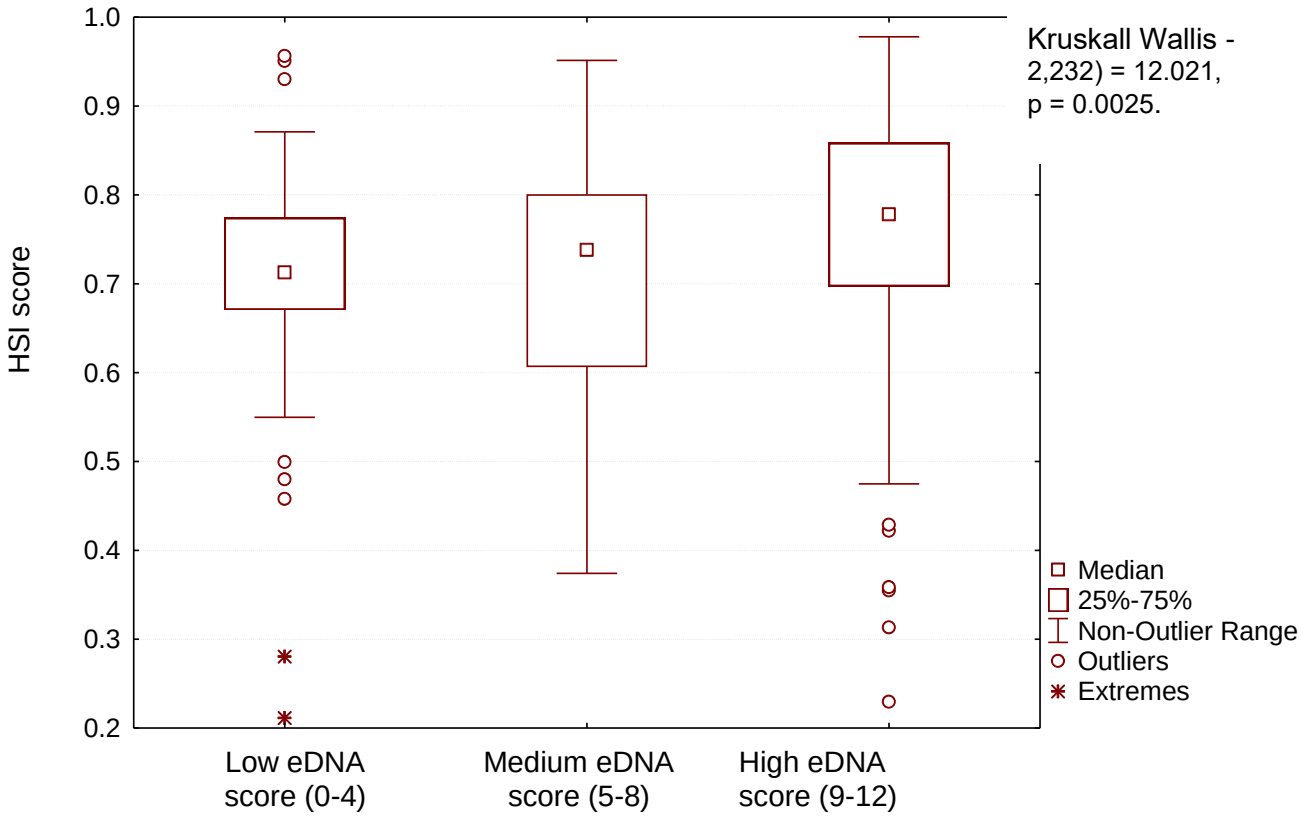


Fig 3.16 Relationship between eDNA groups (low, medium, high) and HSI score in volunteer survey

(ii) Environmental factors potentially influencing eDNA detection

Understanding of the influence of environmental factors on eDNA detection is still at a very early stage as little is known of the ways in which commonly varying environmental factors affect eDNA. We therefore undertook a largely exploratory analysis seeking to screen a variety of potentially influential environmental factors to assess their influence on eDNA detection.

Spearman rank correlation analysis, with Bonferroni correction, indicated that only the overall HSI score was significantly correlated with eDNA score. No other environmental factors were significantly correlated with eDNA score (i.e. had p values of 0.004 of less), although the absence of fish was slightly, but non-significantly, correlated with eDNA score (p=0.027).

Overall, the results suggests that, at a national scale, the single most important factor influencing the eDNA score is the presence of Great Crested Newts, reflected by the HSI score and hinted at by the weak relationship with the absence of fish. Amongst the environmental variables for which we were able to obtain data there was little evidence of a direct impact on eDNA score. However, it should be noted that we have no data on water quality (water quality in the HSI score is a subjective assessment), amounts of organic matter or light climate, all of which might influence DNA breakdown.

Table 3.2 Summary of significance tests for relationships between environmental factors and eDNA score

Environmental factors	Spearman's rank correlation	P (significant values in bold)	n
Overall HSI score	0.221	**0.001**	231
Shade	0.106	0.109	230
Fish (absence)	0.146	0.027	230
Terrestrial habitat quality	0.071	0.282	230
Presence of waterfowl	0.018	0.781	230
Number of adjacent ponds	0.049	0.463	230
Water quality	0.095	0.150	230
Pond dries	0.015	0.817	230
Pond area	0.023	0.727	230
Abundance of aquatic vegetation	0.006	0.930	230
Altitude	0.026	0.688	237
Environmental factors	**Kruskal-Wallis H**	**p**	**n**
Range areas of Great Crested Newt: A, B and C	4.6 (df = 2)	>0.1	230

3.5.3 Methodological influences on the detection of Great Crested Newt using eDNA

(i) Influence of sampler

The quality assurance samples provided a preliminary indication of the influence of sampler on eDNA detection. Resurvey of volunteer sampled ponds by a professional surveyor gave the same result in 92% of cases. There appears to be a limited influence of sampler on the repeatability of eDNA sampling.

(ii) Influence of season of sampling

The detailed methodological analysis (see section 3.1) indicated that there was no difference in eDNA detectability during the main breeding season.

Volunteer samples also showed no evidence of season being a factor in the amount of eDNA detected. eDNA scores did not differ significantly during weekly periods throughout the time that samples were collected (Figure 3.17).

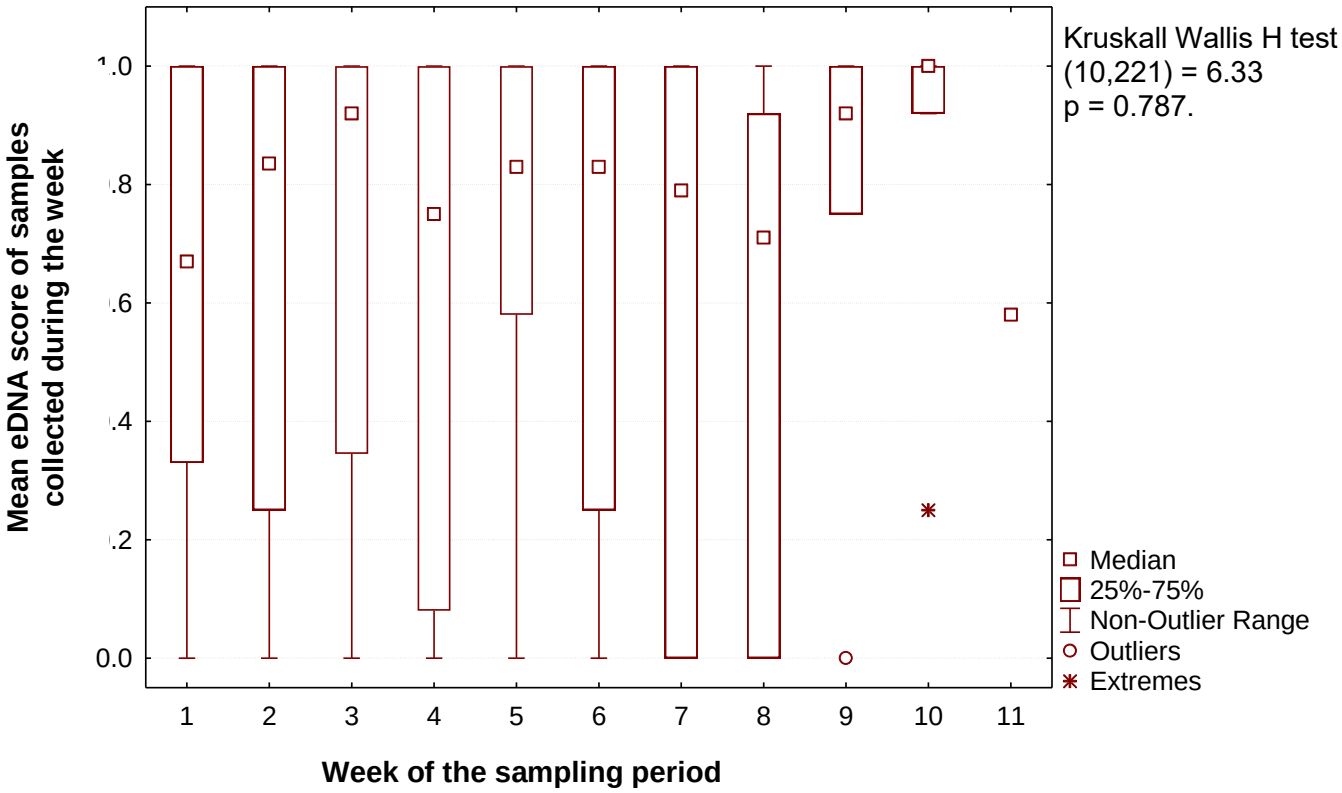


Figure 3.17. Median eDNA scores of samples collected during weekly periods of the sampling programme.

Further autumn samples are currently being collected and analysed to explore whether eDNA could be detected later in the autumn. Results will be reported separately (Biggs *et al*. 2014).

(iii) Influence of cross contamination

There was no evidence of any false positives indicating that cross contamination is not a significant problem in the detection of Great Crested Newts.

4. Results Part B: Statistical support for producing GB trends for the Great Crested Newt

4.1 Introduction

Part B of the project provides statistical support for the design of robust sampling strategies for three parameters: (i) pond turnover, (ii) habitat suitability i.e. HSI score, and (iii) pond occupancy by Great Crested Newts.

There are a number of previous and on-going surveys which have collected data on pond turnover, habitat suitability and pond occupancy by Great Crested Newts. The aim of this report is to determine how much larger these networks/surveys would need to be and how they can be integrated to ensure that they have sufficient power to detect low levels of change. To date there are only a handful of surveys which have collected this type of information which has been invaluable in providing stock data and an indication of the variability between sites and between years. In many cases the data were collected for purposes other than assessing change in occupancy and condition of Great Crested Newt habitats. With a wider network and greater number of samples points, both spatially and temporally, confidence in these data for Great Crested Newt models will increase.

Throughout Part B of the report we use a number of statistical terms to describe the results of power analysis. Box 1 briefly summarises the meaning of these terms.

Box 1. Statistical terminology used to describe the results of power analysis

Power (1-β): the probability of detecting an effect if one exists in the population, largely dependent on sample size N, effect size and levels of variance in sample groups σ^2.

Beta (β): in a power analysis, is the probability of accepting the null hypothesis, even though it is false. This produces a so called Type II when there is a failure to reject the null hypothesis, even though the alternative hypothesis is true.

Alpha (α); the confidence that the observed results are statistically different from the random variation seen in the environment.

Type I errors: as the size of alpha increases (i.e. above the conventionally adopted 0.05), so does the risk of detecting a significant result when one does not exist.

Overall, robust experimental designs reduce the risk of Type I and Type II errors occurring, but at the same time should minimise the cost of analysing too many samples unnecessarily

4.2 Pond numbers

4.2.1 The pond layer from MasterMap

The cleaned water layer of ponds up to 2 ha in area is shown in Figure 4.1. A total of just over 670,000 waterbodies is contained in the pond layer.

Figure 4.1 The cleaned Ordnance Survey pond layer derived from MasterMap data.

4.2.2 Sample size required to achieve different levels of power to detect change in pond numbers at country, England + Wales and Great Britain levels

(i) Background

There is a strong correlation between Great Crested Newt populations and high pond densities (Swan and Oldham 1993, Grayson 1994, Gleed-Owen 2007) and one of the major underlying causes for the decline in Great Crested Newts is the loss of breeding sites (Beebee 1975), i.e. populations may become less viable when pond density drops below 0.7 ponds per square km (Langton 2009).

There have been various estimates of the change in pond numbers over differing time periods (Countryside Survey ponds report (Williams *et al*. 2007)):

- In England and Wales pond numbers decreased by around three quarters during the 20th Century from a maximum of about 800,000 estimated from map counts in the late 19th century to around 200,000 by the 1980s (Rackham 1986, Barr *et al.* 1994, Biggs *et al.* 2005).
- The historic numbers of ponds and rate of pond loss in Scotland are not known, but rates of loss are probably lower with maximum estimated losses of only 7% between the 1950's and the 1980's (Swan and Oldham 1993 and Swan *et al*. 1994).
- Based on National Amphibian Survey estimates, pond loss in Britain since the Second World War was of the order of 38%, a loss of just under 1% per annum (Swan and Oldham 1993).
- Increased declines in breeding ponds were identified from 1966-1974, estimated at 50% by Beebee (1975).
- Countryside Survey results in 1990 suggested losses of around 1% per annum for the period 1984 to 1990 (Barr *et al.* 1994).
- In contrast, the MAFF Survey of Environmental Topics on Farms, investigating trends during the period 1980 to 1985, concluded that there had been a net increase in ponds in England and Wales of approximately 3% over that period (MAFF 1985).
- Countryside Survey data suggest that from the 1990's pond numbers began to rise with an increase of around 6% (0.8% per annum) between 1990 and 1998 (Haines-Young *et al.* 2000).

The Countryside Survey 2007 (Williams *et al*. 2010) found that:

- The number of ponds in Great Britain is estimated to have increased significantly by 12.5% from 425,000 to 478,000 ponds between 1998 and 2007. This equates to a change in average pond density from 1.86 to 2.10 ponds per km^2.
- The number of ponds also increased in all three countries. However, the percentage change in pond numbers was significantly higher in England and Wales (18% and 17% respectively) than in Scotland (5.5%).
- There was a high turnover of ponds between 1998 and 2007, with an estimated 18,000 ponds lost and 70,600 new ponds created, resulting in an annual increase of 1.4% in Britain (Williams *et al*. 2010).

Estimates of pond turnover are dependent on the quality of the datasets used to estimate change. Previous estimates based on field surveys (e.g. early Countryside Survey data from the 1990s) have often underestimated the number of ponds, overlooking ponds in woodland, man-made ponds, temporary ponds, ponds within other wetland habitats, or through lack of surveyor skill in identifying pond features. Remote sensing methods may underestimate the number of ponds due to poor resolution in identifying temporary ponds and those under tree cover or they may overestimate the number of ponds by failing to identify pond features which have since been lost.

There are two main approaches to monitoring strategies for detecting change in pond numbers and therefore pond turnover:

(i) On the ground mapping of ponds within a network of 1 km grid squares - which can be scaled up (e.g. using Land Classes) to give the total turnover for country, England + Wales and Great Britain levels. This is the 'Countryside Survey' approach, which is more onerous than using remote mapping but also more accurate (Williams *et al*. 2010).

(ii) Remote surveys, using Ordnance Survey data at different spatial scales (1 km grid square, land classes, national or GB levels), which could then be ground-truthed periodically to give an estimate of the error.

(ii) Countryside Survey data

Whilst there were issues with data collection and collation in the Countryside Survey (Williams *et al.* 2010), these data can be used to determine:

1. The power of the Countryside Survey analysis.
2. The sample size required to detect different levels of change at different levels of power using the same monitoring strategy.

Pond numbers were collected from 544 1 km grid squares in 1998 and again in 2007 by professional surveyors. The survey was stratified so that the number of squares in each land class was proportional to the area of that land class. The average number of ponds per 1 km grid square within each land class was calculated and then multiplied by the area of each land class. National and GB level estimates were then produced by addition of the totals for each land class.

Standard errors and confidence intervals for square level data in Countryside Survey were estimated using bootstrapping, resampling (c.1000 times) from the sample population (n=544) to give an approximation of the distribution of these data. This allows for the non-normality of these data which were heavily skewed towards the majority of 1 km grid squares with very low pond density, in contrast to a few squares with very high pond density.

In Countryside Survey "stock" refers to data collected in each survey year (e.g. t_1), which can be made up of *any number of 1 km grid squares chosen at random within each land class* which may or may not have been visited in past or future years. These data can be used to report on estimates of each feature of interest for that year. e.g. the number of ponds in Great Britain at t_1 = 480,000 within confidence limits.

But a different approach in the sampling design is required to detect "change" between years. In theory it would be possible to visit the same or different randomly selected 1 km grid squares in each survey year, but this causes some analytical problems:

- *If the same 1 km grid squares are visited each year*, it becomes a repeated measures analysis, which reduces variation and increases statistical power. But, any squares lost or gained from the sampling strategy each year become difficult to analyse (and are often dropped) because there are missing values in subsequent or future years.
- Sampling strategies which *select a new random set of 1 km grid squares each year* overcome this issue, but will have a large amount of variation because of variation in the number of ponds between 1 km grid squares. The amount of statistical power is reduced still further if the design is unbalanced, if more 1 km grid squares are sampled at t_1 than at t_2.
- In Countryside Survey a model was created which would make possible the analysis of both types of data within the same analysis to estimate change between years, allowing for both random and repeated effects. However, it was found that this did not work for ponds because of the very non-normal distribution of these data. The same is likely to be true of Great Crested Newt population data. With more work it may be possible to produce a model specifically for freshwater data, but this was beyond the scope of the present project.

Power of the Countryside Survey approach. Countryside Survey has reported on changes in mean pond density between 1998 and 2007 at Great Britain and national levels (Figure 4.2). There were statistically significant increases in ponds at Great Britain level ($p<0.05$; see Carey *et al.* 2008). Calculations of power were based on estimates because it is not possible to access the raw data on which the mean values were calculated. For the same reason it has not been possible to group England and Wales data to give an estimate for the region.

As would be expected, Countryside Survey has acceptable levels of power and at Great Britain level could detect a 13% change in pond density with 73% power ($\alpha_{0.05}$). Power was also good in England, but less so in Scotland and Wales (Table 4.1).

Table 4.1 Power of the Countryside Survey to detect change in pond numbers between 1998 and 2007

Great Britain
CS '98 Mean (estimated StDev) = 1.86 (9.199), 95% CI = 1.410, 2.540 CS '07 Mean (estimated StDev) = 2.10 (9.196), 95% CI = 1.640, 2.780 Data were from matched pairs, data were not normal - therefore non-parametric tests apply. CS '98-'07 average pond density (ponds per 1 km grid square) increased by 0.24 ponds per 1 km^2 (13%)
Power of this analysis ($\alpha_{0.05}$) = 72.50% Power of this analysis ($\alpha_{0.10}$) = 81.95%
England
CS '98 Mean (estimated StDev) = 1.55 (4.112), 95% CI = 1.300, 1.810 CS '07 Mean (estimated StDev) = 1.83 (4.923), 95% CI = 1.530, 2.140 Data were from matched pairs, data were not normal - therefore non-parametric tests apply. CS '98-'07 average pond density (ponds per 1 km grid square) increased by 0.28 ponds per 1 km^2 (18%)
Power of this analysis ($\alpha_{0.05}$) = 99.95% Power of this analysis ($\alpha_{0.10}$) = 99.99%
Scotland
CS '98 Mean (estimated StDev) = 2.35 (23.312), 95% CI = 1.250, 4.100 CS '07 Mean (estimated StDev) = 2.48 (23.635), 95% CI = 1.370, 4.300 Data were from matched pairs, data were not normal - therefore non-parametric tests apply. CS '98-'07 average pond density (ponds per 1 km grid square) increased by 0.13 ponds per 1 km^2 (6%)
Power of this analysis ($\alpha_{0.05}$) = 8.40% Power of this analysis ($\alpha_{0.10}$) = 14.91%
Wales
CS '98 Mean (estimated StDev) = 1.91 (19.849), 95% CI = 0.850, 3.310 CS '07 Mean (estimated StDev) = 2.24 (19.922), 95% CI = 1.230, 3.700 Data were from matched pairs, data were not normal - therefore non-parametric tests apply. CS '98-'07 average pond density (ponds per 1 km grid square) increased by 0.33 ponds per 1 km^2 (17%)
Power of this analysis ($\alpha_{0.05}$) = 36.71% Power of this analysis ($\alpha_{0.10}$) = 49.08%

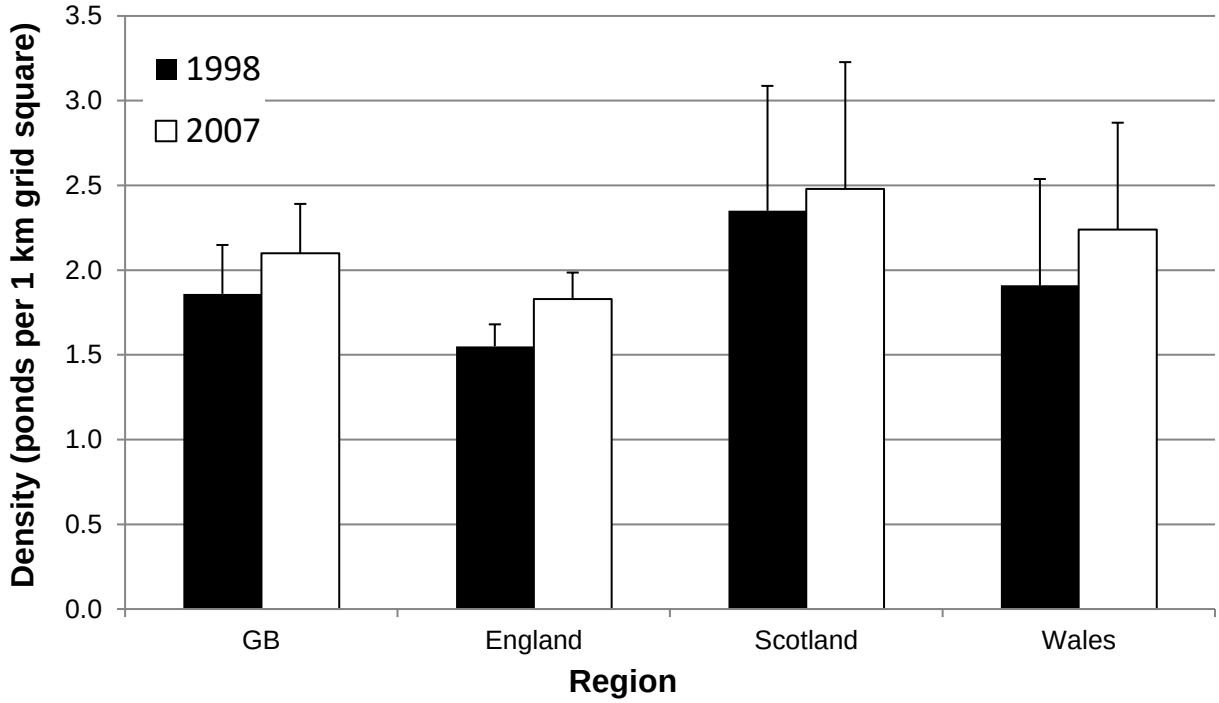


Figure 4.2 Differences in average pond density between 1998 and 2007 based on visiting the same 1 km grid squares at t_1 and t_2.

