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Molecular monitoring and conservation genomics of the tadpole shrimp, *Triops cancriformis*

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by

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Declaration of Authorship

I declare that the work herein is intellectually my own. Data chapters received contributions from my supervisors (Africa Gómez; AG, Bernd Hänfling; BH and Larry R. Griffin; LRG) and collaborators (Cristina Di Muri; CDM and Michael R. Winter; MRW) as stated below. Any additional assistance has been acknowledged at the end of each chapter.

Chapter 2: I led and designed the project, performed the laboratory work, analysed the data and wrote the paper. AG, BH and LRG designed the project, wrote and reviewed drafts of the paper.

Chapter 3: I led and designed the project, performed the laboratory work with MRW, analysed the data and wrote the paper. AG and LRG designed the project, wrote and reviewed drafts of the paper.

Chapter 4: I led and designed the project, performed the experiments and laboratory work with CDM, analysed the data and wrote the paper. AG, BH and CDM wrote and reviewed drafts of the paper.

Chapter 5: I led and designed the project, performed the experiments and laboratory work with MRW, analysed the data and wrote the paper. AG, BH and MRW designed the project, wrote and reviewed drafts of the paper.

Chapter 6: I led and designed the project, performed experiments and laboratory work, analysed the data and wrote the paper. AG and BH designed the project, wrote and reviewed drafts of the paper.

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Table of Contents

Abstract

The tadpole shrimp, *Triops cancriformis* (Bosc, 1801) (Branchiopoda; Notostraca), is a temporary pool specialist invertebrate native to Europe classified as endangered across its distribution. Populations are threatened by habitat destruction through land development and urbanisation. There are just two remaining populations in the UK, but traditional methods are inefficient for discovering and monitoring populations. Furthermore, the genetic relationships between both populations is unknown.

This thesis focuses on the design, development and application of effective molecular methods to address the shortcomings of current methods for the detection and monitoring of *T. cancriformis* populations. To this end I designed a species-specific PCR assay that reliably and efficiently identified extant *T. cancriformis* populations and, perhaps equally importantly, determined viable egg bank densities within them. Application of this method to historic sites on the Solway Firth, south west Scotland, confirmed the species' absence. To increase the scope of experiments in molecular studies, I developed a single, modular and cost effective alternative DNA extraction method to those of the commercial kits available. To better serve the conservation of *T. cancriformis*, I created a highly effective process for the rapid spot testing of multiple sediment samples for viable *T. cancriformis* diapausing eggs. Finally, using a genomewide approach I identified and genotyped SNPs from multiple individuals (RAD-seq) to ascertain genetic diversity and population genetic structure of *T. cancriformis* across its European distribution.

Utilising these newly developed methods I present a molecular 'toolkit' for future *T. cancriformis* conservation. Additionally, I add to existing evidence that *T. cancriformis* is indeed extinct at historic sites on the Solway Firth, Scotland. Finally, I determine that the two remaining extant populations of *T. cancriformis* in the UK have very little genetic diversity and are not genetically differentiated, but they are distinct from the other European populations analysed.

Chapter 1

General introduction

1.1 The temporary pool environment

Temporary or ephemeral pools are abundant habitats throughout many different landscapes and ecosystems worldwide (Williams et al. 2004, Oertli et al. 2005, Céréghino et al. 2008). They are shallow, non-permanent water bodies filled by rainwater or snow melt, that dry out for part or most of a year, or even a period of many years (De Meester et al. 2005). Temporary pools can be very dynamic, forming in almost any kind of depression, and, depending on surrounding environment, can infill and disappear within short time periods (Williams et al. 2010). Disturbance from human activity creates many temporary pools, for example, agricultural and military vehicle tracks can become accidentally formed pool habitats supporting diverse flora and fauna (Armitage et al. 2012). Temporary pools can also be older than permanent water bodies, recurring time and again for possibly thousands of years, as their drought phase reduces silt buildup and prolongs the pools lifetime (Williams et al. 2010). Over short time periods, communities can develop within a temporary pool with historic events determining their current structure (Jeffries 2010), and, with the extended persistence of these pools within a landscape, can lead to the creation of markedly distinct biological communities.

The unpredictable hydroregime of temporary pools place ecological pressures upon the aquatic invertebrates that inhabit them. Organisms that cannot actively disperse, such as crustaceans (copepods, branchiopods) and rotifers, have adaptations that allow them to persist over drought phases. These organisms produce a diapausing or resting egg stage that is resistant to environmental extremes and able to remain dormant for decades, forming 'egg banks' of future generations (Brendonck & De Meester 2003). Resting eggs may not all hatch in a single hydroperiod but instead remain dormant until subsequent hydroperiods as a bet-hedging strategy (Simovich & Hathaway 1997). This stabilises fluctuations in following generations and spreads reproductive risks over time (Seger & Brockmann 1987). The passive dispersal of diapausing eggs, transported in sediment attached externally to grazing animals or ingested by waterfowl (Thiéry 1997, Green & Figuerola 2005, Green et al. 2005, Sánchez et al. 2007, Vanschoenwinkel et al. 2011, Muñoz et al. 2013), can allow for long distance colonisation of new habitats. The specialist environment, with the extreme pressures on

organisms for successful reproduction and dispersal, leads to them supporting fewer aquatic invertebrate species than more permanent water bodies; however they have distinct species assemblages with higher incidences of rare and endangered species (Bratton 1990).

As a habitat, ponds and pools, both permanent and seasonal, have been largely ignored by freshwater biologists in the past, with little or no direct conservation actions specifically targeting them and the organisms they support (Biggs et al. 2005). Temporary pools remain abundant across the UK and Europe, yet are increasingly in decline (Williams et al. 2010). Land development for agriculture and urbanisation has resulted in the loss of, and increased risk to, this habitat (Céréghino et al. 2008). Agricultural land management practices causes land to drain more rapidly, removing water essential for the formation of a temporary pool (Williams et al. 2010) and the use of pesticides, fertilisers and livestock drugs can cause eutrophication, pollution and contamination of existing pool sites (Hughes 1997, Brönmark & Hansson 2002, De Meester et al. 2005). Groundwater abstraction for domestic use affects pool hydroperiods (Serrano & Serrano 1996), reducing their duration and lowering the success of key organisms that require longer hydroperiods to develop, resulting in local extinctions (Schneider & Frost 1996). In particular, temporary pools have also been removed by infilling or deepened to create more desirable permanent ponds (Bratton 1990, Biggs et al. 2010), changing the ecology and leading to the loss of ephemeral pool specialists (Nicolet et al. 2004, Jeffries 2010). One such group of temporary pool specialists affected are the large branchiopods: primitive freshwater crustaceans that are ecologically important and found across the globe (Brendonck et al. 2008). Detrimental changes in land usage and management has caused local extinctions of large branchiopods over recent decades in central Europe (Eder & Hödl 2002). The destruction of large numbers of temporary pools has led to patchy distributions of species across a region, reducing the sustainability of the metapopulations needed for the conservation of threatened large branchiopod diversity (Gołdyn et al. 2012).

Restoration and management of ponds and pools across the European landscape are currently the prime conservation efforts for these habitats (Zacharias et al. 2007, Sayer et al. 2013). Focussing on a landscape scale, rather than solely on localised sites, reveals ponds as biodiversity hotspots connected across a larger pondscape (Biggs et al. 2005, Hill et al. 2018). However, the effective management of temporary pools can require site specific preservation of hydroregimes and natural succession to conserve environmental variability and biodiversity (Zacharias et al. 2007, Biggs et al. 2010). As an addition to protection and management of existing sites the creation of new temporary pools is a cost-effective and accessible method for pondscape recreation or restoration (Biggs et al. 2010).

1.2 Triops cancriformis

The Eurasian tadpole shrimp, *Triops cancriformis* (Bosc, 1801), is a large freshwater invertebrate (Branchiopoda; Notostraca) and an ephemeral pool specialist native to Europe (Hughes 1997, Zierold et al. 2007). With the exception of Antarctica, *Triops* species are present on all continents and are often associated with drier regions of the world (Fryer 1988, Williams & Busby 1991, Kuller & Gasith 1996). There are over 10 *Triops* species and multiple cryptic species have been discovered in previously recognised lineages (Korn & Hundsdoerfer 2006, Korn et al. 2010, Meusel & Schwentner 2017). In addition to *T. cancriformis* there are other species of *Triops* in Europe; *T. granarius* and *T. mauritanicus* (Brtek & Thiéry 1995). However, there are potentially more as *T. mauritanicus* is comprised of distinct, yet cryptic species (Korn et al. 2010) as may *T. granarius* (Korn & Hundsdoerfer 2006).

Figure 1. The Eurasian tadpole shrimp, *Triops cancriformis*. Image courtesy of A. Gomez, used with permission.

As a temporary pool specialist, *Triops* have a rapid life cycle adapted to the unpredictability of pool hydroperiods. Only living for a maximum of four months after hatching, reproductive age is reached at as little as 13 days in some *Triops* species and egg laying individuals deposit frequent and numerous eggs clutches for the remainder of their life span (Meintjes 1996). Like other temporary pool specialists, *T. cancriformis* produce diapausing eggs that accumulate in the sediment of the pool and are capable of remaining dormant for up to 30 years (Schönbrunner & Eder 2006, Feber et al. 2011). *T. cancriformis* exhibits differing sexual systems across the species' distribution; gonochoristic (male and female in similar incidence, 100% outcrossing), androdioecious (hermaphrodite with low male incidence, intermediate outcrossing rate) and hermaphroditic (no male incidence, 100% selfing) (Zierold et al. 2007). Populations from the southern end of the distribution are gonochoric whereas those further north are androdioecious or selfing hermaphrodites (Zierold et al. 2009). The two populations in the UK are toward the northernmost extent of the species' range and are reported as being

composed of self-fertilising hermaphrodites as no males have ever been found (Zierold et al. 2009).

1.3 Ecology of *T. cancriformis*

Shortly after a temporary pool refills with water, *Triops* diapuasing egg stages start to hatch and the nauplii begin an active life in the water column. Temperature is a major hatching cue for *Triops* species across its global distribution (Scott & Grigarick 1979, Kuller & Gasith 1996). Light exposure, neutral water pH and low salinity are also strong hatching cues for the genus (Scott & Grigarick 1979, Kuller & Gasith 1996, Schönbrunner & Eder 2006, Kashiyama et al. 2010). For *T. cancriformis* the optimal temperature for hatching is around 15 - 20°C in the UK (Feber et al. 2011) but this can vary as, for example, it is higher for the populations found in Israel (Kuller & Gasith 1996). Temporary pool inundation later in the year, where the ground temperature is below the optimal temperature (15°C for UK populations), results in no evidence of *T. cancriformis* hatching (Feber et al. 2011).

T. cancriformis is a keystone species that can directly influence the pool's community composition (Waterkeyn et al. 2016). Once hatched, *Triops* nauplii develop rapidly (Møller et al. 2003) and in early instars feed upon phytoplankton (Tietze & Mulla 1989). As *Triops* grow their preference for larger prey items increases (Tietze & Mulla 1989) and their feeding regime is dependent on available flora and fauna (Golzari et al. 2009). *T. cancriformis* is mainly a predatory species that, as an adult, feeds on detritus, plant matter, large zooplankton (Boix et al. 2006) and the resting eggs of other invertebrates that includes cannibalising its own (Waterkeyn et al. 2011b). The strong predatory habits of adult *T. cancriformis* effectively reduces competition (Waterkeyn et al. 2011a) and results in lower densities of zooplankton dominated by fewer, larger species (Waterkeyn et al. 2016). The presence of T. cancriformis has been found to trigger adaptive morphological changes in *Daphnia* species, making them more difficult to manipulate as prey items (Petrusek et al. 2009, Rabus et al. 2011). Despite having strong impacts on community structure of temporary pools, *Triops* coexist with other large branchiopods, such as fairy shrimp (Anostraca) and clam shrimp (Spinicaudata) (Brtek & Thiéry 1995, Petrov & Cvetković 1997). This same community and coexistence is evidenced in 365 million year old fossils (Gueriau et al. 2016).

Adult *T. cancriformis* are bioturbators and ecosystem engineers that cause a regime shift from a clear to turbid water state (Waterkeyn et al. 2016). This has detrimental effects on other organisms as suspended sediment can fill the stomach and effectively starve filter feeding species (Rellstab & Spaak 2007). *Triops* also disturb and feed upon seedling aquatic and terrestrial plants associated with temporary bodies of water (Takahashi 1994, Boix et al. 2006),

this potentially extends the lifetime of a pool by delaying the onset of macrophyte coverage (Waterkeyn et al. 2016). As a secondary effect of the species behaviour, bioturbation by *T. cancriformis* decreases water transparency which may reduce the predation of individuals by birds (Waterkeyn et al. 2011a).

1.4 Historic *T. cancriformis* sites in the UK

T. cancriformis has a long history in the UK, but the records and sites where it has been reported are extremely rare. The species was first discovered in the UK in Kent by Reverend Littleton A. Brown in 1736 (Brown 1738). Additional records came from Christchurch, Hampshire, by Leach in 1816 and then Bristol by William Clayfield, Esq in 1831 (Fox 1949). The species was later recorded in Powick, Worcestershire, in 1841 by John Evans reporting in *The Entomologist* vol.1 (1840 - 1842). At the beginning of the 20th century *T. cancriformis* was discovered at sites on the Solway Firth (Balfour-Browne 1909, 1948). The finding of these populations attracted high interest suggesting that, even in the 18th and 19th centuries, the species was not widely distributed in the UK, and was potentially already in decline. The recorded species' range in the UK has shown a decline over the last centuries, likely due to the loss of available temporary pool habitat being lost (Williams et al. 2010). All the above mentioned sites have yielded no records of the species presence in recent decades, and only the sites of Balfour-Browne (1909, 1948) have been resampled in attempts to rediscover *T. cancriformis* populations (Foster 1993, Adams et al. 2014).

1.5 Conservation of *T. cancriformis* in the UK

T. cancriformis is classified as an endangered species across Europe (Eder & Hödl 2002). Sites at Godshill (Hampshire, southern England) and the Wildfowl and Wetlands Trust Caerlaverock reserve (Dumfriesshire, south west Scotland) hold the two remaining populations of *T. cancriformis* known to exist in the UK (Feber et al. 2011). *T. cancriformis* is protected under Schedule 5 of the Wildlife and Countryside Act 1981 and has a Biodiversity Action Plan (BAP) (JNCC 2010). Key aspects of the BAP and conservation efforts are to discover new populations, monitor existing ones and create suitable habitat (Feber et al. 2011). Detecting the species' presence or absence more reliably will result in a better representation of its distribution in the UK, while monitoring existing sites can give insight to stressors that affect populations. Creating suitable habitats allows for the potential spread of the species either naturally (via passive dispersal) or through translocation and reintroduction attempts. *Ex-situ* breeding programmes to conserve the population in captivity, spread across multiple institutions, are

designed to act as an insurance against the species' extinction in the wild (Hughes 1997), and could allow for its translocation to newly created habitat.

1.6 Conventional detection of *Triops* populations

Surveying for new populations and monitoring existing ones, as stated in the species' BAP, is the main conservation effort for *T. cancriformis* in the UK. Kick-sampling and water column netting are routinely employed to survey water bodies for assessing and identifying aquatic invertebrate communities (Williams et al. 2004, Stark 1993). Variations on these methods have been used worldwide in many studies to survey large branchiopods (Martin et al. 2016), including *Triops* (Sassaman et al. 1997, Zierold et al. 2007). These methods rely on the capture of adult individuals from the water column. However, changes in hydroregime or abiotic factors can create unfavourable conditions for resting *T. cancriformis* eggs to hatch (Hughes 1997), resulting in extended periods with no records of *T. cancriformis* even in recorded population sites (Khalaf 1978, Feber et al. 2011).

An alternative to water body sampling is to hatch *Triops* diapausing eggs from the pool sediment. This involves using hatching approaches widely used on other aquatic invertebrates such as rotifers or cladocerans (May 1986, Carvalho & Wolf 1989). Collected sediment is dried then rehydrated in aquaria, emulating the filling of a pool and incubation of diapausing eggs, inducing hatching and allowing the study of *Triops* hatchlings in the laboratory (Sassaman et al. 1997, Obregón-Barboza et al. 2001, Schönbrunner & Eder 2006, Harper & Reiber 2006, Zierold et al. 2007). Incubation of sediment has been used to survey for *T. cancriformis* within the UK (Hobson & Omer-Cooper 1935), and has been successful in discovering new population sites (Adams et al. 2014). However, hatching of *Triops* eggs in this manner depends on simulating favourable conditions for hatching (Kuller & Gasith 1996, Eder et al. 1997, Schönbrunner & Eder 2006, Kashiyama et al. 2010). Despite ideal conditions, some, or all, eggs could remain dormant as a bet-hedging strategy (Takahashi 1976).

Conventional methods are confounded by the non-uniform hatching of *Triops* in a given hydroperiod. As adults or hatched nauplii are the basis for the discovery of an extant *T. cancriformis* population, bet-hedging strategies may result in no hatching events from sediments that contain viable eggs.

1.7 Molecular methods for detection

All work on *T. cancriformis* thus far has employed conventional methods for the detection of aquatic macroinvertebrates. An alternative is to use molecular approaches, based on polymerase chain reaction (PCR) amplification of extracted DNA from a sample, for the detection of the species. DNA obtained directly from environmental samples, (environmental DNA or eDNA), has been widely used to detect endangered or invasive species from water samples (Biggs et al. 2015, Jerde et al. 2011, Dejean et al. 2012, Rees et al. 2014, Robinson et al. 2018, Blackman et al. 2018, Harper et al. 2019). A proof of concept for large branchiopods is the use of species-specific assays to monitor endangered freshwater biodiversity across Europe, which was successfully used to detect, *Lepidurus apus* another notostracan species (Thomsen et al. 2012). In comparison to conventional methods, molecular approaches have been shown to have increased sensitivity for detection of low abundance or rare organisms (Jerde et al. 2011, Smart et al. 2015, Valentini et al. 2016, Hänfling et al. 2016). The application of sensitive, species-specific detection would greatly facilitate conservation efforts for *T. cancriformis* across its range.

1.8 Genetic diversity and conservation

The genetic differentiation between *T. cancriformis* populations has been ascertained by several studies. Studies using mitochondrial DNA (mtDNA) regions from multiple populations of *T. cancriformis* showed that there is little genetic diversity present within and between populations (Zierold et al. 2007, Mantovani et al. 2008). This has been interpreted as resulting from postglacial colonisation of northern Europe as temperature increased after the last glacial maximum. However, population genetic analysis of microsatellite loci highlighted strong differentiation between populations and low genetic diversity within populations (Mantovani et al. 2008, Zierold et al. 2009). This was probably due to little gene flow and strong genetic drift as populations may be founded by as few as a single individual in the case of the hermaphroditic populations (Mantovani et al. 2008, Zierold et al. 2009). The studies using microsatellites relied only on a few loci and did not include the Scottish population. Therefore, to date, genetic diversity between the UK populations of *T. cancriformis* has only been assessed via mtDNA analysis, where they were shown as closely related within a large clade that encompasses all European populations (Zierold et al. 2007).

The application of population genomics has immense potential for conservation biology (Hendricks et al. 2018, Meek & Larson 2019). It can allow for the detection of evolutionary effects such as genetic drift, local adaptation and gene flow that can shape the genetic

variation of geographically distinct populations across a species' distribution (Cutter & Payseur 2013, Edwards et al. 2016, Franch-Gras et al. 2018). Population genomics relies on the identification of single-nucleotide polymorphisms (SNPs) across the genome of individuals from separate populations. SNPs are a reliable and highly informative method to determine differentiation between populations of a given organism (Helyar et al. 2011). Genotype by sequencing (GBS) uses restriction enzymes to reduce genome complexity, e.g sample the genome, creating a reduced representation genomic library (Narum et al. 2013). As a GBS method, restriction-site associated DNA sequencing (RAD-seq) generates fragments of DNA adjacent to restriction enzyme recognition sites which are used to generate a reduced representation genomic library (Baird et al. 2008). In combination with next generation sequencing (NGS) this allows the genotyping of thousands of SNPs in hundreds of individuals from such a genomic library (Baird et al. 2008). GBS approaches such as RAD-seq have been employed successfully in multiple population and conservation genomics studies (Davey & Blaxter 2010, Narum et al. 2013, Jeffries et al. 2016) and have also been used to identify the basis of sex determination and structure of sex chromosomes in *T. cancriformis* (Mathers et al. 2015, Orr 2017).

Conservation measures rely on awareness of intraspecific genetic diversity to maintain evolutionary potential (Thakur et al. 2018). Managing this natural variation as genetically distinct evolutionary significant units (ESU) can preserve localised diversity and adaptive potential (Fraser & Bernatchez 2001). Determining diversity through population genomics can ultimately be used to inform the management of conservation units (CUs) (Paz-Vinas et al. 2018, Waples & Lindley 2018), leading to the preservation of ESUs. As previous *T. cancriformis* population studies were based on mtDNA and microsatellite data, a higher resolution genomic approach would detect any genetic differentiation between the two UK populations and determine if they should be considered as two separate CUs.

1.9 Thesis aims and overview

In this thesis I aim to develop and apply molecular approaches that address the current shortcomings of conventional *T. cancriformis* detection and further the understanding of genetic diversity within and between the UK populations. The findings of this thesis will be informative for future efforts and function as complementary additions to the already existing measures for conservation of the species in the UK. Several of the approaches developed have the potential to be used for the monitoring and conservation of the species as a whole.

Chapter 2

Conservation of *T. cancriformis* in the UK is primarily directed towards discovering and monitoring populations. Current detection of the species relies upon netting or hatching individuals, but both approaches are confounded by the organism's behavioural strategies. To this end I design a species-specific PCR assay to directly identify the isolated resting eggs of *T. cancriformis*. This molecular approach would reliably and efficiently identify extant populations and, importantly, determine viable egg bank densities within them.

Chapter 3

Along the Solway Firth (Dumfriesshire, south west Scotland) there are two historic records of *T. cancriformis* (Balfour-Browne 1909, 1948). These sites are 20 km west of the currently extant population at WWT Caerlaverock and both historic sites have been extensively surveyed for the presence of *T. cancriformis* with no successful detection (Foster 1993, Adams et al. 2014). I resample these historic locations and apply the molecular approach developed in Chapter 1 to assess multiple temporary pool sites for the presence of the species resting egg banks. Reliably determining the species' presence or absence at historically recorded sites would allow for an informed conservation strategy, for example licensed reintroduction or translocation attempts of *T. cancriformis* within its former range.

Chapter 4

Molecular approaches are increasingly used for sensitive and non-invasive detection of organisms from environmental samples. Tissue samples are also used to identify species and to describe food webs via DNA analysis of gut contents. All molecular work relies on the successful extraction of DNA from a sample type, yet this initial step can represent a major cost in any project and forms a limiting factor when budgets are limited. Here I design and develop a single cost effective alternative DNA extraction method to those of the commercial kits available. The application of the method in any given study would still achieve similar results to those of the commercial options but for a much reduced cost. This approach could be applied to water or sediment samples in potential *Triops* sites.

Chapter 5

To better serve the conservation of *T. cancriformis* a rapid method to detect the species presence from pool sediments would be desirable. Here I develop a highly effective process for

the rapid spot testing of multiple sediment samples for viable *T. cancriformis* diapausing eggs. A combination of species-specific detection (Chapter 2) and cost effective DNA extraction (Chapter 4) are used to create an optimised high sensitivity method to detect viable diapausing eggs within samples. This increased efficiency for the detection of *T. cancriformis* will benefit the conservation effort for the species across its distribution range.

Chapter 6

To date the genetic diversity within the UK populations of *T. cancriformis* is poorly known. To further the understanding of the species' genetic diversity I compare the two populations of the UK (Godshill and Caerlaverock) to two from continental Europe (Espola, Spain, and Königswartha, Germany). Using a genomewide approach to identify and genotype SNPs from multiple individuals (RAD-seq) I will estimate individual and population level genetic diversity and population genetic structure of *T. cancriformis*. This will determine genetic differentiation between populations and demonstrate any genetic diversity that could be used to inform future conservation management efforts for the species in the UK.

Chapter 7

Finally, the findings of the thesis are brought together and their implications for the conservation of *T. cancriformis* are discussed. In this chapter I describe key points for consideration in a revised species action plan and how molecular detection would be a solid addition to the conservation effort for the species. Here I also highlight the importance of habitat management and *ex-situ* breeding for the future of the species in the UK.

Chapter 2

A new molecular diagnostic tool for surveying and monitoring *Triops cancriformis* populations*

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2.1 Abstract

The tadpole shrimp, *Triops cancriformis* (Bosc, 1801), is a freshwater crustacean listed as endangered in the UK and Europe living in ephemeral pools. Populations are threatened by habitat destruction due to land development for agriculture and increased urbanisation. Despite this, there is a lack of efficient methods for discovering and monitoring populations. Established macroinvertebrate monitoring methods, such as net sampling, are unsuitable given the organism's life history, that include long lived diapausing eggs, benthic habits and ephemerally active populations. Conventional hatching methods, such as sediment incubation, are both time consuming and potentially confounded by bet-hedging hatching strategies of diapausing eggs. Here we develop a new molecular diagnostic method to detect viable egg banks of *T. cancriformis*, and compare its performance to two conventional monitoring methods involving diapausing egg hatching. We apply this method to a collection of pond sediments from the Wildfowl & Wetlands Trust Caerlaverock National Nature Reserve, which

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holds one of the two remaining British populations of *T. cancriformis.* DNA barcoding of isolated eggs, using newly designed species-specific primers for a large region of mtDNA, was used to estimate egg viability. These estimates were compared to those obtained by the conventional methods of sediment and isolation hatching. Our method outperformed the conventional methods, revealing six ponds holding viable *T. cancriformis* diapausing egg banks in Caerlaverock. Additionally, designed species-specific primers for a short region of mtDNA identified degraded, inviable eggs and were used to ascertain the levels of recent mortality within an egg bank. Together with efficient sugar flotation techniques to extract eggs from sediment samples, our molecular method proved to be a faster and more powerful alternative for assessing the viability and condition of *T. cancriformis* diapausing egg banks.

2.2 Introduction

The tadpole shrimp, *Triops cancriformis* (Bosc, 1801), is a large freshwater branchiopod of the order Notostraca native to Europe (Hughes 1997, Zierold et al. 2007). As with passively dispersed ephemeral pool specialists, such as other branchiopods and rotifers, *T. cancriformis* has adaptations to persist over unpredictable drought periods. These organisms produce diapausing eggs resistant to environmental extremes that can remain dormant for decades, accumulating in the pool sediment to form 'egg banks' of future generations (Brendonck & De Meester 2003). In this stage the eggs can be passively dispersed by animal vectors (Thiéry 1997, Green & Figuerola 2005, Vanschoenwinkel et al. 2011, Muñoz et al. 2013), colonising potential new habitats over great distances. In addition, a bet-hedging hatching strategy is an adaptive feature in the life history of aquatic invertebrates from ephemeral ponds (Simovich & Hathaway 1997, Allen 2010), including *Triops* (Takahashi 1976). Not all eggs hatch in a given hydroperiod, some remain dormant until future hydroperiods so spreading reproductive risk over time (Seger & Brockmann 1987). Finally, *T. cancriformis* has a rapid life cycle. Reproductive age is reached in as little as 12 days and egg laying individuals deposit numerous egg clutches for the remainder of their life span (Feber et al. 2011). Across the European distribution of *T. cancriformis*, populations exhibit differing sexual systems; southern populations contain similar proportions of males and females whereas those further north are mostly selfing hermaphrodites (Zierold et al. 2009).

