SureScreen Scientifics

Technical Bulletin
Issue 5

e-DNA FOR GREAT CRESTED NEWT 'EVIDENCE OF PRESENCE'

The Great Crested Newt, *Triturus cristatus*, or GCN, is a newt in the family Salamandridae whose numbers are declining in most of Europe due to loss of habitat, fragmentation, deteriorating pond systems or poor water quality.

As with other protected species, the GCN is a valuable marker of the local ecological balance. It is a protected species under Sch.5 of the Wildlife and Countryside Act 1981, with equivalent legislation in Northern Ireland. It is also a European Protected Species and it has additional protection in the UK under Regulation 39 of the Conservation (Natural Habitats etc.) Regulations 1994. As a result, planning laws require an evaluation of any site where GCN presence is possible.

In 2014 the use of eDNA to record species occupancy in ponds was approved in some EU countries for a range of species that included GCN.

eDNA is DNA that is collected from the environment in which an organism lives, rather than directly from the organism itself. 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. Furthermore, using this technique it is possible to detect multiple species from the same water sample.

Thomsen et al (1) showed that GCN eDNA in water degrades in the pond in about 20 days, so a positive result shows the species has been present recently.

eDNA BENEFITS

- Evidence of presence valid for previous 20 days
- Sample any time of day
- More accurate than surveys
- Less Intrusive and safer
- Perfect for 'murky water'
- Accurate for 'evidence of presence'
- Negatives give good guidance
- One sample, multiple species
- qPCR technique may determine population densities
- Future data possibilities.

The eDNA technique has a number of potential advantages, summarised in the Table. The GCN is an example of a fairly cryptic pond species, with a relatively low detection rate using traditional methods of sampling. That's why several negative repeat samples of the same site are needed to provide a reasonably high confidence that the species is truly absent from the site. With eDNA a negative result will be a much stronger indication of true absence, and any individual GCN that is in the pond has a higher likelihood of being detected, even in conditions that are not conducive to traditional sampling (e.g. murky waters).

WHEN CAN WE TEST?

Theoretically, tests can be done at any time, and a positive detection of eDNA is a sure sign of GCN occupancy. However, because GCN activity is mostly land-based and ponds are used for displaying and breeding, negative results will only mean the pond is not a GCN habitat within that breeding period.

Currently, for presence/absence surveys, eDNA can only be sampled between 15 April and 30 June. Although samples taken outside this period can show presence (say, if larval newts are in a pond) such samples cannot be used to prove absence. Currently, these dates are determined in advance and do not take account of weather and regional variations. It seems likely then, that as we develop the methodology further, regional variations in seasons could be addressed. In fact ecologists are well aware of activity stirring after Winter and might be able to make their own decisions within a wider window using the more exact methodology of eDNA.

Although this is an exciting new technological advance for environmental consultancy, at the moment eDNA will not provide an accurate and reliable idea of population size (i.e. numbers of newts present) so, where licencing requires it conventional survey techniques are likely to be more appropriate. However our analytical methods with quantitative PCR certainly opens up this possibility in future. This is something we're going to be researching in conjunction with Dr Michael Sweet, from the College of Life and Natural Sciences, at the University of Derby.

Although a licence is not required to take water samples, care is needed, and Natural England has advised that eDNA water samples would need to be collected by a licenced newt surveyor if survey results are to be provided in support of an European Protected Species (EPS) licence application (presumably to demonstrate proof of absence, where relevant).

You're breaking the law if you:

- capture, kill, disturb or injure great crested newts (deliberately or by carelessness).
- damage or destroy a breeding or resting place (even accidentally).
- obstruct access to their resting or sheltering places (on purpose or by not taking enough care).
- possess, sell, control or transport live or dead newts, or parts of them.
- take great crested newt eggs.
- If you're found guilty of an offence you could be sent to prison for up to 6 months and be fined £5,000 for each offence.

HOW LONG DOES IT TAKE?