Power of the Countryside Survey approach to detect smaller changes. Using Countryside Survey data it is possible to calculate the number of 1 km grid squares which need to be surveyed at t_1 and t_2 to achieve different levels of power, at different levels of confidence, to detect different levels of change (Appendix Tables A2.1 – 2.4). Sample squares would be randomly selected, but stratified within land class to allow for stock estimates e.g. the number of ponds in the survey year, and change estimates e.g. the difference in the number of ponds between survey years. The survey design assumes that the same ponds are surveyed at t_1 and t_2.

If we assume that we are hoping to identify a 10% change in pond density over a c.10 year period, sample size each year would need to be:

- Great Britain level - 1996 1 km grid squares (<1% of the Great Britain total number of squares)
- England - 920 1 km grid squares (<1% of the England total number of squares)
- Scotland - 8218 1 km grid squares (10% of the Scotland total number of squares)
- Wales - 8912 1 km grid squares (40% of the Wales total number of squares)

The alternative would be to look for bigger changes or accept less power in Scotland and Wales, due to the high levels of variability in these countries, or to use a different sampling strategy which can detect smaller changes with greater power.

(iii) Ordnance Survey MasterMap data

Countryside Survey was based on a relatively small number of 1 km grid squares. If the exercise could be carried out remotely using existing remote mapping techniques it would provide a more comprehensive picture of pond numbers. The OS MasterMap freshwater layer provided by JNCC was manipulated to produce a pond layer using existing protocols developed by Amphibian and Reptile Conservation.

Confidence intervals for OS Mastermap data were calculated by re-sampling statistics (1000 subsamples) from the national and Great Britain datasets (Table 4.2). Estimates were calculated because of uncertainties in the data layer which are likely to have over and/or underestimated the number of ponds.

Table 4.2 Pond number and pond density estimates based on Countryside Survey data 2007 and OS MasterMap data 2013.

	Pond Density (per km^2)		Pond Numbers('000)	
	Countryside Survey 2007	OS MasterMap 2013	Countryside Survey 2007	OS MasterMap 2013
Great Britain	2.1 (1.64, 2.78)	2.39 (1.86, 2.93)	478 (374, 634)	581 (451, 710)
England	1.83 (1.53, 2.14)	2.68 (2.26, 3.11)	234 (195, 272)	362 (304, 419)
Scotland	2.48 (1.37, 4.30)	1.92 (1.14, 2.70)	198 (110, 344)	86 (98, 233)
Wales	2.24 (1.23, 3.70)	1.49 (1.19, 1.80)	47 (26, 78)	33 (26, 39)
England + Wales	-	2.59 (2.16, 3.02)	-	157 (338, 472)

The density (Figure 4.3) and number of ponds estimated by OS MasterMap was not significantly different from the estimates produced in Countryside Survey 2007, i.e. the confidence intervals for each region overlap. The mean estimates based on OS MasterMap were higher at Great Britain and England levels, and lower at Scotland and Wales levels than the Countryside Survey estimates.

The accuracy of the extraction method was tested by randomly selecting 10% of 1 km grid squares in the New Forest National Park, England (66 squares), Conwy, Wales (89 squares) and Caithness, Scotland (168 squares). The technique did miss a proportion of ponds in each square either through errors in extraction or because the ponds were not detected by the original OS MasterMap freshwater layer.

- In the New Forest errors were detected in 49% of squares: a total of 75 ponds (32%) were missed and 10 ponds (4%) added when there were none.
- In the Conwy errors were detected in 28% of square: a total of 36 ponds (29%) were missed and 14 ponds (11%) added when there were none.
- In Caithness errors were detected in 24% of squares: a total of 30 ponds (3%) were missed and 43 ponds (4%) were added.

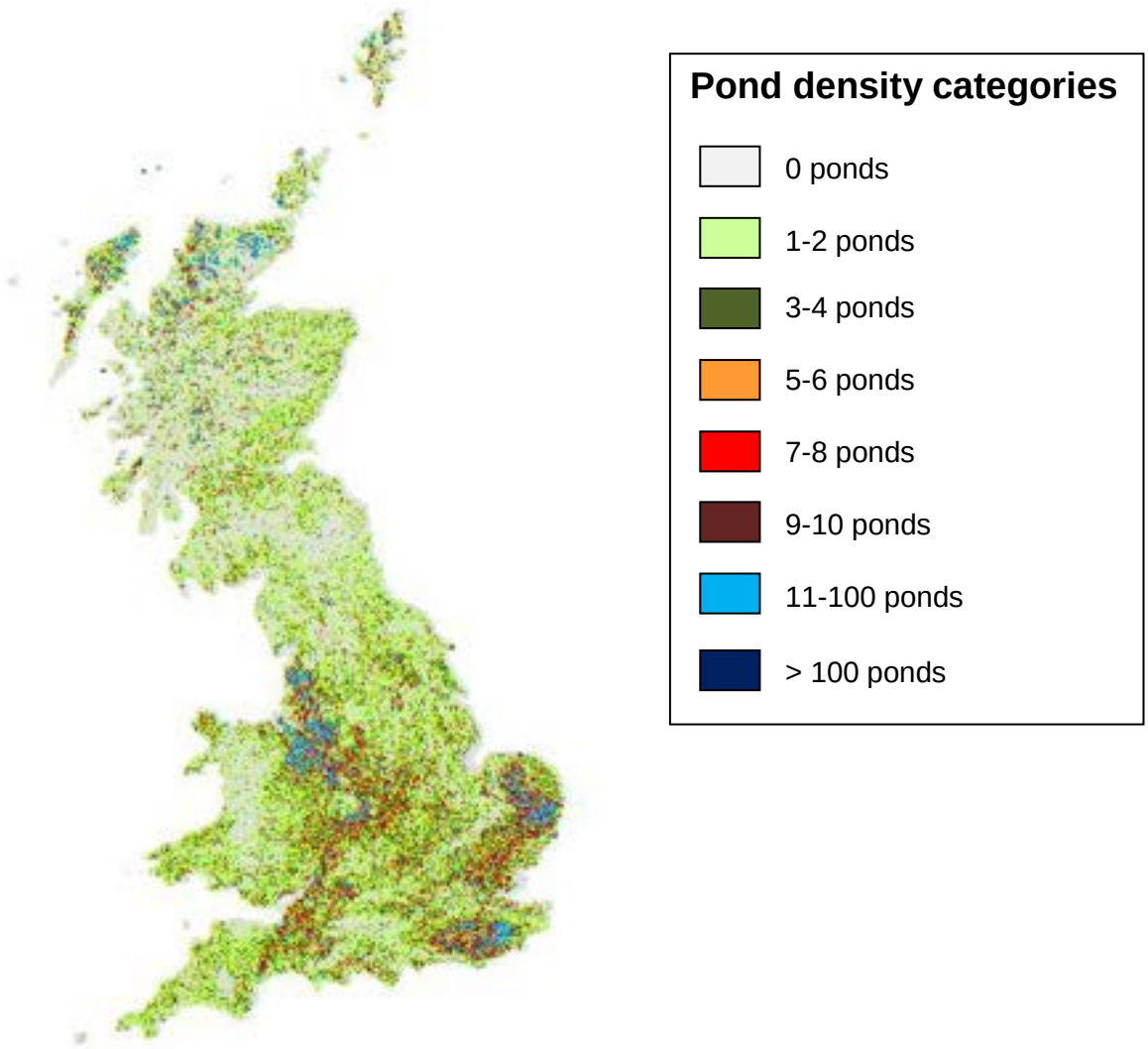


Figure 4.3 Map of pond density based on the number of ponds per 1 km grid square.

A sampling strategy to detect a change in pond numbers could be based on a number of different approaches.

1. Random sampling of 1 km grid squares at national and Great Britain levels (visiting the same or different ponds at t_1 and t_2).
    a. If the same ponds are surveyed again in t_2, analysis depends on matched pairs - power analysis determines the sample size in terms of the number of pairs to be sampled.
    b. If ponds are chosen entirely randomly in t_1 and t_2 , analysis is based on independent samples - power analysis determines the total sample size $t_1 + t_2$, and specifies the proportion of samples to be surveyed in t_1 and the proportion samples in t_2.
2. Random stratified sampling - stratified to ensure that a greater proportion of 1 km grid squares which contain ponds are represented in the sample (visiting the same or different ponds at t_1 and t_2) or restricted selection of 1km grid squares to within Great Crested Newt range.
3. Random sampling which compiles data over several years.

In the following section of the report, these three approaches were examined in detail in a series of power analyses.

1. Random sampling of 1 km grid squares at national and Great Britain levels (visiting the same or different ponds at t_1 and t_2).

Table 4.3 presents the results of different survey strategies to detect change in pond density (ponds per 1 km grid square) at 30% power and 10% change (α=0.05). The optimum strategy would be to undertake national surveys of randomly selected 1 km grid squares surveyed in t_1 with repeat surveys of the same squares in t_2. If unpaired i.e. different random samples were collected in t_1 and t_2 then the optimum allocation would be to split the samples equally between the sample years. The more unbalanced the sample strategy, the greater the number of samples required overall.

Results tables are shown in Appendix Table A2.5 – 2.18.

Table 4.3 Comparison of different survey strategies assessing sample size required to detect a 10% change in the number of ponds per 1 km grid square (80% power, α=0.05) based on (a) paired and unmatched sample squares using Countryside Survey and OS MasterMap data (number of 1 km grid squares for survey each year), and (b) on unmatched data with different allocation ratios using OS Great Britain data only (number of 1 km grid squares for survey each year and total number of 1 km grid squares for survey over 2 years).

(a)	Survey Strategy		
	Countryside Survey - paired	OS - paired	OS - unpaired $t_2/t_1 = 1$
GB	1996	1068	2133
England	920	547	1090
Scotland	8218	3520	7036
Wales	8912	882	1760
England + Wales	-	593	1184

(b)	OS GB data only - unpaired t2/t1 = 1		
Allocation Ratio	**Sample number t_1**	**Sample number t_2**	**Total sample size**
1.00	2133	2133	4266
0.66	1481	2963	4444
0.43	2164	2886	5050
0.25	1666	4999	6665
0.11	1316	10531	11848

2. Random stratified sampling as a monitoring strategy to detect change in pond numbers per 1 km grid square

Results tables are shown in Appendix Table A2.19 – A2.48.

To increase the power of the analysis the sample design can introduce different levels of stratification to ensure that a greater proportion of 1 km grid squares which contain ponds are represented in the sample (visiting the same or different ponds at t_1 and t_2) or stratification can be applied to restrict sample squares to within Great Crested Newt range.

2a. Remove all zero values in the dataset. In this analysis all squares were removed where no ponds are recorded from the sample (Table 4.4). This would give the change in pond numbers between years from the existing resource. The disadvantage of this approach is that any ponds created in squares which did not previously support ponds would not be included in the analysis.

Table 4.4 Proportion of 1 km grid squares with and without ponds - to determine the level of stratification required in each region, if sample squares to detect change in the number of ponds per 1 km grid square are stratified to reflect the proportion of squares with and without ponds in each region.

	Total number of 1 km^2	Number of squares with no ponds	% squares with no ponds	Number of squares with ponds	% squares with ponds
Great Britain	242851	101813	41.92	141038	58.08
England	134702	44014	32.68	90688	67.32
Scotland	86197	47504	55.11	38693	44.89
Wales	21952	10295	46.90	11657	53.10
England+ Wales	156654	54309	34.67	102345	65.33

The following analyses are based on the 141038 1 km grid squares at GB level which were identified by OS MasterMap as containing one or more ponds. Data were analysed as matched pairs, i.e. the same ponds surveyed at t_1 and t_2 and different ponds randomly chosen at t_1 and t_2.

Results are shown in Appendix TablesA2.19 – A2.28

2b. Remove some zero values from the dataset. Some squares are removed where no ponds are recorded from the sample (Table 4.34). The proportion of squares with and without ponds included in the analysis is based on the proportion which are known to support ponds at national and Great Britain levels. The disadvantage of this approach is that the proportion could change in each survey season and would not fit easily with a matched pairs design.

Detailed results are shown in Appendix Tables A2.29 – A2.38.

2c. Restrict the analysis to 1 km grid squares within Great Crested Newt range. This approach would specifically investigate change in pond numbers within the range which is most relevant to Great Crested Newt conservation. As an additional benefit it would reduce variability as it is likely to exclude some areas which have exceptionally high pond density but which are not suitable for Great Crested Newts, e.g. upland squares which tend to skew data because of the large number of bog ponds.

Detailed results are shown in Appendix Tables A2.39 – A2.48.

2d. Summary of options

For ease of comparison, Table 4.5 presents the results of different survey strategies to detect change in pond density (ponds per 1 km grid square) at 80% power, 10% change, α=0.05. The optimum strategy would be to undertake national surveys of randomly selected 1 km grid squares surveyed at time t_1 with repeat surveys of the same squares at time t_2. This approach has limitations and is likely to be an underestimate of the number of sample squares required because of changes to the network caused by e.g. refusal of landowner permission over time, etc. A better approach would be to base the strategy on different randomly selected 1 km grid squares in each sample year.

To make this option feasible, stratification can be applied to the design to reduce the number of samples required. The option with the smallest sample size required is the exclusion of zero values by restricting the survey to only include squares which are known to contain ponds, but this would then fail to detect new ponds created in the proportion of squares which were not monitored, which could then fail to detect improvement for Great Crested Newt.

Stratifying sample squares to reflect the proportion of squares which are known to contain ponds increased, rather than decreased, sample size in most of the regions because a large proportion of the squares in each region do not contain ponds, thereby increasing rather than decreasing variability.

The best strategy to detect change in pond numbers which might impact upon Great Crested Newts would be to stratify the selection of 1 km grid squares to within Great Crested Newt range. The sample sizes required for independent samples, i.e. different squares sampled at t_1 and t_2, are still large using this approach and may be beyond the scope of national monitoring programmes. Table 4.6 shows that a reasonable network of 1 km grid squares based on stratification to within Great Crested Newt range would be able to detect a 20% change in the number of ponds per 1 km grid square.

Table 4.5 Summary of alternative sampling strategies for assessing a 10% change in the number of ponds per 1 km grid square (80% power, α=0.05) – sample sizes are the number of 1km grid squares required each year.

	CS	Raw OS data		Pond only 1 km grid squares		Pond squares proportional to non-pond squares		Only 1km grid square within GCN range	
	Paired	Paired	Un-paired $t_2/t_1 = 1$	Paired	Un-paired $t_2/t_1 = 1$	Paired	Un-paired $t_2/t_1 = 1$	Paired	Un-paired $t_2/t_1 = 1$
GB	1996	1068	2133	167	332	3011	6020	241	479
England	920	547	1090	77	151	451	898	294	585
Scotland	8218	3520	7036	227	451	-	-	269	534
Wales	8912	882	1760	100	196	18626	37248	415	828
England + Wales		593	1184	97	190	187	370	303	602

Table 4.6 Summary of sample size required to detect different levels of change in the number of ponds per 1 km grid square (80% power, α=0.05) when the design stratifies selection of sites to within Great Crested Newt range (random selection of independent sample squares each year) – sample sizes are the number of 1km grid squares required each year.

	Percentage change in the number of ponds per 1 km grid square				
	10%	20%	30%	40%	50%
Great Britain	479	121	54	31	20
England	585	147	66	38	25
Scotland	534	135	61	35	23
Wales	828	208	93	53	34
England + Wales	602	152	68	39	25

3. Random sampling which compiles data over several years

To take account of within and between year variation, for a survey strategy compiling data over several years to compare results between survey periods, the data should be analysed using a nested design, with sub-years (t_1, t_2, t_3, t_4 . . . .) nested within survey periods (p_1 and p_2). For non-parametric data this could be analysed using a nested Kruskal-Wallis analysis – a new approach suggested by Orom and Hoff (2006). Estimates of power for this would be based on simulation, with the proportion of simulated tests returning significant results to indicate the power of the sample size. However, this analysis would require better information about variation between successive sample years, data which will be one of the outputs of PondNet.

It is sufficient to say that the number of squares needed would exceed the number required for tests between two separate survey years (t_1 and t_2) to overcome the variability within and between survey periods in the nested design.

However, the benefit of compiling data over multiple years will be the potential to analyse rates of change over time e.g. 1% change per year over a 10 year survey programme. Sample size within year should remain the same i.e. for England, 121 1 km grid squares.

4.2.3 Interim conclusion for network to monitor change in pond numbers for Great Crested Newt

To effectively monitor pond numbers for Great Crested Newt specifically (rather than as a general statistic, as is obtained in the Countryside Survey) the selected strategy should:

- Survey the number of ponds per 1 km grid square
- Monitor change at national scale (i.e. England, Scotland, Wales)
- Randomly select new 1 km grid squares for each survey year (independent sampling)
- Stratify the sample to only include 1 km grid squares within the Great Crested Newt range

- Aim to detect a 20% change in pond numbers requiring the following number of survey squares:
    - England – 147 1 km grid squares
    - Scotland – 135 1 km grid squares
    - Wales – 208 1 km grid squares
- Use data collected from the first few years of the survey to understand variation in trend data
- Ideally, introduce pond counts per 1 km grid square into NARRS to maximise the benefits of the volunteer effort in this survey.

4.3 Habitat suitability

4.3.1 Sample size required to detect change in Habitat Suitability Index at two time periods using different sampling strategies

We used the datasets described in Section 2.2.1(ii) to evaluate within year variability in HSI scores (which is critical to determining power), to describe real levels of change seen in HSI over periods of 5-10 years, and to determine the sample sizes needed to detect different levels of change in HSI score at different levels of power. The datasets were:

1. DICE/FHT HSI repeat surveys of ponds in Kent and Wales from 2007 and 2013

2. Countryside Survey repeat surveys of ponds from 1996 and 2007, used to approximate HSI scores

3. Amphibian and Reptile Conservation NARRS random surveys of ponds in 2007 and 2012.

For each dataset we described HSI variation and change over 5-10 years, and used these data to calculate the number of ponds that should be surveyed to achieve different levels of power to detect change in HSI scores.

(i) Comparison of HSI scores in DICE/FHT, Countryside Survey and NARRS datasets

Mean HSI values for the DICE/FHT sites in Kent and Wales, and for the Countryside Survey, were not significantly different, ranging from 0.59 to 0.67 ($p=0.736$ and $p=0.716$ for Kent and Wales, respectively). However, HSI scores from the NARRS survey in 2007 were significantly lower than the DICE/FHT surveys and the Countryside Survey with a mean value of 0.49 (Figure 4.4 and Table 4.7).

Very little change was detected in HSI scores between survey years in the DICE/FHT and Countryside Survey datasets (Figures 4.5a, b). The DICE/FHT Kent dataset and the Countryside Survey showed small, but non-significant, increases in HSI scores over periods of 6 and 11 years respectively. The DICE/FHT Wales dataset showed a small non-significant decline in HSI scores over the same period (Table 4.7). In contrast, NARRS data showed a substantial and statistically significant increase in mean HSI over the 5 year period, with mean HSI increasing by 19% over this time. This led to substantial differences in the level of change recorded by the three surveys over longer time periods: Countryside Survey and DICE/FHT data suggest that HSI scores change slowly whereas NARRS suggests a much more rapid rate of change (Table 4.8).

It is not possible to assess independently which of these results is ‘correct’ and no analysis of NARRS data (Wilkinson and Arnell 2013) has concluded anything about change in HSI from these results. The sample sizes for 2007 and 2012 were small and the significant result could have occurred by chance. Likewise it is not possible to conclude that ponds in the DICE/FHT survey are representative of wider countryside changes as they are biased towards a specific location. Although Countryside Survey results lead to the same conclusion as the DICE/FHT survey, the issues with estimates of HSI in the first year of the survey again add uncertainty to the findings. It is our experience that, unless ponds have been subject to substantial management interventions, change in HSI values occur quite slowly and over a 5-10 year period would be relatively modest. The modest improvement in HSI scores here is at odds with other studies finding that pond quality for Great Crested Newts has been in general decline recently (see references in Jehle *et al.* 2011). It would be useful to examine this finding in more detail, but it is not relevant to power analysis for this report. It has been agreed between the agencies and conservation organisations that a meaningful change in HSI scores would be in the order of 10% change. Changes of greater than 10% would be considered to be ecologically damaging or beneficial to Great Crested Newt populations, depending on the direction of change. Although it would be interesting to detect changes in HSI below the 10% level, there is a trade-off between detecting ever smaller changes and sample size. All combinations of change and power have been included in the Appendix A3 of the report for comparison.