Throughout Europe and the United Kingdom ephemeral pools have been lost to, and are increasingly at risk from, land development for agriculture and urbanisation (Serrano & Serrano 1996, Céréghino et al. 2008, Williams et al. 2010). As such *T. cancriformis* is classified as endangered in many European countries (Eder & Hödl 2002) and in the UK it is protected

under Schedule 5 of the Wildlife and Countryside Act 1981 with a Biodiversity Action Plan (BAP) (Feber et al. 2011). The New Forest (Hampshire, Southern England) and the Wildfowl & Wetlands Trust Caerlaverock Wetland Reserve (Dumfriesshire, South West Scotland), discovered in 2004, are the two locations of remaining populations of *T. cancriformis* known in the British Isles. Both are remnants of a historically wider distribution recorded in the south and south west of England (Fox 1949) and south west Scotland (Balfour-Browne 1909, 1948). These two UK populations are toward the northernmost extent of the species range and are comprised of hermaphroditic individuals (Zierold et al. 2009). Given the ephemerality and passive dispersal of *T. cancriformis*, it is likely that undiscovered *T. cancriformis* egg banks and populations exist across the British Isles (as suggested by Adams et al., 2014).

Surveying methods such as water column netting and kick-sampling are conventionally employed to identify and assess aquatic macroinvertebrate communities within a water body (Williams et al. 2004, Stark 1993). Many variations of these methods have been used worldwide to study large branchiopods (Martin et al. 2016), including *Triops* (Sassaman et al. 1997, Zierold et al. 2007). However they rely on finding adult individuals within a water body. Differences in abiotic factors and a pools hydroregime can result in long periods with no records of *T. cancriformis* even within a known population site using such standard methods (Feber et al. 2011). Alternative sampling methods, more suited for the ephemeral nature of *Triops* life history, target the diapausing eggs. As the viability of a *Triops* egg cannot be visually discerned, unlike with rotifers (García-Roger et al. 2005), viability estimates rely on successful hatching of diapausing eggs. Rehydration and incubation of sediment containing diapausing *Triops* eggs has been used for the study of hatchlings in the laboratory (Sassaman et al. 1997, Obregón-Barboza et al. 2001, Schönbrunner & Eder 2006, Harper & Reiber 2006, Zierold et al. 2007). Additionally, collected sediment can also be progressively sieved through finer meshes to isolate, identify and hatch the eggs of *Triops* and other species it contains (Kuller & Gasith 1996). A further method for the isolation of eggs is that of sucrose flotation (Gómez & Carvalho 2000). This is a very efficient method which substantially reduces the time needed to find eggs in sediment. Of these alternative sampling methods only the incubation of sediment has been used to discover new *T. cancriformis* populations within Britain. Adams et al. (2014) surveyed 86 pools consisting of both extant and historic *Triops* population locations on the Solway Firth, UK, including the WWT Caerlaverock Wetland Reserve. Despite the large effort involved, the study only produced three hatched *T. cancriformis* nauplii from two of the sampled sites, over a period in excess of seventy days. The study however did discover a new population of *T. cancriformis* on the Solway Firth. The current methods used are all confounded by the non-uniform hatching of *Triops* at the beginning of a hydroperiod. Hatching

of *Triops* eggs is dependent upon simulating favourable hatching conditions in the laboratory (Kuller & Gasith 1996, Eder et al. 1997, Schönbrunner & Eder 2006, Kashiyama et al. 2010) and some, if not all, of the eggs present could remain dormant as a bet-hedging strategy (Takahashi 1976).

A molecular approach can be applied to the discovery and identification of *T. cancriformis* populations in the UK, removing the associated deficiencies of conventional surveying methods. DNA barcoding using 'universal' primers and sequencing has been used extensively for species identification (Hebert et al. 2003). Environmental DNA (eDNA) has been employed to monitor endangered freshwater biodiversity across Europe, including another notostracan species: *Lepidurus apus* (Thomsen et al. 2012). Given that DNA degrades rapidly after an organism's death (Hofreiter et al. 2001), amplification of a large DNA fragment could potentially be used to assess egg viability in aquatic invertebrates. A species-specific amplification technique applied to isolated diapausing *T. cancriformis* eggs, amplifying a suitably large region of mtDNA, could both determine egg viability and species identity. Such an approach would remove the uncertainty of bet-hedging giving more reliable estimates of *T. cancriformis* egg bank viability. Conversely, species-specific primers designed for much shorter fragments, associated with degenerated mtDNA, could be used simultaneously to identify degraded non-viable eggs. Although small fragments of DNA can persist *post mortem* for long periods of time this preservation requires rapid and prolonged desiccation or very low temperatures (Lindahl 1993, Hofreiter et al. 2001). These conditions are unlikely to be met or maintained in the environment of temporary pools. Although the degeneration of DNA in water is greatly accelerated, small fragments can remain detectable for up to a month (Dejean et al. 2011). However, intracellular DNA, like that within a degraded egg, could be somewhat more protected from abiotic and biotic factors and degenerate at a slower rate (Nielsen et al. 2007). The identification and counts of these degraded eggs could be used as a proxy for the overall condition of an egg bank, presenting a view of recent mortality rates in the diapausing eggs.

Here we developed species-specific DNA barcoding of isolated eggs to identify viable *Triops cancriformis* diapausing eggs from sediments. We compared the results obtained with this method with two conventional alternatives: sediment hatching and isolation hatching over two hydroperiods to account for bet-hedging. We applied the three methods to 12 sediment samples collected from ephemeral pools at the WWT Caerlaverock Wetland Reserve, including pools where *Triops* had been previously recorded plus some potential new sites. We estimate diapausing egg bank size, egg viability and condition in these pools. In addition, from the collected mtDNA data we also describe the genetic diversity of the Caerlaverock populations in

the context of available data from other European populations. Our method could be used as a time efficient strategy for discovering and monitoring the viability and health of *T. cancriformis* egg banks across Europe.

2.3 Materials and Methods

All work was carried out under Scottish Natural Heritage licence number 42854.

Sample collection and preparation

We sampled 12 temporary pool sites on the WWT Caerlaverock reserve from the 10th to the 11th of September 2015. Eight sites, including the site of the species rediscovery in 2004, were located on the Eastpark Farm holding of the reserve along the cattle grazed scrub and grassland bordering the Solway Firth estuary mudflats. The other four sites were on cattle grazed pasture on the Powhillon Farm holding on the north of the reserve. Sites consisted of either temporary pools where *Triops* had been recorded before (either through presence of *Triops* or where past experiments yielded *Triops* hatchings) or sites with no previous *Triops* records but apparently suitable *Triops* habitat in that they had regular hydroperiods and had been recorded to dry out at least once a year.

At each site GPS coordinates were obtained from the centre of the pool using an eTrex Camo GPS device (Garmin Ltd, USA). Using a stainless steel spoon around 500 g of superficial sediment (ca. top 2.5 cm) was collected from eight uniformly distributed sample points, four around the pool centre and four midway to the pool boundary. Sampling spoons were thoroughly cleaned of all sediment and debris after each site to avoid cross-site contamination. Collected sediment from a site was placed directly into large labelled Ziploc bags, which were immediately placed into another identical bag to further prevent cross-site contamination. Once in the laboratory, collected sediment samples were placed in separate open topped 2 l plastic jars and left to dry out over a period of four weeks at 20°C. Once completely dry the samples were gently crumbled into a finer state by hand. Three subsamples of 20 g were then taken from each sample to be used in sediment hatching, isolation hatching and DNA barcoding, respectively.

Comparison of methods

DNA barcoding of isolated *T. cancriformis* diapausing eggs was compared to conventional survey methods of sediment hatching and isolation hatching. Each method gave an estimate of

viable eggs per site. Total egg counts were achieved via diapausing egg isolation from sediment. Viable and total egg counts were recorded from all three methods per site and compared to evaluate our molecular approach (Figure 1). These counts also allowed for the calculation of proportion viability and egg bank density (eggs/kg) per site. Additionally unhatched eggs from the sediment and isolation hatching methods were tested for viability with the DNA barcoding method. Estimates of the time and costs involved in each method were also compared.

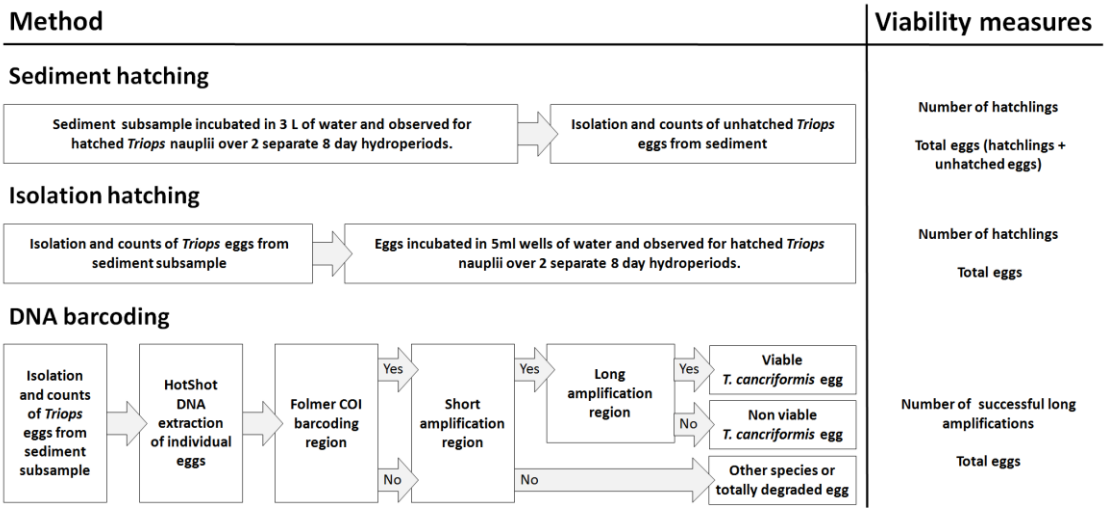

Figure 1. An overview of the three methods used in this study: sediment hatching, isolation hatching and DNA barcoding. Shown are the simplified steps undertaken for each procedure. "Yes" and "No" in the DNA barcoding flow chart refers to successful and unsuccessful amplifications, respectively. The viability measures obtained for each site were used for comparison to determine the value of our molecular approach and to calculate proportion viability and egg bank density (eggs/kg) per site.

Isolation of diapausing eggs from sediment

Eggs were isolated from sediment in all of the three methods of this study as a means for DNA extraction of individual eggs for DNA barcoding, actual isolation of eggs for isolation hatching and the counting of remaining unhatched eggs after sediment hatching experiments had concluded. Identification of *T. cancriformis* eggs was achieved through comparison to known example specimens and collections within the laboratory and to those within the literature (Kuller & Gasith 1996). Diapausing *T. cancriformis* egg isolation from collected sediment samples followed a sucrose flotation method adapted from Gómez & Carvalho (2000). In short, 5 g of sediment was added to 50 ml sucrose solution (50/50 w/v sugar/water). This was thoroughly mixed by vortexing before being centrifuged at 700 rpm. Resting eggs were then captured from the supernatant with a 50 µm Nytal filter. Washed and rehydrated filtrate was observed under a stereoscopic microscope for *T. cancriformis* eggs. Identified *T. cancriformis*

eggs were collected using a 200 µl Gilson pipette and transferred to a sterile small welled cell culture plate (Corning Costar 3526, 24 well, flat bottomed culture plate). Isolated eggs were further checked under a stereoscopic microscope to determine if they were hatched or unhatched. Hatched eggs were discarded. The number of unhatched eggs isolated was recorded for each subsample.

Hatching experiments

Hatching experiments took place from the 16th of November to 19th of December 2015 in conditions optimised for *Triops* hatching: incubated in purified water in a temperature controlled growth room at ~20°C (Eder et al. 1997, Schönbrunner & Eder 2006) with a 12/12 day/night cycle (Kuller & Gasith 1996, Kashiyama et al. 2010) under white fluorescent light tubes. To account for possible bet-hedging strategies of *T. cancriformis*, hatching was undertaken in two short hydroperiods of eight days, each with a seven day drying period in between. To make best use of available space samples were run in batches of six, A to F and G to L, staggered weekly such that as one batch was drying the other was undergoing a hydroperiod. Both sediment and isolation hatching for each site were run alongside one another so as to be under the same lighting and temperature variations over the observation periods.

Sediment hatching

A 20 g subsample of sediment from each site was added to a 6 l lid-less acrylic tank (L = 30 cm, D = 20 cm, H = 20 cm) filled with 3 l of purified water. The sediment was allowed to settle for an hour before being evenly distributed across the tank bottom using a large spatula. The water of each tank was gently agitated with a constantly running air pump during the duration of the experiments. Hatchlings were searched for in each tank daily for a period of about ten minutes. Any hatched *Triops* nauplii were removed using a 1000 µl Gilson pipette, placed in a separate Petri dish for each site and counts recorded. Removed nauplii were destroyed in 70% ethanol or underwent HotShot DNA extraction (Montero-Pau et al. 2008) for future use. Observations were carried out over an eight day period. On the last day after observations the tanks were drained using a thin tube to siphon the water through a 50 µm Nytal filter device. A separate filter was used for each tank. The filters were then examined under a stereoscopic microscope and any hatchlings present were added to the recorded count for the day and destroyed. Any eggs found were placed back in the corresponding tank sediment. The sediment was left in the tanks to completely dry out over a period of seven days before being refilled with 3 l of purified water and the above process repeated for the second hydroperiod.

After the drying period following the second hydroperiod was completed the sediment was removed from the tank. Any remaining eggs were isolated from the sediment using the sucrose flotation method described above. Unhatched egg numbers were recorded and added to the total number of hatchlings for each site as a proxy for the total number of initial eggs present in the subsample.

Isolation Hatching

Eggs were isolated from a 20 g subsample of the dried sediment from each site using the sucrose flotation method as described above. Immediately after being isolated, eggs from each site were placed in 1.5 ml of purified water in a sterile cell culture plate (Corning Costar 3526, 24 well, flat bottomed culture plate), in groups of up to five per cell. The plate cover was placed on top to reduce evaporation. Over the following eight day period ten minute observations of each plate were performed daily and any hatched *Triops* nauplii were removed using a 1000 µl Gilson pipette, placed in a separate Petri dish for each site and counts recorded. Removed nauplii were preserved in 70% ethanol or underwent HotShot DNA extraction for future use. On the eighth day after observations the wells were carefully drained using a 1000 µl Gilson pipette. The plate wells were left with the covers removed to completely dry out over a period of seven days before being refilled with 1.5 ml of purified water and the above process repeated for the second hydroperiod.

DNA barcoding

The molecular method of DNA barcoding of isolated *T. cancriformis* eggs was designed to produce simple PCR steps to identify viable eggs and the diapausing egg bank condition (Figure 1). Two species-specific primer pairs were designed for this study. A primer pair to amplify a large 2500 bp target region of mtDNA (long amplification) so that presumably only intact, viable *Triops* eggs amplified. A second primer pair to amplify a short 132 bp target region of mtDNA (short amplification) to act as a species identifier for degraded DNA, potentially found in *T. cancriformis* inviable eggs. DNA extraction samples from all individual isolated eggs were first amplified with the universal DNA barcoding primers LCO1490 and HCO2198 (Folmer et al. 1994) to give a ~650 bp fragment of cytochrome c oxidase subunit I gene (COI). This first step was aimed at determining the taxonomic identity of all samples that failed to be identified as *T. cancriformis* via subsequent short and long amplifications. All samples then underwent PCR with the short amplification primers, identifying which samples had *T. cancriformis* mtDNA present. Those samples with successful COI amplifications underwent PCR with the long amplification primers. This step would confirm both the designed primers of this study to be

species-specific, as any sample with a long amplification should have a complementary short amplification. Samples with a successful short amplification and no long amplification present were considered to be degraded *T. cancriformis* eggs.

Finally, after the completion of the second hydroperiods for both sediment and isolation hatching, all remaining unhatched eggs were removed and underwent the DNA extraction protocol (see details below) and underwent PCR for the long amplification region. To check if all viable eggs had hatched in our hatching experiments and to confirm the suitability of long amplifications to identify viable eggs, all DNA extractions from unhatched eggs from the hatching methods were amplified for the long amplification region.

DNA Extraction of Isolated Eggs

T. cancriformis eggs from a 20 g sediment subsample were isolated as described above. Genomic DNA was individually extracted using the HotShot DNA extraction protocol from Montero-Pau et al. (2008). 50 µl of lysis buffer was aliquoted into 0.2 ml Eppendorf tubes. A single isolated *T. cancriformis* egg was transferred into each tube using a 200 µl Gilson pipette. The egg was crushed on the side of the tube within the lysis buffer with a sterile 10 µl Gilson pipette tip. Tubes were incubated at 95°C for 30 min followed by cooling on ice for 5 min. 50 µl of neutralising solution was then added to each tube then vortexed and centrifuged. All HotShot extractions were stored at -20°C until required.

Primer design

Species-specific primers were designed and tested in silico with Primer BLAST (Ye et al. 2012) using the complete *T. cancriformis* mitochondrial genome as a reference sequence (Genbank accession number AB084514.1) (Table 1). The long amplification region was located from $tRNA_{Tyr}$ to ATP8. This region encompassed the whole Folmer COI region for comparison to existing *T. cancriformis* COI sequences. The short amplification region was located across $tRNA_{Ala}$ and $tRNA_{Asn}$ after the ND3 gene.

All primers were tested in vitro on three species of *Triops* (*T. cancriformis*: Caerlaverock, Scotland; Espolla, Spain; Königswartha, Germany, *T. mauritanicus*: Doñana, Spain and *T. newberryi*: Triop World (Interplay UK, Marlow, Buckinghamshire, UK)) and several freshwater invertebrates specimens from Caerlaverock and other UK locations (*Daphnia sp*., Ostracoda and Copepoda). DNA templates were from HotShot DNA extractions of hatched specimens and collected tissue samples. The long amplification primer pair amplified only *T. cancriformis* and its sister species *T. mauritanicus*. The short amplification primer pair was found to be

completely specific to the target species. PCR cycling conditions were optimised for both long and short amplification primer pairs.

Table 1. Primers designed and developed in this study. Primer sequences and product size are given.

Primer pair name	Primers	Primer sequences (5' - 3')	Product size (bp)
Long amplification	GS-Tyr-1349F	AGGGGAAACTCCCATATTTAGATT	2500
	GS-ATP8-3806R	TACTAGGGGCTATTTGGGGG	
Short amplification	GS-trnaS-5881F	TGCATTCAAAAGGTACTACCAAAA	132
	GS-trnaS-5971R	TGCCGATCATTGGCTTCAA	

PCR amplification

All PCRs were performed on Applied Biosystems Veriti 96-Well Thermal Cyclers in a 25 µl final reaction volume composed of 2 µl template DNA, 12.5 µl MyTaq^{tm} Red Mix (Bioline), 8.5 µl ddH_2O and 1 µl of each 10 µM primer. PCR products were visualised on 1.5% agarose gels. COI PCRs were run under the cycling conditions: 180 s at 94°C, 37 x (30 s at 94°, 60 s at 52°C, 90 s at 72°C), 600 s at 72°C. Short amplification PCRs were run using the designed primers GS-trnaS-5881F and GS-trnaS-5971R, under the cycling conditions: 180 s at 94°C, 37 x (30 s at 94°C, 30 s at 55°C, 30 s at 72°C), 600 s at 72°C. Long amplification PCRs were run using the designed primers GS-Tyr-1349F and GS-ATP8-3806R, under the touchdown cycling conditions: 180 s at 94°C, 10 x (30 s at 94°C, 60 s at 70°C [-1°C per cycle], 105 s at 72°C), 27 x (30 s at 94°C, 60 s at 60°C, 105 s at 72°C), 600 s at 72°C. Faint amplifications were rerun with a 1:20 template dilution to reduce any PCR inhibition or DNA overloading. Positive (previously successful *T. cancriformis* nauplii extractions) and negative controls were used in each PCR batch.

DNA sequencing

To confirm the specificity of our designed primers, PCR products from five samples with successful short amplifications and five with successful long amplifications were sequenced. To discover possible discrepancies over the COI region, a further 20 samples with successful COI and long amplifications had both PCR products sequenced. Finally, in order to validate our molecular diagnostic tool and to verify the identity of eggs and identify those that could be confused with *Triops*, all samples not identified as *T. cancriformis* via a long amplification that had successful COI fragments were sequenced. COI and long amplification products were

sequenced using the LCO1490 primer. Short amplification products were sequenced using the GS-trnaS-5881F primer. All sequencing was performed by Macrogen (Seoul, South Korea). Sequences were manually edited using CodonCode Aligner (CodonCode Corp., Dedham, MA, USA). End clips were performed to remove low quality regions from both ends of the sequences (end regions containing more than 3 bases with lower quality than 20 within a 25 bp window were trimmed). Sequences shorter than 50 bp after clipping were discarded as poor quality. Furthermore, samples with COI and long amplification fragments shorter than 100 bp were also discarded as poor quality for this size region is unreliable for successful COI identification (Meusnier et al. 2008). Remaining sequences were put through NCBI BLASTn for sequence identification. All good quality *T. cancriformis* COI and long amplification sequences were submitted to GenBank (accession numbers: KY769474 - KY769517).

mtDNA population network

A population network was created to compare the COI haplotypes from the WWT Caerlaverock population to other *T. cancriformis* sequences across Europe. Sequences in this study identified as *T. cancriformis,* from either a COI or long amplification of an individual sample were aligned to all *T. cancriformis* COI sequences available from Genbank. *T. mauritanicus* was used as an outgroup. Sequences were aligned and trimmed to 512 bp using Aliview (Larsson 2014) and any shorter sequences were discarded. POPART (http://popart.otago.ac.nz.) was used to create a TCS statistical parsimony network (Clement et al. 2002).

Egg bank density, viability and condition

Egg bank density was estimated to measure the number of eggs per kg sediment in a site. Proportion viability was estimated to measure the overall viability of the egg bank in a site and was the primary measurement used for the statistical comparison of the three methods. Viable egg counts from all three methods per subsample were used to calculate a proportion viability for each method per site (see Figure 1). For sediment and isolation hatching, the number of viable eggs was estimated as the total number of hatchlings over two hydroperiods in a site subsample. For DNA barcoding the number of viable eggs in a site subsample was estimated as the number of successful long amplifications. Estimated egg bank density for each site was calculated from the average total egg counts per site from the three methods.

Using the barcoding method described here, the condition of an egg bank can be inferred through the proportions of viable (samples with long amplification), degraded (samples with a

short amplification and no long amplification) and totally degraded eggs (samples with neither long nor short amplifications) present. For each site the proportion of viable, degraded and totally degraded eggs were calculated and combined with egg bank density (eggs/kg sediment) to present a measure of egg bank condition. Egg bank density was estimated from the total number of eggs isolated per site from DNA barcoding.

Statistical analyses

We tested for statistical differences between the estimated viability per site across the three tested methods (isolation hatching, sediment hatching, DNA barcoding). We used a general linear mixed model of viable against nonviable egg counts with binomial errors implemented in R (R Core Team, 2016) (version 3.2.5, package "lme4" (Bates et al., 2015)). "Site" was used as a random variable and "method" as a fixed variable. To determine if method was a significant factor in any variances in measures of egg viability, we compared this model to the same model with no fixed variable using a chi-squared test of the likelihood of models. Overdispersion was tested for in both models (R version 3.2.5, package "blmeco" (Korner-Nievergelt et al 2015)).

2.4 Results

Sample collection

Sample sites were located in grazing pasture with cattle present. Sites D, E and F were located in wheel ruts along tractor trails linking grazing pastures. At the time of sampling sites B, C, H and L had water up to a depth of 10 cm remaining. Sites A, B, C, I and L had sparse vegetation growth within the pool boundaries. All other sites were dry, or drying, exposed sediment. Two weeks prior to sampling a tidal surge up the Lochar Water, a river that runs through the reserve, had breached its small defence walls and flooded the eastern side of the Powhillon Farm field area that included sample sites J and K. At the time of sample collection water samples taken from a remaining large pool, adjacent to sites J and K, had a salinity of 17.5 ppt. Estuarine and marine species were found alive within these pools or exposed on the drying pool sediments, *Crangon crangon* (brown shrimp) in drying sediment at site J, *Pungitius pungitius* (ninespine stickleback) within the large saline pool, and juveniles of *Carcinus maenas* (green shore crab) at site K. During sampling there was evidence of *Triops* presence in one of our sampled sites (site K) where no records existed before, with many exuviae present in caked sediment.

Isolation of resting eggs from sediment

Triops cancriformis eggs were isolated from all sites, therefore all sampled sites held a *T. cancriformis* egg bank of varying density. Two sites had distinctly larger egg banks than the other sites sampled: site G, the site of *T. cancriformis* rediscovery at the WWT Caerlaverock Wetland Reserve in 2004, and site J on the Powhillon Farm holding of the reserve. All identified *T. cancriformis* eggs were not in similar condition: many having begun to lose the external coating of fine sediment particles or appearing flat and misshapen.

Hatching experiments

Six of the 12 sample sites produced *T. cancriformis* nauplii from sediment and isolation hatching methods, however not all sites exhibited hatchlings from both methods (Appendix 1: Table 1). Sites G and J had the highest hatching rates. Site K, with no previous records of *T. cancriformis* presence, had a single recorded isolation hatchling. Site E had previous records of *T. cancriformis* presence but had no hatchlings recorded from either hatching method. Just four nauplii hatched in the second hydroperiod of the hatching experiments overall, one in site G sediment hatching and three in site J isolation hatching, indicating low bet-hedging strategies in these populations.

Over the first hydroperiod, hatched nauplii from both hatching methods were recorded within a small time window over the eight day observation period (Appendix 1: Figure 1). The first hatchlings were recorded after a 48 hour incubation period. Most hatchlings appeared on days two to five across both methods. As sediment hatchlings were more difficult to spot compared to those of isolation hatching, a small number of sediment hatchlings may have been overlooked and only discovered on later observation days than those of isolation.

DNA barcoding of isolated eggs

A total of 226 individual eggs were processed using DNA barcoding, of which 153 yielded positive amplifications with at least one of the primer pairs (Table 2). Samples from all sites yielded positive Folmer COI region PCR amplifications yet those with positive long and short amplifications were only present in the six sites with recorded nauplii in hatching experiments: sites D, F, G, I, J and K (Table 2). Short amplifications were associated with samples that had a successful COI amplification with the exception of a single sample from site I. All samples with successful long amplifications also had successful short amplifications.

Table 2. Outcome from *T. cancriformis* diapausing egg DNA barcoding. Counts of samples with COI, long and short amplification PCR combinations and total eggs processed for each site are given. The six sites with recorded hatchlings from sediment and or isolation hatching are marked with asterisks.