Although the sample is preserved in alcohol, eDNA deteriorates with time, so samples are best kept chilled in a cool box, placed in the fridge overnight, and couriered to us with an ice pack. Our sample kit is guaranteed DNA-free and simplifies everything. We extract the DNA from the sample as soon as we receive it and freeze the extract until we test it. Although the testing process takes a day, many laboratories wait until they have enough samples to make the cost economic, so results can sometimes take months. That's a problem because you then have no option but to continue with surveys while you wait for the answer. We appreciate time is critical so we analyse samples within a week, and even offer a premium service that gives you result within three days of receipt of sample. This service is unique and we can only offer this fast turnround because we routinely carry out other forms of DNA analysis in our laboratory.

HOW ACCURATE IS IT?

The process of detecting eDNA provides positive evidence of presence, so this is a great tool in your portfolio to prove GCN are in that pond without the uncertainty of surveys. Protocols were developed with a great deal of research, (2) which showed an accuracy of 99.3% compared to surveys which were only 75% accurate.

eDNA appears to be a highly effective method to determine whether Great Crested Newts are present or absent during the breeding season.

At the present time, a negative result can't be used as conclusive evidence that GCN are NOT present. The reasons for this centre around the uncertainty of complete sampling, especially where access round the whole pond is limited. In those cases, routine surveys or more eDNA samples taken periodically are necessary. Even so, this is a valuable tool in the ecologist's portfolio that is bound to become more accepted and more prevalent with time. Our research is helping that process.

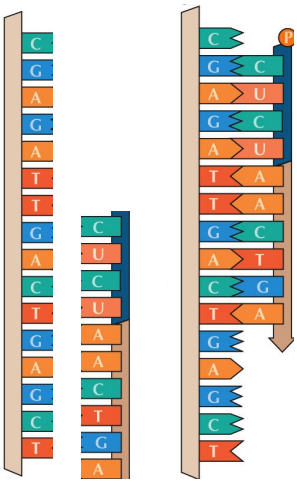
eDNA - HOW IS IT DONE?

All species have unique DNA, constructed from amino acid 'rungs' on a complex carbon ladder system. That sequence of billions of amino acids is what makes every species unique. Subtle variations or polymorphisms in that sequence are what make you and I different. We all leave traces of our DNA around us, just like GCN presence in a pond leaves DNA traces in the water.

Once the unique sequence is known for a species, we can manufacture de-novo sections of that DNA sequence known as a *primer* that will bond to the RNA and initiate a replication sequence. We use a Polymerisation Chain Reaction (PCR) to separate the strands of DNA into its two ladder strings of RNA, allowing primer bonding, then we amplify the DNA in the chain reaction and

analyse it. If GCN DNA is present, the primer reacts with it and produces readable levels of newt DNA. If none is present, the primer has nothing to bond with so no amplification occurs. The rate of amplification that is detected depends on the amount that was present, so theoretically an estimate could be made of the population density, but this depends on accurate sampling of the pond. Early work used traditional PCR which was read at the 'end point' of the process for presence and absence. We only use the protocol approved by Natural England which requires real-time quantitative PCR in which the sample is analysed continuously by the instrument, known as qPCR.

This drawing shows how a primer works. On the left is a tiny portion of a DNA sequence unique to GCN. In the centre is a 'de-novo' or 'assembled' primer made specifically to pair with that unique portion of DNA. The right hand image shows that primer paired with the unique newt DNA, which once amplified and read, provides absolute evidence of that unique eDNA in the sample. Only the GCN has that unique code so there can be no false positives provided sampling avoided cross contamination.



We're used to suiting up for a crime scene, to avoid spreading DNA around. In the case of eDNA, the technology is well proven, and it is the care of the ecologist that provides the best accuracy. This is achieved by avoiding passing eDNA from one pond to another, and ground eDNA into water, or stirring up old DNA in mud or sediment. We've simplified Natural England's advice in the Table opposite. Follow these guidelines in conjunction with our forensic skills and advice, you'll serve your clients better, get results quicker, and provide a state of the art service that will be hard to match in accuracy, speed of turn round and value for money.