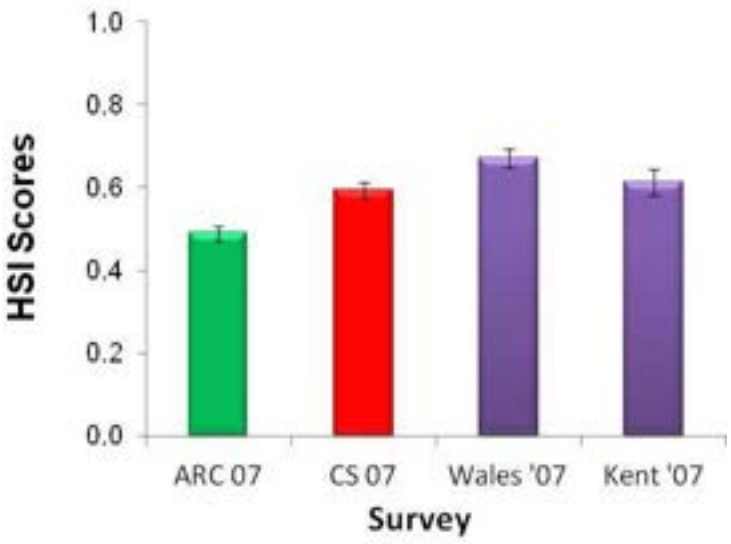


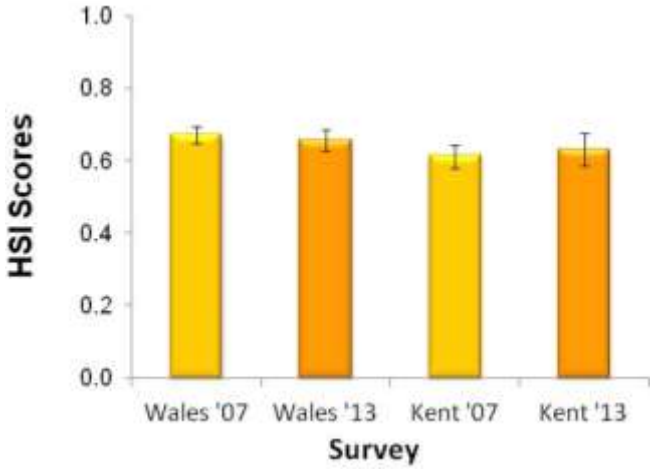
Figure 4.4 Comparison of HSI scores calculated in 2007 from three different surveys: ARC NARRS survey, using only 2007 data (ARC 07), Countryside Survey 2007 (CS 07) and DICE/FHT surveys in Kent and Wales in 2007 (Wales '07, Kent '07)

Table 4.7 Mean values of HSI scores in the DICE/FHT, CS 20007 and NARRS 2007 surveys

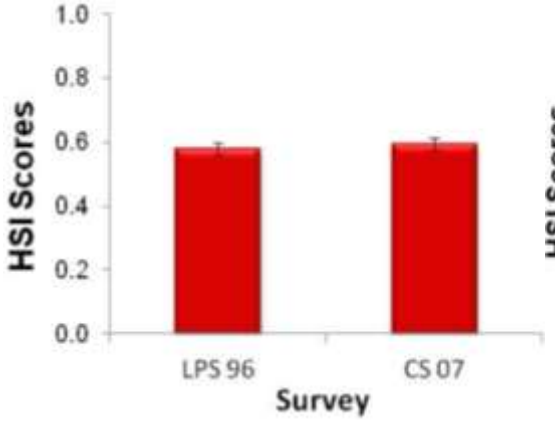
Survey	Mean (StDev)	95% CI
DICE/FHT Kent 2007	0.613 (0.1527)	0.550, 0.675
DICE/FHT Wales 2007	0.670 (0.1187)	0.624, 0.717
CS 2007	0.593 (0.1592)	0.557, 0.629
NARRS 2007	0.489 (0.1824)	0.455, 0.523

- The mean HSI scores for CS 2007 were the same (Figure 1) as the scores collected by DICE 2007 (Kent: T=-0.35, P=0.730, DF=39, Wales: T=0.37, p=0.716, DF=45)
- The mean HSI scores for ARC 2007 were significantly lower (Figure 1) than the scores collected by DICE 2007 (Wales: T=6.15, p<0.001, DF=53, Kent: T=3.40, P=0.002, DF=36)

(a)



(b)



(c)

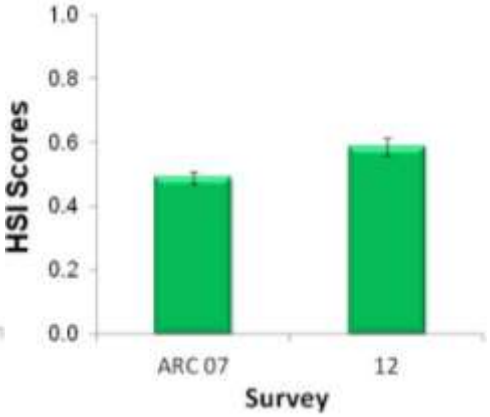


Figure 4.5 Change in HSI scores collected in different time periods and by different survey designs.
(a) Change in mean HSI scores between years in Kent and Wales - DICE/FHT data 2007 - 2013, (b) Countryside Survey 1996 - 2007, matched pairs and (c) ARC NARRS data 2007 - 2012, independent samples.

Table 4.8 Change in HSI scores and the period of time over which those changes were observed.

Survey	Years over which change occurred	Change in HSI (%)
DICE/FHT Kent 2007-2013	6	0.019 (3.12%)
DICE/FHT Wales 2007-2013	6	-0.014 (-2.09%)
CS 1996-2007	11	0.02 (2.63%)
ARC 2007-2012	5	0.096 (19.69%)

(ii) Power to detect change using different sampling strategies

The power to detect change using different survey methods was evaluated using the three study datasets: (a) DICE/FHT Kent and Wales survey data, (b) Countryside Survey 1996 - 2007 data and (c) NARRS 2007 - 2012 data.

(a) Power of the DICE/FHT Wales and Kent analysis, and overall power to detect change in HSI score, as indicated by this dataset

DICE/FHT data were from matched pairs and were normally distributed. In Kent there was a slight increase in HSI score over the 6 year period, and in Wales a slight decline. However, neither difference was statistically significant and, because of the small sample, both surveys had limited power to detect change: In Kent, 7.3% power at $\alpha_{0.05}$ and 13.4% at $\alpha_{0.10}$ and in Wales, 8.5% power with $\alpha_{0.05}$ or 15.2% with $\alpha_{0.10}$.

Using the Kent and Wales data it is possible to calculate sample sizes needed to detect change in HSI scores between years, given the level of variability between sites observed in this dataset. In Kent only 84 ponds would need to be monitored to detect a 10% change in HSI scores (80% power, α=0.05). This assumes that the same 84 ponds would be surveyed at time t_1 and t_2.

In Wales only 28 ponds would need to be monitored annually to detect a 10% change in HSI scores (80% power, α=0.05).

Detailed results are shown in Appendix Tables A3.1 – A3.2***.***

(b) Power of Countryside Survey 1996-2007 analysis, and overall power to detect change in HSI score, as indicated by this dataset

Detailed results are shown in Appendix Tables A3.3.

Data from the Countryside Survey comprised results of the Lowland Pond Survey 1996 and data from the full 2007 Countryside Survey. Separate analysis of these data is justified as the results can give estimates of the variability of HSI scores between ponds at larger spatial scales that the DICE/FHT data and are likely to be more representative of future monitoring networks.

A relatively small number of ponds (n=77) were surveyed in both years. Lowland Pond Survey results were not normally distributed (Figure 4.6) so non-parametric tests were used to analyse changes between years. The non-normality of HSI scores may indicate an issue with the approximation of HSI score in 1996 (see Williams *et al.* 2010 for further explanation) as all other HSI scores have normal distributions.

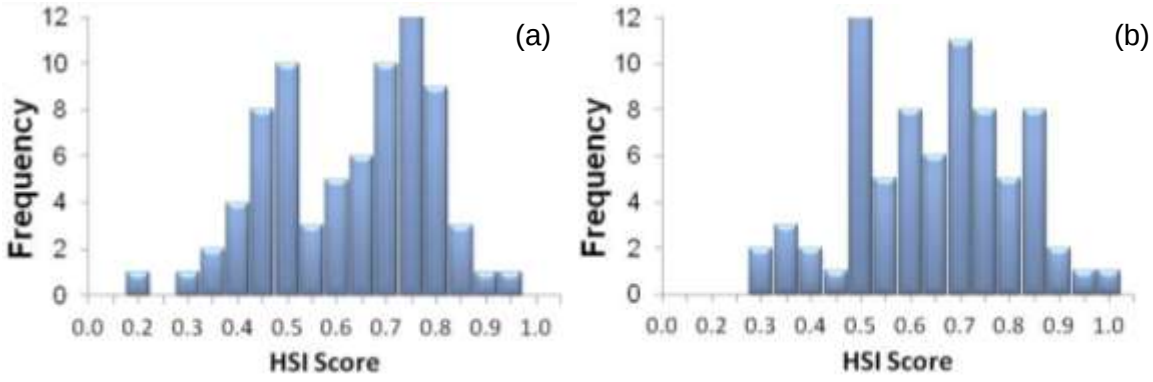


Figure 4.6 Histograms showing distribution of HSI scores for (a) Lowland Pond Survey 1996 and (b) Countryside Survey 2007.

There was a 2.6% increase in approximated HSI score over the 11 years between the two Countryside Survey datasets, but the difference was not statistically significant (W_{72}=1176, p=0.439).

Post hoc, power analysis indicates that at $\alpha_{0.05}$ the sample of 77 sites only had 21.2% power. At $\alpha_{0.10}$ the power of the analysis would be higher (31.7%). Based on repeat surveys of the same ponds, to detect a 10% change in HSI scores between years (80% power, α=0.05), 32 ponds would need to be surveyed. This estimate lies somewhere between the DICE/FHT results.

The small amount of variation within the sample groups and the matched pairs design of Countryside Survey means that the number of ponds that would need to be surveyed is practically very achievable (c.f. the number of sites needed for a survey design in which different ponds are visited each year).

(c) Power of NARRS 2007-2012 analysis, and overall power to detect change in HSI score, as indicated by this dataset

NARRS data were used to investigate power of a sampling programme based on visiting new sites on each sampling occasion. For analysis of existing data, the analysis is based on an unbalanced design (because the nature of volunteer surveys means that unequal numbers of sites are often surveyed each year). This could have important implications for determining whether 'missed' sites need to be mopped up each year by professional surveys to maintain a balanced design.

There was an overall of 19.7% increase in mean HSI score between 2007 and 2012. The difference between the two survey years was statistically significant (T=2.88, df=70, p=0.005) (Table 4.9).

Power analysis indicates that with $\alpha_{0.05}$ the design has 81.3% power to detect the observed change in mean HSI in GB between sample years. This level of power reflects the relatively large change between years. To detect a 10% change in HSI score at 80% power (α=0.05) 549 ponds would need to be surveyed in total (i.e. 346 ponds in year 1 and 203 ponds in year 2). Note the reason for the large sample size compared to DICE/FHT and Countryside Survey results is because different ponds are chosen at random each year and because of the unbalanced design – a different number of ponds surveyed each year.

To increase power, the total number of ponds should be divided into two approximately equal fractions over the first and second surveys. To detect 10% change in HSI scores between years (80% power, α=0.05) with a balanced design but different ponds selected at random each survey year, a total of 434 ponds would need to be surveyed (217 per year).

The more unbalanced the design, the lower the level of power. Careful thought needs to be given as to how the network can be populated with enough volunteers each year to prevent heavily unbalanced designs occurring, or gaps will have to be filled by professional surveyors.

Detailed results are shown in Appendix Tables A3.4 and A3.5

Table 4.9 Mean values of HSI scores in the NARRS survey in 2007 and 2012

Survey year	n	Mean	StDev	95% CL
2007	109	0.49	0.1824	0.455, 0.523
2012	40	0.59	0.1804	0.529, 0.641

Increase in HSI score between 2007 and 2012 = 0.096 (19.69%)

4.3.2 Sample size required to detect change in HSI at two time periods at country (England, Scotland, Wales) and Great Britain levels

To investigate sample sizes needed to detect differences in HSI between two time periods, at country (England, Scotland, Wales) and Great Britain levels, we reanalysed data from the NARRS survey which had sufficient samples to undertake this analysis. To achieve a large enough sample size 2007/8 and 2011/12 data were combined to give a starting time, t_1, and an end time, t_2 (Figure 4.7).

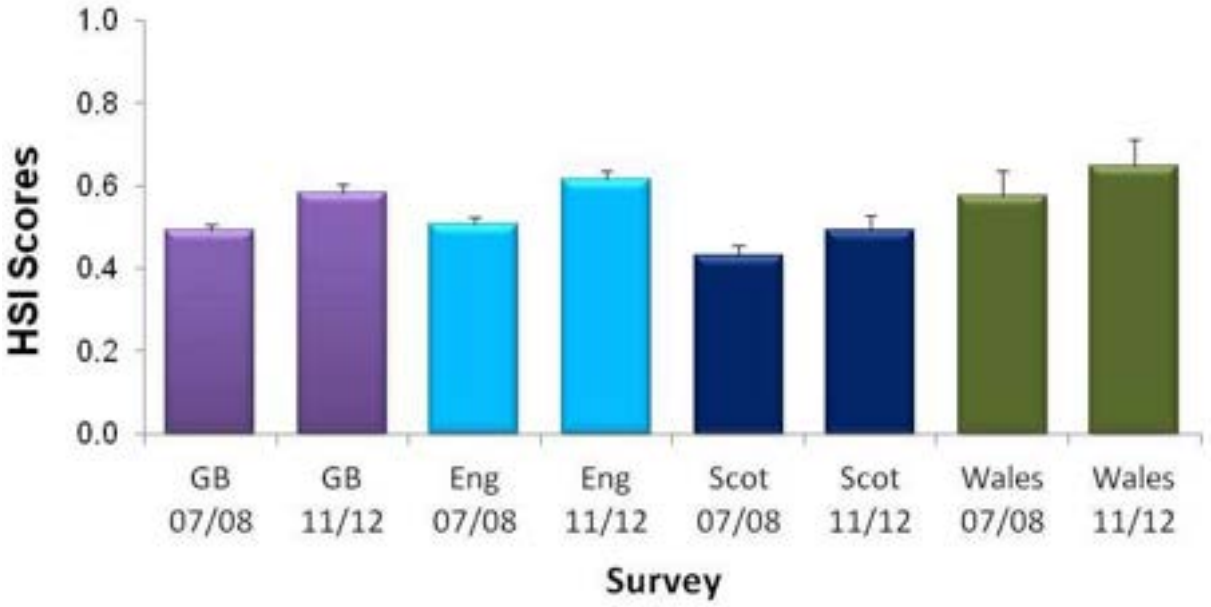


Figure 4.7 Mean HSI scores between sample years 2007/8 and 2011/12 for Great Britain (GB), England, Scotland and Wales based on NARRS data.

For each country, and for Great Britain as a whole, we assessed the (i) actual power of the existing survey strategy to detect change, and (ii) the theoretical power to detect a change in HSI score at different levels of power, % change and significance (α=0.05, α=0.10).

(i) Power of the existing NARRS survey strategy to detect change in HSI between years in each of the regions

The difference in NARRS HSI mean values in England between 2007/8 and 2011/12 was comparatively large, with a change of 21% (Table 4.9) with 96.4% power for $\alpha_{0.05}$ and 98.3% for $\alpha_{0.10}$.

In Scotland and Wales the differences in HSI between the two time periods was a little lower than in England, being respectively, 15% and 13%. In addition, sample sizes were substantially smaller and therefore the power of the existing sampling strategy was much lower, being less than 30% in both Scotland and Wales at $\alpha_{0.05}$ (Table 4.10).

Overall, these results indicate that if large changes in HSI occur (in the region of 20% or more over a given time period) current sampling designs will be able to detect these changes. However, to detect ecologically important smaller changes in HSI changes (e.g. 10% change), larger sample sizes are required.

Table 4.10 Summary statistics of NARRS data used for power analysis of changes between 2007/8 and 2011/12 for England, Scotland, Wales and Great Britain

England				
Survey year	**n**	**Mean**	**StDev**	**95% CL**
2007/8	125	0.508	0.1758	0.477, 0.539
2011/12	51	0.615	0.1649	0.570, 0.660
Increase in HSI = 21% Power of this analysis at $\alpha_{0.05}$ = 96.36% Power of this analysis at $\alpha_{0.10}$ = 98.28 %				
Scotland				
2007/8	44	0.430	0.1704	0.380, 0.481
2011/12	23	0.493	0.1751	0.422, 0.565
Increase in HSI = 15% Power of this analysis at $\alpha_{0.05}$ = 28.38% Power of this analysis at $\alpha_{0.10}$ = 40.19%				
Wales				
2007/8	8	0.574	0.1758	0.452, 0.696
2011/12	8	0.648	0.1878	0.514, 0.778
Increase in HSI = 13% Power of this analysis at $\alpha_{0.05}$ = 11.77% Power of this analysis at $\alpha_{0.10}$ = 19.92%				
Great Britain				
2007/8	177	0.492	0.1776	0.466, 0.518
2011/12	82	0.584	0.1775	0.545, 0.622
Increase in HSI = 19% Power of this analysis at $\alpha_{0.05}$ = 97.16% Power of this analysis at $\alpha_{0.10}$ = 98.70%				

(ii) Sample sizes needed to detect change in HSI score at different levels of power, change and significance in different regions

The network to assess change in HSI score would be based on surveys of one pond (which could be chosen at random (PondNet) or from the south-west corner (NARRS)), within randomly selected 1 km grid squares to maintain independence between pond sample units. Different 1 km grid squares, and therefore different ponds, would be chosen at random each year.

Table 4.10 compares the sample sizes required to achieve 10% and 20% change in HSI score (80% power, α=0.05). As expected sample sizes are larger at the regional level to achieve similar levels of power as the Great Britain survey.

In England the framework suggested for monitoring 20% change in the number of ponds per grid square (Section 4.2) would be sufficiently large to detect 20% change in HSI score (55 ponds), but the network would need to increase to 215 1 km grid squares to detect changes in HSI score of 10%.

In Scotland, mean HSI scores were significantly lower than in England, Wales and Great Britain as a whole. This is to be expected because of the score given to the region as edge of range habitat for Great Crested Newt compared with England. HSI means also differed between sample years (Two-way ANOVA: Country: $F_{3,510}$=5.58. P=0.001; Sample year: $F_{1,510}$=10.09, p=0.002; Interaction: $F_{3,510}$=0.24, p=0.868). Compared to England, more ponds would need to be surveyed to assess change in HSI score at the same level of power – 282 ponds per year to detect 10% change and 71 ponds to detect 20% change (Table 4.11). These analyses are based on NARRS data (sample size 2007/8 = 44, sample size 2011/12 = 23). We also analysed ARC data collected for SNH in 2012 (123 ponds). These estimates of variability were less and suggest that only 118 ponds need to be surveyed each year to detect a 10% change at 80% power. This could be achieved within the 135 1 km grid square framework suggested to detect change in the number of ponds per grid square (Section 4.2).

In Wales, using the NARRS data to provide estimates, 159 ponds would be required to detect a 10% change with 80% power. This could be achieved within the framework suggested to monitor change in pond number per grid square in Wales (208 1 km grid squares). Only 8 ponds were surveyed in 2007/8 and 8 ponds in 2011/12 in Wales as part of NARRS, therefore it is possible that variability is higher than indicated which might lead to a revision of sample size estimates.

Detailed results are shown in Appendix Tables A3.6 and A3.10.

Table 4.11 Summary statistics of sample size (number of ponds) required to detect 10% and 20% change in HSI scores using data from different sources and in different regions to undertake power analysis at 80% power (α=0.05) to assess which sampling strategy produces the most parsimonious result to develop the survey network

% change	DICE/FHT - paired		CS - paired	NARRS GB - Unpaired		NARRS - Unpaired $t_2/t_1 = 1$			
	Kent	Wales	GB	t_2/t_1 = 0.37	t_2/t_1 = 1	England	Scotland NARRS	Scotland SNH	Wales
10%	84	28	32	346/ 203	109	215	282	118	159
20%	22	9	10	88/ 51	55	55	71	30	41

n.b. the unbalanced design of NARRS data with allocation ratio of 0.37 means that different number of ponds will be surveyed in each year e.g. 346 in year 1 and 203 in year 2.

4.3.3 Effect of additional repeat surveys on power to detect changes in HSI score

(i) What power can be achieved if some of the sample squares are repeats and others are different squares each year?

In general, as the analyses have shown here, substantially greater power to detect change is achieved through paired analysis of sites (c.f. Countryside Survey) rather than randomly reselecting sites on each survey occasion (c.f. NARRS). There are no practical advantages to mixing the two methods together – indeed, doing so creates datasets that present significant methodological difficulties as discussed below.