PCR combination			Site										
COI	**Long**	**Short**	**A**	**B**	**C**	**D***	**E**	**F***	**G***	**H**	**I***	**J***	**K***	**L**
✓	✓	✓	-	-	-	1	-	1	25	-	3	22	2	-
✓	-	✓	-	-	-	-	-	-	5	-	-	2	5	-
-	-	✓	-	-	-	-	-	-	-	-	1	-	-	-
✓	-	-	2	1	4	1	2	9	25	6	6	22	5	3
		Total eggs	18	11	23	5	6	10	60	6	17	51	13	6

DNA sequencing

All five short amplification and four of the five long amplification sample sequences were all good quality and identified as *T. cancriformis*, confirming the specificity of our designed primers (Appendix 1: Table 2). The 20 samples with both COI and long amplification products all had good quality COI sequences that were identified as *T. cancriformis* (Appendix 1: Table 3). Of the long amplification sequences, eight were of poor quality and discarded. The remaining 12 were of good quality and all identified as *T. cancriformis*. There were no discrepancies between results as the Folmer COI region from long amplification and COI sequences were identical across all samples, further confirming the suitability of the long amplification for species identification. Out of the 97 samples with COI amplifications (with no long amplification) sequenced 41 were of poor quality and discarded. The top hits of the NCBI BLASTn returns for the remaining samples showed eight *T. cancriformis* sequences and 48 non-*Triops* sequences (Appendix 1: Table 4). All the *T. cancriformis* COI sequences were from five of the six sample sites with recorded hatchlings from this study (sites D, G, I, J and K). All non-*Triops* sequences were identified to species with no similar egg morphology to *Triops*. Two samples from site G had short amplifications with no long amplification and a non *Triops* COI sequence identified. Three samples, from site I, G and K, had a short amplification with a non-existent or poor quality COI sequence.

Population mtDNA network

A total of 115 COI sequences of individual *T. cancriformis* isolates, including 26 from this study, with one *T. mauritanicus* isolate as an outgroup were used to produce the TCS mtDNA haplotype network (Accession numbers and sample ID in Appendix 1: Table 5). We found a single COI haplotype in our Caerlaverock *T. cancriformis*, which is identical to a common

haplotype found in a large number of isolates from Europe, including isolates from the other British population in the New Forest (Figure 2). Intriguingly, the only previously analysed Scottish sample contained a haplotype differing from those of this study in one base pair.

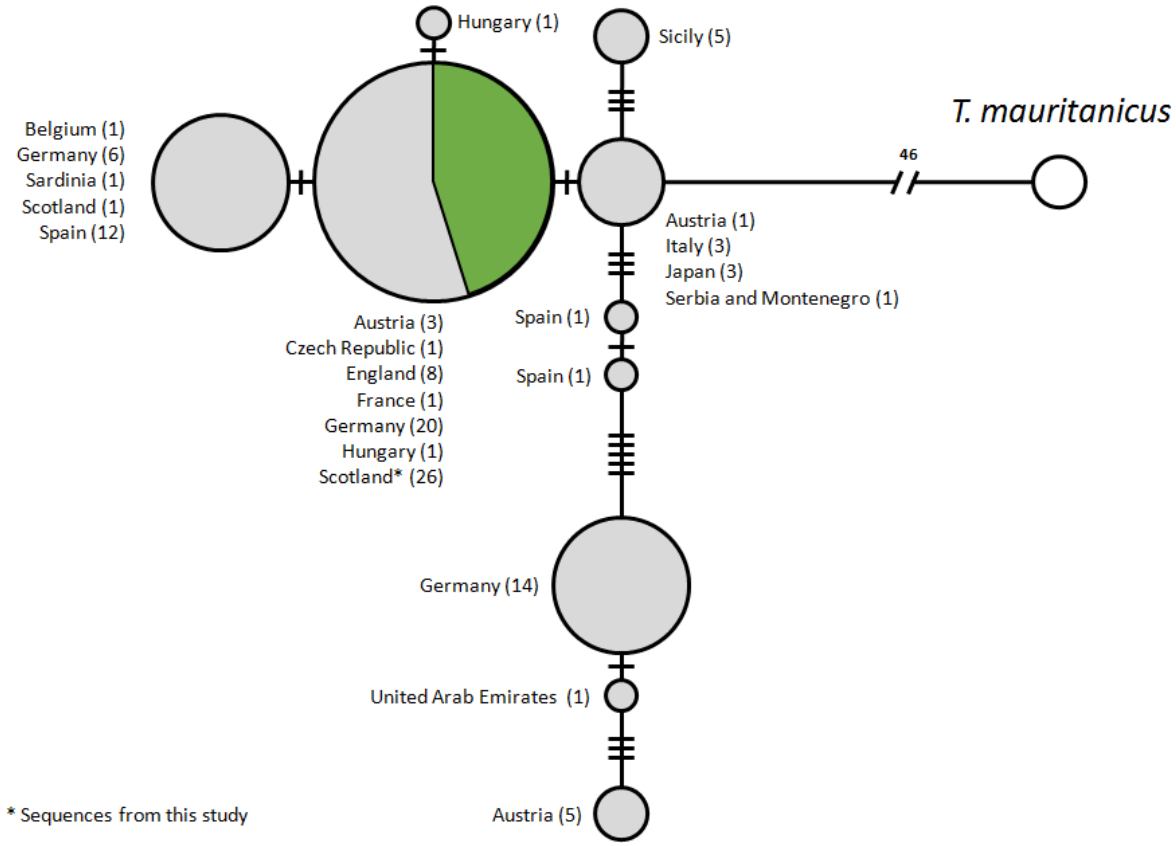

Figure 2. Statistical parsimony network of COI sequences from *T. cancriformis* isolates with *T. mauritanicus* as an outgroup. Those in green are from this study. Countries of origin and the number of isolates are given next to each node. Ticks on linkages indicate number of mutations between nodes.

Comparison of methods for determining egg bank viability

Estimates of proportion viability of egg banks varied between sites, with only 6 out of the 12 sites showing viable egg banks, with the maximum viability found in site G (Figure 3). DNA barcoding was the most powerful method to detect sites with viable eggs (6 sites) compared to isolation hatching (5 sites) and sediment hatching (4 sites). The three methods gave similar results in sites with larger more uniform egg counts: sites G, I and J. There was no significant difference in egg viability estimates between the three methods across all sites (X^2 = 1.7995, df = 2, p = 0.4067) with no overdispersion in either model. Therefore, successful DNA barcoding of long amplifications can be used as a reliable measure of viability in *T. cancriformis* resting eggs.

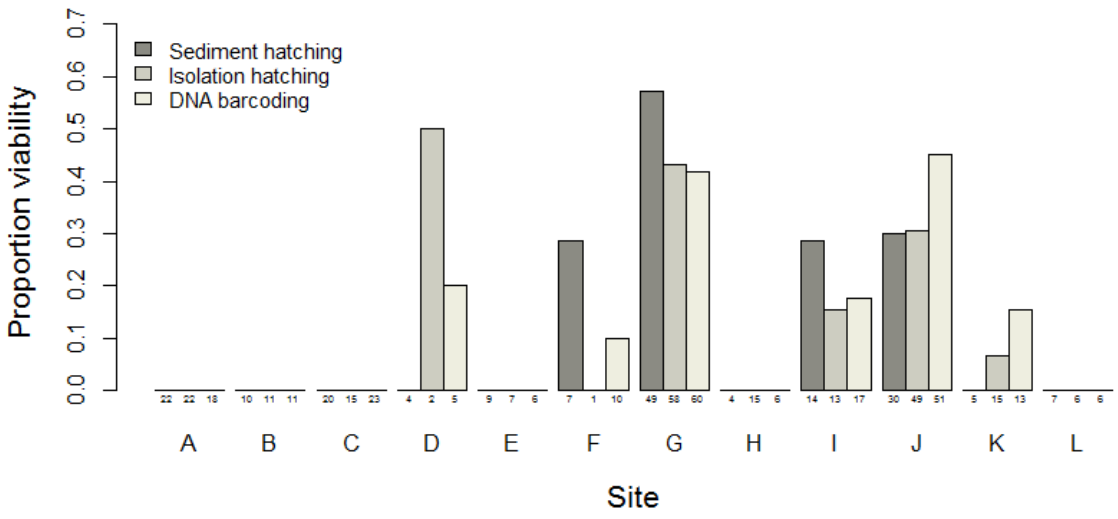

Figure 3. Proportion of *T. cancriformis* viable eggs per site from the three methods employed (sediment hatching, isolation hatching and DNA barcoding each from a 20 g subsample). Total number of eggs per method are listed under the columns.

DNA barcoding of unhatched eggs

DNA barcoding using the long amplification primers on extractions from the unhatched eggs remaining after sediment and isolation hatching experiments was in general unsuccessful. Only 12 eggs out of 308 samples had successful long amplifications (Appendix 1: Table 6). These were in sites with the larger sample sizes (G and J) suggesting some bet-hedging in these populations that the hatching methods failed to detect over the two hydroperiods. Site J isolation hatching showed the highest number of unhatched eggs amplifying the long amplification primers, with eight identified. As we wanted to use long amplification as a proxy for viability, and to determine if there would have been any effect upon the estimated viability between methods had these eggs hatched during the experiments, the GLM analysis was rerun with adjusted results. There was again no significant difference between the three methods used to determine viability (X^2 = 0.6954, df = 2, p = 0.7063) with no overdispersion in either model.

Comparison of time expenditure and costs

Given the budget constraints of environmental monitoring, we estimated time expenditure and equipment cost for each method to produce an egg bank viability estimate based upon a single high egg count subsample (60 eggs per subsample) (Appendix 1: Table 7). Drying of collected sediment was not factored into the comparison. The methods were divided into processes. Each process was evaluated by the time to its completion and the maximum time a

researcher would have to expend executing it. Times for hatching setups were ignored as they were either part of a previous procedure, as for isolation hatching, or considered negligible (less than 2 minutes), as for sediment hatching. The PCR time was calculated for running a single 60 sample PCR preparation and amplification using long amplification primers. Consumables costs were based upon approximate retail values of materials used that could not feasibly be reused for the same process. Salary times were not costed, just time expenditure calculated.

Both sediment and isolation hatching take several weeks to complete (over 32 days and 24 days respectively), considerably longer than the DNA barcoding method to achieve the same result (7.5 hours). Although all three methods require a similar input of time to process (around 4 hours of a researcher's time), this is spread over a much greater time frame for both hatching methods than for DNA barcoding. In contrast to time efficiency, consumables costs for the hatching methods are a minimal amount (0.20 GBP for the sugar used) compared to those of DNA barcoding (30.00 GBP).

Egg bank density, viability and condition of Triops cancriformis populations at Caerlaverock

All sites had an egg bank present based upon calculations from the three methods, yet egg bank density (eggs/kg sediment) estimates varied between sites. Two sites had higher densities: sites G and J (Appendix 1: Table 8). Viable egg banks are found across the reserve but are clustered around the two higher density sites G and J (Figure 4). From the molecular method the proportion of viable eggs (long amplifications), degraded eggs (short amplifications) and totally degraded eggs (those with neither amplifications) were combined with egg bank size to give a representation of overall condition (Figure 4; Appendix 1: Table 9). The 12 sites had differing proportions of viable, degraded and totally degraded eggs.

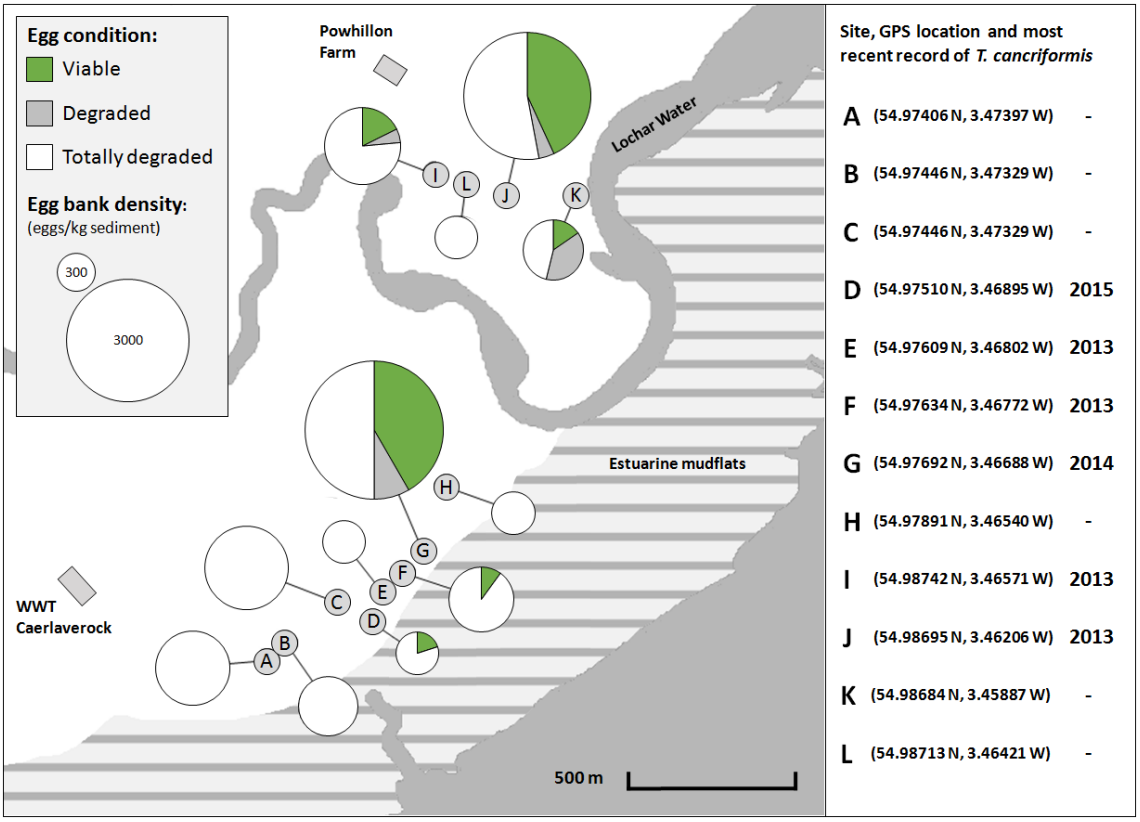

Figure 4. Location and condition of *T. cancriformis* egg banks sampled across the WWT Caerlaverock Wetland Reserve. Shown are proportions of viable, degraded and totally degraded eggs per site as determined by our molecular method. Chart size proportional to estimated egg bank density (eggs/kg sediment) as determined by the molecular method. The GPS location and year of the most recent recorded *T. cancriformis* presence (adults or hatchlings) for the sites are shown for the period up to the time of sampling in September 2015 (right).

Overall, there was a high proportion of totally degraded eggs but six of the sites had egg banks in a totally degraded condition, that is, non-existent (sites A, B, C, E, H and L). In contrast, sites G and J had high proportions of viable eggs. Sites D and F had no degraded eggs present. Sites G, I and J showed low proportions of degraded eggs in comparison to that of viable eggs. Site K had a much higher proportion of degraded eggs than that of viable eggs. Sites with high proportions of viable eggs and low proportions of degraded eggs were interpreted as having *T. cancriformis* egg banks in good condition. Sites with higher proportions of degraded eggs than viable were of poor condition. Sites with no viable *T. cancriformis* eggs, might have held populations in the past, but the species can be considered to have become extinct. All but one of the sites with viable *T. cancriformis* egg banks determined from this study had recent records of the species: site K. In this site we found exuviae at the time of sampling and it is a new location for the species. In site E *Triops* adults were recorded in 2013, however we failed to find viable eggs from all methods employed, suggesting that this population might have become extinct.

2.5 Discussion

This study describes a powerful and efficient molecular technique that can identify viable *T. cancriformis* eggs isolated from sediment samples outperforming conventional incubation methods, therefore helping to discover new populations and monitor existing ones. Primarily, a single PCR using species-specific long amplification primers on DNA extracted from eggs isolated from a sediment sample gave an estimate of viable eggs present. Secondarily, a further PCR using the short amplification primers on the same DNA extractions confirmed the species as *T. cancriformis* and could be used to estimate the number of degraded eggs present: those with no successful long amplification. The combination of these results with the total number of isolated eggs from sediment samples provided an overview of egg bank condition. All good quality long amplification sequences were identified as *T. cancriformis* and the viability estimates obtained from the molecular approach were not statistically different from the sediment and isolation methods across all sites. Most eggs that remained unhatched in both hatching experiments after two rounds of hydration failed to amplify with the long amplification primers, validating the use of our molecular technique to estimate diapausing egg bank viability. However, the fact that a few of these eggs did amplify suggested the presence of a certain amount of bet-hedging in these *T. cancriformis* populations. These were not included in the hatching methods viability measures so reduced the estimates of viability for the sites. This means that our molecular method produces a viability measure for *T. cancriformis* diapausing egg banks, removing any uncertainty of bet-hedging for a complete viability estimate.

The molecular method, as with the hatching methods, relied upon initial morphological egg identification from samples. Our visual identification of *T. cancriformis* was confirmed via the COI DNA barcoding of samples, with most good quality COI and long amplification sequences belonging to *T. cancriformis*. Other good quality COI sequences obtained did not include groups with diapausing egg morphology similar to *T. cancriformis*. Non *T. cancriformis* COI sequences were mostly of bacteria, microalgae and water moulds associated with ephemeral pools that inhabited, were adhered to or present within the sediment attached to a degraded egg (Appendix 1: Table 4). Our data also show that environmental DNA from larger organisms found in and around the habitat pervaded the sample.

Both hatching methods showed a similar pattern of emergence and numbers of hatchlings. Therefore sucrose flotation of *Triops* eggs used in the isolation hatching method had no effect upon hatching rates of resting eggs, as recently supported by Lukic et al. (2016). Our results from the hatching methods suggest that the Caerlaverock *T. cancriformis* populations exhibit a

low level of bet-hedging. This is further supported by the few successful long amplifications found in the remaining unhatched eggs of the hatching methods.

Previously the estimated condition of a species' diapausing egg bank had only been achieved with rotifers via visual inspection of individual egg appearance (García-Roger et al. 2005). Unlike the conventional survey methods used for *T. cancriformis* monitoring, the molecular method used in the current study can similarly estimate the condition of a *T. cancriformis* egg bank through the identification of viable, degraded and totally degraded eggs. Estimating the condition of a population could be based around the relative proportions of egg states. Egg banks in the six sites with high proportions of viable eggs (samples with long amplifications) can be considered to hold good condition, viable *T. cancriformis* populations. Mortality rates within an egg bank can be inferred from the proportion of degraded eggs (samples with only short amplifications) present. As these eggs have relatively recently deteriorated it can be used as a proxy for mortality events from external factors, be they biotic or abiotic. Some sites had small sample sizes due to low egg bank densities and would require larger sample sizes to get better representations of condition. In contrast, the remaining six sites, with only totally degraded eggs, do not currently hold *T. cancriformis* populations. During this study we discovered a new population of *T. cancriformis* on the WWT Caerlaverock reserve (site K) and also determined that a previously recorded population (site E) might now have become extinct. This suggests a certain degree of dynamism in population persistence, potentially reflecting the existence of dynamic metapopulations in the area, as it is the case on other temporary pool branchiopods such as *Daphnia* (Ebert et al. 2002, Haag et al. 2005).

We used the sequences obtained to validate our methods by comparing Caerlaverock samples to other *T. cancriformis* populations. Our analysis showed that Caerlaverock mtDNA belongs to the most common European COI haplotype of *T. cancriformis* (Figure 4). The fact that the only previously sequenced Caerlaverock sample from Zierold et al. (2007), belonging to a different haplotype, could potentially reflect diversity not sampled in our study.

The molecular method of this study is a more efficient method for determining the presence of a viable *T. cancriformis* egg bank than the conventional and standardised methods of sediment and isolation hatching. Additionally with the use of species-specific primers the cost of sequencing is removed, both in terms of time and money, setting our method apart from other molecular approaches that rely upon sample sequencing to determine species identity. A successful amplification viewed via gel electrophoresis can be used to confidently identify the organism as *T. cancriformis* and, as with the long amplification, the viability of a resting egg. As a direct comparison of time frames involved in this study the molecular analysis of a single site, from egg isolation to gel electrophoresis, took a matter of hours (Appendix 1: Table 7),

whereas the hatching experiments took over three weeks to complete (four in the case of sediment hatching). When dealing with much greater sample sizes, as with Adams et al. (2014), the time expenditure can be greatly reduced using our molecular method. The major drawback to the method is the consumables cost. With the hatching methods the only consumable was the sugar used in the sucrose flotation method to isolate the diapausing eggs. This is distinctly inexpensive when compared to the consumable costs for the molecular method which were many times greater than those of the hatching methods (Appendix 1: Table 7). Salary costs were not included as the staff time for each method was very similar. However during the extended time frame of the hatching methods there are periods of daily observations to be undertaken requiring a researcher's presence, which would increase the overall economic costing of the hatching methods.

Molecular approaches, in particular eDNA, are increasingly used to determine the presence of endangered species in freshwater habitats, as with the Great Crested Newt, *Triturus cristatus*, in the UK (Rees et al. 2014) and multiple species in Europe (Thomsen et al. 2012), and can detect secretive or rare species more effectively than conventional methods (Valentini et al. 2016, Hänfling et al. 2016). The molecular method presented in this study not only efficiently detects viable *T. cancriformis* populations, directly addressing the needs for heightened surveillance for *T. cancriformis* populations as raised by Adams et al. (2014), but provides better estimates of egg bank density and condition. Our methods have conservation implications not only for British *T. cancriformis* populations, but more widely as they were designed and tested on European populations. The implementation of an effective method for determining the presence and condition of viable *T. cancriformis* populations across the species' distribution reduces the time costs considerably. The ease of processing many samples, with bet-hedging uncertainties removed, will give accurate, reliable and rapid results for implementation of relevant conservation measures. Additionally our molecular method can be used for the sister species of *T. cancriformis*: *T. mauritanicus*, meaning that the diagnostic tools presented here would be useful for the monitoring of viable *T. cancriformis* and *T. mauritanicus* populations.

2.6 Conclusion

The potential accelerated loss of habitat suitable for endangered *T. cancriformis* populations across the species distribution requires an effective survey method for its conservation. We present a powerful alternative molecular method that, through the amplification of mtDNA extracted from isolated eggs using species-specific primers, can reliably and efficiently

determine the presence, condition and viability of *T. cancriformis* egg banks. The complications of passive dispersal, extended diapause and bet-hedging are removed as, unlike conventional survey techniques, our method does not rely on observations of hatched or adult individuals to discover an extant *T. cancriformis* population. The increasing success and decreasing cost of molecular techniques for ecological conservation and diversity monitoring (Thomsen et al. 2012, Lawson Handley 2015) make them viable alternative approaches. The use of designed species-specific primers alleviates the cost of sequencing, further reducing the costs. Implementation of our molecular method will present a cost-effective and efficient tool for the discovery and monitoring of *T. cancriformis* populations in the UK and Europe. From the results of this study, the current management of WWT Caerlaverock is ideal for maintaining the dynamic metapopulation of *T. cancriformis* that appears to be present across the reserve.

2.7 Acknowledgements

We would like to thank Dr James Kitson and Dr Paul Nichols for advice regarding molecular protocols, Dr Amir Szitenberg for guidance with molecular data analysis tools, Dr James Gilbert for statistical assistance, Dr Lori Lawson Handley and Dr Lesley Morrell for advice and feedback. We thank the Wildfowl & Wetlands Trust for support during the sampling trip to Caerlaverock.

Chapter 3

Surveying for historical *Triops cancriformis* populations in the Southwick area of Dumfries and Galloway, south west Scotland

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3.1 Abstract

The tadpole shrimp, *Triops cancriformis* (Bosc, 1801), is an endangered crustacean in the UK. Despite a wider, if scattered, distribution in the early 19th century in Britain, only two populations are currently known. The Southwick area of the Solway Firth, south west Scotland, historically supported populations of *T. cancriformis* in the first half of the 20th century. Since then, no records have been noted despite intense survey effort. In this study we apply a reliable method for *T. cancriformis* discovery to ephemeral pool sites located close to the historic sites on the RSPB Mersehead and West Preston reserves. Despite the presence of many suitable temporary pools, no *T. cancriformis* populations were detected at any of the locations sampled, suggesting that the species became locally extinct. However, we highlight the potential for the species' reintroduction to this part of its former range.

3.2 Introduction

The tadpole shrimp, *Triops cancriformis* (Bosc, 1801) is an endangered crustacean in the UK, protected under Schedule 5 of the Wildlife and Countryside Act 1981. As with other temporary pool specialists, *T. cancriformis* populations survive adverse periods in the form of diapausing eggs. In this manner the eggs can endure in a viable stage in pool sediments for decades until a suitable hydroperiod (Brendonck & De Meester 2003, Radzikowski 2013). Diapausing eggs can be passively dispersed, transported in sediment attached to animal vectors (Green & Figuerola 2005, Vanschoenwinkel et al. 2011, Muñoz et al. 2013), leading to the colonisation of distant new habitat. *T. cancriformis* can be found throughout Europe and the UK is at the northern extent of its global distribution (Zierold et al. 2007). Despite a wider distribution in the early 19th century, only two populations are now known in the UK, one in the New Forest, England, and another on the Solway Firth, Scotland (Feber et al. 2011). Whereas an irregular, but continuous population has been found in a roadside shallow ephemeral pool in the New Forest since 1934, the history of the Solway Firth Triops is more complex. Populations of *T. cancriformis* were first recorded on the Solway in 1907 and, after a 40 year hiatus, again in 1948 (Balfour-Browne 1909, 1948). However, by the 1960's these sites were thought to have succumbed to coastal erosion and the species thus lost from the Solway area. Subsequent attempts to locate *T. cancriformis* populations in areas proximal to these historical Scottish records and elsewhere on the Solway were unsuccessful (Foster 1993). This thereby seemed to confirm its likely extinction on the Solway (and Scotland) until its rediscovery in 2004 at the Wildfowl and Wetlands Trust (WWT) reserve at Caerlaverock (Feber et al. 2011). Since then, all recent *T. cancriformis* discoveries in the Solway Firth area have been confined to the Eastpark and Powhillon Farm holdings of the WWT Caerlaverock reserve (Adams et al. 2014, Sellers et al. 2017; Chapter 2). Given that the original habitat of shallow, temporary pools (as described by Balfour-Browne (1909, 1948)) are still present across the RSPB Mersehead and West Preston reserve, it is possible that *T. cancriformis* populations could still exist at this historically recorded location.

There have been repeated efforts to locate *T. cancriformis* in the historic sites around RSPB Mersehead and West Preston. The Foster's (1993) survey of 24 ponds and Adams *et al.* (2014) later survey of an additional 48 pools around the historical Mersehead sites failed to find evidence of the species' presence in the area. Both Foster (1993) and Adams et al. (2014) used conventional sediment incubation methods for large branchiopods which consisted of incubating sediment in water conditions suitable for hatching, and examining the water for nauplii. This methodology is labour intensive and presents multiple problems. Primarily, favourable conditions are required for hatching eggs (Kuller & Gasith 1996, Eder et al. 1997,

Schönbrunner & Eder 2006, Kashiyama et al. 2010). Secondarily, eggs may still remain dormant as a bet-hedging strategy (Takahashi 1976) to spread reproductive risks over time (Seger & Brockmann 1987). Both can greatly reduce the successful detection of the species. An action of the current Biodiversity Action Plan (BAP) (JNCC 2010) for the species in the UK is to carry out surveys to find any new sites. Given the limitations of the technique used in previous surveys, and the need to assess the presence of relic populations in the area prior to any translocation attempt, a new survey of the historic sites around RSPB Mersehead and West Preston was needed.

