Natural England Commissioned Report NECR284

Developing a robust technique to detect populations of endangered native white-clawed crayfish *Austropotamobius pallipes*, invasive signal crayfish *Pacifastacus leniusculus* and crayfish plague *Aphanomyces astaci* in lotic systems using innovative eDNA approaches

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Foreword

Natural England commission a range of reports from external contractors to provide evidence and advice to assist us in delivering our duties. The views in this report are those of the authors and do not necessarily represent those of Natural England.

Background

DNA based applications have the potential to significantly change how we monitor and assess biodiversity. These techniques may provide cheaper alternatives to existing species monitoring or an ability to detect species that we cannot currently detect reliably.

Natural England has been supporting the development of DNA techniques for a number of years and has funded exploratory projects looking at different taxonomic groups in a range of different ecosystems and habitats.

This report presents the development of a technique using eDNA from water samples to detect populations of crayfish and crayfish plague in the River Dove. It compares the findings with records from traditional monitoring.

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Further information
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Executive summary

eDNA sampling was carried out on known populations of white-clawed crayfish *Austropotamobius pallipes* and invasive signal crayfish *Pacifastacus leniusculus* using different sampling techniques. A strategy was devised and used to locate populations of white-clawed and signal crayfish in the Upper Dove. The technique was also extended to other areas including donor and ark sites.

Evidence of the presence of target crayfish species by the current assay was found to be consistent with information obtained by non-eDNA methods.

Crayfish plague was also screened for at the same time as the crayfish species. Crayfish plague detection needs further consideration.

The project helped to develop a robust eDNA technique to sample crayfish in the lotic environment and was able to provide evidence to locate remnant native crayfish populations in the Upper Dove.

Table of Contents

Aims

- To develop and test a robust eDNA technique to sample crayfish in the lotic environment.
- To locate remnant native crayfish populations in the Upper River Dove, Staffordshire / Derbyshire border.

Objectives

- Carry out eDNA tests on known populations using different sampling techniques.
- Devise a sampling strategy to meet the aims.
- Continue sampling upstream to locate remnant populations.
- Screen samples for noted species.
- Extend the technique to other local areas.
- Extend the technique for community eDNA monitoring in the lotic environment.

Project Description

(See also Appendix 5)

This is a partnership between Natural England and the Peak District National Park Authority (PDNPA) working through the South West Peak Landscape Partnership (SWPLP). The purpose of the project is to build on a well-recognised approach of using eDNA techniques to survey for great crested newts in a lentic environment and develop this for the lotic environment. Should this methodology prove reliable then this approach could have far-reaching conservation purposes for, in particular, the globally endangered white-clawed crayfish *Austropotamobious pallipes*.

The South West Peak Landscape Partnership (see **Appendix 3**) is comprised of 14 organisations, led by the Peak District National Park Authority. The key members of the partnership involved in this particular study are Staffordshire Wildlife Trust, Natural England, Peak District National Park Authority, and the Environment Agency. The Partnership was offered an earmarked grant from the Heritage Lottery Fund in 2014. An initial development phase during 2015-2016 secured lottery funding towards the partnership's programme of work to be delivered from 2017 to 2021. The partnership operates over an area of 354 square km in the South West Peak. The outcomes are being addressed via a suite of 18 projects focused on natural heritage, cultural heritage, farming heritage and community.

The Crayfish in Crisis project (see **Appendix 4**) is committed to the conservation of the white-clawed crayfish in different catchments of the South West Peak. This includes surveying and monitoring to assess the extent of resident populations, and any non-indigenous crayfish populations. The risks to the viability of populations can be assessed to create the right conditions for translocations and assisted migrations of native crayfish from areas which are threatened with extinction and from other donor sites.

The white-clawed crayfish (WCC), *Austropotamobius pallipes* is the UK's only indigenous freshwater crayfish. It is globally endangered and is a species 'of principal importance for the purpose of conserving biodiversity' covered under section 41 (England) and section 42 (Wales) of the NERC Act (2006). It is one of the UK's largest freshwater invertebrates and an important component of our aquatic ecology. They are becoming increasingly rare through the impact of non-indigenous competitors introducing disease and through habitat degradation and loss. WCC is also listed under Annex II of the Habitats Directive.

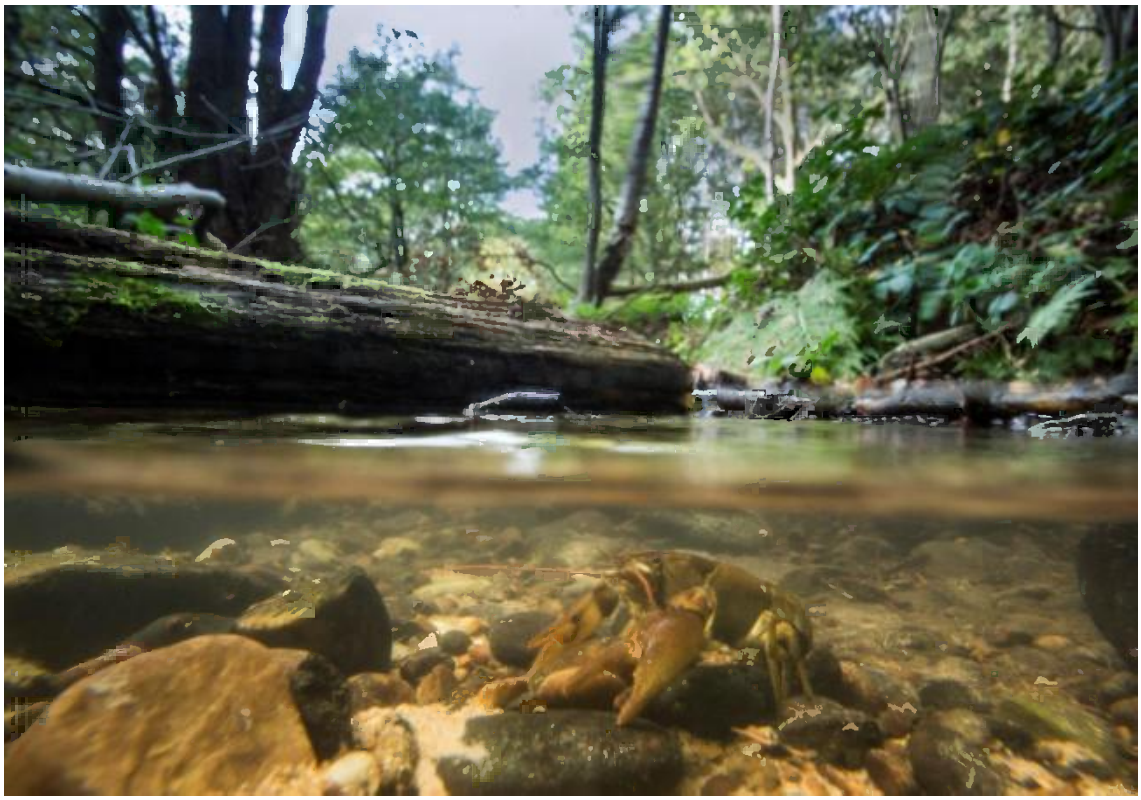

Plate 1 – White-clawed crayfish. Photo - Paul Hobson

Native white-clawed crayfish were thought to have died out in the River Dove over ten years ago as a result of crayfish plague *Aphanomyces astaci*. However, since then several individuals have been found prompting a search for remnant populations (see **Appendix 4**).

Shortly after the discovery of an individual WCC in 2015 during a biodiversity day facilitated by the author, a hand search of the immediate area was carried out but no other individuals were found. Natural England commissioned a further search and only one WCC was discovered (Mott, 2015). A population of invasive American signal crayfish *Pacifastacus leniusculus* was also discovered in the River Dove at the top of Beresford Dale and the river keeper started to set traps under licence to catch the establishing population. During trapping, a native WCC was found and identified at the site implying two mixed or overlapping populations.

A monitoring programme by the PDNPA and SWT was started upstream to detect the location of the population, but at the time failed to find any individuals.

The national distribution of the species is shrinking and is becoming reliant on small, isolated headwaters and 'still' waters where the risk of transmission of disease and the spread of the non-native North American signal crayfish is reduced through natural barriers and natural isolation.

The South West Peak is an area that has ideal conditions for the conservation of WCC having numerous small, isolated headwater streams and ponds. These habitats have excellent conditions for WCC with plenty of features such as calcareous rocks, roots, pools, overhanging banks and good water quality and quantity.

Building on the approach of using eDNA to survey for great crested newts in the lentic environment, there has been increasing interest in using eDNA techniques to monitor freshwater invertebrate communities in the lotic environment. This could be potentially used to assess water quality and biodiversity. After some preliminary research SureScreen laboratories were discovered to be working on an assay for white-clawed crayfish and so were approached to discuss collaborating on initial commercial trials before the launch of the assay.