However, although setting out to create such datasets is undesirable, repeat surveys involving paired sites almost invariably become a mixture of squares which are repeated and small number of squares which are not repeated. This arises when, for example, a landowner does not grant permission for a second visit, a pond is completely destroyed, a square/pond is missed for some other unavoidable reason or a new site is added. This is a problem which has been encountered in the Countryside Survey and is unavoidable (Scott 2008).

The problem can simply be overcome by omitting sites which do not have the second visit. Thus in the Countryside Survey estimates of stock pre-2007 were calculated using all the data from a particular survey while change was calculated from only the more limited sample of repeated measurements across pairs of surveys. This approach has the problem that it both fails to use all the data collected in each survey for change estimates and results in mismatches between estimates, i.e. change in stock estimates are not the same as change estimates. In the Countryside Survey an alternative modelling approach was adopted which, in effect, estimates missing values in paired analyses.

This approach worked well for most Broad Habitat categories but, crucially, failed to predict change in freshwater habitats effectively. The problem appeared to be due to high variability (Scott 2008).

Currently there are two alternative solutions to this problem: analyse data using paired sites only and accept that some data will not be used for this analysis (this is effectively the approach that has been adopted by the Countryside Survey); or alternatively, carry out further exploration of a modelling approach. Although beyond the scope of the present work, further exploration of a modelling approach might be appropriate as part of further work on Great Crested Newts, or as part of the Countryside Survey.

To monitor HSI change as part of a surveillance network for Great Crested Newt we recommend that different sites (1 km grid squares) are chosen at random each year, but the same number of 1 km grid squares should be surveyed each year as far as is practicable.

(ii) The effect on power if data are collected over several years - combining data to look at several years as a single time point

Using NARRS data collected between 2007 and 2012 it is possible to compare sample sizes and power of surveys based on two separate time points, t_1 and t_2, with surveys based on combining data over two time periods, p_1 and p_2. The former is the strategy adopted by the Countryside Survey. The latter has been shown by NARRS data to be the outcome of volunteer surveys because volunteers cannot be forced to visit a site and may not return their results at the end of the survey season.

Detailed results are shown in Appendix Tables A3.11 and A3.12.

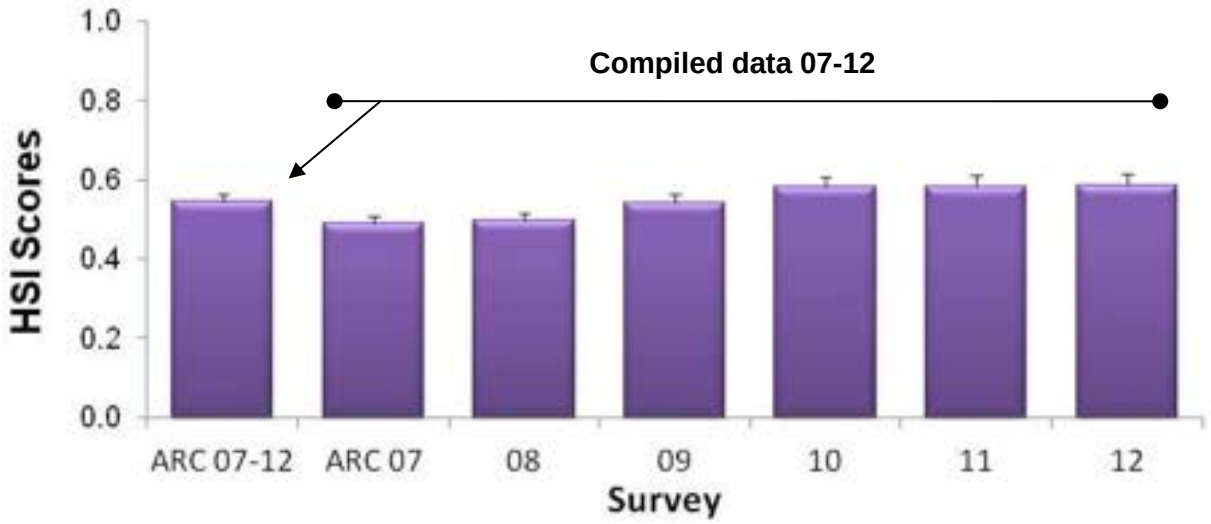


Figure 4.8 Mean HSI scores within sub-groups (individual years) and mean HSI of compiled data (NARRS data 2007-12).

NARRS data were collected every year for 6 years from 2007 to 2012 (Figure 4.8, 4.9), with different samples sizes each year – termed statistically an 'unbalanced' design. Data in each year were normally distributed allowing parametric tests to be applied. This is a nested analysis, with years nested within survey periods.

To assess numbers of samples needed, change is calculated between two time periods, p_1 and p_2, each consisting of 6 years. In a two-level nested ANOVA, one null hypothesis is that the subgroup years nested within each sample period have the same means; the second null hypothesis is that the sample periods (p_1 and p_2) have the same means. Power is calculated at 2 levels - between survey periods and between years.

Real data only exist for p_1 (the years 2007-12), so theoretical data were created for p_2 assuming the same level of variance as in p_1,and assuming the same level of change between p_1 and p_2 as between 2007 and 2012. For NARRS data the time period p_1 is 2007-12 with a mean HSI of 0.546. The increase in HSI score between NARRS p_1 2007-12 and a theoretical p_2 running from 2013-18 was approximately 20%. The power of this analysis would be 96.9% ($\alpha_{0.05}$) or 98.9% ($\alpha_{0.10}$). In total c. 370 ponds were surveyed over the course of the 6 year survey period. To detect a change of 10% between the two periods, power analysis using the nested design suggests that 2376 ponds would have to be surveyed per year or 14258 ponds in total. This is much higher than the estimates for other survey strategies.

Figure 4.9 Distribution of NARRS sites at which HSI scores were available from 2007 to 2012.

If the difference in mean HSI score between sample periods is large, effect size is large and the number of years over which the survey should span is small e.g. with 20% change in average HSI score between p_1 and p_2, at 95% power and α=0.05, number of years in each sample period = 5 (Appendix table A3.12, 95% power, 20% change).

If the difference in mean HSI score between sample periods is small (10% to detect ecologically meaningful change), the number of years in each sample period will increase e.g. with 10% change in average HSI score between p_1 and p_2, at 80% power and α=0.05, the number of years in each sample period is 11 (Appendix table A3.12).

Table A3.12 is an approximation because the greater the between year variation in sub-groups within each sample period, the greater the number of years required in order to ensure that if a difference between sub-groups exists it will be detected and taken into account.

(iii) Random sampling which compiles HSI data over several years to detect trends

As with pond occupancy, the benefit of compiling data over multiple years will be the potential to analyse rates of change over time e.g. 1% change in HSI score per year over a 10 year survey programme. NARRS data showed a 3.9% increase in HSI score per year over 6 years. This was a slight but significant increase (adj. R^2 = 0.044, $F_{1,370}$=17.89, P < 0.001).

Ecologically damaging rates of change are likely to be smaller than this e.g. 2% change per year over 6 years. The total sample size required to detect this level of change at 80% power (α=0.05) would be 661 ponds, 110 ponds per year (110 1 km grid squares). This is the same as the sample size required to detect a difference between individual sample years (Table 4.10).

Detailed results are shown in Appendix Tables A3.13

4.2.4 Interim conclusion for network to monitor change in HSI score for Great Crested Newt

Overall, a network to monitor change in HSI score for Great Crested Newts should:

- Survey one pond in each 1 km grid square in the surveillance network
- Monitor change at national scale
- Randomly select new 1 km grid squares for each survey year (independent sampling)
- Stratify the sample to only include 1 km grid squares within Great Crested Newt range
- Aim to detect a 10% change in HSI score requiring the following number of survey squares:
    - England: 215 1 km grid squares, which is larger than the sample size required to detect change in pond number. This makes it necessary to increase the network size sufficient to assess pond numbers to this total (215) to include HSI score.
    - Scotland: 118 1 km grid squares, which is smaller than the sample size required to detect change in pond number. This means that the network for pond number assessment is also sufficient for assessing HSI values.
    - Wales: 159 1 km grid squares, which is also lower than the sample size required to detect change in pond number. As for Scotland, this means that the network for pond number assessment is also sufficient for assessing HSI values.

4.4 Great Crested Newt occupancy

4.4.1 Background

There is a need to develop a robust sampling strategy for Great Crested Newts because, to date, the lack of systematic recording makes it difficult to make accurate estimates of population status and trends.

Power analysis to develop a robust sampling strategy must use existing data on Great Crested Newt occupancy to explore the sample sizes required to achieve different levels of power to detect change at different spatial scales using different sampling strategies.

Such a monitoring strategy for Great Crested Newts could be structured using any of the following sampling strategies:

(i) The number of ***occupied ponds*** per 1 km grid square at national and Great Britain levels, which would require all ponds within a 1 km grid square to be surveyed (PondNet approach).

(ii) The proportion of ***occupied 1 km grid squares*** at national and Great Britain levels which would only require the presence of Great Crested Newts to be confirmed in one pond in the grid square, but all ponds would need to be surveyed to return a verdict of Great Crested Newts being absent

(iii) The ***proportion of occupied ponds*** with ponds selected by choosing one pond in each 1 km grid square in the survey and scaling up to national and Great Britain levels. This approach requires only one pond in each grid square to be surveyed (NARRS approach)

In addition, the following design permutations are also possible, with a range of statistical and practical advantages and disadvantages:

(i) The same (repeat survey) or different (independent) ponds or squares could be surveyed at times t_1 and t_2.

(ii) The selection of ponds could be random or stratified to exclude squares which do not contain ponds, or to give greater representation to squares known to support Great Crested Newts.

(iii) Data could be collected in one survey year (t_1) and compared with data collected in a separate survey year (t_2) or data could be compiled over a survey period (p_1) made up of a number of years and compared with a future survey period (p_2).

(iv) Undertake complete surveys at regular intervals and analyse to detect trends over time.

The following power analyses, and the sampling strategies suggested, assume that the survey technique or techniques used will detect Great Crested Newt occupancy or will be robust enough to have confidence in negative results. We have assumed that all survey methods are equivalent to the detection power of eDNA or a combined 4 visit combined torch count and bottle trap survey i.e. exceeding 95% certainty of detection or confirmation of a negative result.

4.4.2 Great Crested Newt data

Two principle datasets were used (Table 4.12): (i) derived from the National Biodiversity Network and local records centres and (ii) from the National Amphibian and Reptile Recording Scheme (NARRS).

(i) National Biodiversity Network and local records centre data 1988 - 2012

Data from the National Biodiversity Network Gateway and local records centres, which had already been collated for the PondNet project, were used for the analysis. PondNet will report on the number of ponds occupied by Great Crested Newts per 1 km grid square - which can be scaled up to give national and Great Britain totals - using a sampling strategy based on selection of random 1 km squares and surveying as many ponds in the grid square as possible.

NBN / records centre data included some records which could not be used in the analysis including those which were poorly resolved (e.g. only 2 or 4 figure grid references), old records from before 1998 or those which did not relate to individual ponds (grid references were well away from water).

A process of data cleaning was required to produce a GIS layer which removed records with poor resolution and applied a date filter to identify current, post 1988, records only. Once filtered, a second GIS layer was produced which identified records associated with ponds by overlaying the cleaned dataset on top of the OS MasterMap pond layer. This identified around 5000 ponds with Great Crested Newts, equivalent to about 1% pond occupancy.

Expert opinion suggests that the number of Great Crested Newt records currently held by the NBN and records centres is an underestimate of the actual population. For example, Swan and Oldham (1993) put estimates of Great Crested Newt pond occupancy at 11%, with NARRS suggesting 12% (Wilkinson and Arnell, 2013).

Therefore, further layers were produced using GIS to identify ponds within 250 m, 500 m and 1 km of the NBN Great Crested Newt records to detect waterbodies which are likely to support Great Crested Newts but for which records do not exist. The GIS analysis identifying ponds within 1 km of NBN records produced estimates of Great Crested Newt pond occupancy close to those obtained by field surveys and was therefore used to give estimates of variability between sites within year for the following power analyses (Table 4.12).

Table 4.12 Percentage of occupied ponds based on data collated from NARRS survey and NBN.

	NARRS	GCN NBN raw data		GCN NBN cleaned data		GCN NBN cleaned data buffered to 1 km	
	GCN ponds (%)	GCN 1 km square (%)	GCN ponds (%)	GCN 1 km square (%)	GCN ponds (%)	GCN 1 km square (%)	GCN ponds (%)
Great Britain	12	3	1	2	1	5	10
England	16	4	1	3	1	9	14
Scotland	<1	<1	<1	<1	<1	<1	<1
Wales	15	2	2	2	2	5	14
England/ Wales	15	4	1	3	1	8	14

(ii) National Amphibian and Reptile Recording Scheme (NARRS) data collected between 2007- 2012

NARRS data (Amphibian and Reptile Conservation) comprised 410 1 km grid squares which were surveyed between 1998 and 2007 (Wilkinson and Arnell 2013). The pond nearest to the south-west corner of each square was surveyed for all amphibians including Great Crested Newts. These data have been used by ARC to report on the proportion of occupied ponds.

There are three features of the NARRS data which may influence its application in the design of surveys:

- The results may ***overestimate*** the number of Great Crested Newt occupied 1 km grid squares because volunteers are less likely to submit negative results.
- Squares supporting Great Crested Newts have, on average, 2 to 3 times more ponds than squares without Great Crested Newts (Figure 4.13). This may lead to ***underestimates*** of the number of occupied ponds at national levels because only 1 pond can be counted in each occupied square.
- Different numbers of records were submitted in different years of the survey leading to a very unbalanced design. To overcome this, the NARRS data were pooled, reducing the variability within each sample period. However, this could lead to an underestimation of the number of sites required to detect change between sample periods.

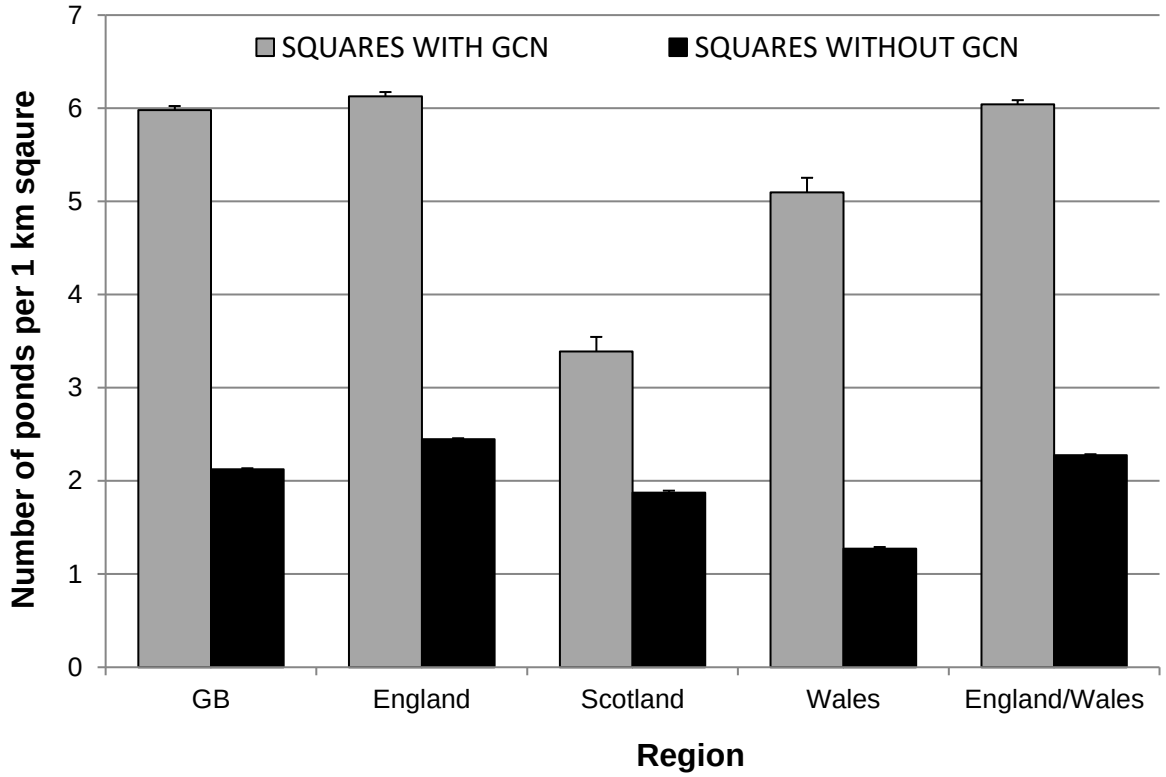


Figure 4.13 The mean number of ponds in 1 km grid square with, and without, Great Crested Newts.

4.4.3 Power analysis of sampling strategies

Due to the high level of variability in occupancy rates for Great Crested Newts, large sample numbers are required for many of the sampling strategies to ensure that significance levels of α=0.05 are achieved at 80% power even to detect relatively large (10%, 20%) changes in pond occupancy. Summary results and comparison are therefore reported at these levels: α=0.05 and α=0.10 with 80% power, to detect 10% and 20% change. However it must be noted that increasing α increases the risk of recording a significant difference when in fact one does not exist, and therefore the design will be less robust than at α=0.05.

In most cases separate analyses have been given for Great Britain, England, Scotland, Wales and England + Wales combined. The results are presented as follows:

(i) NARRS data: power analysis of alternative approaches to assess change in the proportion of occupied ponds (one pond surveyed per 1 km grid square) (Tables A4.1 – A4.5)

(a) Power of existing NARRS surveys

(b) Different randomly selected ponds (1 per grid square) are surveyed each sample year one (i.e. independent samples at t_1 and t_2): Great Britain, England, Scotland, Wales, England + Wales

(c) Different randomly selected ponds (1 per grid square) are surveyed each year and the data compiled over separate survey periods (p_1 and p_2): Great Britain analysis only.

(ii) NBN data: power analysis of alternative approaches to assess change in the proportion of occupied 1 km grid squares and the number of occupied ponds per grid square following survey of all ponds in a square

(a) Change in the proportion of occupied 1 km grid squares - *no stratification* (Tables A4.6 – A4.15)

- Paired samples - the same 1 km grid squares sampled at t_1 and t_2: Great Britain, England, Scotland, Wales, England + Wales
- Independent samples - different 1 km grid squares sampled at t_1 ant t_2: Great Britain, England, Scotland, Wales, England + Wales

(b) Change in the proportion of occupied 1 km grid squares - *stratification to include only squares which are known to contain ponds* (Tables A4.16 – A4.25)

- Paired samples - the same 1 km grid squares sampled at t_1 and t_2: Great Britain, England, Scotland, Wales, England + Wales
- Independent samples - different 1 km grid squares sampled at t_1 ant t_2: Great Britain, England, Scotland, Wales, England + Wales

(c) Change in the number of occupied Great Crested Newt ponds per 1 km grid square: *no stratification* (Tables A4.26 – A4.35)

- Paired samples - the same 1 km grid squares sampled at t_1 and t_2: Great Britain, England, Scotland, Wales, England + Wales
- Independent samples - different 1 km grid squares sampled at t_1 ant t_2: Great Britain, England, Scotland, Wales, England + Wales

(d) Surveys which are stratified to increase the proportion of 1 km grid squares which are known to support Great Crested Newts. Four subsets of analyses within this option are evaluated:

Subset 1: Change in the number of occupied Great Crested Newt ponds per 1 km grid square: *only squares known to support Great Crested Newts* are included in the pool from which random squares are selected - Great Britain, England, Scotland, Wales, England + Wales (Tables A4.36 – A4.40).

Subset 2: Change in the number of occupied Great Crested Newt ponds per 1 km grid square: *50% of sample squares known to support Great Crested Newt*: Great Britain, England, Scotland, Wales, England + Wales (Tables A4.41 – A4.45)

Subset 3: Change in the number of occupied Great Crested Newt ponds per 1 km grid square: *75% of sample squares known to support Great Crested Newt* - Great Britain, England, Scotland, Wales, England + Wales (Tables A4.46 – A4.50)

Subset 4: Change in the number of occupied Great Crested Newt ponds per 1 km grid square: *90% of sample squares known to support Great Crested Newt* - Great Britain, England, Scotland, Wales, England + Wales (Tables A4.51 – A4.55).

(iii) **NBN data: power analysis of a trend analysis approach assessing change over time in the number of occupied ponds per grid square following survey of all ponds in a square.**

(i) NARRS data: power analysis of alternative approaches

The current design of the NARRS project gives a measure of the proportion of ponds occupied by Great Crested Newts based on a survey of 1 pond in each 1 km grid square. Change can be reported as a change in the proportion of occupied ponds between one time period (2007-2012) and some future time period. For comparison these data have been analysed assuming (a) they were collected in a single year (t_1 and t_2) as independent samples, revisiting randomly selected different ponds in t_1 and t_2, and (b) over a known time period (p_1 and p_2), with each period made up of a number of years.

(a) Power of existing surveys

Table 4.13 summarises analyses of the power of the ***existing*** NARRS survey results to detect change in pond occupancy, for Great Britain and the separate country options. Returns from volunteers were low for Great Crested Newts which results in low levels of power to detect change at 20%. Note, however, that obtaining data for country-specific analyses - with their necessarily larger sample sizes – was not a specific objective of NARRS. In addition, NARRS is a survey of all amphibian and reptiles and is not targeted to any one species. A good next step, therefore, would be to recruit more volunteers to NARRS to achieve the required sample sizes for Great Crested Newt surveillance.

Table 4.13: Analysis to determine the level of power achieved to detect change at 20% and 30% in pond occupancy by Great Crested Newt (1 pond surveyed per 1 km grid square) using real data for the sample size currently attained by the NARRS project. Levels of power are given as a %.