In this study we carried out an intensive survey of temporary pools at the Mersehead and West Preston locations using the method developed by Sellers et al. (2017; Chapter 2). With this method we directly target the resting eggs of *T. cancriformis*, rather than hatched nauplii, to determine the presence of viable diapausing egg banks of the species.

3.3 Methods

Temporary pool sites from across the RSPB Mersehead and West Preston reserves were sampled during 13 - 14 June 2016 respectively (Figure 1; Appendix 2: Table 1). Both locations were chosen as 1) they are close to the historic recorded *T. cancriformis* sites of Balfour-Browne (1909, 1948) and 2) they contain multiple temporary ponds which appear to be potentially suitable habitats for the species. The chosen pools partially overlap with those sampled by Foster (1993) and Adams et al. (2014), but also include new, previously unsampled sites. In total, 24 potential pool sites were sampled in a range of wider habitats including: saltmarsh/merse (sites 1 - 9), managed grassland (sites 10 - 15) and grazed pasture/wet meadowland (sites 16 - 24).

Sediment from each pool was collected following the method of Sellers et al. (2017; Chapter 2). In short, GPS readings were taken from the centre of each pool and superficial sediment was taken from eight uniformly distributed sample points within the pool boundary. A maximum of 500 g was collected from each site. Samples were then transported to the University of Hull where they were dried. Once dry, 20 g subsamples from each site underwent sucrose flotation to isolate organic matter from the sediment. The organic matter was then examined under a dissecting microscope for *T. cancriformis* diapausing eggs. DNA from any *T. cancriformis* eggs identified would then be extracted and used in a species-specific PCR assay to ascertain the viability and confirm the identity of each egg.

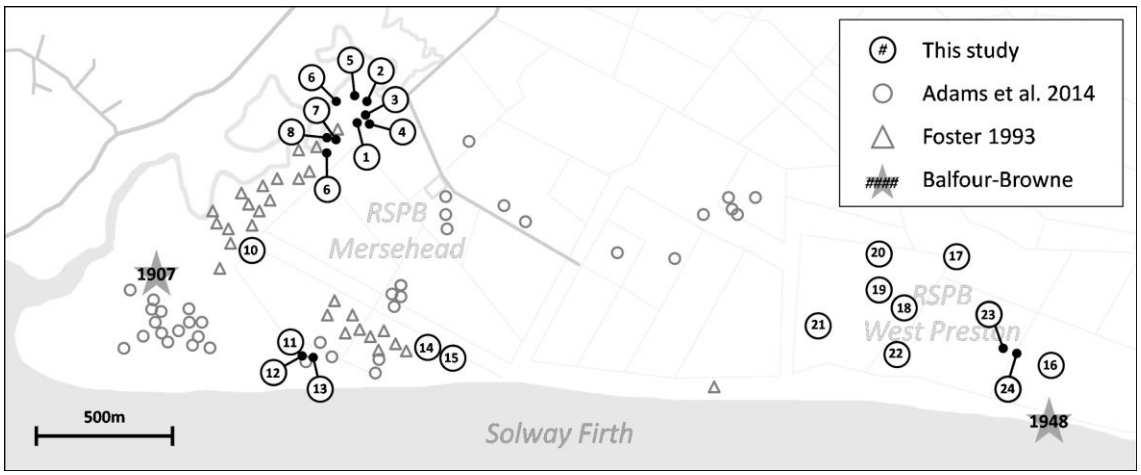

Figure 1. Location of the 24 sample sites of this study at RSPB Mersehead and West Preston. Locations for all sites previously sampled by Foster (1993) and Adams *et al*. (2014) are shown. Additionally, the approximate locations of historically recorded sites of Balfour-Browne (1907 and 1948) are indicated.

3.4 Results

No *T. cancriformis* eggs were found in the organic matter separated from the sediment at any of the sites sampled. This was confirmed by a second round of sucrose flotation using a further 20 g subsample from each sample. As no eggs were discovered the molecular aspect of the Sellers et al. (2017; Chapter 2) method was not employed. With the exception of site 17, all sites contained diapausing eggs from other organisms associated with temporary pools, such as ostracods, copepods and cladocerans.

3.5 Discussion

We applied a reliable method for *T. cancriformis* egg discovery to ephemeral pool sites located close to the historic sites on the RSPB Mersehead and West Preston reserves. Despite the presence of many suitable temporary pools there were no diapausing eggs of *T. cancriformis* detected via sucrose flotation at any of the locations sampled, confirming the high likelihood that this species has become locally extinct in the area.

Reliability of method

The sporadic nature of *T. cancriformis* discovery on the Solway was mentioned by Balfour-Browne (1948) and highlighted in the later studies. Prior to 2015, all surveys for the species on the Solway used conventional net or sediment incubation methods. For example, on 4 September 2008, after a large emergence of *T. cancriformis* at WWT Caerlaverock, upper saltmarsh pools along the length of the Southwick Burn at RSPB Mersehead were hand netted

(by LRG) to search for adult individuals - again this method identified no *T. cancriformis*. These coarse survey methods can easily be confounded by the adult organism's benthic habits, rapid life history, short habitat life span, and bet-hedging strategies in terms of whether or not viable eggs will hatch given the right conditions.

Although this study utilised a reliable method for *T. cancriformis* egg isolation from sediment samples (Sellers et al. 2017; Chapter 2), the positive detection of the species relies on the presence and identification of eggs from a relatively small sediment sample. The non-homogenous distribution of eggs within the pool boundary (Thiéry 1997) may have led to eggs being entirely missed, yet this should have been mitigated for by the sampling strategy from multiple sites across the pool. Each sample underwent two treatments of subsamples utilising the highly effective sucrose flotation method of Sellers et al. (2017; Chapter 2) (adapted from Gómez & Carvalho (2000)), and the likelihood of any collected eggs in a subsample being missed is extremely low. However, the success of all *T. cancriformis* sampling methods relies heavily on large egg banks with sufficient egg density, and therefore, the potential to produce adults in a suitable hydroperiod, so increasing the species' detectability. It is possible that relict egg banks of low density exist in Mersehead. However, considering the extensive surveys carried out (Foster (1993), Adams et al. (2014) and the current study), the number of suitable temporary pools identified and sampled through time (Figure 1), it seems extremely likely that *T. cancriformis* is actually extinct at these historically recorded sites. In contrast, the species has been readily discovered in new pools in the Caerlaverock area using a variety of different techniques across a number of years. If *T. cancriformis* were present in the Southwick area then it seems sampling temporary pools so exhaustively using all these methods would have resulted in its rediscovery.

Suitability of habitat and management

At the time of sampling, RSPB Mersehead was undertaking a habitat restoration regime to maintain existing temporary pools and create new ones for natterjack toads (*Epidalea calamita*) (Colin Bartholemew, pers. comm.). This method of land management and grazing stock rotation, to provide optimal over-winter feeding conditions for migrating wetland birds such as geese, has seen an increased incidence of *T. cancriformis* discoveries across the WWT Caerlaverock reserve (LRG, pers. obs., Sellers et al. (2017); Chapter 2). All of the sampled pools of this study, with the exception of site 17, showed an existing or emerging community suitable for *T. cancriformis*. This was evidenced by ostracod, copepod and cladoceran diapausing eggs within the sediment, pond organisms that are known prey of *T. cancriformis* (Boix et al. 2006).

The future: Triops cancriformis reintroduction/translocation?

T. cancriformis is found across Europe and the Solway Firth is on the northern limit of the species' global distribution (Zierold et al. 2007). The loss of the original Balfour-Brown site (1907) by the mouth of the Southwick water (Feber et al. 2011) demonstrates the vulnerability of the Solway population to coastal squeeze. Since its loss to the sea this site is now located on saltmarsh beside the Southwick water, evidencing the dynamic nature of the Solway coast. Translocation or reintroduction of the species to historic or new sites along the Solway coast would allow for the species' persistence in the face of habitat loss due to storm events or future sea level rise. Translocation attempts of the New Forest *T. cancriformis* population have had mixed success. Two deliberate introductions in the 1970s were monitored for three subsequent years, yet the species was only detected 30 years later at one of the sites (Feber et al. 2011). *Ex-situ* conservation breeding of *T. cancriformis* has also been employed (Hughes 1997), creating captive egg banks to better preserve the species against habitat loss. This opens up the potential of captive egg banks or reared adults being used for licensed translocation or reintroduction. Following the Best Practice Guidelines for Conservation Translocations in Scotland (National Species Reintroduction Forum 2014), reintroduction of *T. cancriformis* to the RSPB Mersehead and West Preston reserves would be a low risk action with considerable benefit. Potentially suitable habitat is already available on the reserve and donor populations of *T. cancriformis* are easily maintained and transported (Hughes 1997). Additionally, the success of the reintroduction can be monitored long term with species-specific detection (see Sellers et al. (2017); Chapter 2). The continued existence of a large *T. cancriformis* metapopulation 20 km further east along the coastal habitats at the WWT Caerlaverock reserve holds promise for future conservation, *ex-situ* or otherwise, and possible reintroduction/translocation attempts in already existing or newly created pools.

3.6 Conclusion

Although no *T. cancriformis* populations or egg banks were discovered across the sampled locations, many potentially suitable temporary pools were identified at RSPB Mersehead and West Preston within which temporary pool specialist guilds of invertebrates were found. These pools could prove to be ideal for any licensed reintroduction attempt of *the species* to these historic locations from the nearby WWT Caerlaverock reserve. The high connectivity between pool sites via waterfowl movements, e.g. of internationally important numbers of Svalbard barnacle geese (*Branta leucopsis*), and grazing livestock could help to increase *T. cancriformis* presence along the Solway coast.

3.7 Acknowledgements

We would like to thank Colin Bartholemew and RSPB Mersehead and West Preston for allowing us to sample the reserve and for the information provided regarding reserve management practices.

Chapter 4

Mu-DNA: a modular universal DNA extraction method adaptable for a wide range of sample types*

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4.1 Abstract

Efficient DNA extraction is fundamental to molecular studies. However, commercial kits are expensive when a large number of samples need to be processed. Here we present a simple, modular and adaptable DNA extraction 'toolkit' for the isolation of high purity DNA from multiple sample types (modular universal DNA extraction method or Mu-DNA). We compare the performance of our method to that of widely used commercial kits across a range of soil, stool, tissue and water samples. Mu-DNA produced DNA extractions of similar or higher yield and purity to that of the commercial kits. As a proof of principle, we carried out replicate fish metabarcoding of aquatic eDNA extractions, which confirmed that the species detection efficiency of our method is similar to that of the most frequently used commercial kit. Our results demonstrate the reliability of Mu-DNA along with its modular adaptability to challenging sample types and sample collection methods. Mu-DNA can substantially reduce the costs and increase the scope of experiments in molecular studies.

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4.2 Introduction

Extraction of double stranded DNA (dsDNA) from samples is essential for molecular studies. However, the inevitable co-extraction of contaminants, in particular humic substances, phenolic compounds and proteins, inhibit polymerase chain reaction (PCR) and other downstream applications (Tebbe & Vahjen 1993, Wilson 1997). Numerous published methods and commercial kits are available for the extraction of high purity DNA suitable for downstream applications. Many published methods are complex and designed for expert use, while commercial kits are readily accessible for those with little experience.

The DNeasy extraction kits (Qiagen) are simple, accessible and widely used. Although designed for specific sample types many studies have adapted their use across sample types. DNeasy PowerSoil, or aspects thereof, has been used for stomach, gut or faecal analysis of invertebrates (Knapp et al. 2010, O'Rorke et al. 2015), fish (Koinari et al. 2013, Bolnick et al. 2014), reptiles (Lau et al. 2013, Colston et al. 2015), birds (Vo & Jedlicka 2014, Lewis et al. 2016), mammals (Parfrey et al. 2014, Ishaq & Wright 2014), and in particular the Human Microbiome Project (Aagaard et al. 2013). DNeasy Blood and Tissue has been used for studies of environmental DNA (eDNA) from water samples (Rees et al. 2014, Spens et al. 2016, Niemiller et al. 2017). Although widely used, commercial kits are expensive and separate kits can be required for different sample types. DNA extraction using commercial kits is therefore a significant cost factor which limits the scope of experiments in molecular studies and increases the costs of genetic biodiversity monitoring.

Here we present a modular universal DNA extraction method (Mu-DNA) to address the issue of the many kits, protocols and expense, for low cost application across multiple sample types. Mu-DNA is a cost-effective and adaptable high-throughput spin column-based protocol for the extraction of high purity DNA from multiple sample types. This is not a *de novo* method but an accessible combination of multiple aspects from recent and classical procedures for DNA extraction and purification. The method is based around easy-to-prepare reagents with an absolute minimum of pH adjustment required. As a modular approach it uses reagent combinations dependent upon the sample type; soil, tissue or water. The method consists of five simple steps, all interchangeable between protocols, based around spin column DNA purification. We compared the performance of our Mu-DNA method, in particular dsDNA yield, purity, downstream inhibition and extracted DNA molecular weight to that of the widely used commercial extraction kits: DNeasy PowerSoil, DNeasy Blood and Tissue and DNeasy PowerWater (Qiagen). Finally we demonstrate the performance of the method in a comparative metabarcoding of fish community composition from lake water DNA extractions.

4.3 Methods

Solutions and reagents

We provide optimised Mu-DNA protocols for soil, tissue and water samples (Detailed protocols can be found at: dx.doi.org/10.17504/protocols.io.nbedaje and Appendix 3: Article 1). Each protocol consists of five stages for DNA extraction: lysis, inhibitor removal, silica binding, wash and elution (Figure 1). Mu-DNA uses a lysis buffer modified from Brolaski et al. (2008). The buffer incorporates guanidine thiocyanate to denature proteins (Pitcher et al. 1989), trisodium phosphate to release adsorbed DNA (Ogram et al. 1987) and ethylenediaminetetraacetic acid (EDTA) to reduce DNA oxidation from metal ions (Lloyd & Phillips 1999). A sodium dodecyl sulphate (SDS) solution is added to the lysis buffer in all protocols to disrupt lipid membranes and degrade proteins. The presence of both EDTA and SDS at the lysis stage inhibits nuclease activity (Williams et al. 1980), greatly reducing the degradation of DNA. For soil extractions the SDS additive includes aluminium ammonium sulphate to reduce humic substances (Braid et al. 2003). For soil and water filter extractions bead milling is performed for unbiased high yield DNA liberation (Robe et al. 2003). Tissue extractions have a Proteinase K incubation period for enzymatic lysis and protein digestion. Soil and water lysates are purified with a contaminant and inhibitor removal solution. This contains ammonium acetate to precipitate proteins (Crouse & Amorese 1987), aluminium ammonium sulphate (Braid et al. 2003) and calcium chloride (Wechter et al. 2003, Singh et al. 2014) to remove contaminants and inhibitors, in particular remaining humic substances and fine sediment particles. DNA is subsequently bound to a spin column silica membrane under chaotropic conditions with guanidine hydrochloride (Davis et al. 1997). Ethanol washes then remove remaining contaminants prior to elution.

The stages of Mu-DNA are designed to be modular and interchangeable between protocols to facilitate optimisation of extraction methods for a given sample type. For example, a bead milling or inhibitor removal stage can be incorporated into a tissue extraction protocol and a tissue wash stage added to a soil or water extraction protocol. All processes are scalable based upon initial sample amount or transferred supernatant volumes.

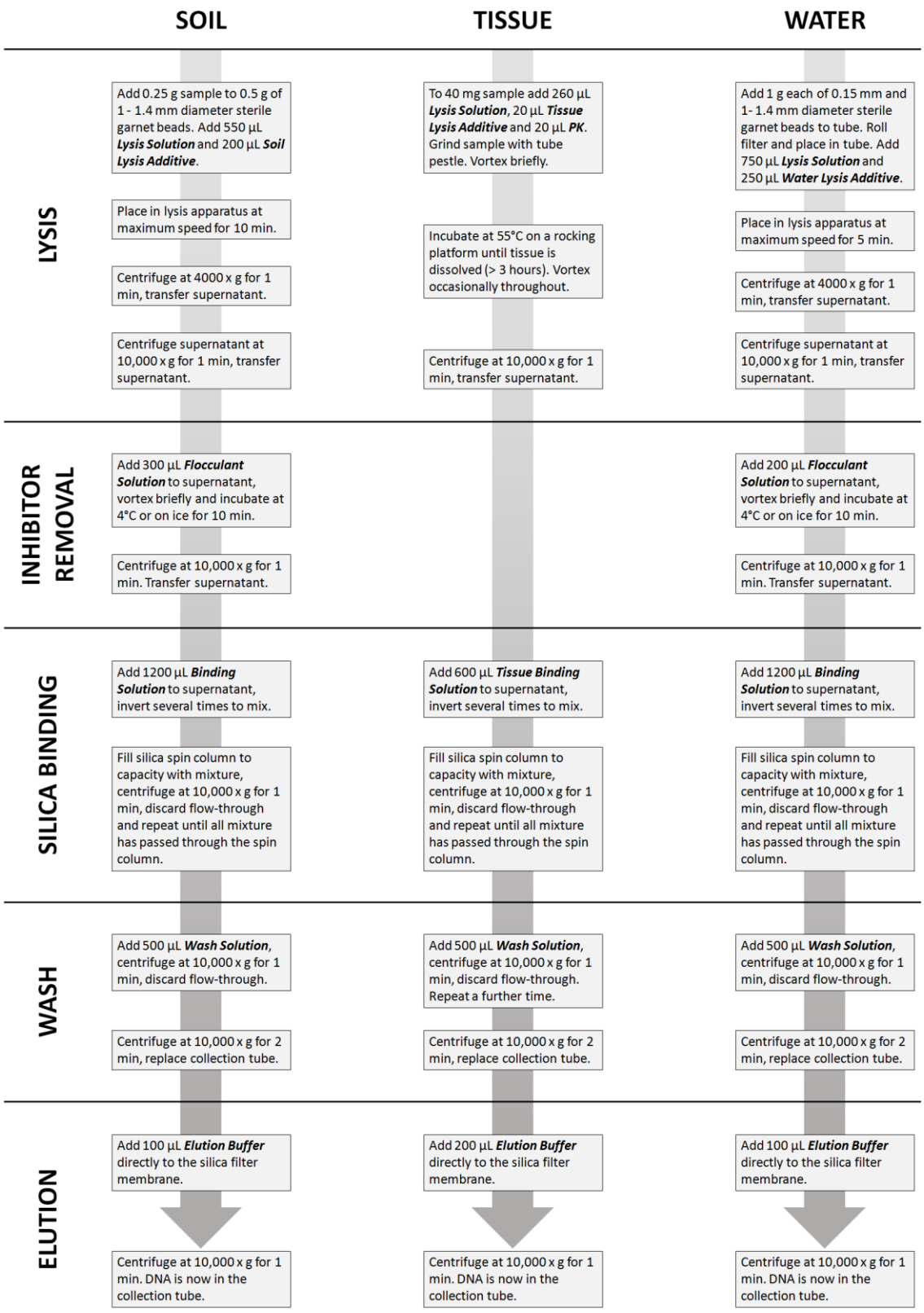

Figure 1. Simplified Mu-DNA extraction protocols for soil, tissue and water samples. All extractions use stock and working solutions and are divided into five interchangeable stages: lysis, inhibitor removal, silica binding, wash and elution.

Comparison of DNA yield and quality

To determine the performance of Mu-DNA, isolated DNA yield and purity was compared to that from the relevant commercial kit across soil, stool, tissue and water samples (Table 1). Molecular weight of extracted DNA from soil, tissue and water samples was compared between respective methods. Three to five biological replicates were performed per extraction method for each sample.

Sample selection

For each sample type three different samples (A, B and C) were selected for comparison (Table 1). Sample A represented a commonly encountered sample of its type whereas B and C were representative of more challenging samples.

Sample preparation

Soil samples were collected from three soil types: A (garden soil; high organic content), B (ephemeral pool sediment; high clay content) and C (diesel polluted soil; high contaminant levels). All samples were loosely mixed at collection. In sterile laboratory conditions 5 g of each sample was put through a 2 mm mesh sieve to remove large particulate debris before being thoroughly homogenised with a pestle and mortar. The homogenate was separated into multiple 0.25 g (wet weight) subsamples and stored at -20°C until required for extraction.

Stool samples were collected from three species with different diets: A (European hedgehog, *Erinaceus europaeus*; omnivore), B (Greylag goose, *Anser anser*; grazer) and C (Otter, *Lutra lutra*; carnivore, high number of volatile organic compounds). In sterile laboratory conditions each sample was thoroughly homogenised with a pestle and mortar. The homogenate was separated into multiple 0.25 g (wet weight) subsamples and stored at -20°C until required for extraction.

Tissue samples were taken from ethanol preserved specimens of three species: A (Cichlid, *Nimbochromis livingstonii*; muscle tissue), B (Woodlouse, *Oniscus asellus*; high chitin content) and C (Earthworm, *Lumbricus terrestris*; mucus rich with soil gut contents). Multiple 25 mg (dry weight) subsamples of specimens were removed and stored at -20°C until required for extraction.

Three water samples types were collected: A (shallow eutrophic lake; high sediment load and faecal matter), B (ephemeral pool mesocosm; turbid, high algal content) and C (deep oligotrophic lake; low particulate matter). After collection, samples were transported on ice and stored at 4°C until filtered. Filtering took place less than 16 hours after collection in sterile

laboratory conditions. Each water sample was thoroughly mixed by pouring, and then split into two subsamples of equal volume. Subsamples were vacuum filtered through sterile 47 mm diameter 0.45 µm Whatman cellulose nitrate membrane filters (GE Healthcare), labelled and stored at -20°C until required for extraction.

Table 1. Samples used for comparison of methods in this study. Shown are the amounts of each sample used per extraction method used: either Mu-DNA or the relevant commercial kit (Qiagen DNeasy).

Sample	Description	Area sampled	Sample amount	Extraction methods	Lysis apparatus	Replicates
Soil A	Garden soil	Topsoil - surface 5 cm	0.25 g	PowerSoil Mu-DNA: Soil	Tissuelyser II	5
Soil B	Ephemeral pool sediment	Topsoil - surface 5 cm	0.25 g	PowerSoil Mu-DNA: Soil	Tissuelyser II	5
Soil C	Diesel polluted soil	All available	0.25 g	PowerSoil Mu-DNA: Soil	Tissuelyser II	3
Stool A	*Erinaceus europaeus*	All available	0.25 g	PowerSoil Mu-DNA: Soil	Tissuelyser II	5
Stool B	*Anser anser*	All available	0.25 g	PowerSoil Mu-DNA: Soil	Tissuelyser II	5
Stool C	*Lutra lutra*	All available	0.25 g	PowerSoil Mu-DNA: Soil	Tissuelyser II	5
Tissue A	*Nimbochromis livingstonii*	Flank muscle	25 mg	Blood and Tissue Mu-DNA: Tissue	NA	5
Tissue B	*Oniscus asellus*	Lateral half	25 mg	Blood and Tissue Mu-DNA: Tissue	NA	3
Tissue C	*Lumbricus terrestris*	Central segments	25 mg	Blood and Tissue Mu-DNA: Tissue	NA	3
Water A	Shallow eutrophic lake	Shoreline surface	150 ml	PowerWater Mu-DNA: Water	Tissuelyser II	5
Water B	Ephemeral pool mesocosm	Surface	50 ml	PowerWater Mu-DNA: Water	Vortex Adapter	3
Water C	Deep oligotrophic lake	Shoreline surface	1 L	PowerWater Mu-DNA: Water	Vortex Adapter	5

DNA extraction

DNA extractions of replicate samples followed the protocol of Mu-DNA for the sample type or the relevant DNeasy kit (Table 1). Protocols were modified as follows: Lysis and DNA purification for all protocols were carried out using identical lysis apparatus and spin columns. Soil and stool samples were lysed in 2 ml microcentrifuge tubes (Starlab) on a TissueLyser II (Qiagen) at 30 Hz for 10 minutes. Water samples were lysed in 7 ml Bijou tubes (Sigma-Aldrich) on either a TissueLyser II at 30 Hz for five minutes or Vortex Genie (Scientific Industries) with Vortex Adapter (Mobio) at maximum speed for five minutes. The DNeasy PowerSoil and DNeasy PowerWater Bead Tube contents were transferred to the new tube type prior to lysis. Tissue samples were lysed overnight for identical time periods and incubated at the temperatures specified per protocol. Where required all available supernatant was transferred and reagent volumes were adjusted accordingly. EZ-10 DNA Mini Spin Columns (NBS Biologicals) were used for DNA purification in all protocols. Elution buffers used in each protocol were added to spin column membranes and left to incubate at room temperature for one minute before final collection. A single elution of the specified volume was performed for each protocol.

Extracted DNA yield, purity and downstream inhibition

dsDNA yield from all extractions was measured with a Qubit 3.0 fluorometer high-sensitivity (HS) dsDNA assay (Invitrogen). Isolated DNA purity was measured with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific) recording A_{260}/A_{280} and A_{260}/A_{230} ratios for all extractions (see Olson & Morrow (2012)). To test for the presence of any inhibiting factors in downstream applications, PCRs were run on all extractions. No PCR additives, such as BSA, were used to enhance PCR amplification. DNA extractions were amplified using the broad range DNA barcoding primers LCO1490 and HCO2198 (Folmer et al. 1994). PCRs were 25 µl final reaction volumes composed of 1 µl template DNA, 12.5 µl MyTaq Red Mix (Bioline), 9.5 µl ddH_2O and 1 µl of each 10 µM primer. All PCRs were performed on Veriti 96-Well Thermal Cyclers (Applied Biosystems) under the cycling conditions: 180 s at 94°C, 37 x (30 s at 94°C, 60 s at 52°C, 90 s at 72°C), 600 s at 72°C, 600 s at 4°C. PCR products were visualised on 1.5% agarose gels. All amplifications were given a PCR index score in comparison to a strong positive as follows: no amplification (0), weak amplification (1), moderate amplification (2) and strong amplification (3). Inhibition was considered present in an extraction if its index was ‘0’. To determine the extent of inhibition exhibited in samples, those with a PCR index of ‘0’ underwent further PCRs at 1:10, 1:100 and 1:1000 dilutions.

Extracted DNA integrity and molecular weight

To assess the integrity and molecular weight of DNA from the Mu-DNA protocols for soil, tissue and water, extractions were compared to those of their commercial counterparts. The highest yielding sample extractions per method were chosen from the highest yielding sample type. 5 µl of the selected extractions were visualised on a 0.5% agarose gel against a GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher Scientific).

Adaptability of Mu-DNA

To demonstrate its adaptability, Mu-DNA was optimised for samples where inhibition (PCR indices of '0') was evident. Optimised protocols were then compared to the relevant commercial kit in fresh extractions from sample remnants.

Fish metabarcoding of lake water DNA extractions

Sample collection and preparation

A minimum of 2 l of water was collected from 13 shore sample sites around Windermere (Lake District, Cumbria, UK). Samples were transported on ice. Under sterile laboratory conditions, samples were thoroughly mixed by pouring and split into paired 1 l subsamples to be filtered. Filtering took place less than 16 hours after collection and filters were stored as above. DNA extractions followed the protocol of Mu-DNA: Water described above or DNeasy PowerWater. Identical lysis and purification conditions for both protocols were maintained: all filters were lysed in DNeasy PowerWater Bead Tubes and MB Spin Columns (Qiagen) were used for purification of all subsamples. Lysis was performed on a Vortex Genie (Scientific Industries) with Vortex Adapter (Mobio) at maximum speed for five minutes.