Initial trials

The background work and pre-trials were carried out prior to securing Natural England funding for the project.

Following a number of discussions and meetings with Troy White from SureScreen and Chris Troth from the University of Derby, it was agreed that a series of commercial trials would be carried out using two proposed sampling methods. The methods are described in the main text and are referred to here as ethanol precipitation (EP) and pump based filtration (F).

Plate 2 - Ethanol Precipitation method carried out by Andrew Farmer. Photo - Nick Mott

The peristaltic pump required for the filtration method was available on loan from the University of Derby from December until March 2018 and so provided the opportunity for some winter sampling. SureScreen provided the sampling kits and filters, and the analysis was carried out by SureScreen in conjunction with Chris Troth as part of his PhD research. The weather in the month of December and early January created continuously turbid conditions in the local watercourses. By late January, the local rivers started to run clear, which is optimum for eDNA collection, and field work commenced. The sites were chosen to give a range of expected crayfish species and sites of interest. Outside of the Dove catchment the sampling at Cannock Chase was assisted by Nick Mott of Staffordshire Wildlife Trust where protocols were established to reduce cross-contamination and to increase biosecurity. Further samples were collected by the author on the dates recorded and an additional control sample was collected by a colleague when conditions permitted at a WCC site in Herefordshire. The first nine sites were sampled using F and EP with the further three sites using EP only.

Pre-trial collection methods

Ethanol precipitation (EP)

eDNA samples were collected using the sample collection protocol based on GCN eDNA testing (Biggs and others, 2014). Using a 50ml ladle and a collection bag, 20 sub-samples were taken at regular intervals along a 5-10m section of river, with the length depending on access and site constraints. Care was taken to ensure that each sample was taken in a consistent manner with minimal disruption of sediment to avoid the disturbance of historical DNA. Subsamples were taken in an upstream direction to avoid the collection of any disturbed sediment. The collected water was homogenized, with 15ml distributed into six ethanol filled tubes (filled with 35ml ethanol/sodium acetate buffer solution). Samples were then refrigerated until arrival in the lab at which point they were stored at -20^{o}C until extraction. See also **Appendix 8**.

Pump Based Filtration (F)

Filtration of the water was achieved by passing a 2L sample from the river through a portable battery powered peristaltic pump (Masterflex E/S Portable Sampler), containing a Millipore Glass fibre filter AP25, 47mm in diameter. The filter was then removed from the pump system and stored in a tube at -20^{o}C before extraction. See also **Appendix 8.**

Table 1. Results of initial pre-trial showing the number of positive samples per site by extraction technique for two crayfish species and crayfish plague (see also plate 7 and Appendix 1 for map and Appendix 2 for grid references)

For ease of interpretation 0/6 or 0/12 results have been left blank.
No sample taken by F is indicated by 'x'.
Unedited notes on these results can be found in Appendix 6

Crayfish eDNA commercial pre-trials					Results					
Sample					Native		Signal		Plague	
ID	Date	Site	River	Expected crayfish	EP	F	EP	F	EP	F
FT01	28/01/2018	Sprink	Upper Dove	Not known at time					2/6	5/6
FT02	30/01/2018	Cannock Chase	Stony Brook	Native	2/6				2/6	3/6
FT03	30/01/2018	Cannock Chase	D/s fishing pool	Signal		1/6		4/6	4/6	6/6
FT04	30/01/2018	Cannock Chase	Bentley Brook	Native 2015 & Signal u/s					6/6	6/6
FT05	12/02/2018	Warslow hall	Manifold trib.	Native Ark site					6/6	6/6
FT06	12/02/2018	Steps	Manifold trib.	Native Ark site	1/6				5/6	6/6
FT07	12/02/2018	Hollinsclough	Upper Dove	Not known at time	3/6	1/6		2/6	1/6	6/6
FT08	12/02/2018	Beresford Dale	Dove	Native & Signal	1/6	1/6			3/6	6/6
FT09	12/02/2018	Jervis	River Hamps	Signal		2/6	2/6	4/6	6/6	6/6
FT10	March 2018	Horsford	Herefordshire site	Native	12/12	x		x	2/12	x
FT11	19/02/2018	Under Whitle u/s	Upper Dove	Native seen 2015		x		x	2/6	x
FT12	19/02/2018	Under Whitle d/s	Upper Dove	Native seen 2015		x		x		x

Conclusions from pre-trial

The pre-trial results raised more questions than answers and led to a process of refinement leading to the current assay: the details of which are pending publication by Troth and others, in summer 2019. Notes prepared shortly after the pre-trials are recorded in **Appendix 6**.

Looking initially at the crayfish species there are many cases where the species are only detected by one or neither of the sampling methods. Where there are negative (0/6 or 0/12) results then it may be considered as showing no evidence of presence. Some of these cases may be 'false' negatives in which case further sampling would be expected to detect the population. No evidence of presence also indicates a potential absence of a species at the site (or upstream), but is not a confirmation of that status. In many of the cases, known populations were detected by either EP or F and only by both on 2 out of 18 results. This implies numerous 'false' negatives especially when compared to known populations. More concerning are the false positives where species are shown to have evidence of presence, but are considered not to be present by non DNA methods (and have subsequently not been detected by the current assay).

The almost universal detection of *Aphanomyces astaci* by this method is unexpected and some aspects of it will be considered in the main discussion.

The pre-trial took place outside of the currently recommended sampling season and mostly used two concurrent sampling methods at each site. The samples were analysed in the laboratory alongside other sample runs. They mostly used 6 technical replicates except FT09, a later sample, which used 12 technical replicates. Every instructed protocol to reduce cross-contamination in the field was followed and exceeded, with any false positives unlikely to have arisen due to prior sampling on the day. The collection method and laboratory protocols have been changed since the pre-trials and so these results are not included in the main discussion. There is a consideration of the evidence of presence of *Aphanomyces astaci* at sites not thought to have a plague presence. The seasonal timing of the sampling may have had an effect on the results, but the nature of this effect is not currently known.

The main project methods are self-contained and include a brief mention of the pre-trials.

Methods

Sampling strategy

Prior to the main eDNA project there was a lengthy discussion to determine if the eDNA approach could be feasibly applied to freshwater invertebrate monitoring. As a result of these investigations an agreement was made to conduct commercial field trials of a crayfish assay at sites of interest to our 'Crayfish in Crisis' project (Appendix 4) which is part of the South West Peak Landscape Partnership (Appendix 3). Once these had taken place an application was made to fund further research with a more robust method. This consisted of 'blind trials' for the laboratory and then two phases of sampling to target particular areas of interest.

Pre-tests

A series of commercial trials were carried out at 12 sites chosen for known populations of the two crayfish species and some sites of unknown presence. All 12 used an ethanol precipitation method as used for great crested newt sampling and nine of the samples used a peristaltic pump and filtration method as developed by the University of Derby. These were conducted in January and February 2018. The results were used to refine the technique and are considered above and in Appendix 6.

Blind trials

After several months of continued development, the laboratory offered five blind samples of the new improved technique (single unit closed system Sterivex™ filters). This followed a lengthy process of research in which three methods were tested against each other (ethanol precipitation, pump filtration and Sterivex filtration) in a number of environments: mesocosm,

river systems and pond systems. The results of this study are currently pending publication by Troth et al, but essentially they demonstrate that filtration is more suitable to obtain higher concentrations of eDNA from the environment than ethanol precipitation. There was little difference in method detection ability between pump-based filters and single use Sterivex filters. The single use filters were therefore chosen due to their simpler in-field applicability. As they are single use they limit the risk of contamination, the spread of crayfish plague and can be carried to and from site more easily than would be possible with a large peristaltic pump. The system involved using sterile single-use syringes and sealed commercially produced filters to collect samples.

Single Use Sterivex™ Filter Based Filtration

20 Sub-samples were collected in order to obtain a representative sample. Water samples were then pressure filtered through a sterile 0.45µM Sterivex™ HV using a 50mL syringe. Samples were passed though the filter until 500mL was filtered or the membrane became clogged and no more liquid could pass through. The total volume filtered was recorded for each sample. At all stages of sample collection, sterile disposable gloves were used and replaced between each sample. All filters were stored in 50mL tubes at -20°C prior to DNA extraction. (See **Appendix 7** for detailed collection methods and FAQs).