	Sample Size (number of ponds / 1 km grid square)	20% change		30% change	
		α=0.05	α=0.10	α=0.05	α=0.10
	n	Power (%)			
Great Britain	**410**	**12.04**	**19.09**	**22.04**	**32.58**
England	**277**	**11.51**	**19.20**	**20.74**	**31.01**
Scotland	**106**	**5.13**	**10.19**	**5.30**	**10.45**
Wales	**27**	**0.06**	**10.80**	**6.29**	**11.90**
England + Wales	**304**	**12.16**	**20.06**	**22.29**	**32.89**

(b) Different randomly selected ponds (1 pond per grid square) are surveyed in year 1 and year 2 at some future time point (independent samples at t_1 and t_2)

Using estimates of variability from analysis of the NARRS results, we used power analysis to calculate the sample size required to detect different levels of change at different levels of power. In other words: how many more ponds should be visited to increase the power of the existing survey (Table 4.12)?

To achieve sufficient power to be able to detect a 20% change in the number of occupied ponds at 80% power (α=0.05) at the level of Great Britain, 2705 ponds would need to be surveyed each year (5410 ponds in total). This is reduced to 2131 ponds per year if α is accepted at 0.10. To detect 10% change, 11351 ponds would need to be surveyed each year (25969 ponds in total). Detailed results are shown in Appendix Tables A4.1.

The sample sizes required to detect change in the regions were higher. To detect a 20% change in the proportion of occupied ponds with 80% power would need in England, 1956 ponds per year; in Scotland, 37120 ponds per year and in Wales, 2065 ponds per year (Appendix Tables 4.2).

The results of this analysis make this approach seem feasible at least at Great Britain level if it is sufficient to detect only large levels of change. However, the sample sizes need to be put in context. For example, if 2705 ponds were visited in year 1 and 317 (12%) were identified as supporting Great Crested Newt, and another 2705 ponds were visited in the second year of survey, we would only have confidence that the sample size could detect the loss of 253 ponds. In this light, 20% loss may not be acceptable using a proportional approach to monitoring.

To be able to detect smaller changes, using the same example, we may want to be alerted to changes in the region of 5%. If this was the case then it would be necessary to increase the sample size to 46450 ponds per year - an option which is clearly not viable.

It is also important to remember that the underlying estimates for this analysis assume that all 410 ponds in NARRS were visited in the same year when in fact they were collated over several years. This will increase the variability still further (see below).

(c) Different ponds (1 pond per km grid square) are surveyed each year and the data compiled over separate survey periods (p_1 and p_2); in this case, each period is comprised of 6 years:

Correct analysis of these data requires an estimate of the between year, as well as between time period, variation. By taking an average occupancy for each year and then comparing the difference in average occupancy between time periods it is possible to estimate the number of years over which the survey data would need to be compiled. Detailed results are shown in Appendix Table A4.6.

If 6 years is considered to be the survey period, with each period surveying 410 samples sites, to achieve 80% power in detecting a 30% change in pond occupancy the survey would need to be continued over 33 sample periods (α=0.05). In other words, change of this magnitude would not be detected over the variability in the data unless the survey ran for over a century. Unless very low levels of power are acceptable, compiling data over sample periods is not a statistically robust design.

To reduce the period of time over which an effect is observed, the sample size within each survey period must increase. These values would exceed the sample size required for discrete surveys because of the variability between years and therefore for Great Crested Newt monitoring this adds an unnecessary level of variability.

(ii) NBN data: power analysis of alternative approaches

(a) Change in the proportion of occupied 1 km grid squares - no stratification

Unlike the NARRS approach, this strategy assumes that all ponds in the survey square are surveyed until Great Crested Newts are confirmed as present or recorded as absent. This will be more onerous than the NARRS approach, but may increase the number of squares which are identified as supporting Great Crested Newt. The advantage is that once one pond has been confirmed as supporting Great Crested Newt, no more ponds in the square require survey. Results of the analyses are shown in detail Appendix Tables 4.7 – 4.16.

Even if the same 1 km grid squares are surveyed in the first and second survey year, without stratification, the variation between sample squares due to the non-normal distribution of Great Crested Newts, and the resulting number of zero values, makes this approach untenable. For example, at Great Britain level to detect a 30% change in square occupancy, with 30% power at α=0.05, 1831 1 km grid squares would need to be surveyed each year (paired samples). This increases to 3866 1 km grid squares for independent samples.

In the individual countries, for paired 1 km grid squares (the same 1 km grid squares surveyed in year 1 and again in year 2) to achieve 30% change with 80% power requires the following sample sizes: England – 858 1 km grid squares per year; Scotland – 26230 1 km grid squares per year; Wales – 1830 1 km grid squares per year and England and Wales combined – 1040 1 km grid squares per year.

If different 1 km grid squares are surveyed in year 1 and year 2 (independent samples) to achieve 30% change with 80 % power the sample sizes become: England – 1804 1 km grid squares per year; Scotland – 55541 1 km grid squares per year; Wales – 3864 1 km grid squares per year and England and Wales combined – 2192 1 km grid squares per year.

(b) Change in the proportion of occupied 1 km grid squares - stratification to include only squares which are known to contain ponds in the first survey year

Using a sample that was stratified to only include squares known to contain ponds in the first year would increase the proportion of Great Crested Newt occupied squares, thereby increasing the level of power for any given sample size. However, ponds created in squares which did not have ponds at t_1 and which may become occupied by Great Crested Newt by t_2, would be overlooked in the analysis. Detailed results of this analysis are given in Appendix Tables 4.17– 4.26.

This approach reduces the number of 1 km grid squares which need to be surveyed each year to detect the same levels of change, but not by much.

If paired 1 km grid squares are surveyed in year 1 and again in year 2 to achieve 30% change with 80 % power the following sample sizes are needed: Great Britain – 1020 1 km grid squares per year; England – 543 1 km grid squares per year; Scotland – 11717 1 km grid squares per year; Wales – 923 1 km grid squares per year and England and Wales combined – 644 1 km grid squares per year.

If different 1 km grid squares are surveyed in year 1 and year 2 (independent samples) to achieve 30% change with 80 % power the following sample sizes are needed: Great Britain – 2148; England – 1139 1 km grid squares per year; Scotland – 24804 1 km grid squares per year; Wales – 1943 1 km grid squares per year and England and Wales combined – 1352 1 km grid squares per year.

The approach of only monitoring at 1 km grid square occupancy also has the added disadvantage that Great Crested Newts have to be lost from all ponds in the square before the change is recorded as a decline.

Summary of strategy to detect change in pond or square occupancy

For ease of comparison, Table 4.14 summarises the results of approaches which use occupancy of the grid square as the focus of the survey, based on NBN data in (ii) a and (ii) b above. For comparison, the number of squares needed to detect change in the % occupied 1 km squares is given for 80% power to detect 10% change with $\alpha = 0.10$.

The optimum strategy at this stage would be to undertake national surveys of randomly selected 1 km grid squares surveyed at time t_1 with repeat surveys of the same squares at time t_2. If unpaired i.e. different random samples were collected at times t_1 and t_2, then the optimum allocation would be to split the samples equally between the sample years. The more unbalanced the sample strategy, the greater the number of samples required overall.

However, repeat sampling of the same squares limits survey design. If squares need to be removed from the network or it becomes desirable to add new squares to the network they cannot be analysed in the matched pairs design.

Table 4.14 The number of 1km squares required to be able to detect change at 80% power with 10% level of change ($\alpha_{0.10}$). GB and national level analyses - based on paired and unmatched (independent) sample squares using NBN GCN data. Squares were either chosen entirely at random or stratified to include only 1 km squares known to contain a pond at t_1 (and at t_2 for independent sample designs).

	1 km squares chosen at random		1 km squares stratified to include only grid squares which contain ponds	
	Paired	Independent	Paired	Independent
GB	1456	3045	811	1692
England	681	1421	432	897
Scotland	20867	94170	9321	19537
Wales	1455	3044	734	1530
England + Wales	827	1727	512	1065

(c) Change in the number of occupied Great Crested Newt ponds per 1 km grid square - no stratification

If the number of occupied grid squares (rather than occupied ponds) is the main focus of the strategy, there is a risk that many occupied ponds within each grid square could be lost before a change was detected. **A better strategy would be to record the number of occupied ponds per grid square**. This would give a more realistic idea of change and could then be used to calculate local, national and Great Britain estimates with confidence intervals for the Great Crested Newt population in terms of number of occupied ponds and as a proportion of all ponds.

Volunteers would visit all ponds within the sample 1 km square and using the best available techniques they would record the presence or absence of Great Crested Newts at each pond. If it were not possible to visit all the ponds within a 1 km square the number of occupied ponds as a proportion of the ponds visited would be calculated for that square.

However, variation in pond numbers between survey squares, and the number of squares with no ponds, means that this option is not feasible as a monitoring strategy. To detect a 30% change at 80% power for both matched pairs and independent samples, the number of 1 km grid squares which would need to be surveyed each year exceeds 1500, both at Great Britain level and within the individual countries. Thus strategies which include squares that have no ponds do not generate practical sample sizes, indicating that stratification of the survey is necessary to reduce the proportion of squares without ponds. Detailed results are given in Appendix Tables 4.27 – 4.36.

(d) Surveys which are stratified to increase the proportion of 1 km grid squares which are known to support Great Crested Newts

To reduce the size of the sample needed to achieve the same levels of statistical power it is possible to stratify the design to ensure that a greater proportion of squares included in the sample are known to support Great Crested Newt. This was done at several levels, from excluding all squares which did not have a record for Great Crested Newt, to selecting 90% of 1 km grid squares randomly from a pool of known 1 km grid squares and 10% from a pool of squares from which Great Crested Newt had never been recorded. The principle would be that non-Great Crested Newt squares would be chosen from within the Great Crested Newt's range in the countries.

This analysis assumes that some of the sample squares are always randomly selected from a pool of squares which are known to support Great Crested Newt. Paired samples are not recommended for this analysis as the proportion of known to unknown squares should remain the same in each sample year. Detailed results are given in Appendix Tables 4.37 – 4.55.

Subset 1: Change in the number of occupied Great Crested Newt ponds per 1 km grid square - 100% of sample squares known to support Great Crested Newt

Subset 1 is based on selecting only squares known to support Great Crested Newts. This option is very effective at reducing sample size, e.g. at the level of Great Britain only 474 1 km grid squares would need to be surveyed in each sample year to detect a 20% change in the number of occupied ponds per grid square with 80% power. However, there are limitations because the technique will be biased towards detecting declines as there is no possibility of recording Great Crested Newts in squares which did not previously have them. Detailed results are given in Appendix Tables 4.37 – 4.41.

Subset 2: Change in the number of occupied Great Crested Newt ponds per 1 km grid square - 50% of sample squares known to support Great Crested Newt

Subset 2, which includes 50% of squares known to support Great Crested newts and 50% unknown squares, is also very effective for reducing sample size (particularly in England) to levels which might be achievable for monitoring. It also maintains the opportunity to detect both population increase and declines. Only 549 1 km grid squares would need to be surveyed in England in each sample year to detect a 30% change in the number of occupied ponds per grid square.

Ponds in Scotland could also be monitored by the same approach with only 513 1 km grid squares would need to be surveyed in Scotland in each sample year to detect a 30% change in the number of occupied ponds per grid square. However, with the remoteness of some Scottish sites and a much smaller pool of Great Crested Newt squares from which to randomly select the 50% of occupied sample squares, we recommend that a 75% approach is adopted (see below). Detailed results are given in Appendix Tables 4.42 – 4.46.

Subset 3: Change in the number of occupied Great Crested Newt ponds per 1 km grid square - 75% of sample squares known to support Great Crested Newt

This analysis assumes that 75% of the sample squares are always selected from a pool of squares which are all known to support Great Crested Newts and 25% from unknown squares. This option was effective for reducing the size of sample required in Scotland, e.g. only 282 1 km grid squares would need to be surveyed in Scotland in each sample year to detect a 30% change in the number of occupied ponds per grid square, with 212 drawn randomly from known Great Crested Newt squares.

This approach could also be used for ponds in Wales, e.g. 389 1 km grid squares would need to be surveyed in Wales in each sample year to detect a 30% change in the number of occupied ponds per grid square, with 292 squares drawn randomly from known Great Crested Newt squares. However, for volunteers this may be too many sites to be a viable monitoring strategy. At present, NARRS volunteer recruitment is lowest in this region. Detailed results are given in Appendix Tables 4.47 – 4.51.

Subset 4: Change in the number of occupied Great Crested Newt ponds per 1 km grid square - 90% of sample squares known to support Great Crested Newt

This analysis assumes that 90% of the sample squares are always selected from a pool of squares which are all known to support Great Crested Newt and 10% from unknown squares.

This approach could also be used for ponds in Wales, e.g. 294 1 km grid squares would need to be surveyed in Wales in each sample year to detect a 30% change in the number of occupied ponds per grid square, with 265 squares drawn randomly from known Great Crested Newt squares

However, although sample sizes are good with this approach, we would advise against using it if possible because it biases the sample heavily towards emphasizing population declines. This is because at 90% of the ponds, the only change possible is for newts to be lost. Gains can only occur in 10% of the sites. Use of eDNA has the potential to identify new sites and testing unknown squares would increase certainty in population estimates. Detailed results are given in Appendix Tables 4.52 – 4.55.

Summary of strategy to detect change in the number of occupied ponds per 1 km grid square

For ease of comparison, the four subsets of Option (d) above are summarised in Table 4.15. The table presents the results of the four subsets of survey strategies to detect change in the number of Great Crested Newt occupied ponds per 1 km square with 80% power, for a 30% change at both $\alpha_{0.05}$ and $\alpha_{0.10}$.

To detect a change in the number of occupied ponds per grid square, the optimum survey strategy would appear to be some level of stratification appropriate to each country, rather than having the same level of stratification in all countries. 'Unknown' squares (i.e. squares where it is not known whether Great Crested Newts occur) would be selected from within the Great Crested Newts range for that country and these squares would be used to evaluate expansion of the population, and to detect new sites. For each 1 km square the number of ponds per square would be recorded along with the number of ponds surveyed, Great Crested Newt occupancy for each pond and an HSI score for each pond in the square.

In England with 50% stratification, to detect a 30% change with 80% power at α=0.05, 549 1 km squares would need to be surveyed (rounded to 550 1 km squares).

In Wales, where 90% stratification is the best option, to detect a 30% change with 80% power at α=0.05, 294 1 km^2 would need to be surveyed (rounded up to 300 1 km squares).

In Scotland, where 75% stratification is recommended, to detect a 30% change with 80% power at α=0.05, 282 1 km squares would need to be surveyed (rounding up, this is 290 1 km squares).

Note that in an earlier draft of this report, initial estimates of squares needed for Scotland were much higher than this because of an anomalous square which supported very large numbers of Great Crested Newt ponds. It is likely that this was due to the way the data were modelled during the OS MasterMap extraction. This square was removed from the analysis to derive this latest set of figures. However, it does raise an interesting problem which we had not considered previously in the development of the network: the effect of exceptionally pond-rich squares. Therefore we recommend that if a randomly chosen square is selected which supports very large numbers of newt ponds (>100 occupied ponds) it should be excluded from the survey. If it is important to monitor the site for conservation purposes it could be included in the survey but excluded from stock and change estimates.

Table 4.15 Great Britain and national level analyses of sample size required to detect 30% change (α=0.05) in the number of GCN occupied ponds per 1 km square - based on paired and unmatched (independent) sample squares using NBN GCN data. Squares were either chosen entirely at random or stratified to include different proportions of 1 km squares known to support Great Crested Newts.

α=0.05	No stratification		Only from squares with records of GCN	50% of squares known to support GCN	75% of squares known to support GCN	90% of squares known to support GCN
	Paired	Independent	Independent	Independent	Independent	Independent
GB	3505	7007	211	604	342	255
England	1982	3960	184	**549**	306	224
Scotland	48282	96562	116	513	**282**	201
Wales	4283	8563	247	675	389	**294**
England + Wales	2148	4293	189	559	312	261
α=0.10	**No stratification**		**Only from squares with records of GCN**	**50% of squares known to support GCN**	**75% of squares known to support GCN**	**90% of squares known to support GCN**
	Paired	Independent	Independent	Independent	Independent	Independent
GB	2761	5519	166	476	246	201
England	1561	3119	143	433	307	177
Scotland	38030	76058	131	404	222	158
Wales	3373	6745	194	532	241	231
England + Wales	1962	3381	149	440	269	206

(iii) Detecting trends over time in the number of occupied Great Crested Newt ponds per 1 km grid square

Rather than making a comparison between two single surveys to determine levels of change, many biological surveys assess rates of population change over time. This is especially useful for populations which naturally fluctuate between years due to environmental factors, e.g. spring temperatures.

For this approach, the simplest model would involve a survey where the number of occupied ponds per 1 km grid square was recorded each year. Sample size would be consistent between years, and the sample years would be equally spaced, e.g. annual surveys. To maintain independence, different ponds would be selected at random each survey year and the model assumes a linear change over time (although this may not be the case e.g. exponential decline).

To increase power, the uncertainty of the measure within year (i.e. occupied ponds) should be reduced by increasing the sampling effort in each year. In trend analysis there is also process variation: the size of the error value of the sample point from the linear trend. This cannot be reduced by increasing sample size within year, but to detect a significant trend above background noise would require more sample years i.e. the more sites visited within year and the more years surveyed, the greater the power to detect ever smaller rates of change per unit time. At a certain point the length of time over which the survey is conducted may introduce too much between-year variation and the subtleties to detect smaller changes between years will be lost. There may, therefore, be an optimum number of years and number of samples within each year required in order to detect change.

To assess the efficiency of different monitoring programs we used Program MONITOR (Gibbs 1995) with sample sizes of 50, 100, 200, 300, 400 and 500 1 km grid squares per year, recording the number of occupied ponds per grid square, and sample years 5, 10, 15 and 20 years from a baseline survey. Estimates of sample variation were based on estimates of pond occupancy compiled for England (NBN dataset - see section 4.4.2.(i)), based on stratification that gave to test two options: (a) that 50% of sample squares were known to support Great Crested Newts and (b) that 100% of sample squares were known to support Great Crested Newts. If sample squares were chosen entirely at random the level of variability within each year made the number of sample squares required unfeasible for a monitoring programme (see 4.4.3.(ii.d)). Estimates of process variation, in other words the variation between years, were based on repeat survey data gathered in Wales at 8 sites between 2006 and 2013 (number of iterations performed – 1000). These were known Great Crested Newt sites and therefore are likely to underestimate variability for more widespread surveys. **Until better baseline data are available these results should be treated with caution**.

Within the 100% occupied strategy (Table 4.16 (a)):

- A 5 year survey period, surveying 50 squares per year would only have 8% power to detect a 10% change if one occurred.
- This would increase to >76% power to detect a 10% change for survey periods greater than 10 years, surveying 50 squares per year.
- A sample size of 300 squares per year would detect changes above 30% in 5 years with >70% power.

As described elsewhere, this approach (100% occupancy) would only be able to monitor change within the existing known distribution of Great Crested Newts and would not allow for detection of range expansion.

Table 4.16 Estimates of the effect of sample size on power to detect different rates of change in pond occupancy by Great Crested Newt over different survey periods, assuming (a) 100% and (b) 50% of squares sampled are known to support Great Crested Newts

Survey period (years)	Average rate of change per year (%)	Power to detect change at different sample sizes (%)					
		50 squares per year	100 squares per year	200 squares per year	300 squares per year	400 squares per year	500 squares per year
(a) 100% of sample squares known to support Great Crested Newts							
5	10	8	7	10	9	13	13
10	10	76	67	93	99	99	99
15	10	99	99	99	99	99	99
20	10	99	99	99	99	99	99
5	20	25	21	39	32	52	50
10	20	99	99	99	99	99	99
15	20	99	99	99	99	99	99
20	20	99	99	99	99	99	99
5	30	59	51	78	70	90	88
10	30	99	99	99	99	99	99
15	30	99	99	99	99	99	99
20	30	99	99	99	99	99	99
(b) 50% of sample squares known to support Great Crested Newts							
5	10	5	5	5	6	5	6
10	10	8	11	11	12	11	12
15	10	25	44	42	51	43	47
20	10	80	96	95	91	96	97
5	20	6	7	6	7	7	7
10	20	36	56	54	63	54	59
15	20	89	99	99	99	99	99
20	20	99	99	99	99	99	99
5	30	8	10	10	11	10	10
10	30	89	98	98	99	98	98
15	30	99	99	99	99	99	99
20	30	99	99	99	99	99	99

Estimates indicate that, as expected, sample sizes required to achieve similar levels of power are larger if the sampling strategy is stratified to include 50% of squares known to support Great Crested Newts, and 50% unknown (Table 4.16 (b)). With the 50% occupied strategy:

- A 5 year survey period is not enough time to detect reasonable levels of change. The power achieved is too low and the number of sample squares required per year is too high.
- A 10 year survey, sampling 50 squares per year, would have 89% power to detect a 30% change. The total number of samples is therefore 500. Undertaking a comparable survey at time t_1 and t_2 as standalone surveys would require a sample size of over 1000, i.e. more than twice as many sites, to achieve the same level of power.
- Extended to a longer timeframe e.g. 15 years, 100 samples per year could detect a 20% change with >95% power. The total number of samples is therefore 1500. An equivalent stand-alone survey, at time t_1, and again at time t_2, would require over 4000 squares.
- Over 20 years, 200 samples per year could confidently (>95% power) detect a change of 10%, requiring a total of 4000 samples. The equivalent stand-alone survey is not feasible, due to the large number of samples required in each year of survey.