Library preparation

A double-indexed library was prepared following a 2-step PCR based protocol (Kitson et al. 2018) using primers for the vertebrate 12S mitochondrial gene region (Riaz et al. 2011, Kelly et al. 2014). In short, an initial PCR reaction amplified the target region using individually indexed 12S primers for each lake water DNA sample. To minimize PCR and sequencing bias, three sets of three PCR replicates per sample were performed to create three technical replicates with individual library indices. Collection blanks were included in PCRs along with positive (*Maylandia zebra*) and negative controls. Replicate PCR products were pooled and purified using double-size selection with Mag-Bind RNXPure Plus beads (Omega Bio-tek) to remove nonspecific products and primer dimers. Final library concentration was assessed via qPCR assay using the NEBNext library quantification kit (New England Biolabs) and diluted as

required to a final concentration of 4 nM. The final library was run at 15 pM concentration with 10% PhiX on an Illumina MiSeq using 600 bp V3 chemistry. A detailed protocol can be found in Appendix 3: Article 2.

Bioinformatics and data analyses

metaBEAT, a custom bioinformatics pipeline (https://github.com/HullUni-bioinformatics/metaBEAT), was used to process sequencing outputs. The workflow consisted of the following steps: (i) demultiplexing; (ii) trimming, merging and quality filtering; (iii) chimera detection; (iv) clustering; (v) taxonomic assignment against a curated database. A low-frequency noise threshold approach was used to remove potential false positives from the metaBEAT data (Hänfling et al. 2016), only records exceeding a minimum proportion (0.001) of read counts in a sample where accepted as "true" positive records. Remaining reads were converted to relative species abundance (%) of assigned reads.

Statistical analysis

All statistical analysis was performed using R 3.2.5 (R Core Team 2016) using the VEGAN package (Oksanen et al. 2017). DNA yield and purity measures for extractions were compared with a linear model using planned contrasts between methods per sample. Metabarcoding of lake water DNA extractions were analysed using an analysis of similarity (ANOSIM) of relative species abundance across all replicates between methods. Non-metric multidimensional scaling (NMDS) ordination was used to visualise differences in extraction methods across all replicates grouped by site and extraction.

Costing of extraction methods

A cost per extraction was calculated for Mu-DNA: Soil, Tissue and Water. Costs per extraction were compared to those of DNeasy PowerSoil, DNeasy Blood and Tissue and DNeasy PowerWater respectively. All costs used for comparisons were based on undiscounted list prices (GBP excluding VAT and shipping) for chemicals, plastics (excluding pipette tips) and Qiagen kits.

4.4 Results and discussion

Extracted DNA yield, purity and downstream inhibition

Our Mu-DNA method exhibited similar, if not significantly higher, dsDNA yields than the DNeasy kits for most extractions (Figure 2, Appendix 3: Table 1). The DNeasy kit achieved significantly higher dsDNA yields than Mu-DNA only for Tissue B (woodlouse) (t = 6.42, p = <0.001). A_{260}/A_{280} ratios for all extractions were similar or significantly higher for Mu-DNA (see Appendix 3: Table 1) except Stool B, which was significantly lower than the DNeasy kit (t = 11.03, p = <0.001). Soil B had higher A_{260}/A_{230} ratios from the DNeasy kit whereas Soil C had a significantly higher value from Mu-DNA (t = -4.95, p = <0.001). Stool A had higher A_{260}/A_{230} values from Mu-DNA whereas Stool B and C had higher values from the DNeasy kit. All A_{260}/A_{230} purity measures for tissue extractions were similar. All water sample extractions had higher A_{260}/A_{230} measures from Mu-DNA. PCR inhibition (PCR index of '0' in some or all extractions) was only detected in commercial kit extractions for Soil C and Mu-DNA extractions for Stool B and C. All other samples exhibited complete PCR success from both methods. Mu-DNA had a higher PCR index than the DNeasy kit for Tissue B despite having lower dsDNA yield. PCR inhibition was overcome by extraction dilution (1:10) for DNeasy kit extractions for Soil C and Mu-DNA extractions for Stool C. However, Mu-DNA extractions for Stool B failed to amplify across all extractions at any dilution tested indicative of high level inhibition.

The DNeasy kits reliably extracted inhibition-free DNA from all sample types except Soil C (diesel polluted soil). Compared to this baseline of extraction success our Mu-DNA protocols, with the exception of two samples (Stool B and C), performed similarly. Therefore the three basic Mu-DNA protocols we provide for soil, tissue and water are highly suitable for many sample types. Our unmodified protocols successfully extracted inhibition-free DNA from 10 out of 12 of the samples tested in this study. Modification of our protocols for the more challenging samples is described later (see Adaptability of Mu-DNA).

We used A_{260}/A_{280} and A_{260}/A_{230} UV absorbance measures via spectrophotometry to determine the quality of DNA extractions as suggested by Olson & Morrow (2012). The ideal measures for pure DNA are shown in Figure 2, yet in some cases they are exceeded. These measures can be influenced by many aspects, such as invertebrate chitin (Athanasio et al. 2016) and RNA. Spectrophotometry of extracted DNA can be affected with the presence of co-extracted RNA by inflating A_{260} values, therefore the ratios used for purity evaluations are skewed upwards. In our study we refrained from the use of RNase so as to give a true representation of the method in an unmodified state. Should RNA-free DNA be required for any sample type we

suggest an RNase A treatment for a short incubation period (< 1 hour) post-lysis. As purity measures can be affected by many factors, extracted DNA quantity and quality can therefore only reliably be ascertained by a combination of high-sensitivity dsDNA assays, gel electrophoresis visualisation of extracted DNA and intensity of PCR amplification success.

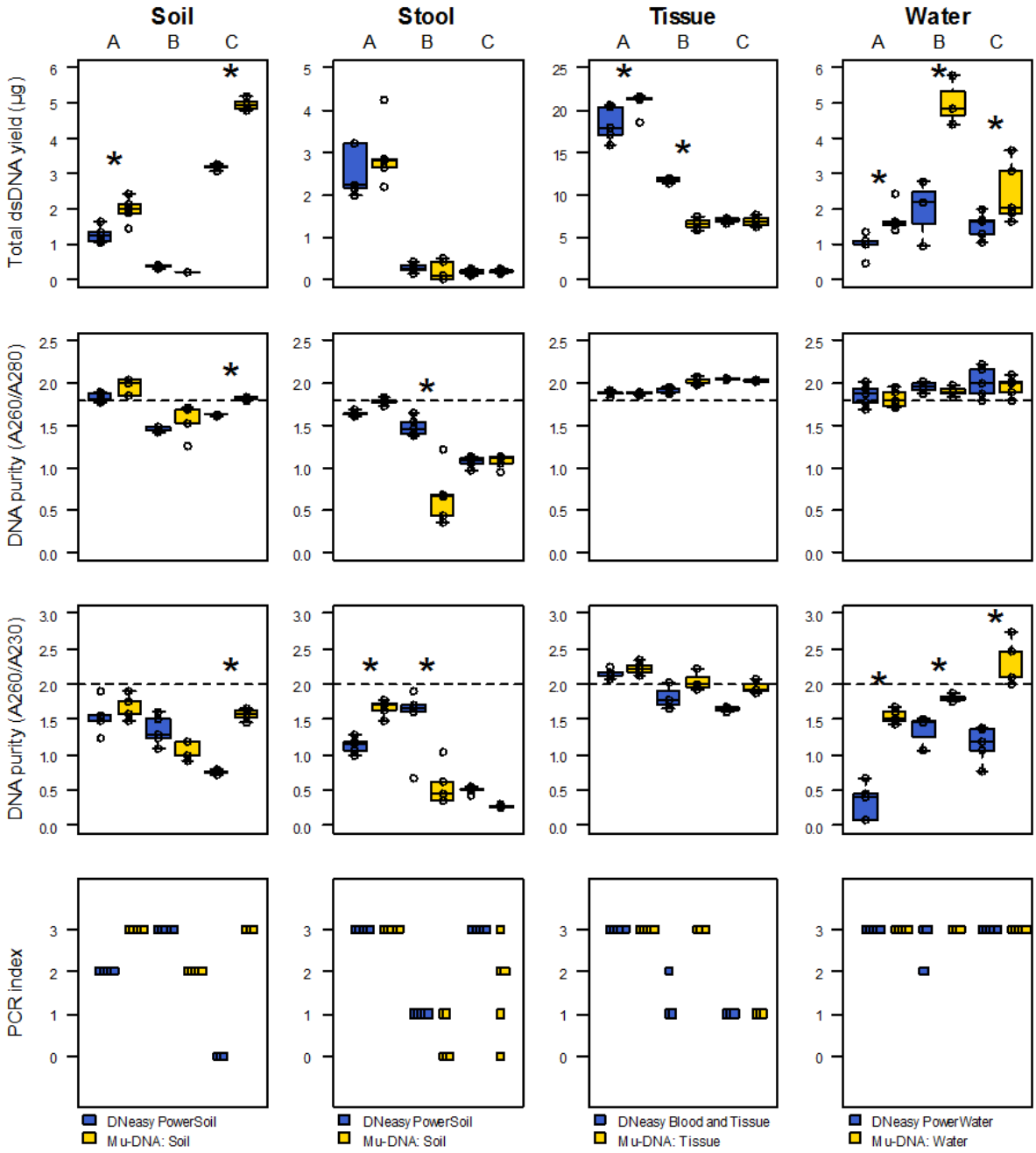

Figure 2. Isolated dsDNA yield, purity and PCR index of samples used in the comparison of methods. Total dsDNA yield, A_{260}/A_{280}, A_{260}/A_{230} ratios and PCR indices are shown for soil, stool, tissue and water samples per method. Horizontal dashed lines indicate ideal measures of A_{260}/A_{280} and A_{260}/A_{230} ratios for pure DNA. Asterisks indicate significant differences between methods (planned contrast linear model, $p<0.05$).

Extracted DNA integrity and molecular weight

The highest yielding extractions per method (Qiagen DNeasy or Mu-DNA) from Soil C, Tissue A and Water B were selected for DNA integrity and molecular weight visualisation (Figure 3). All extractions had a molecular weight of approximately 10 kbp or higher. Similar integrity of extracted DNA was observed in the soil and water samples, however DNeasy Blood and Tissue had poor integrity compared to Mu-DNA: Tissue.

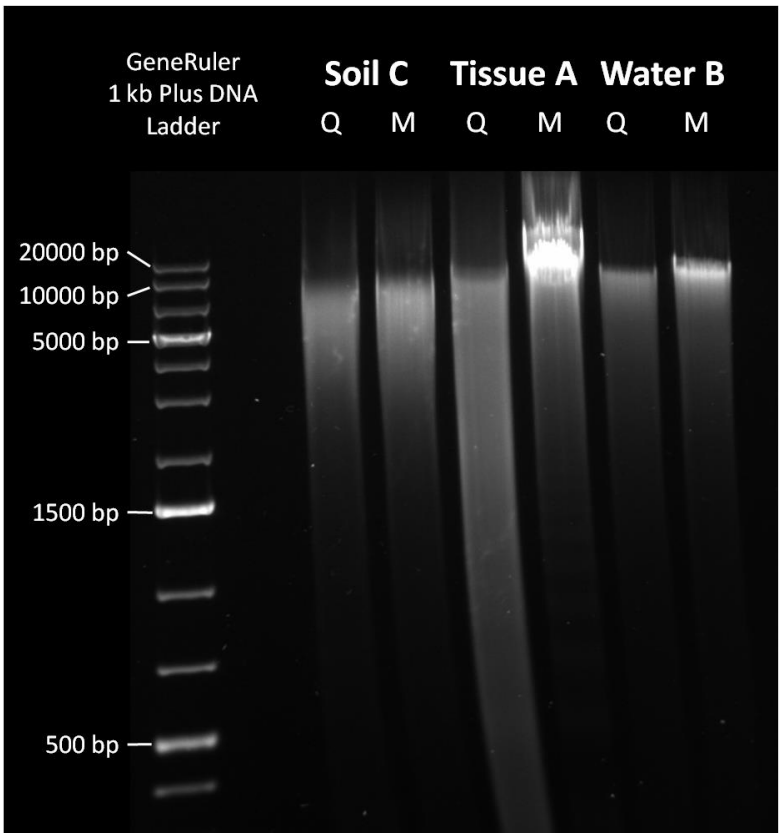

Figure 3. Integrity and molecular weight of soil, tissue and water sample extractions from the methods compared in this study. Shown are the highest yielding extractions per method from Soil C, Tissue A and Water B. Extractions are indicated by relevant method for sample type; DNeasy (Q) or Mu-DNA (M).

High molecular weight DNA extraction is desirable for many next generation sequencing (NGS) studies. It also allows for long range PCR amplification of whole mitochondrial genomes from eDNA samples (Deiner et al. 2017). Bead milling lysis has been shown to cause shearing of nucleic acids, resulting in low molecular weight of extracted DNA (Bürgmann et al. 2001). Our method yielded DNA of ≥ 10 kbp (Figure 3) in bead milled extractions but shearing is still present, evident in an extended smear. However, Mu-DNA protocols exhibited increased concentrations of higher molecular weight DNA than their commercial counterparts. Reducing bead milling times or enzyme digestion temperatures are both possible with Mu-DNA to reduce DNA shearing depending upon user end requirements. Additional measures can be

taken to reduce the effects of physical and enzymatic shearing of DNA during sample preparation, extraction and even handling (see Klingstrom et al. (2018)), yet these could become time consuming for very large sample numbers.

Adaptability of Mu-DNA

PCR inhibition was present in DNA extractions of two samples for the Mu-DNA protocol: Stool B and C. The modular aspect of the Mu-DNA method was employed to optimise extractions for each of these samples to achieve complete initial PCR success. For Stool B, a tissue lysis stage that incorporated bead milling was used. 0.25 g of the sample was added to 0.5 g of 1 - 1.4 mm garnet beads. A 2.5 x volume tissue lysis mixture was added. Soil protocol bead milling was performed followed by overnight tissue protocol incubation. The extraction then followed the soil protocol with a tissue protocol wash stage. For Stool C, the soil protocol was modified with a tissue protocol wash stage. These modifications improved DNA purity for both sample types with successful PCR amplification (Figure 4).

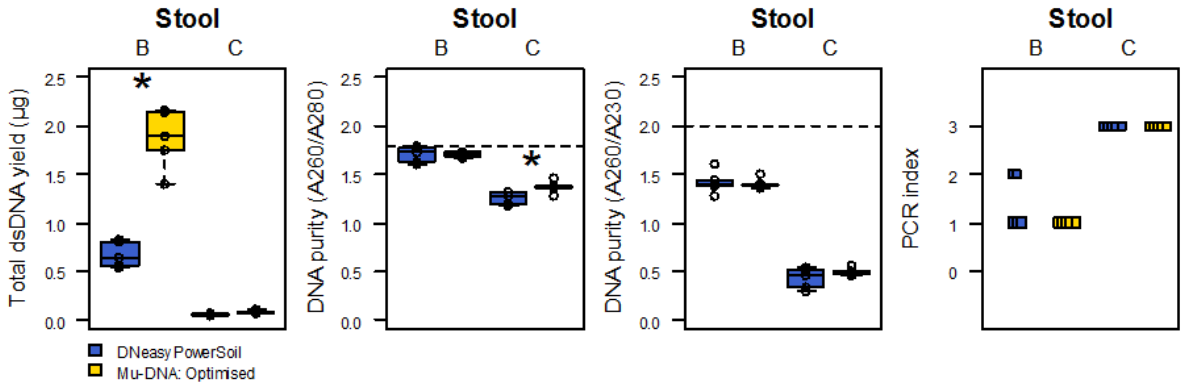

Figure 4. Optimised Mu-DNA protocols for stool samples that previously failed to achieve inhibition-free DNA. Optimised protocols are compared to DNeasy PowerSoil. Total dsDNA yield, A_{260}/A_{280}, A_{260}/A_{230} ratios and PCR indexes are shown for stool samples B and C. Horizontal dashed lines indicate ideal measures of A_{260}/A_{280} and A_{260}/A_{230} ratios for pure DNA. Asterisks indicate significant differences between methods (planned contrast linear model, $p<0.05$).

The modular adaptability of Mu-DNA allows for its application across different sample types or integration into existing protocols. For example, Spens et al. (2016) made use of DNeasy Blood and Tissue in their protocols for water filters, including Sterivex filters. We adapted these protocols and found them to be easily changed to use a Mu-DNA: Tissue/Water protocol. For this we recommend beginning with a tissue protocol lysis, adjusting the volumes as required, then following the water protocol from inhibitor removal through to elution. In this way contaminants are greatly reduced and there is no need for extra purification of extractions (unlike with Niemiller et al. (2017)). However we found that neither DNeasy Blood and Tissue

or the adapted Mu-DNA: Tissue/Water protocol could achieve inhibition-free DNA from turbid, algal rich waters (Water B) as effectively as DNeasy PowerWater or Mu-DNA: Water (GSS pers. obs.). Solid phase reversible immobilization (SPRI) DNA purification, based on Rohland & Reich (2012), can achieve higher DNA yield and purity than spin column based protocols (Vo & Jedlicka 2014). The Mu-DNA method can be easily converted to SPRI purification by replacing the silica binding step with an SPRI protocol. However, Vo & Jedlicka (2014) found SPRI to only have improved performance with less contaminated samples, such as avian oral and cloacal swab extractions. SPRI DNA purification is therefore best reserved for relatively clean environmental sample types, in particular clear lake and stream waters, or tissue samples (see Mayjonade et al. (2016)).

Our modular approach to DNA extractions is not a new concept. Lever et al. (2015) developed a modular extraction method for multiple environmental samples. Although a more complex protocol it is nonetheless highly efficient and many aspects of the study can be applied to Mu-DNA. For example, fine tuning of pH and phosphate concentration for lysis of specific sample types could lead to increased DNA yields. Our method uses chemical flocculation of inhibitors from extracted DNA and is pH sensitive (see Dong et al. (2006)). For this reason we did not explore the higher pH lysis of Lever et al. (2015) and it remains an aspect open for future investigation.

Metabarcoding of Lake water DNA extractions

After the application of noise filtering thresholds to read count data, both methods detected the same 15 fish species, 14 of which were previously recorded in Lake Windermere (Hänfling et al. 2016). Broadly, individually sequenced samples cluster by site when visualised with NMDS ordination with some variance between replicates (Appendix 3: Figure 1). Although species detected varied between method replicates per site (Appendix 3: Figure 2) there was no significant difference between methods in overall species relative abundance (ANOSIM: R = -0.02, p = 0.93) and both methods produced high similarity species profiles for the lake as a whole (Figure 5). This shows that Mu-DNA produces DNA of sufficient quality for metabarcoding approaches even when target DNA concentration is low and that no bias is introduced through the choice of extraction method.

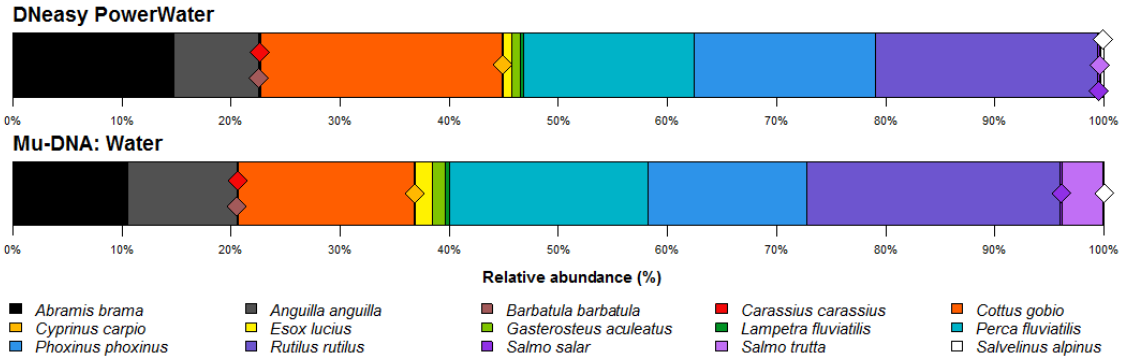

Figure 5. Species profiles of Windermere from metabarcoding of extractions using the compared methods of this study. Relative species abundance (%) of assigned reads is given per method; DNeasy PowerWater or Mu-DNA: Water. Positioning of species is arbitrary and arranged alphabetically. Diamonds indicate the position of low abundance species in the profiles for both methods.

Costing of extraction methods

Mu-DNA protocols cost less per extraction than the commercial kits to which they were compared (Table 2). Initial consumable costs for our method are higher than purchasing a single commercial kit yet the number of extractions covered by this cost is considerable (Appendix 3: Dataset 1). For the cost comparison institutional discounts were not considered. Were they to have been taken into account the cost of Mu-DNA would be appreciably lower. Lower costs, combined with a modular application across multiple sample types, makes the method an attractive alternative to commercial kits.

Table 2. Cost per extraction for Mu-DNA protocols and the commercial kits compared in this study.

	Cost per extraction (GBP)
DNeasy PowerSoil (100)	5.24
Mu-DNA: Soil	0.71
DNeasy Blood and Tissue (250)	2.92
Mu-DNA: Tissue	0.67
DNeasy PowerWater (100)	7.03
Mu-DNA: Water	0.83

4.5 Conclusion

The DNA extraction method presented here, Mu-DNA, achieved high purity DNA yields suitable for PCR and other downstream applications. Mu-DNA is an exploration of the concept of a rapid, modular approach to DNA extraction from a wide range of sample types. Our modular

approach to DNA extraction performed as well as, if not better than, the commonly used commercial kits even across challenging samples. This modular adaptability has the potential to be applied to any sample, creating a bespoke DNA extraction to achieve the desired results for the user. As a single, cost effective and comparable alternative to multiple commercial kits, the reliable performance of Mu-DNA allows it to reduce the costs and increase the scope of molecular studies and experiments.

4.6 Acknowledgements

Many thanks to Dr James Kitson for the inspiration to design Mu-DNA. Dr Dave Lunt for advice on extraction optimisation. Dr Peter Shum for advice on multiple extraction techniques and sample donation. Marco Benucci, Rosetta C. Blackman, Dr Robert K. Donnelly, Lynsey Harper and Jianlong Li for sample donation and participation in initial protocol optimisation and comparisons. Robert Jaques, Alan Smith and Vic Sweetez for sample collection. Dr Michael A. Braim for sampling permission. Dr Hayley Watson for assistance in field water sample collection. Dr Lori Lawson Handley for feedback on manuscript drafts. Dr James Gilbert for R advice and proofreading. Lisa Malm for adopting our method and the feedback given. Dr Ian Winfield and the Windermere FBA site for laboratory facilities during fieldwork.

Chapter 5

A rapid molecular assay for detection of viable *Triops cancriformis* diapausing egg banks from multiple samples

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5.1 Abstract

The tadpole shrimp, *Triops cancriformis* (Bosc, 1801), is a temporary pool specialist invertebrate native to Europe classified as endangered across its distribution. Surveying existing and potential sites is one of the main conservation strategies for the species however, this is laborious using traditional means. To better serve the conservation of *T. cancriformis* a rapid method to detect its egg banks deposited in temporary pool sediments is required. Here we develop and present a highly effective process for the rapid spot testing of multiple sediment samples for viable *T. cancriformis* diapausing eggs. We provide an optimised sucrose flotation technique and a bespoke Mu-DNA extraction protocol for optimal DNA capture from resting *T. cancriformis* eggs. We also demonstrate the sensitivity of the method to detect a single viable egg within a sample. This increased efficiency for species-specific molecular detection of *T. cancriformis* will benefit the conservation efforts for the species across its distribution range.

5.2 Introduction

The tadpole shrimp, *Triops cancriformis* (Branchiopoda; Notostraca) (Bosc 1801), is a temporary pool specialist invertebrate native to Europe (Hughes 1997, Zierold et al. 2007). The species is classified as endangered across its distribution (Eder & Hödl 2002). In the UK, where only two populations are known, it is protected under Schedule 5 of the Wildlife and Countryside Act 1981 and has a Biodiversity Action Plan (BAP) (JNCC 2010). Action points of the *T. cancriformis* BAP include surveying to identify new sites and monitoring of existing ones. Sellers et al. (2017; Chapter 2) outlined an effective molecular method to detect viable *T. cancriformis* resting eggs isolated from sediment samples for detection and monitoring purposes. This method is easily applied to a small number of sediment subsamples and is ideal for monitoring sites known to hold *T. cancriformis* populations. However, for detection and discovery of new populations, especially from potentially large sample numbers, it becomes increasingly inefficient. The time required to isolate, identify and PCR amplify DNA extracts from eggs is considerable when applied to large scale sample collections. Additionally, due to the non-homogeneous distribution of diapausing eggs in a pool's sediment (Thiéry 1997), and therefore within a sample, replication is required to increase the chance of detection.

To better serve the conservation of this endangered species a rapid method to detect the species presence in temporary pool sediments is required. To this end we adapted the Mu-DNA: Soil extraction method of Sellers et al. (2018; Chapter 4) to be applied to the sucrose flotation filtrate isolated from temporary pool sediment samples of Sellers et al. (2017; Chapter 2) (adapted from Gómez & Carvalho (2000)). This eliminates the need for searching for individual eggs in the sucrose filtrate and allows for multiple samples to be processed in parallel. Additionally, this approach greatly reduces the amount of material to be processed for DNA extraction, with further increases time and cost efficiency. In this study we utilise the species specific detection of Sellers et al. (2017; Chapter 2) as a single PCR assay to detect *T. cancriformis* resting eggs from DNA extracted from sediment subsample replicates of a sampled site. To achieve this we:

1) Optimise the sucrose flotation to allow for the capture of resting eggs from a sediment subsample while reducing the amount of non-target organic matter.

2) Design a bespoke Mu-DNA extraction protocol for optimal DNA capture from sucrose flotation filtrates.

3) Demonstrate the sensitivity of the method to detect a single viable egg in a single subsample.

As an end result we present a detailed protocol for the rapid screening of multiple subsamples for viable *T. cancriformis* diapausing egg presence. The efficiency of the optimised method is illustrated with its application to sediment samples with and without diapausing egg banks and different egg bank densities.

5.3 Methods

Sample selection

To develop and test the protocol presented here we used negative and positive samples (Table 1). For negative samples, temporary pool sediment samples were used from three sites with no recorded *T. cancriformis* egg banks. Negative samples were screened three times using the method of (Sellers et al. 2017; Chapter 2) to confirm the absence of *T. cancriformis* eggs. To ascertain the presence of an existing temporary pool community, screened samples were also checked for presence of other aquatic invertebrate eggs (e.g. anostracans, copepods, cladocerans and ostracods). For positive samples, pool sediment samples from three known populations of *T. cancriformis* were used. Populations were selected based on holding a range of viable egg bank densities; low, medium and high (see Table 1). Viable egg bank density estimates were based on three rounds of screening using the Sellers et al. (2017; Chapter 2) method or from nauplii counts achieved from incubation of 20 g subsamples.