At each stage careful consideration was given to reduce or eliminate the possibility of cross contamination in the field. Sampling kits were stored in the vehicle segregated from used kits and at least 'double-bagged' and boxed. Sterile kit was not allowed to touch any object including clothing or skin and only opened immediately prior to use and at least sealed and double-bagged before removal from the site. Gloved hands only came into contact with sterile equipment or river sample water and care was taken not to touch clothing or face and hair. Sealed filters were stored in a freezer before transport to the laboratory in a heavily iced cool box. The ice packs were put in fresh bags before placing in the box. Used kit was stored in a separate location and left untouched until the samples had arrived at the laboratory. (See also **Appendix 7**)

Plate 3 – Sample collection by the author: Photo - Nick Mott

DNA extraction

For ethanol precipitation, the six sub-samples were centrifuged at 14000g (30 min at 4^{o}C). The eDNA samples were then extracted following the protocol outlined in the GCN eDNA methodology (WC1067, Biggs and others, 2014). Samples were stored at -20^{o}C until analysis. For all filtration methods, all filters were extracted according to Spens and others, (2017).

Quantitative PCR (qPCR)

The detection of *A. pallipes, P. leniusculus* and *A. astaci* was conducted using three separate qPCR protocols, each one specific to the intended target species.

White-clawed crayfish

A real-time qPCR assay was set up in a 25µl reaction containing: 12.5µl TaqMan® Environmental Master Mix 2.0 (Life Technologies®), 6.5µl DH20, 1µl (10µM) of each primer (forward and reverse), 1µl (2.5µM) of probe with the addition of 3µl template. qPCRs were performed with 12 replicates of each eDNA sample on the ABI 7500 qPCR System (Applied Biosystems) under the conditions: 50^{o}C for 5 min, denaturation at 95^{o}C for 8 min, followed by 50 cycles of 95^{o}C for 30 seconds and 55^{o}C for 1 min. 6 x NTC's (no template controls) were prepared using RT-PCR grade water alongside a 10x serial dilution of *A. pallipes* DNA standard for each qPCR plate that was run.

Signal crayfish

For *P. leniusculus* detection, a qPCR assay was set up using the same reagent concentrations and conditions as used for white-clawed crayfish with the altered annealing temperature of 56^{o}C. Full protocol and primers including method development can be found in Mauvisseau and others, (2017).

Crayfish plague

Analysis for the crayfish plague was conducted using primers and conditions designed by Vrålstad and others, (2009). A qPCR assay was set up in a 25µl reaction containing: 12.5µl TaqMan® Environmental Master Mix 2.0 (Life Technologies®), 4.5µl DH20, 1µl (10µM) of each primer (forward and reverse), 1µl (2.5µM) of probe with the addition of 5µl template. qPCRs were performed with 12 replicates of each eDNA sample on the ABI 7500 qPCR System (Applied Biosystems) under the conditions: 50^{o}C for 5 min, denaturation at 95^{o}C for 8 min, followed by 50 cycles of 95^{o}C for 15 s and 58^{o}C for 1 min. 6 x NTC's were prepared using RT-PCR grade water alongside a 10x serial dilution of *A. astaci* control DNA standard for each qPCR plate that was run.

Species presence within a site was inferred by the positive amplification of target species' eDNA.

The sites were selected based on the knowledge of populations, a variety of expected outcomes and potential leads for phase one and two. They were given anonymous serial names for laboratory analysis. These trials were carried out in mid-September 2018 with Tim Brooks from the Environment Agency acting as field assistant.

AF01 Steps Farm	SK 08987 59812	Manifold Tributary
AF02 Hollinsclough	SK 06245 66851	Upper Dove
AF03 Under Whitle	SK 11017 64259	Upper Dove
AF04 Beresford Bridge	SK13090 58390	Dove
AF05 Jervis Arms	SK 05016 55236	Hamps

(See plate 7 and Appendix 1 for map of sites).

Results of blind trials

Table 2. Species recorded from observations and eDNA results at each site

Site		Species					
		Native		Signal		Plague	
		Known records/ observations	eDNA	Known records/ observations	eDNA	Known records/ observations	eDNA
AF01	Steps Farm	Present on day of sampling	11/12	Not found by non DNA methods	0/12	Not expected	8/12
AF02	Hollinsclough	Not found by non DNA methods	0/12	Not found by non DNA methods	0/12	Not known	0/12
AF03	Under Whitle	Two individuals found in 2015	0/12	Not found by non DNA methods	0/12	Not known	0/12
AF04	Beresford Bridge	Known presence: small population	2/12	Known presence: large population	12/12	Not known	0/12
AF05	Jervis Arms	Not found by non DNA methods	0/12	Established presence	12/12	Expected	12/12

Overall the evidence of crayfish presence was entirely consistent with current knowledge by non-DNA methods. The presence of crayfish plague in an apparently healthy population of WCC was not expected.

The **Steps Farm** site was an Ark site established the previous year by the Crayfish in Crisis project, using a population from a donor site on Cannock Chase, and had a confirmed presence of white-clawed crayfish (WCC) on the day. Routine checks by the project officer also confirmed the presence of a healthy population of WCC. Results of eDNA analysis showed 11/12 technical replicates with evidence of presence of WCC - confirming the known population. Laboratory analysis also showed 8/12 presence for crayfish plague, raising issues covered in the discussion. This site is on a tributary of the River Manifold.

The following three sites lie on the River Dove and are ordered from upstream to downstream.

The **Hollinsclough** site was negative for all three targets, which confirms observations on the ground using torchlight surveys and refuge traps upstream. This site is below the confluence of several headwater tributaries and could potentially steer more targeted sampling if WCC were detected.

The **Under Whitle** site was also negative for all three targets. A single WCC had been found at this site in July 2014 by the author who was running a ‘biodiversity day’ session, with a further different individual found after extensive searching of the area by a team in September of the same year.

Beresford Bridge was positive for signal crayfish 12/12 and WCC 2/12. The site is known for the occurrence of signal crayfish from torchlight surveys and trapping by the river keeper. Unexpectedly, a native white-clawed crayfish was found in a trap in October 2016 with the identification confirmed by the author in-vivo and by others from photographs. Other individuals were found before trapping was suspended.

Plate 4 – WCC found by river keeper in October 2016. Photo - Andrew Farmer

The next site is on the River Hamps which has an established population of invasive American signal crayfish.

The **Jervis Arms** site is well known for having a strong population of signal crayfish and was picked up by eDNA at 12/12. Crayfish plague was detected in 12/12 of the technical replicates. This provided a control for the detection of signal crayfish.

October 2018 sampling

Following the results of the blind trials, it was decided to carry out a series of samples in the catchment. The optimum number in a batch of samples is seven and so it was decided to carry out two sets of samples with the second batch informed by the results of the first in an iterative approach. This enabled laboratory costs to be reduced and the work to be carried out within the recommended sampling season and project budget.

Phase one

The first six sites were chosen to give an even spread of sites from the headwaters of the River Dove down to Beresford Bridge. The seventh site is downstream of a recent ark site.

Table 3. Results for phase one (See plate 7 and Appendix 1 for map)

Site		Species		
River	**Name**	**Native WCC**	**Signal Crayfish**	**Plague**
Dove - upstream	Washgate	0/12	0/12	0/12
Dove	Stannery	0/12	0/12	10/12
Dove	Beggar's bridge	0/12	0/12	3/12
Dove	Pilsbury	2/12	0/12	0/12
Dove	Sprink	7/12	1/12	0/12
Dove - downstream	Hartington bridge	3/12	1/12	0/12
Blakebrook	Lumppool	0/12	0/12	0/12

Once again, the evidence of crayfish presence was entirely consistent with existing knowledge by non-DNA methods at the time of sampling. The presence of crayfish plague at **Stannery** and **Beggar's Bridge** was not expected as there is no evidence of crayfish presence. The lack of evidence of presence of WCC at **Lumppool** was unexpected.

Plate 5 – eDNA kit. Photo - Nick Mott

The River Dove sites in order from the headwaters downstream.

Washgate is a site approximately one kilometre upstream of Hollinsclough and was negative for all three targets. This was chosen as a complementary sample for the Hollinsclough site but further upstream to investigate particular tributaries. It is approximately two kilometres downstream of the source of the River Dove and would be a good candidate for repeat samples in the future to negate the chance of false negatives. Conversely, a positive WCC result would indicate a small remnant population. There is anecdotal evidence of 'crayfish' upstream in recent years, which has not been confirmed. This site however may have been wiped out by plague infection or by diesel stripping solvent from the Axe Edge layby in the early 2000's with the possibility of some persistent toxicity.

Stannery was positive for plague 10/12 with no eDNA evidence for the presence of either species of crayfish. The site is about one kilometre downstream of Hollinsclough.

Beggar's Bridge was positive for plague 3/12. No eDNA evidence for presence of either species of crayfish.

The evidence for the presence of plague at these sites has implications in the light of recent developments in the knowledge of plague pathogenicity.