As expected, the more samples taken each year, combined with taking a longer view over more years, results in the ability to detect smaller changes with greater power. But the main point to emphasise about the pros and cons of comparing a monitoring design between two years or gathering the data over multiple years is that, after undertaking a two point survey t_1/t_2, it is possible to report immediately whether a change has occurred. In a trend analysis following a multi-year survey, it would only be possible to report confidently on levels of change at the end of the survey, e.g. after 10 years. Therefore it is sensible to try and detect change following the shortest survey possible by increasing the number of samples per year.

A second caveat is that because of the long view, if there is an increasing population, it becomes easier and easier to detect change as the population gets larger (on average more occupied ponds per grid square). But on the other hand, if the population is declining, then it will become harder to detect the changes, due to the variation both within and between years. This is not the case when comparing two stand-alone surveys at t_1 and t_2.

Estimates for trend analysis are more speculative than those for t_1/t_2 surveys because of the lack of existing data on which to base models. However, a survey of 50 squares per year over 10 years should be sufficient to detect a 30% change in the average number of occupied ponds per 1 km square. However, we recommend that this approach is adopted only after stand-alone surveys are initially undertaken over a period of 5-6 years to provide both a definite result initially and data which can be used to refine the trend analysis model.

(iv) Summary

The pros and cons of all the survey approaches described above are summarised below in terms of the sampling unit (Table 4.17) and the survey design (Table 4.18).

The recommended sampling unit is:

- Recording the number of Great Crested Newt occupied ponds per 1 km grid square

The recommended features of sampling strategies for describing pond occupancy are:

- Separate national surveys (England, Scotland, Wales)
- Independent analysis - different random squares are monitored each survey year
- Stratify to select a greater proportion of Great Crested Newt occupied squares.

Table 4.17 The advantages and disadvantages of different sampling units - number of occupied ponds per 1 km grid square, proportion of occupied ponds and proportion of occupied 1 km grid squares.

Sampling unit	Advantages	Disadvantages
Recording the number of Great Crested Newt occupied ponds per 1 km grid square **RECOMMENDED**	A useful measure of both pond numbers and Great Crested Newt occupancy	May be time consuming for volunteers to survey more than one pond per 1 km squares
	Can identify areas of high and low newt density	Accuracy is compromised if it is not possible to visit all the ponds in a 1 km square, although estimates can be made depending on the proportion of visited ponds which were occupied
	It can be scaled up at local, regional, national and Great Britain levels to give population estimates	
Proportion of occupied ponds (based on survey of 1 pond in each grid square)	An existing dataset which could already provide estimates of change if they exist	If no change is observed it may be due to small sample size because the experimental design lacks power
	Can be scaled up to national and Great Britain levels to give population estimates	Does not take account of pond density and therefore may underestimate occupancy in areas of high pond density
Proportion of occupied 1 km squares	Once a pond in a grid square has been shown to support Great Crested Newt, no more ponds in that square need to be searched	To have confidence that the square is unoccupied, all the ponds within the grid square need to be surveyed
	Can be scaled up to national and Great Britain levels to give estimates of population extent	Lacks the detail to report on population size i.e. number of occupied ponds.
		Newts must become absent from all the ponds in a square before loss is reported – this may hide declines

Table 4.18 The advantages and disadvantages of different sampling strategies – including: GB vs national surveys; paired vs independent samples; random vs stratified sample location; and complete survey in one year vs several years data collated into one sample point.

Survey design feature	Advantages	Disadvantages
Separate national surveys **RECOMMENDED**	Useful for reporting at national levels Sample size sufficiently large to be able to report at England+Wales and Great Britain levels as well	Requires a larger sample size
Great Britain survey; England+Wales survey	Useful for reporting at European level Smaller sample size needed than doing separate national surveys.	Hides population change in Scotland and Wales
Independent analysis - different random squares are monitored each survey year **RECOMMENDED**	Flexibility is the design means that if permissions are refused or more squares need to be added to the design it does not affect the analysis	Larger sample size required
Matched pairs analysis - the same ponds are monitored each survey year	Higher levels of power can be achieved with a smaller sample size	Design is fixed - analysis becomes difficult if squares are lost from the network
	Volunteers become attached to 'their' square and are more likely to submit results	
Fully random deign - no stratification - sample squares can be selected from anywhere in the region	The most flexible design which makes no assumptions about the distribution of the data	Large number of zero values in the analysis - results in the need for very large sample sizes to achieve sufficient levels of power
Stratify to select sample squares from those which are known to contain ponds	Removes some areas which are unlikely to ever be suitable for Great Crested Newt	Inflexible design - new pond creation could allow populations of Great Crested Newt to expand, this would not be detected using this sample design
Stratify to select a greater proportion of Great Crested Newt occupied squares **RECOMMENDED**	A 50:50 design allows for a statistically robust design - change can be detected with smaller sample sizes - expansion into new areas will be detected	Not possible with a matched pairs design - as the proportion of sample squares in each category cannot change over time

Table 4.18 (continued) The advantages and disadvantages of different sampling strategies – including: Great Britain vs national surveys; paired vs independent samples; random vs stratified sample location; and complete survey in one year vs several years data collated into one sample point.

Survey design feature	Advantages	Disadvantages
All sample squares are visited in the same survey year **RECOMMENDED**	Statistically more robust as there is no between year variation within the sample period to account for in the design	Intensive survey effort required
	Trends can be seen over time	May miss fluctuations in populations in the years between the survey years.
Data are compiled over several years	Trends can be seen over time	Only able to report on changes after the end of the survey period.
	Fewer samples needed per year to detect similar levels of change.	If the population is declining it become increasingly hard to detect change.

5. Discussion, conclusions and recommendations

5.1 Part A: eDNA

5.1.1 Legislative background

The Great Crested Newt is strictly protected under EU and domestic legislation. Consequently, the UK is required to report on the status of the species across its range in the UK – i.e. in England, Wales, and Scotland. Sampling also needs to be proportionate, as the countries need to make the best use of resources, and balance surveillance against conservation action for the Great Crested Newt, and surveillance and action for other species and habitats.

The UK statutory conservation agencies have agreed (Defra 2012) that in order to be able to report on the national status of Great Crested Newt, data are required (on an ongoing rather than one off basis) for three key parameters:

1. Pond turnover (i.e. balance of losses and gains in the number of ponds)
2. Habitat suitability for Great Crested Newt, principally through use of the 'Habitat Suitability Index'. This is focussed on ponds, but also includes the quality of surrounding habitat
3. Pond occupancy by Great Crested Newt.

These three parameters together are expected by the statutory conservation agencies to be able to fill the biggest current information gaps in reporting for Article 17 of the Habitats Directive – trends in population and habitat. There are other pieces of information required for Article 17 reporting too, but trend in population is key to demonstrating if the species is doing well or not, and monitoring of habitat can give an early warning and suggest causes of change.

The present work aimed to evaluate eDNA as a new potential technique for assessing Great Crested Newt pond occupancy, a key measure of population trend, and to provide further advice on the statistical design of survey strategies for detecting change in habitat extent, habitat quality and population size (respectively, pond numbers, Habitat Suitability Index and pond occupancy by Great Created Newt).

5.1.2 eDNA in the context of previous survey work on Great Crested Newts

Historically, information on the status of Great Crested Newts for nature conservation purposes has been derived from four main sources: opportunistic 'natural history' sightings (Jehle *et al*. 2011), questionnaire surveys of herpetologists to assess trends in populations (e.g. Beebee 1975), co-ordinated regional surveys (e.g. Hollinshead *et al.* 2008) and co-ordinated national surveys.

Large scale, co-ordinated, national field surveys in the UK of Great Crested Newts, and amphibians generally, began with the National Amphibian Survey of the 1990s (Swan and Oldham 1993), and were more formally implemented with the National Amphibian and Reptile Recording Scheme (NARRS), organised by Amphibian and Reptile Conservation, which was started in 2007 (Wilkinson and Arnell 2013).

Over this period various recommendations emerged to standardize surveys so that comparisons between sites could be made in a quantitative way, particularly the 'peak count', now used as an index of population size for over two decades. The peak count involves undertaking several surveys and using the maximum number of newts observed during any one session, and by any method, as a measure of population status. Populations are classed as 'small' (<10), 'medium' (10-100) or 'large' (>100) (e.g. English Nature 2001). An alternative scoring method that was based on confidence limits calculated from a sample of Great Crested Newt ponds was proposed by Griffiths *et al.* (1996). This used population

densities rather than total counts, and allowed a comparison between different methods. Neither peak counts nor population densities take into account factors that may affect detectability at a given site, so the relationship between these indices and the actual number of newts present at a site is not straightforward. Consequently, there is a risk that large populations may be underestimated when detectability is low.

NARRS uses volunteer-based efforts to monitor and report on the status of amphibians and reptiles, including the five widespread native amphibian species: Common Frog (*Rana temporaria*), Common Toad (*Bufo bufo*), Smooth Newt (*Lissotriton vulgaris*), Palmate Newt (*L. helveticus*) and Great Crested Newt (*Triturus cristatus*). NARRS primarily uses trained volunteer surveyors who carry out presence-absence surveys using a standard protocol.

Further refinements to volunteer-based surveys were proposed by Sewell *et al* (2010), leading to the recommendation of a 'four visit'/four method' protocol of visual searching, torch counting, netting and bottle trapping in the surveying of amphibians in order to achieve reliable assessments of the presence or absence of species. This protocol was based on an occupancy modelling approach that goes some way to resolving issues concerned with variation in detectability that can lead to 'false absences' in presence-absence surveys. The proposed protocol is a compromise between rigour and simplicity, necessary for volunteer-based field work.

Reporting on the first six years of NARRS Wilkinson and Arnell (2013) provided estimates of current pond occupancy by Great Crested Newts over the 5 year 2007-12, indicating that 12% of ponds were occupied by Great Crested Newts.

Survey of amphibians to asses status and trends in populations have made substantial progress since the first work co-ordinated by Swan and Oldham in the 1990s. However, the natural variability of Great Crested Newt populations and the relatively small pool of surveyors available for survey work, still present substantial challenges to obtaining national surveillance data on Great Crested Newts. Thus Wilkinson *et al.* (2013) commented that "Current (NARRS) survey sample sizes will not detect useful levels of change in occupancy rate [of Great Crested Newts] at anything other than low power. An unacceptably large second sample size (many thousands of surveys) would be required to remedy this in the second NARRS survey cycle. Even detection of 30% change in occupancy with a less rigorous $\alpha=0.2$ would require over 1,500 surveys between 2013 and 2018" (Wilkinson *et al.* 2013). However, it should be noted that NARRS does provide a satisfactory baseline for detecting change in pond occupancy for other widespread amphibians, at standard levels of power (80% and above), and for occupancy changes of 30% or more, and with $\alpha=0.1$.

The present project suggests that eDNA could add a valuable new technique to the collection of information on the Great Crested Newt. In particular, it may reduce the number of 'false absences' when traditional methods are used at sites where detectability may be an issue.

5.1.3 The performance of eDNA techniques to determine the presence of Great Crested Newt in a wide variety of pond habitats across Great Britain

Geographical extent of the survey. We believe the present survey is the first to apply the eDNA technique to a wide range of sites throughout the national range of a species. For example, the work by Ficetola *et al*. (2008), Thomsen *et al*. (2012) and Pilliod *et al.* (2013) provide methodological proofs of concept dealing with a geographically restricted range of sites, the most extensive survey so far being the work of Dejean *et al*. (2012), who surveyed 49 ponds with eDNA for the Bullfrog in the south of France. In the present work we surveyed ponds throughout the range of the Great Crested Newt in Great Britain, reaching the extremities of the species' range in England, Scotland and Wales.

Effect of environmental factors on eDNA. An objective of the present study was to make a preliminary assessment of the impact of major environmental factors (e.g. climate, geology,

water quality) that could affect eDNA detection. Currently there are few published studies of eDNA approaches that have investigated the influences of environmental factors or pond characteristics on eDNA detection. However, it is well known that algae, leaves and organic matter may contain PCR inhibitors, and a number of fluids and tissues have been identified as PCR inhibitors including bile salts and complex polysaccharides in faeces, collagen in food and tissue samples, heme in blood, humic substances in soil, melanin and myoglobin in tissue, polysaccharides and tannic acid in plants, proteinases and calcium ions in milk, and urea in urine (Rådström *et al.* 2004). Humic acid and tannins are likely co-extracted from water samples and they interact with the PCR by binding to DNA (humic acid) or act as Taq inhibitors (tannins) (Opel *et al*. 2010). In the only specific study of environmental factors affecting DNA so far undertaken Pilliod *et al*. (2013) founds that, in mesocosm systems, eDNA in both full-sun and shaded treatments degraded exponentially to <1% of the original concentration after 3 days. eDNA was no longer detectable in full-sun samples after 8 days, whereas eDNA was detected in 20% of shaded samples after 11 days and 100% of refrigerated control samples after 18 days.

Although our evaluation of potential environmental impacts was essentially correlative, rather than experimental, we found little evidence to suggest that eDNA detection was seriously impaired by environmental factors. The only significantly correlated factor, HSI score, is clearly related to the presence or absence of newts. Although we cannot rule out the possibility that one of the environmental factors that make up the HSI score influenced DNA persistence directly, it seems more likely that the effect was simply due to the occurrence of newts.

However, given that environmental factors have been shown to affect eDNA persistence in other studies, better understanding of the factors influencing the detection of DNA in water would be highly desirable if the method is to be widely adopted. We propose areas for further research below (Section 5.1.7).

False positives and false negatives. In the present study we found no evidence of false positives. Likewise Pilliod *et al*. (2013) found no evidence of false positives at three sites tested which were outside the range of their target species, the Rocky Mountain Tailed Frog (*Ascaphus montanus*) and Idaho Giant Salamander (*Dicamptodon aterrimus*). A similar result was obtained by Thomsen *et al*. 2012 who found that negative results were obtained for each of the six species investigated (including Great Crested Newt) from three control ponds where the respective species were known to be absent.

False negatives i.e. failing to detect the species when it is present, seem a more likely risk, and we recorded 1% such errors in the detailed methodological study, and c10% errors in the volunteer survey. We suspect that there is a difference in the error rates because of the nature of the sites surveyed: the methodological study sites were surveyed by experienced professional surveyors or by mixed amateur/professional teams with considerable experience. Additionally, all the sites in Wales were specially designed Great Crested Newt mitigation ponds. The shorelines of the sites were generally highly accessible enabling water samples to be collected from virtually all of the pond margins without difficult. The 'volunteer' sites were inevitably more heterogeneous: we speculate that the greater rate of error was primarily due to sampling problems or very small populations, or a combination of the two factors. However, it is also worth noting that volunteer surveyors received very limited training, being provide only with the written instructions shown in Appendix 1.

Additionally, it might be expected that false negatives would be a feature in ponds which are only used intermittently by newts. We do not know what proportion of the ponds we surveyed were used in this way but Great Crested Newts are well known to use some ponds – especially small ones – for foraging only (not breeding) (Jehle *et al.* 2011). In such cases, they may only visit sporadically during the spring and summer, increasing the chance that the time of an eDNA survey would not coincide with presence of newts.

Conversely, the Great Crested Newt is one of the few European newts where the juveniles regularly occupy ponds during the spring at some sites – in most species, they only return to the ponds after reaching sexual maturity – and Great Crested Newts also have a longer larval period. These are both factors which could make the Great Crested Newt more suitable for eDNA survey, with a greater chance of detection compared to species where there are fewer life stages in the water at the same time. At present, however, we have no data on the detection of other species of newts using eDNA with which to evaluate this possibility.

Overall, it is clear that better understanding of the issues leading to false negatives is likely to be needed if the eDNA method is to be widely employed.

5.1.4 The performance of eDNA techniques in the prediction of Great Crested Newt abundance

A key project objective was to assess how well eDNA performed in predicting the abundance of Great Crested Newts. We evaluated the relationship between eDNA score and counts of newts at the sites where the detailed methodological study was undertaken (n=35). We also compared eDNA scores with peak count data from 30 ponds around the Dew's Farm SAC provided by Tom Langton.

All of our detections of Great Crested Newt eDNA were below the Level of Quantification (10^{-4} ng/l), the concentration below which it is not currently possible to quantify with precision and accuracy the amount of eDNA in a sample. However, it is a working assumption of the DNA method that the number of qPCR replicates that are positively amplified is related to the quantity of DNA in the sample and therefore that the 'eDNA score' is a reflection of the amount of DNA in the sample. This then provides a potential link to the abundance (or more strictly, the counts) of newts.

There was some evidence of a relationship between eDNA score and the abundance of newts, as shown by peak counts, in the south Hampshire and Suffolk datasets. The relationship appears to depend on there being sites with a full range of eDNA scores (i.e. from 0/12 to 12/12), as occurred in the south Hampshire and Suffolk sites. In Wales, although newt counts were no higher than in south Hampshire, eDNA values were higher, with no site scoring less than 9/12. With this small range in eDNA scores there was little chance of a significant correlation occurring between eDNA score and newt counts. Although the relationship between eDNA and newt abundance is noisy, it is possible from our results to state that low eDNA scores are always associated with small counts of newts. However, the converse, that a high eDNA score means a larger newt count, does not always hold true: there were a number of sites where a high eDNA score was obtained with low newt counts. There are a number of reasons why this could occur.

Other studies have provided similar evidence of a rather weak but positive relationship between eDNA quantity and the abundance or counts of animals. For example, Thomsen *et al*. (2012) reported a significant correlation between eDNA abundance and Great Crested Newt density (Figure 5.1).

For riverine amphibians, Pilliod *et al*. (2013) found eDNA concentration was positively related to field-measured density and biomass (Figure 5.2a,b). Pilliod *et al*. (2013) measured the biomass and density of Rocky Mountain Tailed Frogs and Idaho Salamanders in 13 streams. At each location they took three replicate water samples to derive a mean eDNA concentration. They found correlations between eDNA concentration and Rocky Mountain Tailed Frog biomass (Figure 5.2), although the strength of correlations was increased by removing outliers.

In this study, prior to analysis, any replicate sub-sample whose eDNA concentration fell outside of the 95% confidence interval for the other samples in a given stream was flagged as an outlier. To decrease the influence of individual sub-samples and to provide an

estimate of the effects of outliers on analyses, results were presented with and without outliers removed. Removing outliers substantially improved correlations (e.g. in Figure 5.2a, which shows the analysis with outliers removed, the correlation between eDNA and frog biomass was increased from an r^2 of 0.36 to r^2 of 0.83).

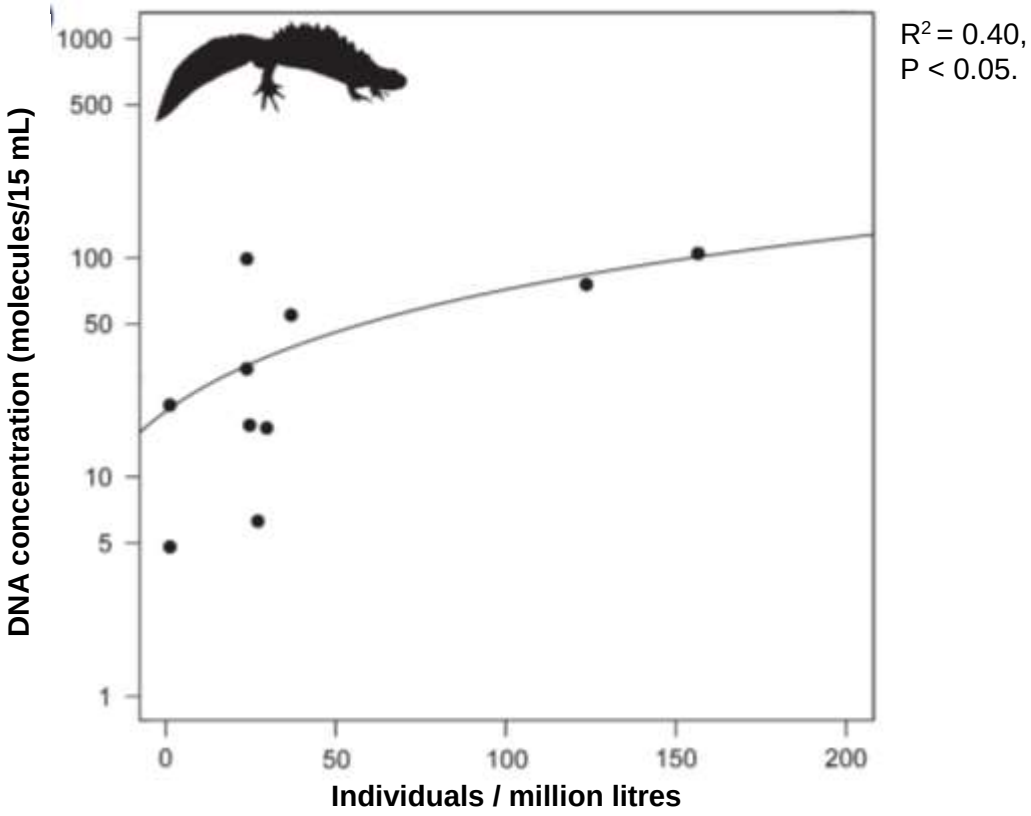


Figure 5.1 The relationship between Great Crested Newt density and DNA concentration. From Thomsen *et al.* (2012).

Inspecting the results of both Thomsen *et al*. 2012 and Pilliod *et al.* (2013) it is clear that much of the correlation between eDNA and amphibian abundance is due to a small number of higher density/biomass locations where eDNA amounts are unequivocally higher. At lower animal densities the pattern appears generally to be one where eDNA amounts may be rather varied. Removing the small number of high values from these correlations substantially reduces them suggesting that, in effect, the results are really distinguishing between low density sites and high density sites, rather than providing a more nuanced interpretation of abundance.