Table 1. Sample ID and location of samples used in this study. Sample type (negative or positive), estimated viable *T. cancriformis* eggs per 20 g subsample and sample composition are shown.

Sample ID	Country	Lat, Long (WGS 1984)	Sample type	Viable eggs/20 g	Composition
EAS	England	53.98178, -0.47544	Negative	0	Humic rich, medium organic load
KIN	England	54.00125, -0.45599	Negative	0	Clay rich, low organic load
MER	Scotland	54.88293, -3.64848	Negative	0	Humic rich, high organic load
MUR	Spain	38.19868, -1.57349	Positive	4*	Clay, low organic load, low egg density
CAE	Scotland	54.98695, -3.46206	Positive	25**	Humic rich, high organic load, medium egg density
KOE	Germany	51.32696, 14.30615	Positive	> 100***	Sand, medium organic load, high egg density

* three rounds of Sellers et al. (2017) method, viable egg count of zero in one subsample.
** estimates taken from Sellers et al. (2017).
*** three rounds of incubation, observed nauplii >100 for all subsamples.

Optimisation of sucrose flotation for isolation of resting eggs

For initial tests we followed the method of Gómez & Carvalho (2000) for isolation of resting eggs from 20 g subsamples of sediment samples. Organic matter from the flotation supernatant was collected on a 50 µm Nytal filter. However, due to the high amounts of organic matter that can be present in a given soil subsample using this method (> 4g wet weight, GSS and MRW, pers. obs.), we optimised the process to improve the resting egg isolation and produce manageable volumes of filtrate for DNA extraction. We experimented with a range of lower molarity sucrose solutions; 0.5 to 0.2 molar in 0.05 increments. The desired sucrose solution would still capture *T. cancriformis* resting eggs but with a significant reduction in non-target organic matter. A high organic matter negative sample (MER) was selected for further testing. Optimal molarity of the sucrose solution was judged by the consistent capture of low amounts of non-target matter isolated from the range of molarities tested. The chosen solution was then tested on the same negative sample (MER) but with five freshly isolated *T. cancriformis* eggs from a positive sample (KOE) introduced. This spiking procedure was tested three times and the number of *T. cancriformis* eggs collected recorded. Finally, to demonstrate the method's wider application, the solution was tested on positive samples that contained resting eggs of anostracans, cladocerans, copepods and ostracods (CAE, KOE and MUR).

Design of a bespoke Mu-DNA extraction protocol

For the extraction of DNA from sucrose flotation of organic matter from sediment samples we began with the Mu-DNA: Soil protocol. As the lysis step is based upon starting sample weight, the optimised sucrose flotation method (above) was used to considerably reduce the lysis volumes required. As diapausing eggs are highly resistant to chemical stress (Makrushin & Lianguzova 2006), only mechanical lysis methods were tested. Garnet grit bead milling in 7 ml Bijou tubes (Sigma-Aldrich) (as per Sellers et al. (2018; Chapter 4)), or 10 ml stainless steel grinding jars (Qiagen) were used. All mechanical lysis was performed on a TissueLyser II (Qiagen) at 30 Hz. Protocol optimisation was performed on positive samples (MUR, CAE and KOE). Subsamples of a high organic matter negative sample (MER) were processed alongside the tests as negative controls. After multiple tests a bespoke Mu-DNA extraction protocol was finalised.

Species-specific PCR assay

We used the species-specific short amplification primers of Sellers et al. (2017; Chapter 2) to ascertain the presence of a viable population. Although intended for shorter fragments of degraded *T. cancriformis* DNA, they were the more species-specific of the two designed primers and successful amplification was only associated with viable egg banks - those with hatched nauplii present (Sellers et al. 2017; Chapter 2: Table 2). All PCR reactions used 2 µl of DNA template following the thermocycling conditions of Sellers et al. (2017; Chapter 2): 180 s at 94°C, 37 x (30 s at 94°C, 30 s at 55°C, 30 s at 72°C), 600 s at 72°C.

Detection sensitivity

Here we tested the sensitivity of the method to detect a single viable egg in a 20 g negative control subsample (the minimum detectable density). Freshly released eggs were collected from an isolated hermaphroditic *T. cancriformis* adult (reared from CAE sediment). Released eggs were collected and stored in a 1.5 ml eppendorf tube at -20 °C until required. After sucrose flotation, multiple negative sample filtrates (EAS, KIN and MER) were spiked with a single viable *T. cancriformis* egg. The filtrate was then processed using the finalised bespoke Mu-DNA extraction protocol. A positive short amplification PCR assay determined the success of the method. Extraction blanks (lysis solution) and sample blanks (negative samples) were processed alongside the sensitivity tests as negative controls. Additionally, positive (*T. cancriformis* DNA) and negative controls were used in all PCRs.

Comparison of processing times

As a demonstration of efficiency, we compared the time for one researcher to process a single 20 g sediment subsample to DNA isolation with the presented protocol to that of Sellers et al. (2017; Chapter 2). Approximated times for processing were based on a medium density egg bank (25 eggs/20 g sediment) and excluded the initial step of sucrose flotation as it was identical in both methods. DNA extraction was calculated for the time to extract 25 eggs using the HotShot protocol (Gómez & Muñoz 2008), or for processing a single sucrose flotation filtrate with the modified Mu-DNA protocol presented here. Additionally we provide approximated processing times for both methods scaled to 10 and 20 subsamples. Sterilisation of apparatus during sucrose flotation and sample homogenisation was not considered during processing samples.

5.4 Results

The final protocol for the isolation and DNA extraction of diapausing eggs from temporary pool sediments can be found in Appendix 4: Article 1.

Optimisation of sucrose flotation for isolation of resting eggs

We found that a 0.2 M to 0.25 M sucrose solution outperformed the 2.5 M solution described in Gómez & Carvalho (2000). For ease of solution preparation a final molarity of 0.23 M was chosen, resulting in an 8% sucrose solution (80 g of sugar dissolved in water to a final volume of 1 l). Maximum filtrate wet weight from any given sample tested was 1.5 g, appreciably lower than >4 g from high organic matter samples using the original protocol (Appendix 4: Table 1). The repeated tests of the spiking procedure resulted in 100% recovery of introduced *T. cancriformis* eggs to the negative control (MER). Resting eggs of anostracans, cladocerans, copepods and ostracods, along with *T. cancriformis* eggs, were also successfully isolated using the optimised sucrose solution from positive samples (CAE, KOE and MUR) (Appendix 4: Table 2).

Design of a bespoke Mu-DNA extraction protocol

As the maximum filtrate wet weight (including filter) from any sample was 1.5 g (Appendix 4: Table 1) the Mu-DNA: Soil protocol was adjusted at the lysis step to accommodate this starting sample weight. All samples, regardless of having a lower starting weight, were treated with this scaled up lysis step. A 600 µl volume of lysate was then processed. Initial tests using garnet bead lysis and processing with the Mu-DNA: Soil protocol were unsuccessful due to PCR inhibition. Optimisation of the protocol for the higher levels of organic matter and humic substances was simply achieved. Adopting the wash and elution steps of Mu-DNA: Tissue to Mu-DNA: Soil produced PCR amplification with no inhibition. However, over three replicates, the medium egg bank density site (CAE) failed to produce successful PCR amplification despite having a known and confirmed viable population present. In fact, visual inspection of the lysates of the positive samples with higher egg densities (CAE and KOE) revealed intact *T. cancriformis* eggs. Therefore, Garnet grit bead milling was insufficient for complete tissue disruption of *T. cancriformis* eggs during lysis. In order to overcome this, we used TissueLyser II 10 ml stainless steel grinding jars (Qiagen) in the protocol, resulting in the full homogenisation of all samples. After processing 600 µl of lysate, all positive control samples gave successful PCR amplification with no indication of inhibition.

Detection sensitivity

All negative sample filtrates spiked with a single viable *T. cancriformis* egg produced successful detection in PCR assays (Figure 1). All negative controls (extraction blanks and sample blanks) had no PCR amplification with strong primer dimer present, demonstrating no evidence of inhibition.

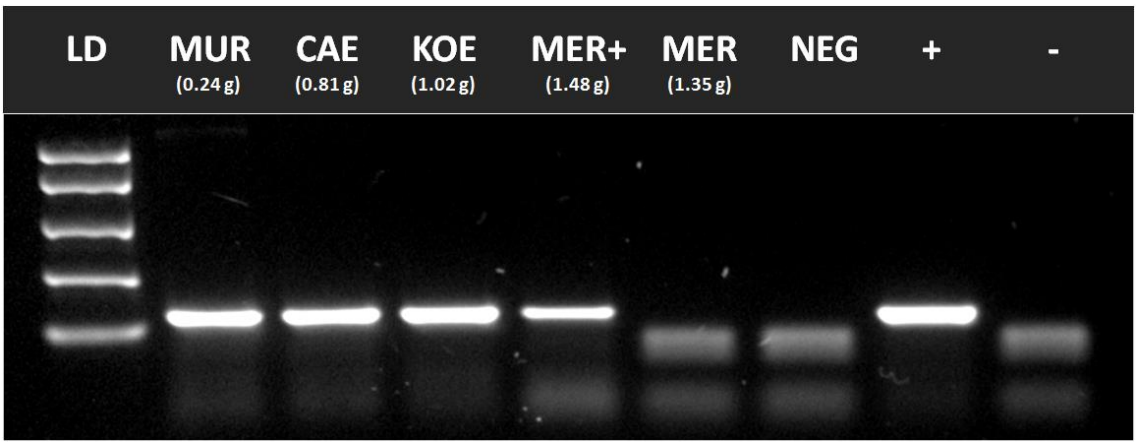

Figure 1. Gel electrophoresis of PCR assay products from a selection of sample types tested in this study. Shown are EasyLadder™ I (Bioline) molecular weight marker (LD), positive samples (MUR, CAE and KOE), spiked negative sample (MER+), negative sample (MER), extraction blank (NEG), PCR positive (+) and negative (-) controls. Where applicable, initial flotation filtrate weights are given below sample names.

Comparison of processing times

The presented protocol took less than half the time to complete for a single subsample than that of Sellers et al. (2017; Chapter 2). This was also applicable to the process when scaled up to 10 and 20 subsamples (Table 2).

Table 2. Comparison of processing times for the Sellers et al. (2017; Chapter 2) and the presented protocol. Approximated times are for processing a single (1 x), or multiple (10 x, 20 x) dried 20 g sediment subsamples from a medium density egg bank (25 eggs/20 g sediment). Sucrose flotation was ignored for comparison as it was identical in both methods.

Process	**Sellers et al. 2017 protocol**			**Presented protocol**		
No. samples	1 x	10 x	20 x	1 x	10 x	20 x
Egg isolation	15 min	2 hr, 30 min	5 hr	-	-	-
Homogenisation	-	-	-	5 min	25 min	50 min
DNA extraction	1 hr	3 hr	6 hr	30 min	2 hr	3 hr
Total time taken	1 hr, 15 min	5 hr, 30 min	11 hr	35 min	2 hr, 25 min	4 hr, 50 min

5.5 Discussion

In this study we designed and developed a highly efficient process for the isolation, DNA extraction and species-specific PCR assay of *T. cancriformis* resting eggs from sediment samples. The final protocol incorporates optimised versions of existing methods (Gómez & Carvalho 2000, Sellers et al. 2017, 2018) resulting in a single streamlined high throughput protocol that allows for the rapid spot-testing of multiple samples in parallel. This leads to an increase in the number of samples screened for the presence of viable *T. cancriformis* egg banks.

The optimised sucrose flotation process was primarily designed to reduce the levels of organic matter for DNA extraction, yet it still successfully isolated resting eggs from other organisms such as anostracans, cladocerans, copepods and ostracods. The application of this sucrose flotation, with its much reduced organic matter load, allows for easier isolation of aquatic invertebrate eggs, such as *Daphnia*. With the adjustment of filter pore size it can be reapplied to smaller invertebrate resting eggs (as was its original purpose in Gómez & Carvalho (2000)). Additionally, the complete homogenisation of the sample in the protocol, using the TissueLyser II 10 ml stainless steel grinding jars (Qiagen), means no resting eggs remain intact and any present are completely lysed. The optimised Mu-DNA extraction of Sellers et al. (2018; Chapter 4) isolated sufficient DNA for strong PCR detection of a single *T. cancriformis* egg in subsample filtrate with higher organic matter loads (Figure 1: MER+). The final DNA extract can be used in PCR assays for many species and could be used in the future for DNA metabarcoding of diapausing egg stages, to assess the biodiversity of temporary pool specialist organisms.

In the comparison of the presented protocol to that of Sellers et al. (2017; Chapter 2) the approximated times are based on a known egg density subsample and could be misleading. The presented protocol has to complete PCR and gel electrophoresis to determine viable egg presence, adding two hours or more to the overall process. If there were no eggs detected with the Sellers et al. (2017; Chapter 2) protocol the processing times are greatly reduced, stopping after egg isolation. However, despite the Sellers et al. (2017; Chapter 2)) protocol being faster to determine egg presence, there remains the chance of an egg being missed due to human error. This error is removed in the presented protocol, there is no need to search for, or identify, *T. cancriformis* resting eggs from the isolated organic matter. This in turn opens up *T. cancriformis* population detection to researchers that have no previous experience in the morphological identification of resting eggs.

The protocol is split into three distinct stages: (i) sucrose flotation, (ii) sample homogenisation and (iii) DNA extraction. The distinction in the stages allows for suitable stopping points after

sucrose flotation and sample homogenisation where the products of these stages can be stored at -20 °C until required for further processing. The process is then completed by the species specific detection using the short amplification primers of Sellers et al. (2017; Chapter 2) in a single PCR assay per subsample. Upon successful detection, sites can then be processed with the complete Sellers et al. (2017; Chapter 2) method to estimate egg bank density and viability.

5.6 Conclusion

Here we present a highly effective process for the rapid testing of multiple sediment samples for viable *T. cancriformis* diapausing eggs. The sensitivity of the method results in the successful PCR detection of a single viable egg from as much as 1.5 g of sucrose flotation filtrate. Additionally, removing the need to identify resting eggs prior to DNA extraction allows samples to be processed by researchers without prior identification experience. Although initially designed for *T. cancriformis*, the presented protocol has wider applications as it can be used for viable egg bank detection of other aquatic invertebrates. The increased efficiency for species-specific molecular detection of *T. cancriformis* will benefit the conservation effort for the species across its distribution.

5.7 Acknowledgements

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Chapter 6

Conservation genomics of the Eurasian tadpole shrimp, *Triops cancriformis*

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6.1 Abstract

A species' resilience to climate change and habitat unpredictability relies on populations holding sufficient genetic diversity. The genetic diversity of a species is affected by sequential colonisation, long distance dispersal and sexual system. These factors are all present in the endangered Eurasian tadpole shrimp, *Triops cancriformis* (Bosc, 1801). This large branchiopod exhibits differing reproductive modes between populations which range from selfing hermaphroditism to gonochorism. The species is endangered in the UK, with two remaining populations but little is known of the genetic diversity of these. Filling this knowledge gap will better inform conservation management efforts for *T. cancriformis* in the UK. In this study we utilise a ddRAD-seq GBS approach to determine the genetic diversity and population differentiation of populations of *T. cancriformis* representing all known sexual systems in the species. Our results show that, in comparison to androdioecious and gonochoristic populations, the selfing hermaphrodites of the UK have extremely low genetic diversity and extreme homozygosity at both population and individual levels. Due to this low diversity we determine the UK populations should be considered a single evolutionary significant unit (ESU) for the purposes of translocation, reintroduction and *exsitu* conservation breeding.

6.2 Introduction

A species' resilience to climate change and habitat unpredictability relies on populations holding sufficient genetic diversity (Hughes et al. 2008, Pauls et al. 2013). Geographically distant populations may hold differences in genetic diversity as adaptive potential and selection pressures are related to environmental heterogeneity (Chevin et al. 2010). The sexual systems within a species can also have a dramatic impact on its genetic diversity (Ellegren & Galtier 2016) with sexual outcrossing organisms expected to have more diversity than self-fertilising organisms (Glémin & Galtier 2012). Self-fertilisation effectively halves the number of gametes sampled for reproduction and decreases effective population size (Glémin et al. 2006), resulting in increased homozygosity and reduced genetic diversity. Sequential colonisation and long distance dispersal can also lead to a reduction in diversity, as with postglacial expansions of species from refugia (Hewitt 1999), and may select for self-fertilisation as a reproductive assurance (Baker 1955).

Genomic approaches have allowed for genetic diversity estimation in populations so that effects such as drift, isolation and gene flow that shape genetic variation of populations can be detected on an improved scale (Morozova & Marra 2008, Cutter & Payseur 2013, Edwards et al. 2016). As conservation measures rely on awareness of intraspecific genetic diversity to maintain evolutionary potential (Thakur et al. 2018), managing this natural variation as genetically distinct evolutionary significant units (ESU) can preserve localised diversity and adaptive potential (Fraser & Bernatchez 2001). The application of population genomics therefore has immense potential to inform conservation biology (Hendricks et al. 2018, Meek & Larson 2019). Determining intraspecific diversity between populations can ultimately inform the management of conservation units (CU) (Paz-Vinas et al. 2018, Waples & Lindley 2018).

The Eurasian tadpole shrimp, *Triops cancriformis* (Bosc, 1801), has a geographic distribution across Europe and is also found in Japan (Zierold et al. 2007, Feber et al. 2011). It is classed as an endangered species in many European countries (Eder & Hödl 2002). In the UK, for example, two populations (Feber et al. 2011) are the last remaining sites of a larger historic distribution (Fox 1949). Until its rediscovery at the Wildfowl and Wetland Trusts (WWT) Caerlaverock reserve in 2004 (Feber et al. 2011), the species was considered to be extant only in the New Forest (Hughes 1997) and its historic populations along the Solway Firth (Balfour-Browne 1909, 1948) having become extinct.

T. cancriformis populations across Europe have differing reproductive modes; gonochoristic (male and female in similar incidence, 100% outcrossing), androdioecious (hermaphrodite with low male incidence, intermediate outcrossing rate) and hermaphroditic (no male incidence,

100% selfing) (Zierold et al. 2007). *T. cancriformis* hermaphrodites, unlike other hermaphroditic invertebrates such as Gastropods (Jarne et al. 1993) or Poriferans (Riesgo et al. 2014), are obligatorily self-fertilising (Zierold et al. 2007) and outcross only with males. In *T. cancriformis*, outcrossing decreases toward the north of the species' distribution with many of these populations being composed of self-fertilising hermaphrodites with low to zero male incidence (Zierold et al. 2007). *T. cancriformis* populations are genetically structured likely due to founder effects, bottlenecks and genetic drift (Zierold et al. 2009, Mathers et al. 2013). Analyses of five microsatellite loci suggested that the species' genetic diversity is linked to its reproductive mode: hermaphroditic and androdioecious populations, with low to zero outcrossing and high degrees of inbreeding, have lower diversity than those that are gonochoristic (Zierold et al. 2009). However these five loci were unable to distinguish between the genetic diversity of hermaphroditic and androdioecious populations (Zierold et al. 2009). The populations of the UK, at the northern limit of the species' distribution (Zierold et al. 2007), are known to be selfing hermaphrodites (Zierold et al. 2009) and, therefore, are expected to have low levels of genetic diversity. Due to the nature of the organism's colonisation of new habitats via passive dispersal (Thiéry 1997, Green & Figuerola 2005, Vanschoenwinkel et al. 2011), the possibility of post-glacial maxima transfer of resting cysts (Mathers et al. 2013) from the southern to the more northern UK site is likely. If this were the case, given both populations are comprised of selfing hermaphrodites, the UK populations would be expected to be genetically very similar. Studies of mitochondrial DNA (mtDNA) regions from multiple populations of *T. cancriformis* show little genetic diversity present within and between populations (Zierold et al. 2007, Mantovani et al. 2008). Individuals from the two UK populations were identical in analysis of COI mtDNA gene (Sellers et al. 2017; Chapter 2) and sit within a large clade that encompasses all European *T. cancriformis* populations (Zierold et al. 2007). In contrast, population genetic analysis of microsatellite loci, which were more polymorphic than mtDNA, highlight strong differentiation between European populations (Mantovani et al. 2008, Zierold et al. 2009), however microsatellite markers were few in number and the results reflected the low genetic diversity within populations. As previous assessments of genetic diversity of *T. cancriformis* studies were based on mtDNA and microsatellite data, a higher resolution genomic approach would prove more effective in ascertaining the population genetic differentiation and the impact of sexual system in genetic diversity. This would determine if populations should be considered separate conservation units in relation to translocation, reintroduction and *ex-situ* conservation breeding, especially in the UK populations.

SNPs, single-nucleotide polymorphisms, are a reliable and highly informative marker to estimate differentiation between populations of a given organism (Helyar et al. 2011). Restriction-site associated DNA (RAD) markers are short fragments of DNA adjacent to instances of a restriction enzyme recognition site (Baird et al. 2008), which generate a reduced representation library of a genome. RAD sequencing (RAD-seq), in combination with next generation sequencing (NGS), allows for the discovery and genotyping of thousands of SNPs in hundreds of individuals (Baird et al. 2008). A modification of the original method, dubbed Double-digest RAD (ddRAD) sequencing uses two restriction enzymes to generate fragments of genomic regions between both enzyme cut sites, so increasing sampling of homologous genomic regions of sequenced individuals (Peterson et al. 2012). Genotype by sequencing (GBS) approaches such as RAD-seq (double-digest or otherwise) have been employed successfully in multiple population and conservation genomics studies (Davey & Blaxter 2010, Narum et al. 2013, Jeffries et al. 2016).

In this study we utilise a ddRAD-seq GBS approach to determine the genetic diversity and population differentiation of populations of *T. cancriformis* representing all known sexual systems in the species. We compare the selfing hermaphroditic UK populations to the populations of Königswartha (Germany, androdioecious) and Espolla (Spain, gonochoristic), both previously identified as being genetically distinct populations by Zierold et al. (2009) and Mathers et al. (2015). As genetic variation and distinction between populations can be a case for them being considered separate conservation units (Fraser & Bernatchez 2001), ascertaining the genetic distance between the UK *T. cancriformis* populations will better inform conservation management effort.

6.3 Methods

Population sampling, hatching and rearing

Sediment samples containing *Triops cancriformis* diapausing eggs were obtained from the two known sites harbouring UK populations; Caerlaverock, Scotland (CAE), and Godshill, England (GOD), under Scottish Natural Heritage licence number 42854 and Natural England licence number 2016-24031-SCI-SCI-1 respectively. Sediment samples from Espolla, Spain (ESP), and Königswartha, Germany (KOE), were sampled and sent to us by associates in each country. In order to hatch and rear *T. cancriformis* individuals for analysis, multiple 20 g subsamples of sediment from each site were mixed with 3 l of purified water in separate acrylic tanks. Tanks were maintained at 20°C and subject to a 12/12 day/night light cycle under daylight fluorescent tubes. *Triops* hatchlings were reared on ground cichlid food pellets. After a period

of four weeks mature individuals were removed from the tanks, sexed and fixed in 100% ethanol. Sexing was based on the presence of ovisacs for females (ESP) and hermaphrodites (CAE, GOD and KOE) and their absence for males (ESP and KOE). Fixed individuals were stored at -20°C until required for DNA extraction.

DNA extraction

Approximately 10 mm of a halved tail section from each individual was used for DNA extraction. Gut contents were flushed with 100% ethanol prior to extraction to remove contamination and reduce inhibitory factors. A modified Mu-DNA: Tissue extraction (Sellers et al. 2018; Chapter 4) incorporating RNase incubation and using a solid phase reversible immobilization (SPRI) magnetic bead capture method (adapted from Rohland & Reich (2012)) to isolate high molecular weight DNA (for full protocols see Appendix 5: Articles 1 and 2). DNA quantification and integrity assessment was performed with visualisation of extractions on 1.5% agarose gels. DNA purity was measured on a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific).

ddRAD library preparation

Library preparation followed a modified protocol of Kess et al. (2016) using *SbfI* and *MseI* restriction enzymes (New England Biolabs). In short, 10 µl (~ 75 ng) from each DNA extraction was digested with 2 units of each restriction enzyme. Cut-site specific adapters were then ligated to the digested DNA prior to a size selection bead purification (adapted from Rohland & Reich (2012)) to reduce adapter dimer and short fragment (< 300 bp) carryover. Samples were indexed in a PCR reaction using Illumina sequencing adapters with unique paired indices for each sample (Appendix 5: Table 1). Indexed samples were then pooled for gel size selection of ca. 300 to 700 bp fragments. Size selection was performed twice on the pooled samples, each becoming a separate sequencing library. Library concentrations were measured with a Qubit 3.0 fluorometer high-sensitivity (HS) dsDNA assay (Invitrogen) and fragment size distribution with a 2200 Tapestation (Agilent). Each library was diluted to 4 mM before being loaded at 10 pM on an Illumina MiSeq using 600 bp V3 chemistry with custom sequencing primers (for the full library preparation protocol see Appendix 5: Article 3). The two final libraries were sequenced separately.

Sequence processing and quality control

Illimina's MiSeq Reporter software was used to demultiplex sequencing reads to sequenced samples. After demultiplexing, Fastq files from the two sequencing runs were merged into

separate forward and reverse files per sample. Sequence quality was assessed across all samples with the R package seqTools (Kaisers 2017) to inform read trailing end trimming. Sample reads were trimmed using Trimmomatic version 0.35 (Bolger et al. 2014) to remove identified low quality trailing end bases, forward reads were trimmed to 150 bp and reverse to 70 bp. fastp (Chen et al. 2018) was used for adapter removal and quality filtering. Reads were again processed with Trimmomatic to a uniform minimum length and to ensure the removal of shorter non-target regions (forward reads: 150 bp, reverse: 70 bp). Finally, as Stacks uses paired end data, fastq files were rewritten using fastq-pair (Edwards 2017) ensuring remaining reads were mate paired and all singletons removed.

SNP calling and population statistics generation

We loosely followed the procedure of Rochette & Catchen (2017) however, using Stacks version 2.0 (Catchen et al. 2013) we were able to process paired end data. As adapter sequences (including cut sites) were already removed during demultiplexing of sequencing reads, Stacks *process_radtags* was primarily used to further quality control the paired end data by discarding reads with remaining low quality or uncalled bases. Sample reads were then aligned to a *T. cancriformis* hermaphrodite reference genome (Orr 2017) with BWA version 0.7.17 (Li & Durbin 2009) *mem* and output in SAM format. SAMtools version 0.1.19 (Li et al. 2009) was used to convert SAM files to sorted BAM format files. Stacks *gstacks* then merged paired ends and called SNPs and genotypes with stringent significance levels (0.01). Stacks *populations* was run for SNPs present in 80% of individuals in a population. SNP site coverage depth was calculated per individual using Samtools *mpileup*. SNP data was then cleaned to reduce SNPs from repetitive regions and sequencing errors. For this, RAD loci identified from the initial *populations* analysis with SNP coverage depth > mean + 2 SD or had > 0.75 observed heterozygosity were blacklisted. A subsequent Stacks *populations* analysis was run excluding blacklisted loci.