Risks	Prevention
False Positives	
Cross contamination	Take care to avoid contact from other ponds via boots, tools etc. Use only Scientifics sampling kits which are eDNA-free. If you use a pole, do not allow it to get wet and contaminate other sites.
Inflows of eDNA from other ponds etc. Unlikely but theoretically possible.	Most GCN ponds have no inflows. Record the presence/absence of inflows in the survey.
Aquatic animals e.g. herons, water voles; transferring eDNA between sites.	Risk is small. Recording habitat details will help us refine the eDNA process and offer more advice. We'll freeze samples for future statistical research too so you'll be helping to improve the protocol.
False Negatives	
Low numbers of newts may result in low eDNA counts.	Follow good field protocol, refrigerate samples overnight and use our courier service for prompt delivery to avoid deterioration. We use qPCR to detect low levels of eDNA, optimizing accuracy in low levels.
Samples from deep ponds may not have eDNA in them even if the pond is occupied.	Gently stir the water as eDNA is believed to sink. Avoid water less than 5-10 cm deep. Tape the ladle to a long pole for deep water. Do not stir sediment which may contain historical eDNA.
eDNA is less likely in densely packed mats of vegetation.	Sample water in areas where vegetation is suitable for egg-laying and open water areas suitable for displaying.
eDNA is less likely to be detected if the whole pond perimeter is not sampled.	Try to access 20 sampling positions, as where 80-90% of pond margins were accessed, testing achieved 99.3% detection rates.

WHO ARE WE?

You may not have heard of SureScreen Scientifics before, because we're usually the backroom boffins behind some of the world's leading brands. In fact many of our clients have their own well equipped laboratories but still utilise our special skills.

Our diagnostics arm manufactures many of today's modern medical tests for issues such as pregnancy, cancer and infectious diseases. Our Life Sciences Lab analyses neurochemistry associated with dementia, schizophrenia and depression, and our forensics lab undertakes examinations of crime scenes & engineering disasters.

Our DNA suite analyses biological materials. We currently have two DNA research projects. The first involves a UK team dedicated to developing a rapid genetic sequencer for tumour tissue that should provide rapid results on the tumour type and its response to chemotherapy in a fraction of the time taken for our standard sequencer and at a fraction of the cost. Our second project, RESPOC undertakes genetic analysis of respiratory diseases such as pertussis (whooping cough) to detect the problem in infants earlier than ever before, because early diagnosis is the key to keeping potentially deadly respiratory diseases at bay especially in the young.

ANY QUESTIONS?

Why are sampling points spread around the pond?
eDNA level depends where GCN have been. Sampling many areas increases the likelihood of collecting their DNA successfully.

Why is the water column mixed before sampling?
DNA tends to sink so there will be more nearer the bottom. However, do not collect sediment as old DNA might be present, causing a greater risk of a false positive.

Why is such a large volume of water collected?
Natural England recommends a larger volume of water than previous methods (1) as it improves accuracy. Mix this quantity and fill the six small tubes in the kit from it to send to us for analysis.

What if duckweed, algae or zooplankton are in my sample?
Small amounts don't matter. We only detect GCN eDNA.

What if I spill the preservative - or the sample tube itself
If you spill some preservative from one of the tubes, just add proportionally less water from your pond sample. We combine all the samples, so it's not disastrous if some sample is lost. The preservative is alcohol, which isn't hazardous.

Will samples degrade in the post?
Keeping the samples refrigerated slows DNA degradation. Add an ice pack if you can, but the rate of decay while posting at ambient temperatures will not degrade the sample completely.

What evidence is there to support the use of this technique?
Defra project WC1067 revealed GCN eDNA 99.3% of the time in ponds where they were known to occur. When used by volunteer surveyors, eDNA detected Great Crested Newts at 91% of ponds where they were known to be present. No false positives were recorded from sites either outside or within the known range of the newt. It is the method, not the laboratory, which is approved, but we are working with the University of Derby to develop a quality control auditing scheme for participating laboratories to keep standards high and develop the technology further.

REFERENCES

1. Monitoring endangered freshwater biodiversity using environmental DNA. Thomsen PF[1], Kielgast J, Iversen LL, Wiuf C, Rasmussen M, Gilbert MT, Orlando L, Willerslev E. Mol Ecol. 2012 Jun;21(11):2565-73. http://www.ncbi.nlm.nih.gov/pubmed/22151771

2. http://randd.defra.gov.uk/Default.aspx?Menu=Menu&Module=More&Location=None&Completed=0&ProjectID=18650 (refer to downloads)

We're well known for delivering advice that is simple to understand and easy to apply. Why not check out our other bulletins, or browse our extensive library of failures on www.surescreenscientifics.com.

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