A similar trend is apparent in our results where the eDNA score associated with lower newt counts is rather variable (there may be both low and high eDNA scores) whereas at higher newt abundance eDNA scores are normally higher (see Figures 3.4, 3.5, 3.8 and 3.9).

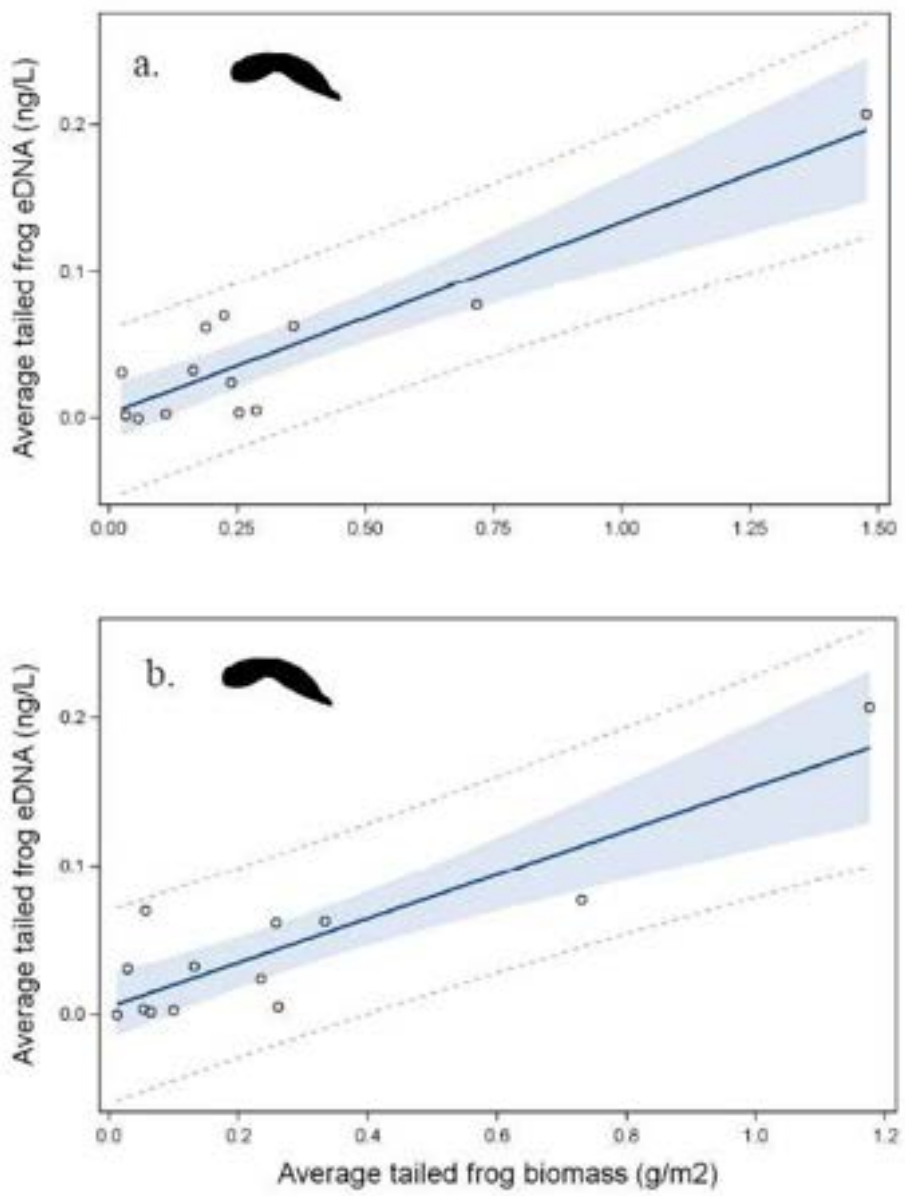


Figure 5.2 Relationships between eDNA concentration and (a) density and (b) biomass of Rocky Mountain Tailed Frogs. From Pilliod *et al*. (2013).

As suggested by both Lodge *et al*. (2012) and Pilliod *et al*. (2013) there is a dearth of knowledge about how different environmental conditions affect the production, degradation, and detection of eDNA. Additionally, factors affecting persistence and limits of detection of eDNA have only just started to be investigated (Dejean *et al*. 2011; Takahara *et al*. 2012).

In terms of abundance estimation our results, and those of others, suggest that eDNA currently gives a rough indication of the abundance of the amphibian species so far examined. With further experience this may allow us to conclude that populations are small or medium /large, following the traditional terminology of ‘low, ‘medium’ and ‘large’ counts applied by English Nature (2001).

Overall, conclusions about the sensitivity of the method, which appears to be high, must be tempered with some caution given the current limits on our understanding.

Recommendations to fill the methodological gaps in understanding are given below (Section 5.1.8).

5.1.5 Implications of eDNA survey for consultants and developers

An important practical issue with eDNA, which will require further investigation to resolve, is the extent to which transient animals visiting a site are detected. This could have a substantial impact on the practical use of the method and, given its apparent sensitivity, could substantially increase the number of sites considered to be supporting Great Crested Newts.

In addition, it seems likely that eDNA will detect smaller populations where there is continuous pond occupancy and/or breeding, compared to traditional methods. There is a parallel here with trends over the years with mitigation surveys: consultants have been encouraged to do more intensive surveys (especially in relation to terrestrial surveys) and this has led to the detection of ever smaller populations.

5.1.6 Volunteer surveys: some practical considerations of volunteer motivation

We conclude that theoretically it would be feasible for either volunteers or professionals to collect eDNA samples as part of a national survey. However, there are additional logistical issues that need to be addressed if eDNA surveys using volunteers, in particular, are to be effective.

First, attitude surveys of NARRS and PondNet volunteers show that volunteers prefer to go to sites which are close to home. Since most volunteers live in urban areas visiting more remote rural sites will inevitably require professional backup to ensure a properly structured set of sites is visited.

Secondly, volunteers are generally unwilling to visit sites where land ownership is not already known. Hence it is essential for a scheme organiser to obtain prior permission for survey. Thirdly, volunteers are time-limited and may not be willing to collect samples from all ponds in a 1 km square if more than two or three ponds are present.

Finally, as many volunteers do pond surveys because they enjoy seeing amphibians, additional explanation and encouragement may be needed to ensure that volunteers find eDNA surveys - during which you do not need to see amphibians - sufficiently rewarding. In particular, it is important to avoid the risk of undermining established volunteer survey programmes (especially NARRS) through demotivation of volunteers who may be more interested in seeing amphibians than collecting water samples.

Thus, as with other wildlife monitoring schemes, a significant element of professional volunteer support, survey work and other logistical backup is likely to be essential for eDNA surveys that involve volunteers. We comment further on the potential for integrating volunteer survey work with eDNA surveys below (Section 5.2).

5.1.7 Areas where further research is needed on the eDNA method

As has been noted by several studies there are still important gaps in our understanding of the eDNA method. Thus Pilliod *et al*. (2013) note that little is known about how processes such as secretion rate, environmental degradation, and time since colonization or extirpation from a given site, affect eDNA measurements.

With respect to Great Crested Newts we have some limited data on eDNA persistence from the Thomsen *et al*. (2012) study. We have no information about secretion rates and only indirect observations (from the present study) on the effect of environmental factors on degradation. We know nothing of the dispersion of eDNA in ponds, although there are hints from the present study that it may sometimes be patchily distributed. It is clear therefore that it would be valuable to have information on the following factors describing the dynamics of eDNA in ponds, ideally before there is widespread implementation of the eDNA method.

- **Persistence of eDNA, including in both field situations and experimental set-ups**. At present, we have only the data from mesocosm studies by Thomsen indicating a fairly

short (up to 2 weeks) persistence of Great Crested Newt eDNA. The study by Dejean *et al*. 2011 indicate slightly longer persistence (up to one month) for Bullfrog tadpoles and the Siberian Sturgeon.

As part of this work is would be important to explore in more detail the environmental factors potentially affecting persistence. In the present study there was little suggestion that environmental factors played a strong role in eDNA detection. However, environmental factors do influence eDNA breakdown and it will be important to quantify the effects.

- **Quantification of eDNA at levels below the current Level of Quantification (LoQ)** Obtaining information on the abundance of Great Crested Newts is time consuming and / or expensive and it would be valuable to policy makers and others if eDNA could provide better information on newt abundance. To achieve this methodological development is probably needed in two areas: methods for increasing the amount of eDNA extracted from the water to increase the precision and accuracy of DNA quantification at lower field concentrations of DNA, and experimentation on sampling strategies to better understand the distribution of DNA in the water. To extract larger amounts of eDNA requires further experimentation on the filtration of water to collect eDNA, collecting larger samples or pooling samples over a period of time, along with better understanding of the distribution of DNA in the water both spatially and temporally. Understanding of eDNA distribution in ponds requires matching intensive water sampling strategies to field data on the abundance and distribution in the pond of newts.
- **Persistence of DNA in other parts of the pond (e.g. sediment vs water)**. eDNA can persist for extremely long periods in some environmental compartments. For example, in Greenland, ancient communities of plants and animals were described using short fragments of 450,000 year old DNA extracted from silty ice samples extracted from the bottom of the Greenland ice cap (Willerslev *et al*. 2007). It would therefore be valuable to understand how newt DNA was distributed within pond compartments other than the open water in order to understand the potential for generating false positive results. Such information could also be valuable for understanding changes in the use of ponds by Great Crested Newts.
- **Differences in eDNA production by life stages**. It is likely that different life stages will produce different quantities of DNA, although at present there is no information available on this issue.
- **Risks of contamination, false positives, and length of time Great Crested Newt needs to spend in a water body for its DNA to be detected**. Related to other issues of detectability it would be valuable to assess experimentally how vulnerable to contamination the method is, and to further explore false positives. False positives are challenging to assess because of the difficulty of proving absence with traditional survey methods. The issue of how long a newt needs to be in a pond before it can be detected could probably be addressed fairly straightforwardly with studies of caged animals added to otherwise newt free ponds.
- **Storage and handling effects**. eDNA methods seem to be fairly robust to storage and handling effects when reasonable care is taken to avoid contamination. In addition to our own practice in the present project, which places considerable emphasis on avoiding contamination, Pilliod *et al*. (2013) conclude that methodological differences appear to have relatively little impact on eDNA detection. However, it would also be useful to investigate the impacts of handling and storage on results to understand better the risk factors.
- **Survey work outside the main Great Crested Newt breeding season**. A small pilot study is in progress to assess the detectability of Great Crested Newts outside the

normal breeding season, in the autumn. Unfortunately the pilot did not begin collecting data until October, later than planned, because of logistical problems. It would be useful to undertake a full version of this work testing detectability of newts from July through to March. Given that Great Crested Newts may be present in the water year round this has the potential to substantially increase the survey season for the species.

In addition to specifically funded work, there are opportunities for PhD work on this subject with members of the present project team. It is perhaps worth noting that for critical pieces of information work should be undertaken by experienced staff (rather than PhD students who are learning skills), with PhDs providing additional information which is less crucial to the successful application of the method.

- **Other species**. Given the success of the method with Great Crested Newt there may well be a case for assessing the ability of eDNA to:

  (a) detect other amphibians: this was originally costed in the project proposal and it may still be valuable to assess the effectiveness of surveys of other amphibian species. It is technically practical to test for the presence of all widespread native amphibians using an eDNA test which costs approximately 50% per sample more than the single species Great Crested Newt test.

  (b) Other vertebrates: it may be valuable to test the potential to detect fish for which methods are also rapidly developing. There are reporting needs for relatively widespread protected species (e.g. Atlantic salmon) and for endangered species (e.g. Arctic Charr) which may be enhanced or made more cost-effective with eDNA. Thomsen *et al.* (2012) demonstrated that eDNA could be used to detect aquatic mammals: species such as Water Vole and Water Shrew which are comparatively cryptic may be amenable to eDNA survey methods.

  (c) Other protected species: There are a number of protected invertebrates where methodological challenges of survey work, or the need to avoid disturbance, could make eDNA survey work attractive if it were reliable possible to detect the species. For example, distribution data on the Pearl Mussel (which often has small and difficult to find populations), the Southern Damselfly (where larval surveys to locate breeding sites could damage the habitat) and the Little Whirlpool Ram's-horn Snail (where very large networks of ditch sites make searching for the species time consuming) might all potentially be enhanced by eDNA methods. The same would potentially be true for many BAP species for which there are national reporting requirements.

  (d) Non-native species: eDNA methods have already been identified as potentially valuable for locating populations of cryptic non-native species (e.g. Bullfrog). For example, cryptic non-native fish which may be released into pond systems may be more easily surveyed by eDNA than traditional fish survey methods which are labour intensive.

5.2 Part B: Design of surveillance monitoring programmes for the Great Crested Newt

5.2.1 Number of grid squares and ponds to be surveyed

We make the following recommendations on network designs for Great Britain, England + Wales and separate national networks for England, Scotland and Wales.

Given the substantial differences in the patchiness of Great Crested Newt populations in England, Wales and Scotland, we recommend that the mixed known/unknown squares approach is used with Great Crested Newt range, but with the proportions of squares known to have newts tailored to each country. This targets sample squares to within the Great Crested Newt's range and reduces the number of squares with zero values. At the same time it still allows new sites to be detected and for range expansion because a proportion of the survey squares will always be of unknown status. This approach works because we are interested in monitoring how the number of ponds per 1 km grid square, and the number of occupied ponds per grid square, changes over time. New sites are randomly selected each year to maintain the ratio of known to unknown sites.

Taken together the three countries' surveys would then provide a view for Great Britain as a whole but would not be analyzed as a single dataset. A single Great Britain-wide strategy is not ideal since such a strategy provides good estimates for England but does not provide reliable data for Scotland and Wales when broken down to country level.

In England we suggest a survey based on 50:50 stratification where half the squares surveyed are known to support Great Crested Newts, and half are squares where status is unknown. This would require a survey of 550 squares to detect a 30% change in occupancy with 80% power, involving an estimated 1100 ponds. This number of squares and ponds would need to be surveyed at both t_1 and t_2 survey times.

In Scotland and Wales we recommend strategies much more focused on known sites. In Scotland we propose a network of 290 squares, with 75% of squares known to support Great Crested Newts, involving an estimated 580 ponds. In Wales we propose that 90% of squares that are surveyed are known to support Great Crested Newts, with 10% selected from the 5 km buffer zone around the known distribution to detect expansion. This would still require a survey of 300 1 km squares to achieve 80% power to detect 30% change, an estimated 600 ponds. This number of squares and ponds would need to be surveyed at both t_1 and t_2 survey times.

The size and shape of this network also allows for statistically robust analysis (80% power) of other key parameters, i.e. habitat condition (HSI score) for Great Crested Newts and pond numbers. A change of 10% in habitat condition, assessed using Habitat Suitability Index scores, and a 20% change in the number of ponds per 1 km grid square could also be detected in each of the national networks given the sample sizes suggested for monitoring Great Crested Newt pond occupancy.

Reporting on NARRS, Wilkinson and Arnell (2013) have also provided estimates of current pond occupancy (based on a network of randomly selected ponds) over the 6 year 2007-12 and have analysed the power of further surveys to detect change in Great Crested Newt and other amphibian populations. They concluded that the current NARRS data could detect a 20% or 30% change in Great Crested Newt pond occupancy with, respectively, greater than 25% and 50% power, with $\alpha = 0.1$. They comment: "Current survey sample sizes will not detect useful levels of change in occupancy rate at anything other than low power. An unacceptably large second sample size (many thousands of surveys) would be required to remedy this in the second NARRS survey cycle. Even detection of 30% change in occupancy with a less rigorous $\alpha=0.2$ would require over 1,500 surveys 2013 – 2018." These values are broadly in line with the estimates made in the present study based on a survey strategy which records the proportion of occupied ponds, although it should be noted that the occupancy methodology adopted in the present study differs from that applied in NARRS.

The reason the NARRS approach requires a significantly larger number of samples to detect change, compared to the approach recommended in this report, is that it cannot be stratified to increase the proportion of samples with Great Crested Newts.

Analysis by DICE, based on occupancy models developed using data collected from high density core range and low density edge of newt range areas, has estimated that with improved detection probabilities (using 4 methods and 4 repeat surveys), it should be possible to detect a 30% decline with 80% power through a monitored network of around 300 randomly selected ponds within Great Crested Newt range (Griffiths and Sewell 2013). There are a number of reasons for the difference in the estimates which need exploring. For example, the proportion of occupied ponds recorded by NARRS appears to be four times lower than the occupancy probabilities used in the DICE model.

5.2.2 Type of survey: should the approach use eDNA alone?

There are potentially three approaches to obtaining data on the presence of Great Crested Newts in ponds.

(i) Traditional survey alone

(ii) eDNA survey alone

(iii) A combination of eDNA and traditional survey methods.

(i) Traditional survey methods

Traditional amphibian survey methods (specifically torch counts and bottle trapping) were able to detect newts about 95% of the time in the present survey. As a combined 4 visit method this approach was only slightly less effective than eDNA. However, the traditional survey approach requires substantially more survey time, and a need for licensing for both professional and volunteer surveyors. For some volunteers, night-time working is also off-putting (although for others it is part of the pleasure of the survey). Current volunteer-collected amphibian data shows that the majority of volunteers were willing to make only one or two survey visits per year, using 1-3 survey methods. Thus although it is theoretically feasible that comprehensive traditional surveys could be undertaken by volunteers, the level of recruitment, organisational challenge and cost would be substantial, and project success not guaranteed.

(ii) eDNA survey method

The current project indicates that eDNA can quickly establish the presence or absence of Great Crested Newts. A single, stand-alone eDNA survey appears to be a practically feasible way to obtain data on the presence or absence of Great Crested Newts. There is also evidence from the present study that eDNA provides a rough indication of the abundance of newts: sites with high eDNA scores (9/12 to 12/12) generally had ‘medium’ or ‘high’ newt counts. Lower eDNA scores were often associated with ‘low’ newt counts.

eDNA surveys undertaken by professional surveyors are eminently feasible. Completion of at least part of the survey by volunteers is also a viable option, and made more achievable because no license is needed to collect the eDNA sample. However, is unlikely to be possible to recruit sufficient volunteers to visit all the sites needed, particularly in areas further away from population centres.

An additional, and currently unknown, consideration in an eDNA-only study design is the motivation of volunteers. As noted above, many amphibian volunteers like to see the animals – understandably, this is often their primary motivation for undertaking surveys. Asking volunteers to adopt a survey approach which does not involve looking for amphibians has the potential to alienate surveyors, reduce volunteer numbers and undermine the existence of current volunteer surveys. There are currently no data to show the extent to which this

effect is likely, and were an eDNA-only approach to be adopted for Great Crested Newt surveillance, we recommend that it is accompanied by investigation in this area.

(iii) Combination of eDNA and traditional methods

Once at a site, professionals and trained volunteers can sometimes rapidly establish the presence of newts by direct observation of adults or eggs, particularly where the survey timing is optimal and there are reasonable newt populations. The process of collecting HSI data (for both eDNA and traditional surveys) also provides an opportunity to quickly search for amphibians.

A 'mixed method' survey option is therefore possible, where professionals or licensed volunteers briefly search for easily detectable newts (if licensed, they could look for eggs). If a positive sighting is made, this is sufficient to prove presence, and an eDNA sample is not needed. If newt traces are not evident, an eDNA sample would then be collected. The advantage of this method is that it directly involves and empowers amphibian surveyors in searching for Great Crested Newts (ii) it saves the cost of processing unnecessary eDNA samples. Sample kits alone cost about £12, so avoiding eDNA analysis would save c£90/site. If 25% of sites did not need eDNA survey, in England this would save about £25,000 in a total analysis cost of c£110,000.

The disadvantages of this approach are: (i) it requires surveyors to be trained and licensed to look for eggs, (ii) surveyors cannot enter the water to undertake searches because this would compromise the eDNA water sample, (iii) there will be a, probably small but unknown, additional source of error caused by surveyor misidentification.

Note also that the generation of datasets using a combination of 'traditional' and eDNA methods has not directly been trialled, although conceptually it is little different to the combined torching and bottle trapping of traditional survey methods

Overall the likely cost savings of this mixed option are modest (the main cost for all surveys is in gaining landowner permission). However, this approach does offer greater potential for volunteers, in particular, to see Great Crested Newts.

5.2.3 Estimated Costs

(i) Assumptions

To provide an estimation of costs we have compared the amount of time required for a 4 visit/2 method (torching and bottle trapping) 'traditional' survey with the time required for an eDNA survey. The present study indicates that the 4 visit/2 method survey is required to achieve the same level of detection as an eDNA sample.

To enable comparisons to be made we have assumed that for professional survey work a day of time costs £350, whether collecting eDNA samples or surveying amphibians. We have costed eDNA samples at the same price as the present work i.e. £100. In this indicative analysis we have not taken travel costs into account but have assumed they would be broadly equivalent for both surveys.

For the eDNA survey we have assumed that 50% of the sample collection time would be undertaken by volunteers. We have assumed that the 'traditional' survey is carried out by professionals. Clearly this leads to substantial costs. We have not provided costs of analysing results since these are broadly similar whether surveys are professional or volunteer, and involve either traditional or eDNA methods.

Table 5.1 shows the time needed for different survey activities. Table 5.2 summarises the overall estimated costs for t_1/t_2 type surveys. These costs would be incurred in each year the survey was undertaken. We have not calculated the cost of trend analysis surveys as there remain considerable uncertainties in our estimates of the numbers of sites that would be needed at present.