Population statistical analysis

To assess genetic diversity, SNP heterozygosity was first investigated at a population level. Heterozygous SNP counts per population were used as a proxy for overall genetic diversity. The inbreeding coefficient (F_{IS}) and minor allele frequency (MAF) were used to highlight the degree of inbreeding, outcrossing and bottlenecks. Genetic diversity was also assessed at an individual level by estimating individual genome-wide heterozygosity to obtain evidence for inbreeding and selfing in the four populations. Pairwise fixation index (F_{ST}) calculations between populations in addition to genetic distance and relatedness were used to highlight

differentiation in population structure. Population level statistics were generated, and all subsequent analysis performed, using the 1st SNP of each RAD locus to reduce linkage associated replication. Population observed heterozygosity and F_{IS} for expected heterozygous SNPs were calculated with Stacks *populations*. MAF were generated using PLINK version 1.9 (Purcell et al. 2007) from the Stacks *populations PLINK* format output. Individual genome-wide heterozygosity estimates were calculated from the Stacks *populations Genepop* format output, using custom Shell scripts, as the proportion of typed loci present in an individual that were heterozygous. Pairwise F_{ST} values were generated using Stacks *populations*. Genetic differentiation of individuals was analysed via principal component analysis (PCA) of allele frequencies generated from the Stacks *populations Genepop* format output with R 3.5.1 (R Core Team 2018) using adegenet version 2.1.1 (Jombart and Ahmed 2011).

6.4 Results

Population sampling

Between 18 and 20 *T. cancriformis* individuals were successfully reared from each of the four populations selected for this study (Table 1; Appendix 5: Table 1). Morphological sexing of individuals revealed males were only present in KOE (0.20) and ESP (0.56). CAE and GOD consisted solely of hermaphrodites.

Sequencing

We sequenced 78 individuals across the four populations (Table 1). Sequencing from the two MiSeq libraries produced a total of 7,206,102 raw paired end reads (14,412,204 raw sequencing reads). Of those, 3,179,645 paired end reads (0.44 of raw reads) were successfully demultiplexed to sample. After quality control and mate re-pairing of sequences 2,124,663 paired end reads remained. 3,462,348 sequence reads (0.24 of raw reads) were mapped to the reference genome assembly with an average 468,383.49 (SD 54,255.49) bases mapped per individual sampled, covering 0.35% of the 135 Mb reference genome.

SNP calling, coverage and density

After SNP data cleaning, Stacks *populations* analysis called 1,525 SNPSs over 408 RAD loci present in at least 80% of individuals within a single population. Mean coverage depth of called SNPs present in all individual samples was 26.66 (SD 15.73). Estimated SNP density (%

polymorphic SNPs/base pair) varied widely across populations with the UK populations having the lowest (Table 1).

Table 1. Sample size (N), SNP calling and estimated SNP density (% polymorphic SNPs per base pair) of the four *T. cancriformis* populations used in this study.

	N	Total sites	SNPs	Polymorphic SNPs	SNP density
CAE	20	178,161	1,413	37	0.021
GOD	20	171,811	1,39`9	32	0.019
KOE	20	157,229	1,372	214	0.136
ESP	18	143,086	1,356	824	0.576

Population genetic diversity and Hardy-Weinberg equilibrium

The UK *T. cancriformis* populations, CAE and GOD, exhibited extremely low numbers of heterozygous SNPs compared to KOE and ESP (Figure 1). GOD had more observed heterozygous SNPs than CAE. In addition, the levels of observed heterozygosity in the UK populations were much lower than that seen in the androdioecious and gonochoristic populations. KOE and ESP had higher numbers of heterozygous SNPs than the UK populations, ESP with the higher of the two. The number of heterozygous SNPs and their observed heterozygosity differed with sexual system and showed an increase with higher male incidence, and hence, outcrossing level. The few heterozygous SNPs found in CAE and GOD did not deviate from Hardy-Weinberg equilibrium (HWE) (Figure 1). KOE had clear deviations of HWE in the genome as a whole, with high levels of inbreeding evidenced by the larger number of SNPs with positive F_{IS}. ESP showed genome-wide HWE, in a normal and more balanced distribution pattern as expected for an outcrossing organism.

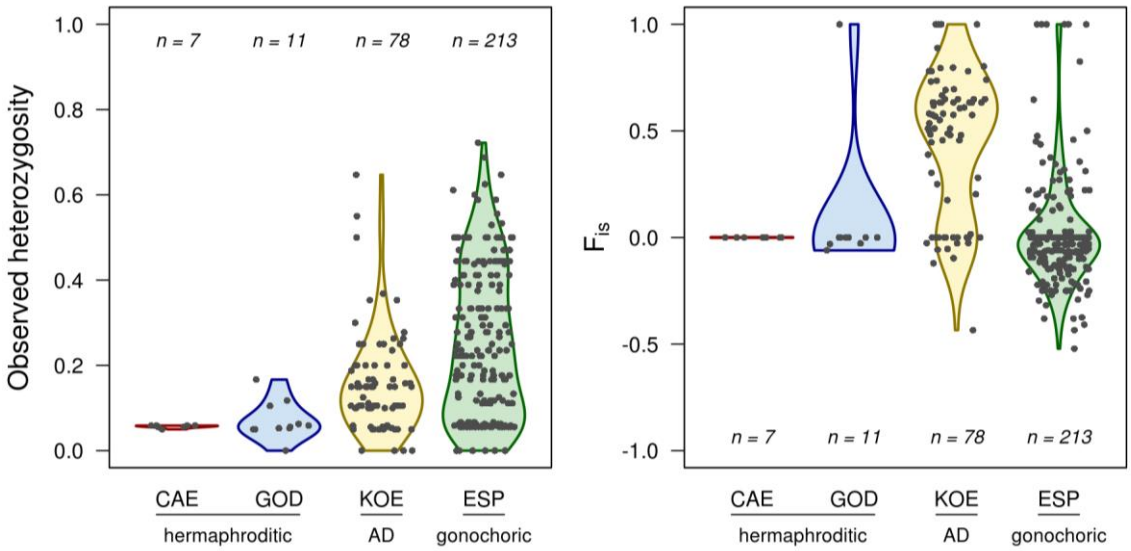

Figure 1. Observed heterozygosity and inbreeding coefficient, F_{IS}, for expected heterozygous SNPS in the four *T. cancriformis* populations used in this study. Number of expected heterozygous SNPs in each population are shown (*n*). Sexual system is shown below each population (AD: androdioecious).

Minor allele frequency

CAE and GOD both had low minor allele frequencies reflecting the levels of heterozygous SNPS in these populations (Figure 2). KOE and ESP had a markedly increased incidence of higher frequencies in comparison to the UK populations.

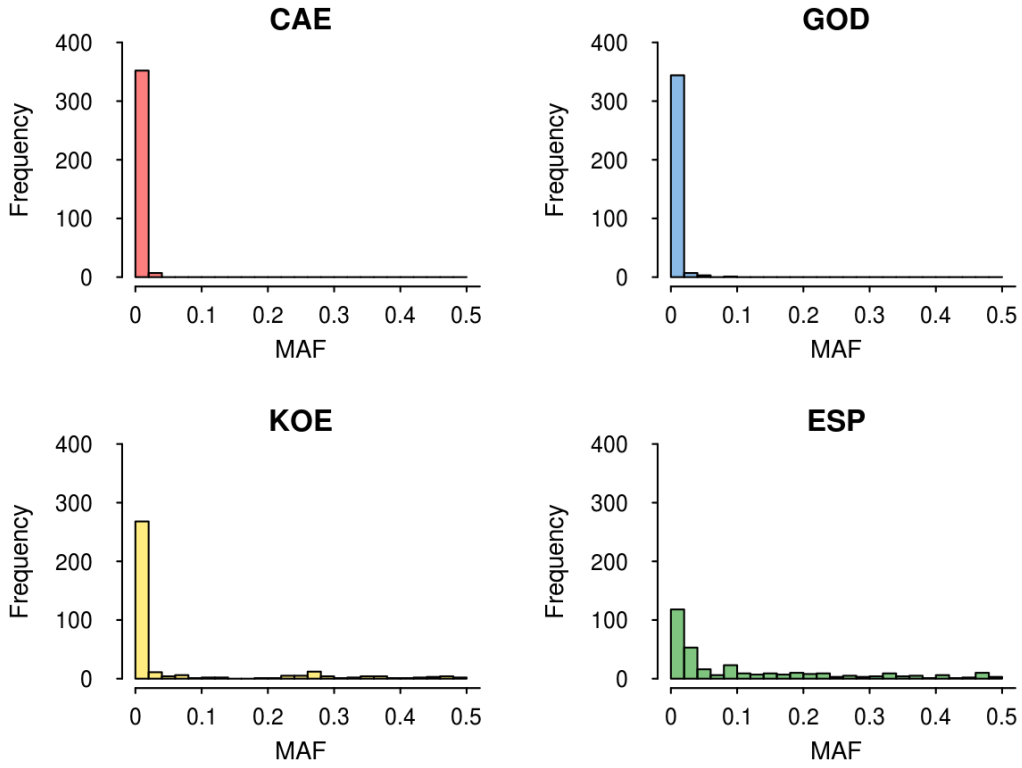

Figure 2. Minor allele frequencies (MAF) found in the four *T. cancriformis* populations used in this study.

Individual genome-wide heterozygosity

Estimated genome-wide heterozygosity for sampled individuals varied within and between populations (Figure 3). Individuals from the UK populations exhibited close to zero heterozygosity, although GOD had slightly higher values for more individuals than CAE. Both UK populations had little variance between samples. KOE and ESP both had higher individual heterozygosity, with ESP having the highest. KOE, the androdioecious population, had lower proportion heterozygosity but had the largest range of values across samples, some individuals had as low heterozygosity as the selfing hermaphrodites, while others reached the levels of some ESP individuals. ESP had the highest heterozygosity of the four populations but had lower range across samples than KOE.

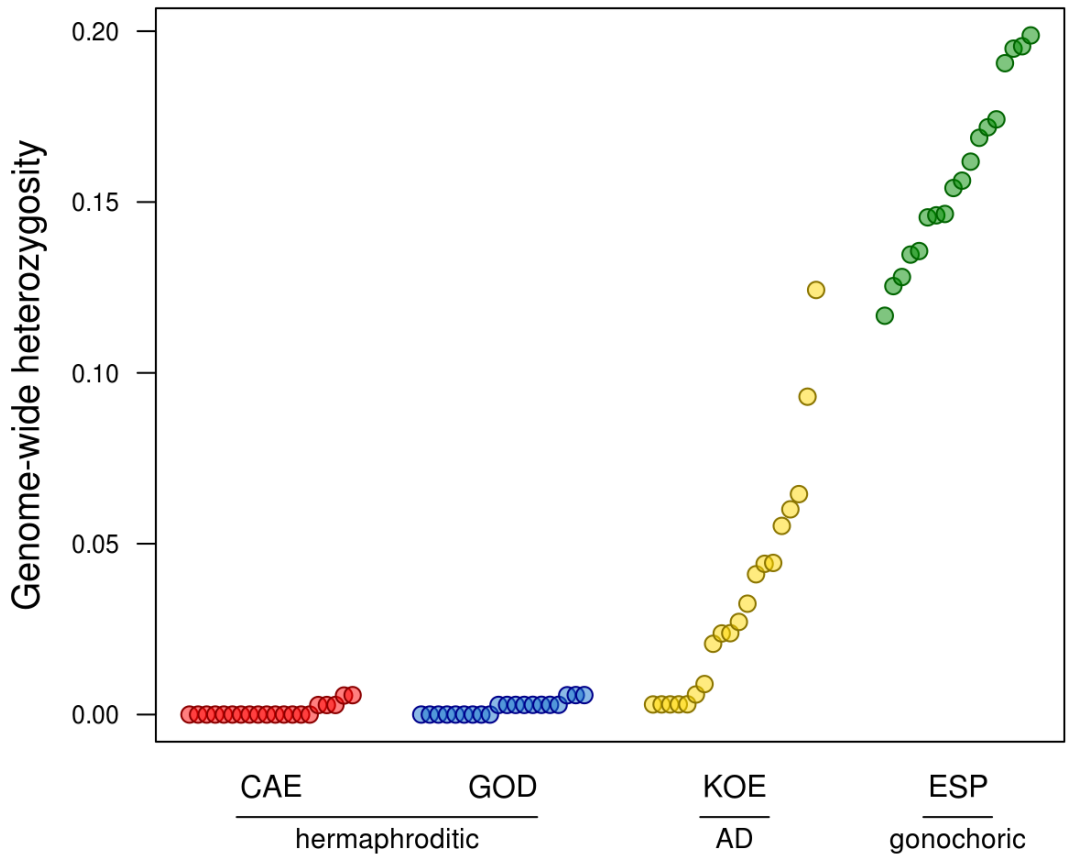

Figure 3. Estimated individual genome-wide heterozygosity in the four *T. cancriformis* populations used in this study. Each circle represents an individual and are ranked by increasing heterozygosity within population. Genome-wide heterozygosity was estimated as the proportion of typed loci present in an individual that were heterozygous. Sexual system is shown below each population (AD: androdioecious).

Population structure

Populations were strongly structured genetically (Table 2; Figure 4). ESP was highly differentiated from the remaining populations. CAE and GOD had the lowest level of

differentiation between them and similar pairwise structured difference to both KOE and ESP. In the PCA analysis, individual samples from KOE and ESP grouped discreetly by population while CAE and GOD clustered tightly together. ESP was distinctly separated from the other populations along PC 1 whereas the distinctions between KOE and the UK populations were only on PC 2.

Table 2. Mean pairwise distance matrix of fixation index, F_{ST}, for the four *T. cancriformis* populations used in this study.

	ESP	GOD	KOE
CAE	0.81	0.09	0.44
ESP		0.80	0.78
GOD			0.41

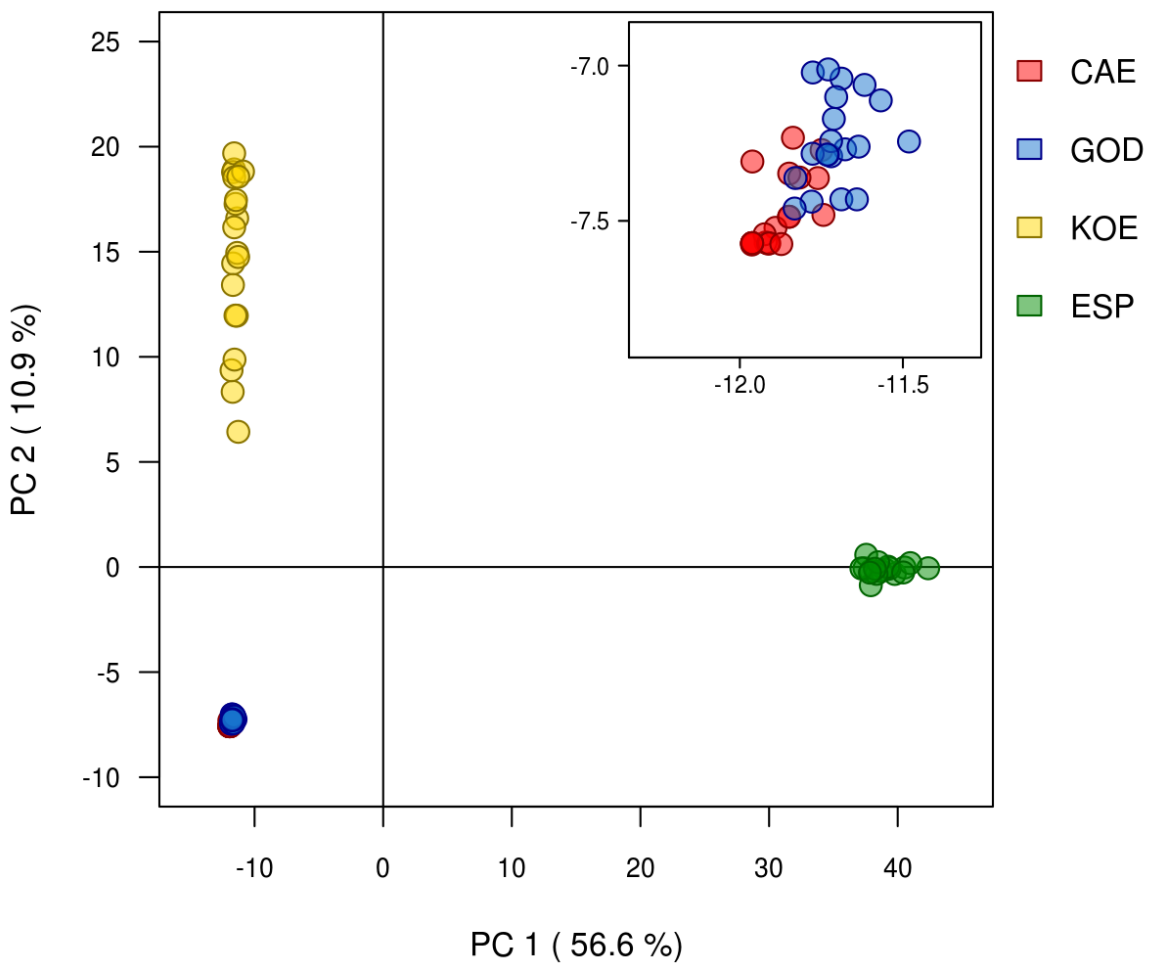

Figure 4. Principal component analysis (PCA) on the sampled individuals of the four *T. cancriformis* populations used in this study. Each individual is coloured by population. Top right inset magnifies the distribution of individuals from the UK populations (CAE and GOD).

6.5 Discussion

Our analysis of genome wide genetic diversity sampled over a thousand variant SNP sites demonstrates there is extremely little genetic diversity and low differentiation in the two UK *T. cancriformis* populations. In comparison to the androdioecious and gonochoristic populations, those of the UK, which are selfing hermaphrodites, showed extremely low SNP heterozygosity at both population and individual levels. Due to this low diversity and extreme homozygosity, the UK populations could be considered to be composed of almost genetically identical individuals.

Genetic diversity, sexual system and colonisation

Across Europe, *T. cancriformis* populations are thought to have expanded from southern European refugia in a serial colonisation pattern resulting in low genetic diversity which hampered analyses using traditional COI sequencing and microsatellite analysis (Zierold et al. 2007, 2009, Mantovani et al. 2008). However, our ddRAD-seq approach identified enough genomic variability across Europe to show that genetic diversity strongly varies with sexual system and is more pronounced in populations in which males, as the only means of outcrossing, are present, i.e. androdioecious and gonochoric populations. The maximum genome-wide heterozygosity values were found in gonochoric populations where outcrossing is obligatory, and lower in AD populations which have a proportion of selfing. On the other extreme, CAE and GOD, selfing hermaphrodites, had extremely low levels of genetic diversity. Estimated SNP density of the UK populations CAE and GOD were also extremely low, amongst the lowest estimated for animals (Leffler et al. 2012). The level of genetic diversity in UK *T. cancriformis* populations puts them on a par with mammals with low genetic diversity such as the Scandinavian wolverine, *Gulo gulo*, and the European lynx, *Lynx lynx*, (Walker et al. 2001, Hellborg et al. 2002, Leffler et al. 2012), mainly due to population reduction through human persecution. The level of genetic diversity is also similar to that of the self-fertilising nematode *Caenorhabditis briggsae* (Cutter et al. 2006, Dolgin et al. 2008, Leffler et al. 2012).

The differentiation between populations, with F_{ST} estimates similar to those of previous studies (Zierold et al. 2009, Mathers et al. 2015), show ESP was more distinctly separated from the UK populations than KOE, a pattern attributed to drift and possibly sexual system. The large genetic differentiation seen between KOE and ESP reflects the genetic diversity seen in *T. cancriformis* (Zierold et al. 2009, Mathers et al. 2015) and is also observed in other *Triops* species (Sassaman et al. 1997). As expected due to the presence of selfing in the population, the androdioecious population, KOE, had strong deviations from HWE (Mathers et al. 2015).

CAE and GOD are hermaphrodites with zero male incidence so have no outcrossing and have low genetic diversity.

The UK *T. cancriformis* populations showed low genetic differentiation. With the low genetic diversity observed in UK *T. cancriformis,* in combination with mtDNA analysis that has shown they are closely related (Zierold et al. 2007, Sellers et al. 2017; Chapter 2), it is highly likely these populations are the result of post-glacial colonisation events. This has been previously suggested for the northern European populations of *T. cancriformis* based on mtDNA and microsatellite analysis (Zierold et al. 2007, 2009). As a reproductive mode hermaphroditism has advantages for colonisation (Baker 1955), as evidenced in *T. cancriformis* by Mathers et al. (2013), with a single organism capable of generating an entirely new population. However, selfing hermaphrodites exhibit a decreased effective population size (Glémin et al. 2006) and this becomes more pronounced through founder events allowing for little adaptive selection. The lack of diversity between the UK populations almost certainly comes from geographic isolation, colonisation by selfing hermaphrodites and no genetic outcrossing with the diverse continental populations. The marginally lower levels of diversity found in CAE than GOD may provide tentative evidence of sequential colonisation of the sites from a historic southern UK population.

Implications for conservation

Genetic differentiation between the UK and those of continental populations would validate the UK populations to be considered as a separate ESU (Fraser & Bernatchez 2001). The low genetic diversity of *T. cancriformis* in the UK could result in future local extinctions due to climate change and reduced adaptive capabilities (Pauls et al. 2013). Effective population size of both UK populations, as selfing hermaphrodites, is practically nil and preserving what little diversity remains may mean translocation of individuals between populations. The WWT Caerlaverock reserve boasts a large meta population of *T. cancriformis*, spread across multiple pools only hundreds of meters apart (Sellers et al. 2017; Chapter 2), whereas the Godshill region has two known pool sites some kilometres apart (Feber et al. 2011). *Ex-situ* conservation breeding can be used to bolster population sizes by creating captive egg banks for future reintroduction (Hughes 1997), yet this will rely on the availability of existing viable egg banks. A demographic approach to the conservation of the species, concentrating in particular on available population size, would make the large meta population at WWT Caerlaverock the better suited of the two as the focus of future *ex-situ* breeding and translocation or reintroduction programmes.

6.6 Conclusion

Geographic distribution and genomic diversity can complicate a species' conservation effort (Coates et al. 2018). The UK *T. cancriformis* populations may be geographically distant, yet they were once part of a more continuous distribution (Fox 1949). This historic distribution is now gone and all that remains are two isolated populations (Feber et al. 2011) containing low genetic diversity with little genetic differentiation between them. In this study we did not look for adaptive markers that could be used for determining independent ESU status for each of the UK populations as we did not discover sufficient heterozygous SNPs in either population to give power to any adaptive marker discovery. The extremely low genetic diversity between and within these two populations would suggest that they should be considered identical, ignoring geographic distance. In the event of the loss of one of the UK *T. cancriformis* populations, based on the findings of the current study, it would be feasible and warranted to rely on the other for reintroduction.

6.7 Acknowledgements

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Chapter 7

General discussion

The conservation of *T. cancriformis* faces a number of challenges relating to the fragmented nature of the remnant populations and the inability of them to expand due to the lack of suitable habitats or dispersal vectors linking them. The extant UK populations of *T. cancriformis* are some 465 kilometres from one another and are remnants of a much larger historical distribution. Historic records of the species across the UK are patchy and spread over centuries (see Fox (1949)). This is likely due to the ephemeral nature of the species' habitat, its additional bet-hedging strategies and benthic habits that make it especially difficult to monitor with traditional methods. Additionally, there was no information on the genetic relationships between the UK populations that could be informative to the species continued conservation.

In this thesis I designed, developed and applied effective molecular methods to address the shortcomings of current methods for the detection and monitoring of *T. cancriformis* populations. Together these studies form an essential 'toolkit' for effective molecular detection of the species that can be used for both reliable identification of extant populations and long term egg bank monitoring of known sites and reintroduction attempts. Additionally, genome-wide analysis has revealed genetic relationships between *T. cancriformis* populations across its distribution. Utilising these newly developed methods I have added to existing evidence (Foster 1993, Adams et al. 2014) that *T. cancriformis* is indeed extinct at historic sites on the Solway Firth (Balfour-Browne 1909, 1948). Finally, I determined that there is strong differentiation between distant populations in Europe and that the two remaining extant populations of *T. cancriformis* in the UK have very little genetic diversity and are not genetically differentiated.

7.1 Molecular detection

The application of the molecular methods presented in this thesis will allow for rapid and reliable testing of multiple samples that results in a significant increase in detection rates in comparison to conventional approaches. Molecular detection of the species is not limited to sediment samples or isolated eggs. The Mu-DNA protocol (Sellers et al. 2018; Chapter 4) describes methods for DNA extraction from diverse sample types, including soil and water, and facilitates the capture of environmental DNA (eDNA). The cost effectiveness and efficiency of

these molecular approaches allow for up-scaling of sampling efforts and can therefore generate more meaningful ecological data sets. Water samples can be collected, filtered and the extracted DNA used in a species-specific PCR assay (as in Sellers et al. 2017; Chapter 2) to determine *T. cancriformis* presence within the water body. However, due to bet-hedging strategies (Takahashi 1976) and dependance on favourable hatching conditions (Kuller & Gasith 1996, Eder et al. 1997, Schönbrunner & Eder 2006, Kashiyama et al. 2010), it suffers from similar constraints as conventional methods: a reliance on individuals within the sampled water column. This method differs though in that it permits the use of a non-invasive, non-destructive and reliable method for detection of the species in a pool without the need to capture a single *T. cancriformis* specimen. Applying this approach to a temporary pool at intervals during the pool's lifetime can give a temporal view of the species' presence throughout a given hydroperiod.

7.2 Implications of genetic diversity for conservation

Genetic diversity within *T. cancriformis* populations is linked to its reproductive mode. Hermaphroditic populations, which are obligatory selfers, have extremely low genetic diversity, androdioecious populations combine selfing and outcrossing and have increased genetic diversity with intermediate inbreeding coefficients, whereas purely outcrossing gonochoristic populations have the highest diversity. This pattern was highlighted in observed heterozygosity of four populations and genome-wide heterozygosity in all individuals from ddRAD sequencing data (Chapter 6; Figures 1 and 3). The gonochoristic population had the highest number of heterozygous SNPs and high genome-wide heterozygosity per individual, the androdioecious slightly reduced and the purely hermaphroditic populations (from the UK) had the lowest in both measures.

The analyses of the UK populations of *T. cancriformis* determined them to be genetically highly similar, both shared the same COI mtDNA haplotype (Sellers et al. 2017; Chapter 2) and exhibited extremely low genetic differentiation (Chapter 6). These populations can, for all intents and purposes, be considered almost genetically identical. Additionally, genetic differentiation observed between the UK and those of continental populations validates the consideration of the UK populations as a separate ESU (Fraser & Bernatchez 2001). However, the low genetic diversity of the UK populations could increase the risk of local extinction due to climate change and reduced adaptive capabilities (Pauls et al. 2013).