Pilsbury has recent sightings (October 2018) of two signal crayfish and a WCC by torchlight survey. The eDNA detected WCC 2/12, but there was no evidence of the presence of signal crayfish.

Sprink has eDNA evidence of both species of crayfish WCC 7/12 and signals 1/12.

Hartington Bridge has eDNA evidence of both species of crayfish WCC 3/12 and signals 1/12.

Although disturbing from a WCC conservation perspective, these results show clear trends and support knowledge of current populations. There are issues raised by the evidence of the presence of crayfish plague which are discussed later in this report. There is a potential false negative for signal crayfish at Pilsbury.

Lumppool is a site just downstream of a translocation and reintroduction of over 400 WCCs less than two weeks previously. The laboratory results were negative for all three targets.

Table 4. Results of blind trials and phase one combined

Site		Species		
River	**Name**	**Native WCC**	**Signal Crayfish**	**Plague**
Dove	Washgate			
Dove	Hollinsclough			
Dove	Stannery			10
Dove	Beggar's bridge			3
Dove	Under Whitle			
Dove	Pilsbury	2		
Dove	Sprink	7	1	
Dove	Hartington bridge	3	1	
Dove	Beresford Bridge	2	12	
Blakebrook	Lumppool			
Hamps	Jervis Arms		12	12
Manifold trib	Steps Farm	11		8

Phase two

Informed by the previous results the following sites were chosen for phase two. The River Dove sites in some way fill in the gaps between the phase one and blind trials sampling to give a spacing of about 1-2km from source to the Beresford Bridge site. This is the distance recommended by the sampling instructions. **(See Appendix 7).**

River Dove sites

Swallow Brook is one of the headwaters of the Upper Dove joining the main river just downstream of Hollinsclough. It was highlighted by several stakeholders as a tributary of interest due to water quality and habitat. None of the three targets were detected by eDNA sampling. There is no other evidence of the presence of the three targets.

Glutton Bridge had evidence of presence of crayfish plague 7/12 which fits in with both the upstream (10/12) and downstream (3/12) site results. No crayfish were detected as expected from previous results upstream and downstream.

Plate 6 – Nick Mott filtering. Photo - Andrew Farmer

Crowdecote had no evidence of presence of any of the three target species and no other recent evidence. The site downstream at Under Whitle also showed no evidence of the presence of the three target species and so shows continuity.

Ludwell is a site between Pilsbury and Sprink which both show evidence of presence of WCC. eDNA detected WCC in 6/12 of the technical replicates. This fits neatly within the other results.

Table 5. Results of Dove sites from blind trials and targeted phases. Alt refers to relative altitude with higher numbers indicating sites further upstream.

Site		Species		
Alt	**Name**	**Native WCC**	**Signal Crayfish**	**Plague**
13	Swallow Brook			
12	Washgate			
11	Hollinsclough			
10	Stannery			10
9	Glutton bridge			7
8	Beggar's bridge			3
7	Crowdecote			
6	Under Whitle			
5	Pilsbury	2		
4	Ludwell	6		
3	Sprink	7	1	
2	Hartington bridge	3	1	
1	Beresford Bridge	2	12	

The results display clear trends in crayfish population presence which is supported by non-DNA methods

Table 6. Species found from sites outside the River Dove

Sites not on the River Dove			Species		
Site name	**OS reference**	**River**	**Native WCC**	**Signal Crayfish**	**Crayfish Plague**
Lower Fleetgreen	SK 06051 61127	Blakebrook	1	0	6
Lumppool	SK 06099 61161	Blakebrook	0	0	0
Stony Brook	SK 02121 16628	Cannock Chase	12	0	0
Jervis Arms	SK 05016 55236	Hamps	0	12	12
Steps Farm	SK 08987 59812	Manifold tributary	11	0	8
Elkstones	SK06508 58001	Warslow Brook	0	0	0

Ark site repeat

Lower Fleetgreen is effectively a repeat sample of Lumppool. WCC detected this time in 1/12, but also plague in 6/12. No plague was detected ten days earlier at Lumppool.

Potential ark site

Elkstones had no evidence of any of the three target species and no other recent evidence. This is a site on a tributary of the River Manifold with potential for ark sites upstream. The brook has historically held large populations of WCC before crayfish plague wiped out the entire known population.

Donor site

Stony Brook is a donor site on Cannock Chase used for translocations to ark sites in the South West Peak. There was a strong presence indicated by eDNA with 12/12 positive results. No other targets indicated. This is consistent with the known presence of a large healthy population of WCC.

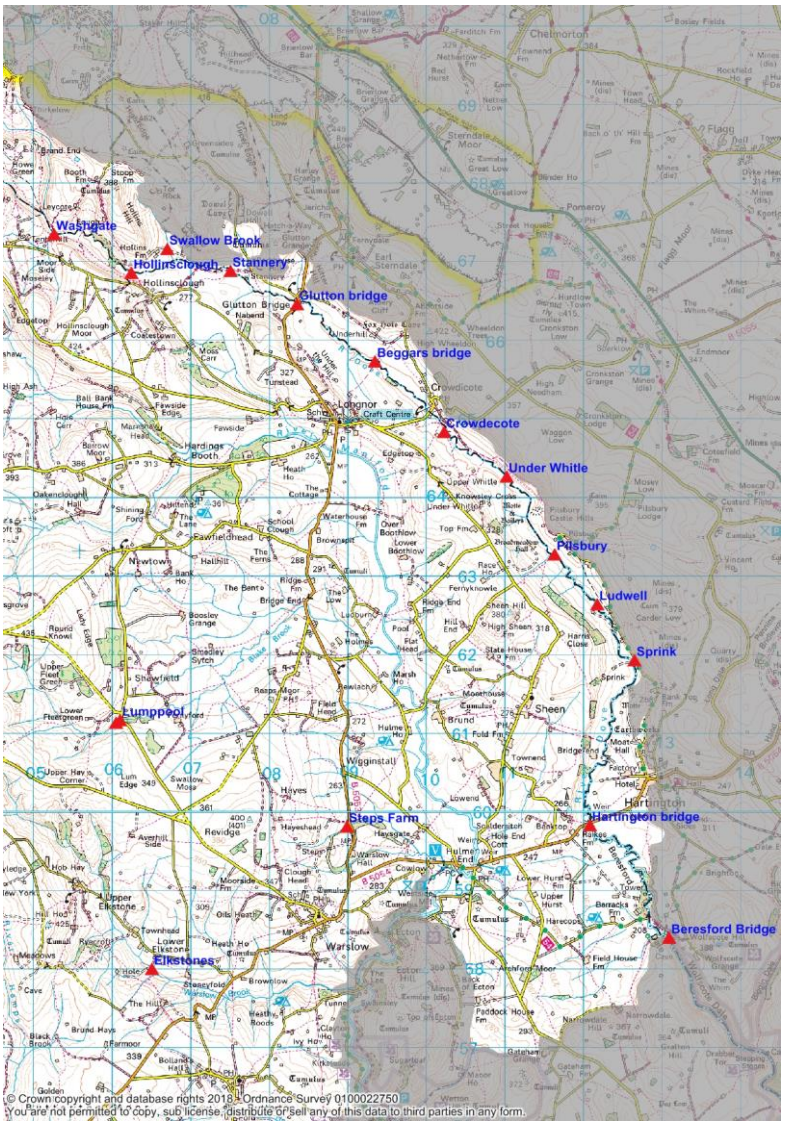

Plate 7 - Sampling sites on the River Dove and Manifold. (Larger version in Appendix 1)

Discussion

The current assay employed to sample the three targets of white-clawed crayfish *Austropotamobius pallipes*, invasive signal crayfish *Pacifastacus leniusculus* and the crayfish plague *Aphanomyces astaci* has produced some strong and positive results. There are a number of limitations identified and also areas of further research which are likely to have important consequences.

Crayfish species on the main River Dove

The distribution of crayfish on the main river shows a very definite pattern from the eDNA studies. The downstream end at Beresford Bridge has a large population of signal crayfish which appear to decrease in number as you travel upstream. The white-clawed crayfish have a concentration around Ludwell and Sprink with fewer positive replicates upstream and downstream. This is a heavily spring fed section of the river and may have an independent water source and possible refuge area. The native population is picked up by eDNA as far down as Beresford Bridge at the downstream end of the study area. There is evidence of the presence of both species at the three downstream sites from eDNA.

One of the original aims of the study was to detect remnant populations of white-clawed crayfish surviving in the upper reaches of the River Dove. The strategy was to detect eDNA on the main river or at the junctions of one or more tributaries during phase one and then to target specific tributaries in phase two. Unfortunately, upstream of Crowdecote no native crayfish have been detected recently by eDNA or other methods. However, on a positive note, no invasive signal crayfish have been detected either. The eDNA results do indicate that the remnant population is concentrated between Hartington and the Pilsbury area. Within this stretch of the river there are limited and fairly short tributaries which would be suitable sites for future sampling.