(ii) Costs

eDNA survey

There are three main costs in an eDNA survey:

- Obtaining site permissions. In an eDNA survey, this is the single largest cost but it is unavoidable whether the survey is done by volunteers or professionals. Obtaining site permissions is particularly important for volunteer engagement as having to seek permission to visit sites is a major barrier to volunteer engagement. Extensive experience of setting up permissions to visit sites obtained during the PondNet work indicates that 2 days / kilometre square should be allowed for this work. Staff engaged in site permission work would also support volunteers collecting samples.
- Visiting the sites to collect the eDNA samples. It takes about 30 – 1 hr minutes to collect an eDNA sample and take an HSI score. We have assumed 1 hour travelling each way to the site (travel to location of the site, and walking to the pond).
- Analysing the eDNA samples. This is currently costed at £100/sample, although for a large survey we might expect some reductions on this.

We have assumed in the cost of obtaining site permissions that this would be done by four regional co-ordinators with a salary of c.£25,000 / year, which have an overall cost including overheads of c£200/day. Hiring a consultancy to do this would lead to higher costs: we have (conservatively) assumed that consultants would charge at least £250/day for work like this.

Overall in England, the cost of a 50% volunteer collected eDNA survey would be about £410,000, of which over half is the cost of obtaining site permissions and supporting volunteers. In Scotland and Wales the overall cost of a 50% volunteer collected eDNA survey is likely to be similar in both countries, with each costing about £180,000. The costs of writing up the results would be additional to the survey costs and would be in the region of £50,000 for the whole of Great Britain. These costs would be incurred each time the survey was undertaken (i.e. at t_1 and t_2).

Traditional survey

The cost of surveying ponds using traditional survey methods, combining torch counting with bottle trapping, are roughly 6 - 10 times greater than eDNA survey costs. It would be possible to reduce costs substantially if all survey time was allocated to volunteers, but we think it is unlikely that sufficient volunteers could be recruited to achieve this.

Mixed method survey

We have not provided a separate costing for a mixed method survey combining volunteers looking for newts, supplemented by eDNA survey.

In practice the costs would be broadly similar to the volunteer survey, except for the expenditure on eDNA sample analysis. As we do not yet have experience of what proportion of sites would ***not*** need the eDNA sample it is not possible to say what cost reduction this would lead to. However it seems unlikely that it would more than about 25% of sites, given that half of the survey will be of waterbodies where the current status of newts is unknown, so are less likely to be known in advance by volunteer recorders, a factor which is likely to contribute to easy detection of newts.

Table 5.1 Times required for Great Crested Newt and eDNA sampling used to estimate overall costs of a national Great Crested Newt survey with traditional or eDNA methods

	Traditional survey methods	Collect eDNA sample
	Hours per visit	
	4 visits	1 visit
Get to/from site (evening for traditional methods; anytime for eDNA)	1	1
Torch count	1	-
eDNA	-	0.5
eDNA sample handling	-	0.5
Put out bottle traps	1	-
Get to/from site (next morning)	1	-
Collect bottle traps	2	-
	Traditional methods require 2 people	eDNA with 1 person
Hours	6	2
People	2	1
Visits	4	1
Total hours	**48**	**2**

Table 5.2 Number of 1 km grid squares visited in each survey year, approximate pond numbers and costs of surveys required to detect a 30% change, with 80% power and 95% confidence, in Great Crested Newt occupancy

Country	No. of 1km grid squares (and approximate pond numbers)	Traditional survey cost Requires 4 visit survey with torch counts and bottle trapping; 2 people per visit needed	eDNA survey cost
		Assumes work done professionally as it is very unlikely that a national 4 visit/2 method survey could be achieved with volunteers	Assumes: 50% eDNA samples collected by volunteers 50% eDNA samples collected by professionals
England	550 (1100)	Site permissions: c 2 days /1 km square: £250,000 Field survey £1,100,000 **TOTAL: £2,714,000**	Site permissions: c 2 days /1 km square: £250,000 Professional eDNA surveyor time: £51,000 Volunteers eDNA survey time: £0 cost eDNA samples: £110,000 Volunteer coordinator: included in site permissions work **TOTAL: £411,000**
Wales	300 (600)	Site permissions: c 2 days /1 km square: £93.750 Field survey £1,100,000 **TOTAL: £1,438,000**	Site permissions: c 2 days /1 km square: £93,750 Professional eDNA surveyor time: £28,000 Volunteers eDNA survey time: £0 cost eDNA samples: £60,000 Volunteer coordinator: included in site permissions work **TOTAL: £182,000**
Scotland	290 (580)	Costs are essentially the same as for Wales because number of sites and ponds is very similar. **TOTAL: £1,430,000**	**TOTAL: £182,000**

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Appendix 1. PondNet information for surveyors on how to collect an eDNA sample

How to collect a water sample to detect Great Crested Newt eDNA

What is eDNA?

eDNA is DNA that is collected from the environment in which an organism lives, rather than directly from the plants or animals themselves. In aquatic environments, animals including amphibians and fish, shed cellular material into the water via their saliva, urine, faeces, skin cells etc. This DNA may persist for several weeks, and can be collected through a water sample, and analysed to determine if target species of interest have been present in the waterbody.

Who's involved in the current project?

Water samples for analysis of Great Crested Newt DNA are being collected by PondNet and NARRS volunteers co-ordinated by Pond Conservation and Amphibian and Reptile Conservation respectively. There is an additional trial, undertaken by John Poland, to collect multiple eDNA from a small number of ponds. The cost of analysis is mainly funded by Defra with a contribution from JNCC and Natural England. The analysis is being undertaken by SPYGEN, who are one of the main groups to have researched and developed the eDNA technique in Europe.

Survey Protocol

Overview

You use the eDNA sampling kit provided, to collect water from a pond known to support Great Crested Newts in May 2013. It usually takes around 30 minutes to collect the sample. But may take double this the first time that you do it. After collection, the sample should be kept in the fridge and then sent off to Pond Conservation as soon as possible. The sample will then be couriered to France for analysis by SPYGEN.

How do I get my eDNA sampling kit?

In most cases, kits will be sent by post to your address in early May.

What do I do when my kit arrives?

- Place the kit in a plastic bag and put it in the kitchen fridge. Don't freeze it.
- Please use the kit within about two weeks of receiving it, and use one kit per pond

Why? The kit already contains a small amount of 'control' DNA - this ensures we will know if the final sample has been poorly stored since this DNA will be lost. Keeping the sample in the fridge stops the 'control' DNA from degrading. Putting the kit in a plastic bag, just stops the kit from coming into contact with DNA from food in the fridge. It's not a major problem, but a good principle to minimise all sources of 'other' DNA in the French labs where the samples will eventually be unpacked and analysed.

Sampling the pond

You can watch a video illustrating the eDNA sampling method at a pond on the Pond Conservation website: http://www.pondconservation.org.uk

1. Don't go in the water - but treading on muddy edges is OK

- Collect your eDNA water sample *before* you do any other surveys at the pond.
- Take the sample whilst standing the pond bank or muddy edges. Don't tread in the pond water itself either before or during collection of the DNA water sample.

Why? There is a considerable risk of contaminating your pond sample by bringing in Great Crested Newt DNA in mud and water from other areas on your boots and equipment. This is a real risk: DNA can remain on surfaces even after they have been dried, and can persist in soil for many years. There are recorded examples of eDNA cross-contaminating pond water samples from surveyor's boots.

2. Walk around the pond, to identify areas where you can take your eDNA samples

- In a moment you will take 20 water samples from around the pond: so roughly plan where you will collect them.
- The aim is to spread the samples out evenly around the pond edge (e.g. one sample every 2m).
- The samples should be taken from both open water and vegetated areas if present.
- If you can't access all areas of the pond (=most ponds!), spread the samples out as best you can without entering the water.

Why: Existing data shows that eDNA can be very patchy depending on where the animals have been. By sampling in many areas you *considerably* increase your chance of collecting their DNA successfully.

3. Collect the sample

- Open your kit. Inside you will find:
  - 1 sterile Whirl-Pak bag
  - 2 pairs of gloves
  - 1 blue sampling ladle
  - 6 conical tubes two thirds full of preserving fluid (mostly alcohol)
  - 1 sterile pipette
  - 1 protocol sheet
- Put on a pair of gloves.
- Open the sterile Whirl-Pak bag by tearing off the clear plastic strip c 1cm from the top (along the perforated line), then pulling the tabs. The bag will stand-up by itself.
- Collect 20 samples of 40 mL of pond water from around the pond (see 2 above) using the blue ladle (fill the ladle), and empty each sample into the Whirl-Pak bag. At the end the Whirl-Pak bag should be just under half full.
- NOTE: Before you take each ladle sample, be sure to mix the pond the water column by gently using the ladle to stir the water from the surface to <u>close to the pond bottom</u> without disturbing the mud in the bottom.

Why: DNA 'sinks' and so will often be present in larger amounts close to the pond bottom. However, it is important not to collect sediment, because DNA can be absorbed in sediment particles and persist for a very long time. If you collect sediment, your sample might show a false positive indicating GCN was present recently, when in fact this was a long time in the past.

4. Preserve the sample

- When you have collected your 20 samples, close the bag securely using the top tabs and shake the Whirl-Pak bag for 10 seconds. This mixes any DNA across the whole water sample.
- Put on a new pair of gloves to keep the next stage as uncontaminated as possible.
- Using the clear plastic pipette provided take c15 mL of water from the Whirl-Pak bag, and pour into one of the six conical tubes with preserving fluid (i.e. fill tube to the 50 mL mark).

- Close the tube. Ensure the cap is tight - leaky samples could later contaminate the analysis laboratory with DNA.
- Shake the tube vigorously for 10 seconds to mix the sample and preservative. Otherwise they will stay as separate layers and the DNA will degrade.
- Repeat for each of the six conical tubes in the kit. NOTE: Before taking each sample from the bag, stir the water in the bag to homogenize the sample - this is because the DNA will constantly sink to the bottom.
- Empty the remaining water from the whirl-Pack bag back into the pond.

5. Label the sample

- Each kit has a single unique identifier letter /number code. This number is printed on the box and on each sample tube label.
- It is essential that you record this number together with the site name, because this is the only way we will be able to tie the DNA sample results to your site.
- To reduce the potential for errors, please record this information in two places.
  1. **Complete the sample information box (grey box) below, and return this information with your sample.**
  2. **Keep a separate record of your own.**

     PondNet surveyors: to do this please write the code number on your amphibian site recording sheet (in the box provided), and enter the data on the PondNet website with your other amphibian results. We'd also be grateful if you could use the Notes Box to provide any feedback about how you found the eDNA sampling!

Returning the kit

- On returning home, store the six preserved sample tubes in their box in a kitchen fridge (i.e. at normal 2-4º C fridge temperature). Put the box in a plastic bag so it does not touch food. Don't freeze the sample.
- **As soon as possible, mail the tubes in their original box, to Pond Conservation using the pre-paid, addressed envelope and the packaging provided.**

If the original packaging is lost please send samples to the following address:
Pond Conservation
c/o Faculty of Health & Life Sciences
Oxford Brookes University, Gipsy Lane, Headington,
Oxford, OX3 0BP

Thank-you very much for helping with the project!

Great Crested Newt eDNA sample record

Sample code number (from the box or tubes):	
Site name:	
Your name:	Sampling date:
Survey type (e.g. PondNet, NARRS, if other please state):	

Please complete and return this slip with your water sample

FAQ's

Only one kit per pond - what about very big ponds?

The sampling protocol has been optimised to detect Great Crested Newt at sites with an area less than 1 hectare. It is unlikely that there will be larger sites sampled as part of PondNet or NARRS, since these large waterbodies are rarely optimal for GCN. However, if your site is larger than 1 ha, alert your regional co-coordinator who will provide an additional kit.

Does it matter if I get things like duckweed, algae or zooplankton in my sample?

No, small amounts don't matter. However try not to collect bottom sediment in the sample, because the DNA can be absorbed by sediment and may give false positive results (see above).

What happens if I spill the preservative - or the sample tube itself

If you spill some of the preservative from one of the tubes, just add proportionally less water from your pond sample. The samples from all six tubes are later combined for the lab analysis, so it's not disastrous if some sample is lost.

Won't my samples degrade in the post?

The preservative (alcohol) in your sample bottle will slow, but not eliminate degradation of any DNA. Keeping the samples in the fridge also slows this process. Sending the samples by post at ambient temperatures will mean the DNA will degrade little faster during this time, but it won't be sufficient to degrade the sample completely.

Out of interest - how much does it cost to analyse an eDNA sample?

It's still quite expensive - the lab costs are currently c£100 per sample (i.e. per pond).

When will I get the eDNA results from my pond back?

The eDNA analysis will be completed by SPYGEN by mid summer. So the *results for all ponds will be circulated to volunteers by early September at the latest.*

Why is this protocol so damn long?

Because it's good to get it right, and interesting to know why. But here's a checklist of the essentials:

1. At the pond put on waterproof gloves and use the blue ladle to take 20 samples from different places around the pond. Don't stand in the water.
2. Before taking each water sample, mix the pond water column. Don't disturb the sediment.
3. Put all 20 samples into the Whirl-pak plastic bag.
4. Then close the bag securely and shake vigorously for 10 secs.
5. Put on a new pair of gloves.
6. Use the pipette to put 15 mL of water from the Whirl-Pak bag into each of the six conical tubes with preserving fluid (fill tube to the 50 mL mark). Mix the bag water before taking each pipette sample.
7. Tighten the six tube caps securely and shake each tube for10 secs to mix well.
8. Double label: (i) fill out the grey information box (p3 of this protocol) and return it with the sample (ii) Keep your own record. PondNet vols: fill in your Amphibian Sheet and submit the data online.
9. On returning home put the boxed samples in a plastic bag in the kitchen fridge. Don't freeze.
10. Use the SAE to return the sample in the post to Pond Conservation ASAP. Thank-you!

Contact us

If you have questions or queries please contact Pond Conservation:

Dr Jeremy Biggs, Jbiggs@pond.conservation.org.uk Tel: 01865 483608
Dr Naomi Ewald, newald@Pondconservation.org.uk Tel: 07793 950441

Pond Conservation
For life in fresh waters

Appendix 2. Volunteers who collected samples for the eDNA project and undertook the detailed methodological study in Wales

eDNA sampling

Volunteer	Programme	Country	No. of kits sent	No. of ponds sampled
1. Adrain, Lorcan	PondNet	England	1	1
2. Allen, Marie	Other	England	3	3
3. Aquilina, Robert	PondNet	England	2	2
4. Atkins, Karen	Other	Wales	3	3
5. Baker, John	Other	England	1	1
6. Baker, Paul	Other	Scotland	4	4
7. Bell, David	Other	Scotland	7	7
8. Bennet, Holly	Other	England	2	2
9. Bignell, Sarah	Other	England	2	2
10. Booth, Polly	PondNet	England	1	0
11. Boraman, Lance	Other	England	1	0
12. Brown, Ruth	Other	Wales	1	1
13. Bruce, Lizzie	Other	England	1	1
14. Chan, Mei	PondNet	England	1	1
15. Cope, Simon	Other	Wales	4	4
16. Corcoran, Stephen	Other	Scotland	1	1
17. Court, Ian	Other	England	1	1
18. Cozens, Mark	Other	England	2	2
19. Davies, Lisa	PondNet	England	1	1
20. Dempsey, Tara	PondNet	England	1	1
21. Dickins, Dennis	Other	England	1	1
22. Dodd, Melanie	Other	Wales	2	0
23. Driver, Alastair	Other	England	2	2
24. Driver, Dorothy	**Other**	**England**	**4**	**4**
25. Dunn, Francesca	**Other**	**England**	**2**	**2**
26. Ewald, Naomi	**PondNet**	**England**	**2**	**2**
27. Ferguson, Margaret	Other	Scotland	3	3
28. Forbes, Neil	Other	England	2	2
29. Ford, Claire	PondNet	England	2	0
30. Foster, Jim	**Other**	**England**	**2**	**2**
31. Gleed-Owen, Chris	PondNet	England	1	1
32. Glover, David	PondNet	England	3	3
33. Gribbin, Karen	Other	England	1	0
34. Griffiths, Bryn	Other	Wales	2	2
35. Griffiths, Richard	**Other**	**England**	**6**	**6**
36. Hammond, Martin	Other	England	9	9
37. Harbutt, Louise	Other	England	1	0

Volunteer	Programme	Country	No. of kits sent	No. of ponds sampled
38. Harmer, Andy	Other	England	4	0
39. Haystead, Zoe	Other	England	1	1
40. Hogan, Mike	Other	Wales	1	1
41. Holmes, Laura	PondNet	England	2	2
42. Hope, Colleen	PondNet	England	2	2
43. Hotchkiss, Alastair	Other	Wales	1	1
44. Hubble, Dave	Other	England	4	4
45. Iles, Emily	Other	England	1	1
46. Jarvis, Helen	PondNet	England	2	0
47. Johnson, Adam	PondNet	England	2	2
48. Jones, Kylie	Other	Wales	3	3
49. Langton, Tom	Other	England	30	30
50. Leach, Peter	Other	Scotland	8	8
51. Leese, Stephanie	Other	England	1	1
52. Lewis, Anthony	Other	Wales	2	2
53. Lewis, Bev	Other	Wales	3	3
54. Little, Nadine	Other	Scotland	1	1
55. Long, Rebecca	PondNet	England	1	1
56. Lowe, Stephen	Other	Wales	4	4
57. Lynes, Sarah	PondNet	England	2	2
58. McIlwraith, Andy	Other	England	1	1
59. McIlwraith, Clare	Other	England	9	9
60. Miles, Zoe	PondNet	England	1	1
61. Millard, Simon	Other	England	1	0
62. Monk, Chris	Other	England	1	1
63. O'Brien, David	Other	Scotland	4	4
64. Osmond, Richard	PondNet	England	1	0
65. Popeley, Pauline	PondNet	England	2	2
66. Price, Sue	Other	Wales	1	1
67. Prina, Mark	Other	England	2	2
68. Reeves, Richard	PondNet	England	2	2
69. Roberts, Rachel	Other	Wales	5	4
70. Rooke, Rosemary	Other	England	1	1
71. Rose, Julie	Other	England	3	3
72. Rothwell, Andy	PondNet	England	2	2
73. Rowe, Robert	PondNet	England	1	1
74. Russell, Vicky	Other	England	1	1
75. Sayer, Kathy	Other	England	1	1
76. Seymour, Tony	Other	Scotland	8	8
77. Shillaker, Richard	Other	England	1	1
78. Simcock, Jenny	Other	England	1	0
79. Simmonds, John	Other	England	1	1
80. Slater, Fred	Other	Wales	3	3

81.	Smith, Tom	PondNet	England	1	1
82.	Spawforth, Lyndsey	Other	England	2	2
83.	Surrey, Kate	Other	Wales	5	5
84.	Tatman/Lyndsay, Overstall Sue	PondNet	England	1	1
85.	Templeton, Larry	Other	Scotland	3	3
86.	Towner, Carolyn	Other	England	1	1
87.	Underhill-Day, Nick	Other	England	1	1
88.	Wadge, Bev	Other	England	3	3
89.	Wallbanks, Guy	PondNet	England	2	2
90.	Walsh, Marcus	PondNet	England	4	4
91.	Webb, Natalie	PondNet	England	1	1
92.	Webster, Lizzy	Other	Wales	2	2
93.	Welch, Andy	Other	England	5	5
94.	**Williams, Penny**	**Other**	**England**	**6**	**6**
95.	**Williamson, Becca**	**Other**	**England**	**6**	**6**
Total				**256**	**238**

Volunteer surveyors who undertook the detailed methodological study in Wales

96.	Balasuriya	Rachel	Conwy County Borough Council
97.	Bagnall	Lee	Volunteer (Atmos)
98.	Batterham	Richard	Volunteer
99.	Brown	Alan	NRW
100.	Body	Stuart	FCC
101.	Butler	Anne	Conwy County Borough Council
102.	Butler	Jenny	volunteer (Bangor University Student)
103.	Cartwright	Mandy	FCC
104.	Conyers	Sally	NWWT Volunteer
105.	Davies	Amanda	FCC
106.	Davies	Megan	FCC
107.	Day	Paul	NRW
108.	Ellis	Matt	NRW
109.	Evans	Rhys	NRW
110.	Evans	Alun	CW&C
111.	Green	Amy	FCC
112.	Hancock	Emma	FCC
113.	Hatton	Alex	Volunteer
114.	Helm	Chloe	Chester Zoo volunteer
115.	Hughes	Laura	CW&C
116.	Hughes	Rhian	North Wales Wildlife Trust
117.	Jones	Aled	NRW
118.	Jones	Glyn D	FCC
119.	Johnson	Sophie	NWWT Volunteer
120.	Kenny	Mr	Volunteer (Andrew's Dad)
121.	Kenny	Andrew	Volunteer
122.	Lee	Betty	Volunteer
123.	Norman	Kim	Volunteer (BHP)
124.	Owsianka	Barbara	Conwy County Borough Council
125.	Purchase	John	FCC
126.	Purchase	Tom	Volunteer
127.	Rees-Jones	Chris	FCC
128.	Reynolds	Nick	Volunteer
129.	Rose	Julie	Volunteer
130.	Sheldrake	Sara	Volunteer
131.	Shepherd	Ruth	Chester Zoo volunteer
132.	Slingsby	Elizabeth	Volunteer (Atmos)
133.	Surry	Kate	NRW
134.	Thomas	Nick	NRW
135.	Watkins	Nia	NRW
136.	Watson	Rachael	FCC
137.	Weale	Vicky	FCC
138.	Webster	Elizabeth	DCC
139.	Webster	Lindsay	Volunteer
140.	Williams	Phil	Ecological Land Management
141.	Wilson	Hannah	Chester Zoo volunteer
142.	Woods	Richie	Ecological Land Management