This distribution has largely been confirmed by non-DNA methods except for a false negative for signal crayfish at Pilsbury. This may be due to a small or recent population and may well be picked up by a repeat sample. There is also a possibility that spring feeds into the base flow from the river bed or banks may mask the eDNA collection.

The consequences of the information gained from these results are significant. The extra confirmation of a previously suspected situation will drive efforts to prioritise protection of the remaining individuals. The native and non-native populations are clearly overlapping and so need immediate intervention. The Crayfish in Crisis project has a working group set up from the steering group and other stakeholders, which is currently investigating a suitable future strategy as a result of these findings. There are also discussions with groups nationally to share knowledge and best practice, including the River Barle Signal Crayfish Project and the MoD.

Although there are no recent crayfish records, further sampling may reveal populations in the headwaters. The fact that there is no evidence of presence does not confirm absence. This is the case in any form of biological monitoring.

Crayfish species in other sites sampled

There were 13 sites sampled on the River Dove leaving six other sites. One was a donor site in Cannock which showed 12/12 for WCC as expected and one a known population of signal crayfish showing 12/12 for signals and 12/12 for plague. The established ark site at Steps showed 11/12 WCC as expected, but also 8/12 for plague which was unexpected considering the presence of an apparently healthy population. The potential ark site on the Warslow Brook from Elkstones showed no target species which fits in with current knowledge. The sample area at Lumppool / Lower Fleetgreen showed no target species less than two weeks after the population was translocated to the site but picked up WCC 1/12 and plague 6/12 ten days later.

Two matters arising from these results concern the detectability of newly translocated crayfish and the implications of the detection of crayfish plague.

Plate 8 – Swallow Brook: Photo - Nick Mott

Levels of detection of small populations or recently introduced populations

There were two known **'false negatives'** in the results.

One was at Lumppool / Lower Fleetgreen and may be attributed to various factors. It is recommended to take repeat samples at a site to reduce the chance of missing a population as is applicable to all sampling programmes. With a limited budget this may not be possible if a wide area needs to be covered. In this case it was possible to repeat the test at a later date which detected the population of WCC. This could suggest that for a recently introduced population insufficient DNA would be released into the environment to be reliably detected. This suggestion may not be valid as it is applied to one site only, and may just have been an example of a case where DNA was not collected in sufficient quantities to be amplified in a sample. There is scope for further work here to assess the detectability of a population following a reintroduction into an area that has had no known crayfish population previously.

The second 'false negative' was at Pilsbury where three signal crayfish individuals were identified the week before. This could be a new or small population which may be picked up by repeat sampling. There is potential here for further study when the recommended sampling season starts again.

For these reasons it would be reasonable to assume that if there is no evidence of presence of a species by one or more eDNA sample, then there would need to be further consideration of all sources of evidence before the species absence could be reasonably confirmed. As the eDNA is tested more rigorously then the validity of a negative result may be more accurately assessed.

Conversely as long as the risk of cross-contamination has been controlled and there is reasonable supporting evidence, then a positive result is likely to indicate the presence of a crayfish species, according to this study. There is a possibility though of false positives as a result of stochastic events such as crayfish parts being transported by birds or other organisms

to the sampling site giving positive evidence of presence without a living population being there. This would be true of otters moving through the system and crossing into new watercourses within their territory. Target eDNA may be present in their spraint. In terms of crayfish species this scenario did not appear to happen in this main study.

Implications of detection of crayfish plague by eDNA

There seem to be inconsistencies in plague detection by eDNA.

At Lumppool / Lower Fleetgreen there was a population of WCC translocated from a donor site with no plague detected. Less than two weeks later there was no plague (or WCC) detected at the ark site. However, evidence was found at the ark site for the presence of *Aphanomyces astaci* ten days later, when the evidence of the presence of WCC was also found by eDNA.

At Steps there was eDNA evidence for plague, but an apparently healthy population of WCC. The site had been populated by a translocation the previous year from a healthy donor site on Cannock Chase. The donor site is regularly monitored and currently shows no evidence of anything other than a healthy population of WCC. Crayfish were seen in the brook on the day of eDNA sampling and also confirmed by routine monitoring at the time.

In the River Dove there is evidence for plague upstream of known populations of crayfish, but not in the populations themselves. This is difficult to explain as it would be expected that fungal spores and other structures would be carried downstream to other areas where they would infect WCCs and be detected in the downstream samples. There is also the expectation that the plague would require a host to concentrate DNA to detectable levels. However there is a much greater probability of detecting plague DNA than crayfish eDNA due partly to the fact that it is the DNA of the fungus that is being amplified and not strictly eDNA released from the organism. (pers. comm. Terentyev/Sweet)

In order to understand these results, it is necessary to explore the issues further.

Areas for further investigation relating to crayfish plague

- Plague can be detected from spores and may have orders of magnitude more detectability than crayfish eDNA. (pers. comm. Yaroslav Terentyev)

- There are different strains of plague *Aphanomyces astaci* with different levels of pathogenicity (Martin-Torrijos and others, 2017).

- There are documented cases of plague resistance in *Austropotamobius pallipes* from Ireland (pers. comm. Luca Mirimin) and the Pyrenees (Martin-Torrijos and others, 2017) implying that there may be resistance in local native populations to certain strains of plague.

- Previously unknown species of *Aphanomyces* may be interfering with the assay in a similar way to the observations as suggested by Viljamaa-Dirks & Heinikainen (2018).

- The crayfish plague assay may not be sufficiently robust to distinguish from other *Aphanomyces* species.

Summary of conclusions

Evidence of the presence of target crayfish species by the current assay is consistent with information obtained by non-eDNA methods.

The project helped to develop a robust eDNA technique to sample crayfish in the lotic environment and was able to provide evidence to locate remnant native crayfish populations in the Upper Dove.

eDNA tests were carried out on known populations using different sampling techniques. A sampling strategy was devised to meet the aims, which was continued to locate populations of white-clawed and signal crayfish. Where possible the technique was extended to other areas including donor and ark sites. Crayfish plague was also screened for at the same time as the crayfish species. Crayfish plague detection needs further consideration.

Further innovative work proposed as a result of this project

Extend the technique for community eDNA monitoring in the lotic environment.

There has been work using eDNA to detect macroinvertebrate community species in the lotic environment (pers. comm. Rosetta Blackman & Kat Bruce, also Elvira Machler), with some success, but requires further development to apply it to water quality monitoring in the UK. Use of reference lists of species at sites in the South West Peak (e.g. from Everall and others, (2010) and more up to date studies) and other areas, in conjunction with developing eDNA techniques including metabarcoding and targeted assays, could be used to develop a water quality monitoring tool. This could be used to augment Water Framework Directive water quality monitoring and to detect rare or invasive species missed by non-DNA methods. Crayfish species and potential plague strains could be targeted at the same time. Details on the progress of this proposal are available on request from the author.

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Appendix 1. Sampling sites on the River Dove and Manifold.

Appendix 2.

Table 7. Grid references and positive replicates out of twelve for all sites

Site name	Species	Result	Positive replicates	OS reference
Washgate	White-clawed Crayfish	Negative	0	SK05259 67347
Washgate	Signal Crayfish	Negative	0	SK05259 67347
Washgate	Crayfish Plague	Negative	0	SK05259 67347
Beggar's bridge	White-clawed Crayfish	Negative	0	SK09346 65732
Beggars bridge	Signal Crayfish	Negative	0	SK09346 65732
Beggars bridge	Crayfish Plague	Positive	3	SK09346 65732
Sprink	White-clawed Crayfish	Positive	7	SK12644 61924
Sprink	Signal Crayfish	Positive	1	SK12644 61924
Sprink	Crayfish Plague	Negative	0	SK12644 61924
Hartington bridge	White-clawed Crayfish	Positive	3	SK12078 59828
Hartington bridge	Signal Crayfish	Positive	1	SK12078 59828
Hartington bridge	Crayfish Plague	Negative	0	SK12078 59828
Stannery	White-clawed Crayfish	Negative	0	SK07508 66872
Stannery	Signal Crayfish	Negative	0	SK07508 66872
Stannery	Crayfish Plague	Positive	10	SK07508 66872
Lumppool	White-clawed Crayfish	Negative	0	SK06099 61161
Lumppool	Signal Crayfish	Negative	0	SK06099 61161
Lumppool	Crayfish Plague	Negative	0	SK06099 61161
Pilsbury	White-clawed Crayfish	Positive	2	SK11634 63275
Pilsbury	Signal Crayfish	Negative	0	SK11634 63275
Pilsbury	Crayfish Plague	Negative	0	SK11634 63275
Glutton bridge	White-clawed Crayfish	Negative	0	SK08356 66455
Glutton bridge	Signal Crayfish	Negative	0	SK08356 66455
Glutton bridge	Crayfish Plague	Positive	7	SK08356 66455
Ludwell	White-clawed Crayfish	Positive	6	SK12170 62635
Ludwell	Signal Crayfish	Negative	0	SK12170 62635
Ludwell	Crayfish Plague	Negative	0	SK12170 62635
Swallow Brook	White-clawed Crayfish	Negative	0	SK06702 67156
Swallow Brook	Signal Crayfish	Negative	0	SK06702 67156
Swallow Brook	Crayfish Plague	Negative	0	SK06702 67156
Lower Fleetgreen	White-clawed Crayfish	Positive	1	SK06051 61127
Lower Fleetgreen	Signal Crayfish	Negative	0	SK06051 61127
Lower Fleetgreen	Crayfish Plague	Positive	6	SK06051 61127
Elkstones	White-clawed Crayfish	Negative	0	SK06508 58001
Elkstones	Signal Crayfish	Negative	0	SK06508 58001
Elkstones	Crayfish Plague	Negative	0	SK06508 58001
Crowdecote	White-clawed Crayfish	Negative	0	SK10223 64827
Crowdecote	Signal Crayfish	Negative	0	SK10223 64827
Crowdecote	Crayfish Plague	Negative	0	SK10223 64827
Stony brook	White-clawed Crayfish	Positive	12	SK02121 16628
Stony brook	Signal Crayfish	Negative	0	SK02121 16628
Stony brook	Crayfish Plague	Negative	0	SK02121 16628
Hollinsclough	White-clawed Crayfish	Negative	0	SK06245 66851
Hollinsclough	Signal Crayfish	Negative	0	SK06245 66851
Hollinsclough	Crayfish Plague	Negative	0	SK06245 66851
Under Whitle	White-clawed Crayfish	Negative	0	SK11017 64259
Under Whitle	Signal Crayfish	Negative	0	SK11017 64259
Under Whitle	Crayfish Plague	Negative	0	SK11017 64259
Beresford Bridge	White-clawed Crayfish	Positive	2	SK13090 58390

Site name	Species	Result	Positive replicates	OS reference
Beresford Bridge	Signal Crayfish	Positive	12	SK13090 58390
Beresford Bridge	Crayfish Plague	Negative	0	SK13090 58390
Jervis Arms	White-clawed Crayfish	Negative	0	SK05016 55236
Jervis Arms	Signal Crayfish	Positive	12	SK05016 55236
Jervis Arms	Crayfish Plague	Positive	12	SK05016 55236
Steps Farm	White-clawed Crayfish	Positive	11	SK08987 59812
Steps Farm	Signal Crayfish	Negative	0	SK08987 59812
Steps Farm	Crayfish Plague	Positive	8	SK08987 59812

Appendix 3

The South West Peak Landscape Partnership

The South West Peak Landscape Partnership (SWPLP) comprises 14 different organisations, led by the Peak District National Park Authority. The Partnership was offered an earmarked grant from the Heritage Lottery Fund in 2014. An initial development phase during 2015-2016 led to securing £2.4m of lottery funding towards the partnership's £4.1m programme of work to be delivered from 2017 to 2021. The Partnership operates over an area of 354 square km in the South West Peak.

The Partnership's Mission Statement is:
By working together in the South West Peak, we will shape a better future for our communities, landscape, wildlife and heritage where trust and understanding thrive.

The Stated Partnership Outcomes are:

Relationships

- Improved understanding and relationship between different communities (farmers, conservationists, residents, visitors, partners)

- **Land management**
- Habitats are more diverse and more resilient
- Populations of key species are supported and more resilient
- A move towards economically and environmentally sustainable land management or 'high nature value farming'
- The landscape is managed for multiple benefits

- **People**
- People have a stronger sense of place, they are engaging with the landscape, have better experiences, and have gained respect and understanding
- People have gained skills and knowledge about the landscape
- People value the landscape and understand the benefits it provides
- People who are currently disconnected from the landscape are supported to build a relationship

- **Landscape character**
- Historic and built elements of the landscape are recorded, understood, valued and restored
- The distinctive mosaic of natural heritage is maintained and enhanced

These outcomes are being addressed via a suite of 18 projects focused on natural heritage, cultural heritage, farming heritage and community.

One of the 18 projects is the Crayfish in Crisis project detailed below.

Appendix 4

Crayfish in Crisis

Project proposed by Nick Mott of Staffordshire Wildlife Trust

Summary

The white-clawed crayfish (WCC) is the UK's only indigenous freshwater crayfish. It is globally-endangered and is a Priority (S41) / Biodiversity Action Plan species. It is one of our largest freshwater invertebrates and an important component of our aquatic ecology. They are becoming increasingly rare through the impacts of non-indigenous competitors introducing disease along with habitat degradation and loss.

The distribution of the species is shrinking and is becoming reliant on small isolated headwaters and 'still' waters where the risk of transmission of disease and the spread of the non-native North American Signal crayfish is reduced through natural barriers and reduction of disease vectors.

The South West Peak is an area that has ideal conditions for the conservation of WCC having numerous small, isolated headwater streams and ponds. These habitats have excellent habitat for WCC with plenty of features such as rocks, roots, pools, overhanging banks and good water quality / quantity.

Project description (purpose)

There is a crisis facing our indigenous crayfish in the South West Peak, in Britain and in Europe. Until 2005 strong populations of white-clawed crayfish were recorded in the 'mainstem' of the Upper River Manifold from Ecton Bridge to Wettonmill, the Lower Hoo Brook, the Lower (and mid-sections) of the Kirksteads Brook, the Lower (and mid-sections) of the Warslow Brook, the Upper Dove from Sprink to Wolfscote Dale (and continuing unbroken downstream to the Manifold confluence near Ilam) and the River Hamps (and the Black Brook) near Waterhouses. Separate crayfish plague outbreaks on the Dove in 2005 and the Upper Manifold in 2008 wiped out all these populations. Follow up surveys failed to find any surviving evidence of white-claws until, encouragingly, a single adult female was confirmed on the Upper Dove near Crowdecote in September 2014. Unfortunately, signal crayfish were recorded the following summer a short distance downstream on the Dove at Wolfscote Dale. The project partners are not aware of any recent records of white-clawed crayfish in the Dane, Goyt and Dean river catchments within the South West Peak.

However, signal crayfish are known to be present on the mainstem of the Dane as far up as Danebridge (and possibly further upstream). Survey and feasibility work is required here to assess these catchments. This project will look to develop conservation action plans for the species in different catchments of the project area which will include surveying and monitoring to assess extent of resident populations, and any non-indigenous populations, assess and mitigate identified risks to the viability of populations and create the right conditions for translocations and assisted migrations of WCC from other areas which are threatened with extinction from a local hatchery (set up in the Dove catchment in 2015) established to provide stock for translocations and from suitable 'donor sites' identified in Staffordshire and Derbyshire.
A crucial element for our community engagement work will also be around awareness-raising and education of biosecurity risks with angling clubs that fish across catchments that include non-native crayfish. We will also seek to raise awareness with landowners, land managers, local residents, local community groups, tourists / visitors and with conservation partners.

Project description (location)

The project will focus primarily on the headwater streams and tributaries of the Dane, Dove and Manifold rivers. There are several streams within these headwaters which have previously supported WCC and retain the necessary characteristics to support populations. Many of these have been previously surveyed to provide initial data and understanding of their ecology and

hydrology to confidently promote their suitability as candidate sites for the translocations / assisted migrations of WCC. The main aim is to identify suitable headwater ARK sites for white-claws above natural or man-made barriers such as waterfalls, sink holes, extensive culverts or weirs.

Project description (site details)

Key candidate sites requiring detailed feasibility checks (see list below):

- Upper Dane tributaries
- Upper Kirksteads Brook sub-catchment
- Upper Oakenclough Brook sub-catchment
- Warslow Brook sub-catchment
- Upper Blake Brook sub-catchment
- Head of Manifold sub-catchment
- Upper Churnet tributaries

Project description (private/ public benefit)

Work will be undertaken predominantly on private land, used in the main for livestock grazing. There are existing pressures on some of these watercourses through lack of buffers which could be resolved through measures such as providing fencing and formalising watering points. Implementing such measures could benefit landowners through improving animal health and safety and better pasture. They would also provide additional benefits for water quality and ecology, providing universal benefits. Catchment Sensitive Farming could be a key partner in delivery of these measures and potentially provide a cost-effective delivery model.

The project will comply with the natural heritage good practice guidelines as published by HLF:

- have a core of good-quality priority habitat or support a significant population of priority species from which to extend out from; this core land must be designated as being of national, regional or local importance;
- enhance and/or expand the extent and quality of habitat that will help to meet UKBAP habitat and species targets;
- contribute to long-term sustainable management of the area; and
- demonstrate a strategic approach to the conservation of a priority habitat or species.

Project description (site management)

The focus of the project will be on watercourses through private land with restricted access, other than on public rights of way. Sites with public access will be less of a priority due to increasing the likelihood of vectors for biosecurity issues. Most of the work will be low impact and unobtrusive which should remove any potential conflict with current or future land management objectives, whether for farming or conservation. In fact measures most likely required, e.g. buffering of watercourses will provide a range of wider benefits. All project activity will follow the correct protocols which will include Natural England licensing, flood defence consent and IUCN guidance.

Similar project work has been undertaken by the project lead in other protected landscape (Cannock Chase AONB / SAC) and lessons learned will be applied. Bristol Zoological Society's Crayfish Conservation in the South West has been delivering a successful project engaging the public in the issues concerning crayfish conservation and establishing Ark sites. We intend to liaise with the project to gain from their experiences and hopefully use some of the interpretation materials to provide efficiencies.

Appendix 5

Project Description (Innovation Fund)

This is a partnership project between Natural England and Peak District National Park Authority, the purpose of which is to build on a now well-recognised approach of utilising eDNA techniques to survey for great crested newts in a lentic environment and further develop this for the lotic environment. Should this methodology prove reliable then this approach could have far-reaching conservation purposes for, in particular, the globally endangered white-clawed crayfish.

Aims:

- To develop and test a robust eDNA technique to sample crayfish in the lotic environment.
- To locate remnant native crayfish populations in the Upper Dove.

Objectives:

- Carry out eDNA tests on known populations using different sampling techniques.
- Devise a sampling strategy to meet the aims.
- Continue sampling upstream to locate remnant populations.
- Screen samples for noted species.
- Extend the technique to other local areas.
- Extend the technique for community eDNA monitoring in the lotic environment.

The Peak District National Park Authority (PDNPA), working through the South West Peak Landscape Partnership (SWPLP), will be carrying out the project. The key members of the partnership involved in this piece of work are: Staffordshire Wildlife Trust, Natural England, Peak District National Park Authority, and the Environment Agency.

All SWPLP partners have signed a partnership agreement concerning the governance arrangements for the Landscape Partnership. For this project, work will be conducted by Andrew Farmer in his capacity as South West Peak Farm Link Worker (hosted in this role by the PDNPA) and also as a freshwater ecologist, and he will be building on his links with the DNA laboratory and a PhD student at the University of Derby who are working towards validating the technique in moving water. Karen Shelley-Jones (SWPLP Manager) will provide project and line management time as an in-kind contribution. Andrew will liaise with partners on at least a quarterly basis (in-kind contribution of time from PDNPA, NE, SWT, EA) to gain expert input to the feasibility study and benefit from their regional and national contacts.

Appendix 6

Notes on Field Trial Results (Unedited)

Table 8. Pre-test notes

Crayfish eDNA commercial pre-trials					Results					
Sample					Native		Signal		Plague	
ID	Date	Site	River	Expected crayfish	EP	F	EP	F	EP	F
FT01	28/01/2018	Sprink	Upper Dove	Not known at time					2/6	5/6
FT02	30/01/2018	Cannock Chase	Stony Brook	Native	2/6				2/6	3/6
FT03	30/01/2018	Cannock Chase	D/s fishing pool	Signal		1/6		4/6	4/6	6/6
FT04	30/01/2018	Cannock Chase	Bentley Brook	Native 2015 & Signal u/s					6/6	6/6
FT05	12/02/2018	Warslow hall	Manifold trib.	Native Ark site					6/6	6/6
FT06	12/02/2018	Steps	Manifold trib.	Native Ark site	1/6				5/6	6/6
FT07	12/02/2018	Hollinsclough	Upper Dove	Not known at time	3/6	1/6		2/6	1/6	6/6
FT08	12/02/2018	Beresford Dale	Dove	Native & Signal	1/6	1/6			3/6	6/6
FT09	12/02/2018	Jervis	River Hamps	Signal		2/6	2/6	4/6	6/6	6/6
FT10	March 2018	Horsford	Herefordshire site	Native	12/12	x		x	2/12	x
FT11	19/02/2018	Under Whitle u/s	Upper Dove	Native seen 2015		x		x	2/6	x
FT12	19/02/2018	Under Whitle d/s	Upper Dove	Native seen 2015		x		x		x

Peristaltic pump filtration and Ethanol precipitation.

Dove catchment/Cannock Chase Crayfish study.
January/February 2018

Unedited notes

FT01 - Sprink
Plague c 2007. White-clawed 2014 - 2 adults. D/s 2016-2017 2 adults. Signal crayfish found downstream. Seems likely that plague is still present and that any remnant native crayfish populations are small and hence crayfish eDNA quantities are undetectable at this site at this time.

FT02
Forestry commission site. Known high density forest stream population of white-claws. As expected to find native crayfish. Detected by EP and actual searching on the day, but not by F. Plague presence unexpected as no evidence seen in population - which is regularly monitored. There may need to be some thoughts about the implications of plague detected amongst an apparently healthy population of native crayfish.

FT03
On-line population of signal crayfish in pool immediately upstream. Samples taken from overflow channel. As expected presence of Signals and Plague. Unexpected native presence, but likely as we have seen mixed populations on the Dove.

FT04
Signal population in lake upstream. Known population of natives surveyed within 3 years along watercourse. Expected plague, but unexpected absence of both species of crayfish. This could be due to winter sampling and dilution; or sensitivity of method; or both. This sample does

reinforce lack of cross contamination of crayfish DNA as the sample was taken on the same day after other positive samples.

FT05 Warslow Hall
Downstream of Ark site. No natives found. Population small and a recent introduction. May be picked up during warmer sampling periods / further replicates.

FT06 Steps Farm
Downstream of Ark site. Introduced population just detected by EP. Alarming presence of plague in both samples, but see above (FT02).

FT07 Hollinsclough
White-clawed found downstream 3 years ago. Snow on ground. As hoped to find presence of native crayfish upstream of Hollinsclough and a very encouraging result for the project. This is the most positive and inspiring result to build on. Unfortunately signal crayfish and plague were also detected (mostly by F). Unlikely to be signal crayfish contamination from previous samples during fieldwork as none were detected earlier in the day. Plague detection proving to be mysterious.

FT08 Beresford Dale
Signals being trapped, but some white-clawed found in traps. Surprising result. Would have expected a significant signal presence. Native presence expected in small quantities as seen.

FT09 Jervis Arms
Established signal crayfish population. Signal crayfish and plague presence confirmed emphatically as expected. Surprising native presence detected by F. No native crayfish have been recorded recently.

(FT10
Sampler (Charlie) encountered unfeasible river conditions at site and has held on to EP kit for a later date.)

FT11/FT12
Site of re-discovery. Crayfish thought to be extinct locally then discovered by AF in 2015 (2 found)
Known very small population of natives found several years ago, so result in keeping with this observation; i.e. none detected. They may be picked up in active season or after more sample time. Encouraging seeing low detection of plague.

Appendix 7

Detailed Sample Collection Method for Crayfish eDNA (figures removed).

1. Identify where 20 sub-samples will be taken from the pond/river.
The location of these should be spaced as evenly as possible around the site. In ponds, samples should be taken from locations around the entire pond perimeter, where accessibility permits. In rivers, samples should be taken against the flow of the stream, working upstream in a diagonal pattern where possible to ensure that any disturbed ancient DNA is not collected, should it be necessary for the collector to enter the watercourse.

2. Open the sterile Whirl-Pak bag and **collect 20 samples of around 50 mL of water** from around the site using the ladle (fill the ladle) into the Whirl-Pak. The water sample should be taken from the middle of the water column. Where possible, avoid any disruption of sediment as this can not only clog the filter quicker, but introduce ancient DNA into the sample. In larger sites it may be necessary to use a telescopic pole.

3. Once 20 samples have been taken, add the vial of spiked DNA in to the bag, close the bag securely using the top tabs and shake the Whirl-Pak for 10 seconds. This mixes DNA across the water sample.

4. Using the sterile syringe, take up 50mL of sample from the Whirl-Pak and then attach the syringe using a half twist action to the Sterivex™ Filter (The syringe will only fit to one end of the filter). Apply pressure to the syringe until all liquid has passed into and through the filter.

5. Remove the Sterivex™ Filter from the syringe and repeat until you have filtered 1L OR you are no longer able to push any liquid through. Record the amount of liquid which has been filtered on the form.

6. Empty the syringe and fill with air, attach this to the filter and push air through the syringe until it is completely free of water.

7. Screw the red-caps to secure both ends of the filter and then place the filter into the 50mL tube provided.

8. Place the sample in a freezer until ready to return to the laboratory. When ready to return, wrap the frozen ice pack around the sample and place inside the clear A4 bag and ship to the laboratory as soon as possible. Keep the sample chilled/frozen until returned to the laboratory for analysis.

From a pdf document (with figures removed) provided by

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Crayfish eDNA: FAQ's

How do I collect a sample?
For a detailed guide on sample collection see our detailed sample collection method for Crayfish on our website. This will also be included in the Crayfish kit along with a summary sample collection method. Samples can be taken from ponds, canals, streams or rivers.

Who can sample?
Anyone can sample! A license is only required if you are conducting a white-clawed crayfish survey additionally to the eDNA survey. If you are just simply taking a water sample then you do not require a licence.

When is the best time to sample?
We recommend that samples should be collected between 1st April and 31st October to coincide with when crayfish are most active (and hence releasing most eDNA). Samples can be taken outside of this window but accuracy does drop. Further to this the sample should be collected when the pond/river/stream is relatively calm, with little turbidity. Try to avoid sample collection from murky rivers/ponds or at sites just after large rainfall as the filter will soon clog and you will be unable to pass a sufficient volume of water through for analysis.

What volume of water do I need to filter?
The filters are designed to process up to 1 litre of fluid. However, in the case of rivers and streams, due to turbidity and sediment load it may not always be possible to filter such a high volume. We recommend the filtration of at least 150mL, the higher the filtered volume the increased chance of obtaining eDNA within your sample (if it is present within the sample site) If you are unable to filter such high volumes – don't worry. Just make a note of the volume which was filtered on the sample collection form.

How many samples do I need to collect?
Usually one. One kit is adequate for a pond less than 1 hectare, any canal, or any stream/river approximately less than 10m wide. One kit will usually detect crayfish up to 1km upstream so as part of your strategy, you may decide to collect samples every 500m or 1km pinpoint the location with a network.

Can you analyse for all three species from one sample?
Yes. Each sample can be analysed for all eDNA target species currently offered by SureScreen. Once the DNA has been extracted from a sample it can then be analysed multiple times: i.e. for white-clawed crayfish, signal crayfish and the crayfish plague.

Is the test specific to the species?
The assays used in the laboratory for the detection of the crayfish species and crayfish plague have by design been developed as species specific. This means that they will only detect and amplify DNA of the target species, thorough testing has been conducted to ensure that this is the case and that each of our assays for *A. pallipes, P. leniusculus, A. astaci* and our other eDNA services do not cross-amplify any other species.

What about biosecurity?
With the crayfish plague being a large problem for white-clawed crayfish in the UK at the moment it is highly important that biosecurity measures are followed when collecting crayfish eDNA samples.

We have tried to design a biosecurity friendly eDNA kit which includes single use components and therefore reduces the risk of transferring plague from site to site. However, it is also important that the end-user thoroughly cleans any additional equipment, wellies and clothes which they take to any site before moving onto a new site to reduce the risk of transferring any plague spores.

Are there any other laboratories that can analyse for the presence of White clawed crayfish?
At the time of writing (August 2018), no other eDNA laboratories offer species specific services for crayfish or crayfish plague.

What are the chances of detecting old/no longer present populations of crayfish?
We have been conducting experiments to determine the rate at which the DNA degrades from the environment once a crayfish population is no longer present. Results indicate that the DNA levels drop below detectable amounts after two weeks individuals have left the system. This means that there is minimal chance of detecting a population of crayfish which have been absent for several months.

From a pdf document provided by

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Appendix 8

Filtration Collection Method:

Method:

1. Put a clean pair of gloves on. Connect the filter holder, inlet tubing (sterile) and outlet tubing together. If possible, place the inlet tubing in the water course. If this is not possible then it may be necessary to remove water in a sterile Whirl-Pak bag (as used with the ethanol precipitation method) and to filter water directly from this bag. Avoid the collection of sediment by placing the inlet tubing at least 10cm above the bottom of the river/lake. Thus, clogging of the filtration system can be avoided.

2. Turn the pump on – follow instructions in the manual for additional information/support with pump. Before pumping water, ensure that the flow direction is switched to pump from the watercourse through the filter holder as opposed to the reverse direction in order to avoid contaminating the sample.

3. Collect the sample by taking 2L of water, if the filter becomes clogged or slows down substantially record the amount filtered and finish collecting the sample. The duration may vary, depending on the water composition and dissolved particles.

4. After the desired amount of water has been filtered and the pump has been switched off, the filter can be removed. Unscrew the filter holder and using a clean pair of gloves, remove the washer, fold the filter and insert into a sample tube. Place this sample in the freezer as soon as possible. It may be beneficial to carry an ice-pack bag to keep the filter cool before freezing.

5. After use, place used inlet tubing and filter holders in 'contaminated/used bag' and flush through the outlet tubing with 10% bleach/water solution.

Notes:
- Record date sample kit sent
- Record unique reference number for each kit
- If in doubt – change gloves
- Flush through tubing with 10% domestic bleach solution and water

Ethanol Precipitation Collection Method:

1. Identify where 20 sub-samples will be taken from the pond/river. The location of sub-samples should be spaced as evenly as possible around the site, and if possible targeted to areas where there is vegetation which may be being used as refuge and open water areas which the species may be using. In rivers, samples should be taken against the flow of the stream, working upstream in a diagonal pattern (see diagram) where possible to ensure that any disturbed ancient DNA is not collected and to avoid contamination from the collector should it be necessary for the collector to enter the watercourse.

2. Open the sterile Whirl-Pak bag by tearing off the clear plastic strip 1cm from the top (along the perforated line), then pulling the tabs.

3. Collect 20 samples of around 50 mL of water from around the pond (see 1 above) using the ladle (fill the ladle), and empty each sample into the Whirl-Pak bag.

4. NOTE: Before each ladle sample is taken, the water column should be mixed by gently using the ladle to stir the water from the surface to close to the bottom without disturbing the sediment on the bed of the pond/river. It is advisable not to sample very shallow water (less than 5-10 cm deep).

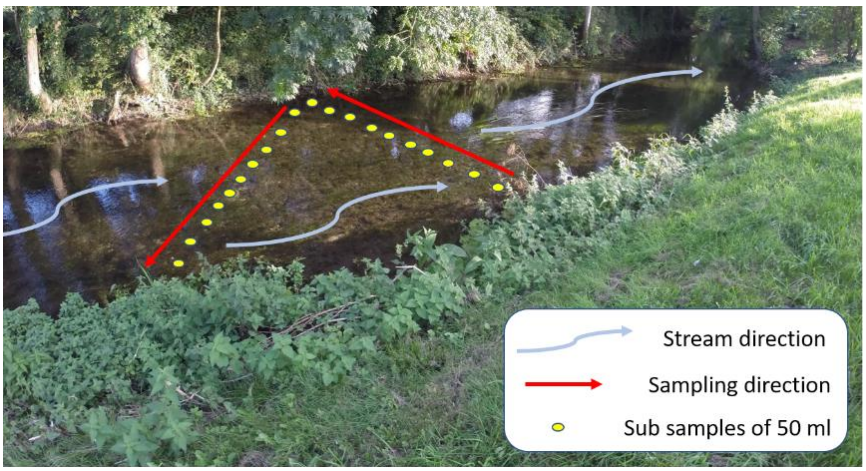
5. Once 20 samples have been taken, close the bag securely using the top tabs and shake the Whirl-Pak bag for 10 seconds. This mixes DNA across the water sample.

6. Using the clear plastic pipette provided take 15 mL of water from the Whirl-Pak bag and pipette into a sterile tube containing 35 mL of ethanol to preserve the eDNA sample (i.e. fill tube to the 50mL mark). Close the tube ensuring the cap is tight.

7. Shake the tube vigorously for 10 seconds to mix the sample and preservative. This is essential to prevent DNA degradation. Repeat for each of the 6 tubes in the kit. Before taking each sample, stir the water in the bag to homogenize the sample - this is because the DNA will constantly sink to the bottom.

8. The box of preserved sub-samples is then returned at ambient temperature immediately for analysis. If batches of samples are collected and stored for longer periods prior to analysis they should be refrigerated at 2-4° C.

Notes:
- Record date sample kit sent
- Record unique reference number for each kit
- If in doubt – change gloves
- Each kit is single use – recycle/dispose of used kit equipment.



SOUTH WEST PEAK
LANDSCAPE AT A CROSSROADS