Genetic rescue, a conservation tool by which gene flow from another population within the species is increased to an inbred population in order to increase genetic diversity and reduce inbreeding depression, has been used to improve evolutionary processes and adaptive

potential (Ingvarsson 2001, Frankham 2015). This intraspecific hybridisation is as an effective conservation management tool (Chan et al. 2019). However, populations of selfing organisms are expected to avoid inbreeding depression through purging of deleterious mutations via nonrandom mating (Glémin 2003, Arunkumar et al. 2015). This is most effective in large populations (Glémin 2003) and given the fecundity of *T. cancriformis*, capable of depositing thousands of eggs per individual (Feber et al. 2011), the effects of deleterious mutation purging should be highly efficient. In the case of UK *T. cancriformis* populations, outcrossing would only serve to increase genetic diversity within them. This would be best performed with males from an androdioecious *T. cancriformis* population, e.g. Germany (KOE), but the success of outcrossing would require substantial research prior to being employed. The UK populations may likely suffer from outbreeding depression through outcrossing leading to a costly reduction in fitness, as has been shown in wild populations of *Caenorhabditis elegans* (Dolgin et al. 2007, Chelo et al. 2014). Should it be determined a viable option, outcrossing would not be possible if UK *T. cancriformis* were to be classed as an ESU. Hybridising with a donor population would alter the genetic traits and sexual system that make the UK populations distinct enough for ESU status. In an ESU scenario the preservation of what little genetic diversity remains may simply be to translocate individuals between populations - spreading risk across populations but achieving no increase in outcrossing or evolutionary adaptive potential.

Genetic diversity in the UK *T. cancriformis* populations may not be an issue in future climate change scenarios. The temporary pool environment may be more at risk (Zacharias & Zamparas 2010), the species could still have adaptive potential but will be lost due to the lack of available habitat. Therefore, conserving and maintaining temporary pools should be the main focus for *T. cancriformis* conservation, with sites populated from *ex-situ* breeding and captive populations or habitat management conducive to natural colonisation of sites.

7.3 A revised species action plan for *T. cancriformis* in the UK

The current BAP for *T. cancriformis* (JNCC 2010) is now nine years old. It is simplistic in nature when compared to the overall conservation effort for the species, as reviewed by Feber et al (2011). To this end I describe some findings distilled from this thesis to better inform the efforts directed towards the species' conservation. A revised action plan for *T. cancriformis* will need to address availability of suitably maintained temporary pool habitats with licensed *ex-situ* breeding for captive populations and reintroduction or translocation. Finally, effective and long term monitoring is required to assess the distribution and success of reintroduction attempts.

The known sites at the WWT Caerlaverock reserve form a dynamic metapopulation (Sellers et al. 2017; Chapter 2) only hundreds of metres apart, whereas the New Forest sites are isolated to two locations (Feber et al. 2011) with kilometres between them. With its few, widely distributed sites the New Forest population may at first appear to be more at risk of local extinction than that of WWT Caerlaverock. However, as the loss of the historic Balfour-Brown site (1907) on the Solway coast (Feber et al. 2011) demonstrates, the Caerlaverock population may be at risk from coastal erosion and extreme storm events. This places both UK population sites at high risk of local extinction directly through habitat loss or destruction. Creation and management of suitable temporary pool habitat combined with reintroduction of the species will allow for a larger distribution of *T. cancriformis* than at present, so spreading the potential risk of local extinction across many populations.

Habitat provision and management

A revised action plan should recommend improving the connectivity between existing populations by creating potential colonisation sites that will form 'stepping stones' across the landscape. This connectedness will increase the success of dispersal of organisms (De Meester et al. 2005, Céréghino et al. 2008) and lead to the creation of meta-ecosystems (Gounand et al. 2018). The creation of low scrapes that could become new temporary pool environments over time would facilitate this and has been undertaken at the RSPB Mersehead and West Preston reserve (Colin Bartholemew, pers. comm.). However, these new sites will not be successfully colonised unless dispersal occurs. Triops dispersal depends on animal traffic to act as vectors between sites (Thiéry 1997, Vanschoenwinkel et al. 2011). Emphasis must be placed upon rotational low intensity grazing by cattle (as per WWT Caerlaverock reserve management; Joe Bilous, pers. comm.) or, at best, other large herbivores, ideally provided with 'scratching posts' at designated locations, to optimise egg dispersal and maintain temporary pool depth and disturbance. Importantly, cattle grazing maintains the temporary pool habitat, the diversity it contains and can mediate against hydroregime disruptions from climate change (Biggs et al. 1994, Marty 2005, Pyke & Marty 2005). Moving grazing animals from one location to another with temporary pool sites will facilitate dispersal of *T. cancriformis* eggs in sediment attached to hooves or the body (Vanschoenwinkel et al. 2011). Wildfowl such as ducks, gulls or otherwise, previously noted as being a concern in the BAP, are possibly an essential asset to the species' distribution between suitable habitat. Ingested eggs of branchiopods (when adults are predated, as described in Balfour-Browne (1948) can be potentially transferred to local or more distant sites effectively through wildfowl vectors (Proctor & Malone 1965, Green et al. 2005, Sánchez et al. 2007, Rogers 2014). Waterfowl are not removing viable eggs through predation of adult *T. cancriformis* individuals, but are instead acting as highly motile vectors of

the extremely resilient eggs to potential new habitat. The availability of suitable habitat in combination with animal vectors, such as cattle and waterfowl, has apparently lead to the creation of the significant *T. cancriformis* metapopulation at WWT Caerlaverock. This issue should be investigated in more detail to ascertain the potential *Triops* dispersal vectors in UK populations.

Detection and monitoring

Utilising the molecular methods presented in this thesis would allow for low cost and highly accurate monitoring of the species' presence within a pool, detecting eggbanks from sediment samples (Chapter 5) and ascertaining the overall viability of resting eggs (Sellers et al. 2017; Chapter 2). Low cost eDNA monitoring of sites, via DNA extracted from the water column (Sellers et al. 2018; Chapter 4) and a species-specific PCR assay (Sellers et al. 2017; Chapter 2), will allow for non-invasive passive detection of the species.

Ex-situ conservation breeding for reintroduction or translocation

Ex-situ management and captive breeding of native *T. cancriformis* could be used as an insurance against its extinction in the wild (Hughes 1997). Collection of samples for captive populations of *T. cancriformis* from Godshill was successfully achieved by Hughes (1997). After the rediscovery of *T. cancriformis* at Caerlaverock in 2004, sediment was collected for the creation of a captive population and a stored diapausing egg bank that is still viable 10 years later (Larry R. Griffin pers. comm.). The life history of *T. cancriformis* can be effectively exploited for *ex-situ* conservation breeding. The long lived and resistant diapausing egg banks can be stored long term and used to rear adults as required. As the UK populations are selfing hermaphrodites, a single individual can potentially create a whole new population and there are few concerns of sampling of the population diversity.

Captive egg banks can be incubated to rear adults for translocation to fresh or newly created habitats, alternatively these sites can be inoculated with sediment containing a captive *T. cancriformis* egg bank. The low costs of rearing *T. cancriformis*, combined with the ease with which suitable habitat can be created (Williams et al. 2007), makes *ex-situ* breeding for translocation to new habitat highly feasible. However, the success of reintroduction or translocation of a species must be assessed and requires reliable monitoring of the species over successive years (Fischer & Lindenmayer 2000). Translocation of *T. cancriformis* adults has been attempted in the 1970's at two pool sites in the New Forest National Park (Feber et al. 2011). One of these introduction attempts was successful and became the second known site of *T. cancriformis* in the area. However, the species was only successfully detected some 30

years later using traditional detection methods (Feber et al. 2011). The species' molecular detection (Sellers et al. 2017; Chapter 2, Chapter 5) can be used to reliably measure the success of reintroduction or translocation attempts and could form the basis of an introduced populations monitoring programme.

7.4 Concluding remarks

The methods and findings described in this thesis provide a solid addition to the conservation effort for *T. cancriformis* across its global distribution. The application of species-specific molecular detection, be it from resting eggs (Sellers et al. 2017; Chapter 2) or eDNA sample collection (Sellers et al. 2018; Chapter 4, Chapter 5), will greatly improve the discovery and monitoring of the species. This in turn will lead to the increased efficacy and application of multiple sample site surveys. In contrast it can also be utilised to determine absence at historic sites (Chapter 3), thus determining if reintroduction is a viable option for expansion of the species range. Through a population genomics study (Chapter 6) it has been ascertained that the UK *T. cancriformis* populations are genetically highly similar and should be considered a separate, and single, ESU to those from continental Europe. Although there is little genetic diversity in the UK populations of *T. cancriformis*, it is worth noting that the temporary pool habitat in which they exist may actually be more at risk than the species (Biggs et al. 1994, Zacharias & Zamparas 2010). To this end, it is important to create, maintain and protect this specialist environment and utilise *ex-situ* conservation breeding, under strict licensing, for reintroduction of the species if necessary. The molecular 'toolkit' presented in this thesis will prove to be a worthy asset for the long term monitoring and conservation of *T. cancriformis* in the face of future climate change and potential habitat loss.

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Appendix 1: Additional information for Chapter 2

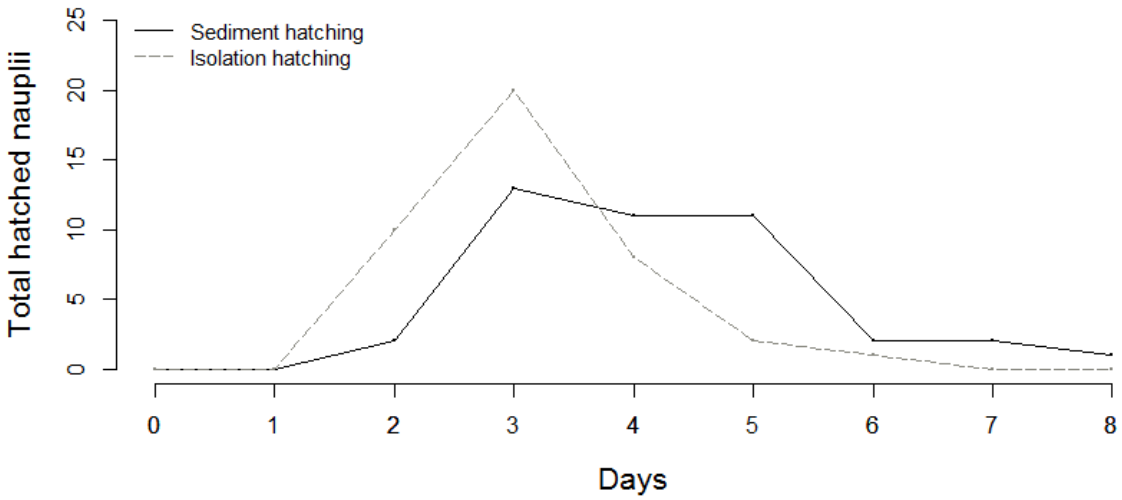

Figure 1: Total *T. cancriformis* hatchlings observed per day over the first hydroperiod for sediment and isolation hatching. Hatchling counts from all 12 sites were combined to give the daily total for each method.

Table 1. *Triops cancriformis* hatching experiment results. Recorded hatchlings from the two hydroperiods and the total eggs present per site in sediment and isolation experiments are given. Hatching rates are given as the proportion of hatched eggs per site over the two hydroperiods for both methods.

Hatching method		Site											
		A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**
Sediment	1st hydroperiod	-	-	-	-	-	2	27	-	4	9	-	-
	2nd hydroperiod	-	-	-	-	-	-	1	-	-	-	-	-
	Total eggs	22	10	20	4	9	7	49	4	14	30	5	7
	Hatching rate	0	0	0	0	0	0.29	0.57	0	0.29	0.30	0	0
Isolation	1st hydroperiod	-	-	-	1	-	-	25	-	2	12	1	-
	2nd hydroperiod	-	-	-	-	-	-	-	-	-	3	-	-
	Total eggs	22	11	15	2	7	1	58	15	13	49	15	6
	Hatching rate	0	0	0	0.50	0	0	0.43	0	0.15	0.31	0.07	0

Table 2: Short and long amplification sequencing results. Top NCBI BLASTn hit for five short amplification and five long amplification sequences used to test the specificity of the designed primers of this study. Sequences are shown in ascending order of sample ID for each amplification region. Samples descriptions marked with '*!*' indicate a poor quality, discarded sequence.

Region	Sample	Description	Query length	Cover	E value	Ident	Accession
Short amplification	F8	*T. cancriformis*	84	98%	2.00E-34	100%	AB084514.1
	G9	*T. cancriformis*	84	98%	2.00E-34	100%	AB084514.1
	I6	*T. cancriformis*	81	98%	1.00E-32	100%	AB084514.1
	J7	*T. cancriformis*	88	98%	1.00E-36	100%	AB084514.1
	K12	*T. cancriformis*	84	98%	2.00E-34	100%	AB084514.1
Long amplification	G28	*T. cancriformis*		100%	0	99%	AB084514.1
	G41	*T. cancriformis*		100%	0	99%	AB084514.1
	J15	*T. cancriformis*		100%	0	99%	AB084514.1
	J16	*T. cancriformis*		100%	0	99%	AB084514.1
	J17	*!*					

Table S3: COI and long amplification sequencing results. Top NCBI BLASTn hit for 20 samples with both COI and long amplifications from the current study. Sequences are shown in ascending order of sample ID. Samples descriptions marked with '*!*' indicate a poor quality, discarded sequence.

	COI										
Sample	Description	Query length	Cover	E value	Ident	Accession	Description	Cover	E value	Ident	Accession
D1	*T. cancriformis*	629	100%	0	99%	JX110644.1	*T. cancriformis*	100%	0	99%	AB084514.1
G7	*T. cancriformis*	563	100%	0	99%	JX110644.1	*!*				
G20	*T. cancriformis*	562	100%	0	99%	JX110644.1	*!*				
G25	*T. cancriformis*	606	100%	0	99%	JX110644.1	*!*				
G30	*T. cancriformis*	625	100%	0	99%	JX110644.1	*T. cancriformis*	100%	0	99%	AB084514.1
G43	*T. cancriformis*	623	100%	0	99%	JX110644.1	*T. cancriformis*	100%	0	99%	AB084514.1
G48	*T. cancriformis*	632	100%	0	99%	JX110644.1	*!*				
G53	*T. cancriformis*	628	100%	0	99%	JX110644.1	*T. cancriformis*	100%	0	99%	AB084514.1
G54	*T. cancriformis*	631	100%	0	99%	JX110644.1	*T. cancriformis*	100%	0	99%	AB084514.1
G59	*T. cancriformis*	630	100%	0	99%	JX110644.1	*T. cancriformis*	100%	0	99%	AB084514.1
I17	*T. cancriformis*	596	100%	0	99%	JX110644.1	*T. cancriformis*	100%	0	99%	AB084514.1
J1	*T. cancriformis*	616	100%	0	99%	JX110644.1	*T. cancriformis*	100%	0	99%	AB084514.1
J8	*T. cancriformis*	602	100%	0	99%	JX110644.1	*!*				
J24	*T. cancriformis*	596	100%	0	99%	JX110644.1	*!*				
J30	*T. cancriformis*	632	100%	0	99%	JX110644.1	*T. cancriformis*	100%	0	99%	AB084514.1
J31	*T. cancriformis*	627	100%	0	99%	JX110644.1	*T. cancriformis*	100%	0	99%	AB084514.1
J39	*T. cancriformis*	624	100%	0	99%	JX110644.1	*!*				
J42	*T. cancriformis*	507	100%	0	99%	JX110644.1	*!*				
J47	*T. cancriformis*	629	100%	0	99%	JX110644.1	*T. cancriformis*	100%	0	99%	AB084514.1
J51	*T. cancriformis*	628	100%	0	99%	JX110644.1	*T. cancriformis*	100%	0	99%	AB084514.1

Table 4: COI sample sequencing results. Top NCBI BLASTn hit for sequences of samples with no long amplification that successfully amplified with the Folmer primers LCO1490 and HCO2198 from the current study. Sequences are shown in ascending order of E value. Sequences identified as other than *T. cancriformis* have Family name included in parenthesis.

Sample	Description	Query length	Cover	E value	Ident	Accession
G24	*Triops cancriformis*	632	100%	0	99%	AB084514.1
G47	*Triops cancriformis*	616	100%	0	99%	AB084514.1
J20	*Triops cancriformis*	405	100%	0	99%	JX110644.1
J49	*Triops cancriformis*	624	100%	0	99%	AB084514.1
K4	*Triops cancriformis*	625	100%	0	99%	AB084514.1
K7	*Triops cancriformis*	399	100%	0	99%	JX110644.1
K13	*Triops cancriformis*	621	100%	0	99%	AB084514.1
J22	*Cyclotella sp. (Stephanodiscaceae)*	527	92%	0	90%	KM202115.1
G44	*Cyclotella sp. (Stephanodiscaceae)*	630	89%	0	90%	KM202115.1
J2	*Cyclotella sp. (Stephanodiscaceae)*	630	89%	0	90%	KM202115.1
K5	*Cunea thuwala (Paramoebidae)*	526	100%	0	89%	KP862852.1
J37	*Cyclotella sp. (Stephanodiscaceae)*	572	95%	0	89%	KM202115.1
J3	*Achlya hypogyna (Saprolegniaceae)*	627	96%	1E-175	83%	KF226724.1
G21	*Cyclotella sp. (Stephanodiscaceae)*	484	99%	4E-175	89%	KM202115.1
L2	*Nannochloris sp. (Coccomyxaceae)*	578	93%	1E-168	85%	KM202120.1
K11	*Triops cancriformis*	327	100%	9E-165	100%	JX110644.1
G34	*Homo sapiens (Hominidae)*	326	100%	3E-164	100%	NG_046602.1
J10	*Pythium iwayamai (Pythiaceae)*	632	96%	2E-154	80%	JX397974.1
G33	*Hartmannella vermiformis (Hartmannellidae)*	532	100%	6E-147	82%	GU828005.1
H4	*Cyclotella sp. (Stephanodiscaceae)*	413	97%	2E-146	89%	KM202115.1
H3	*Calosilpha brunneicollis (Silphidae)*	302	100%	7E-146	99%	HM180488.1
G14	*Navicula minima (Naviculaceae)*	488	87%	5E-142	87%	HM449704.1
L6	*Pythium cylindrosporum (Pythiaceae)*	537	99%	2E-134	80%	GU071824.1
I13	*Invertebrate environmental sample*	623	98%	2E-134	77%	GU070917.1
F9	*Invertebrate environmental sample*	566	99%	3E-132	79%	GU070917.1
J40	*Mitrella tuberosa (Columbellidae)*	629	89%	7E-127	79%	KF643804.1
J35	*Invertebrate environmental sample*	633	88%	3E-126	78%	GU070901.1
G19	*Paralagenidium karlingii (Pythiaceae)*	296	100%	1E-112	91%	KC767953.1
G39	*Triops cancriformis*	281	100%	3E-107	91%	JX110644.1
J50	*Roya obtusa (Mesotaeniaceae)*	351	97%	1E-98	84%	KF060943.1
A1	*Phytophthora boehmeriae (Pythiaceae)*	315	99%	4E-98	86%	HQ261251.1
G27	*Invertebrate environmental sample*	323	99%	5E-97	85%	GU070904.1
I12	*Hymenoptera sp.*	507	87%	3E-94	78%	KM564452.1
J36	*Calyptogena ponderosa endosymbiont*	366	96%	1E-67	77%	FJ899955.1
G12	*Cyclotella sp. (Stephanodiscaceae)*	239	99%	6E-58	82%	KM202118.1
J27	*Albugo laibachii (Albuginaceae)*	207	99%	5E-53	83%	FR832888.1
F1	*Thiomonas sp. (Comamonadaceae)*	226	99%	7E-51	81%	LK931622.1
J29	*Legionella oakridgensis (Legionellaceae)*	233	100%	1E-48	79%	CP004006.1
G2	*Pinnularia neomajor (Pinnulariaceae)*	167	95%	2E-46	87%	JN418687.1
J38	*Calyptogena ponderosa endosymbiont*	274	98%	6E-46	76%	FJ899955.1
E5	*Chaetosoma scaritides (Chaetosomatidae)*	116	98%	2E-38	93%	EU877951.1
F2	*Roseiflexus castenholzii (Chloroflexaceae)*	606	71%	8E-38	69%	CP000804.1
F3	*Roseiflexus castenholzii (Chloroflexaceae)*	419	91%	1E-35	70%	CP000804.1
G36	*Scytosiphon lomentaria (Scytosiphonaceae)*	211	100%	3E-31	75%	AB747604.1
J6	*Echiura sp.*	171	76%	9E-31	85%	KT383422.1
I5	*Bivalvia environmental sample*	155	99%	1E-29	81%	KP136604.1
J45	*Durvillaea sp. (Durvillaeaceae)*	119	100%	1E-17	79%	HQ386098.1

H6	*Legionella longbeachae (Legionellaceae)*	141	56%	5E-15	86%	FN650140.1
A5	*Roseiflexus castenholzii (Chloroflexaceae)*	289	95%	6E-14	68%	CP000804.1
G58	*Pseudomonas sp. (Pseudomonadaceae)*	304	32%	2E-08	77%	KJ885299.1
G37	*Pseudomonas sp. (Pseudomonadaceae)*	329	29%	2E-08	77%	KJ885299.1
F5	*Streptomyces sp. (Streptomycetaceae)*	123	79%	6E-08	76%	CP015098.1
C11	*Mesorhizobium loti (Phyllobacteriaceae)*	202	38%	2E-07	79%	CP016079.1
C2	*Pseudonocardia dioxanivorans (Pseudonocardiaceae)*	145	71%	2E-06	74%	CP002593.1
G56	*Micromonospora coriariae (Micromonosporaceae)*	152	54%	3E-05	76%	LT607412.1
G16	*Roseiflexus sp. (Chloroflexaceae)*	179	41%	0.053	75%	CP000686.1
E4	*Roseiflexus castenholzii (Chloroflexaceae)*	133	40%	0.64	80%	CP000804.1

Table 5: Sequences used for mtDNA population network. Accession numbers are given for all COI sequences from Genbank. Sample ID is given for COI sequences from the current study. Species name and country of origin is also included for each sequence.

Accession No./Sample ID	**Species**	**Country of origin**
EF675900.1	*T. mauritanicus*	Spain
DQ148291.1	*T.cancriformis*	Italy
DQ369312.1	*T.cancriformis*	Austria
DQ369313.1	*T.cancriformis*	Italy
DQ369314.1	*T.cancriformis*	Italy
DQ369315.1	*T.cancriformis*	Sardinia
DQ369316.1	*T.cancriformis*	Sicily
DQ369317.1	*T.cancriformis*	Spain
EF189678.1	*T.cancriformis*	Austria
EF675826.1	*T.cancriformis*	Japan
EF675827.1	*T.cancriformis*	Japan
EF675828.1	*T.cancriformis*	Japan
EF675829.1	*T.cancriformis*	Germany
EF675830.1	*T.cancriformis*	Germany
EF675831.1	*T.cancriformis*	Germany
EF675832.1	*T.cancriformis*	Germany
EF675833.1	*T.cancriformis*	Germany
EF675834.1	*T.cancriformis*	Germany
EF675835.1	*T.cancriformis*	Germany
EF675836.1	*T.cancriformis*	Germany
EF675837.1	*T.cancriformis*	Germany
EF675838.1	*T.cancriformis*	Germany
EF675839.1	*T.cancriformis*	Germany
EF675840.1	*T.cancriformis*	Germany
EF675841.1	*T.cancriformis*	Germany
EF675842.1	*T.cancriformis*	Germany
EF675843.1	*T.cancriformis*	Germany
EF675844.1	*T.cancriformis*	Germany
EF675845.1	*T.cancriformis*	Germany
EF675846.1	*T.cancriformis*	Germany
EF675847.1	*T.cancriformis*	Germany
EF675848.1	*T.cancriformis*	Germany
EF675849.1	*T.cancriformis*	Hungary
EF675850.1	*T.cancriformis*	Czech Republic

EF675851.1	*T.cancriformis*	Austria
EF675852.1	*T.cancriformis*	Austria
EF675853.1	*T.cancriformis*	Austria
EF675854.1	*T.cancriformis*	England
EF675855.1	*T.cancriformis*	England
EF675856.1	*T.cancriformis*	England
EF675857.1	*T.cancriformis*	England
EF675858.1	*T.cancriformis*	England
EF675859.1	*T.cancriformis*	England
EF675860.1	*T.cancriformis*	England
EF675861.1	*T.cancriformis*	England
EF675862.1	*T.cancriformis*	Germany
EF675863.1	*T.cancriformis*	Germany
EF675864.1	*T.cancriformis*	Scotland
EF675865.1	*T.cancriformis*	Germany
EF675866.1	*T.cancriformis*	Germany
EF675867.1	*T.cancriformis*	Germany
EF675868.1	*T.cancriformis*	Germany
EF675869.1	*T.cancriformis*	Spain
EF675870.1	*T.cancriformis*	Spain
EF675871.1	*T.cancriformis*	Spain
EF675872.1	*T.cancriformis*	Spain
EF675873.1	*T.cancriformis*	Spain
EF675874.1	*T.cancriformis*	Spain
EF675875.1	*T.cancriformis*	Spain
EF675876.1	*T.cancriformis*	Spain
EF675877.1	*T.cancriformis*	Spain
EF675878.1	*T.cancriformis*	Spain
EF675879.1	*T.cancriformis*	Hungary
EF675880.1	*T.cancriformis*	Sicily
EF675881.1	*T.cancriformis*	Sicily
EF675882.1	*T.cancriformis*	Spain
EF675883.1	*T.cancriformis*	Spain
EF675884.1	*T.cancriformis*	Germany
EF675885.1	*T.cancriformis*	Germany
EF675886.1	*T.cancriformis*	Germany
EF675887.1	*T.cancriformis*	Germany
EF675888.1	*T.cancriformis*	Germany
EF675889.1	*T.cancriformis*	Germany
EF675890.1	*T.cancriformis*	Germany
EF675891.1	*T.cancriformis*	Germany
EF675892.1	*T.cancriformis*	Germany
EF675893.1	*T.cancriformis*	Germany
EF675894.1	*T.cancriformis*	Germany
EF675895.1	*T.cancriformis*	Germany
EF675896.1	*T.cancriformis*	Germany
EF675897.1	*T.cancriformis*	Germany
EF675898.1	*T.cancriformis*	Austria
EF675899.1	*T.cancriformis*	Austria
FN691430.1	*T.cancriformis*	United Arab Emirates
FN691431.2	*T.cancriformis*	Austria

FN691432.2	*T.cancriformis*	Serbia and Montenegro
GQ144445.1	*T.cancriformis*	Austria
JN175234.1	*T.cancriformis*	Belgium
JN175241.1	*T.cancriformis*	France
JX110644.1	*T.cancriformis*	Spain
D1_Long	*T.cancriformis*	Scotland
G30_Long	*T.cancriformis*	Scotland
G43_Long	*T.cancriformis*	Scotland
J47_Long	*T.cancriformis*	Scotland
G53_Long	*T.cancriformis*	Scotland
G54_Long	*T.cancriformis*	Scotland
G59_Long	*T.cancriformis*	Scotland
I17_Long	*T.cancriformis*	Scotland
J1_Long	*T.cancriformis*	Scotland
J15_Long	*T.cancriformis*	Scotland
J16_Long	*T.cancriformis*	Scotland
J30_Long	*T.cancriformis*	Scotland
J31_Long	*T.cancriformis*	Scotland
J51_Long	*T.cancriformis*	Scotland
G7_COI	*T.cancriformis*	Scotland
G20_COI	*T.cancriformis*	Scotland
G24_COI	*T.cancriformis*	Scotland
G25_COI	*T.cancriformis*	Scotland
G47_COI	*T.cancriformis*	Scotland
G48_COI	*T.cancriformis*	Scotland
G53_COI	*T.cancriformis*	Scotland
G54_COI	*T.cancriformis*	Scotland
J8_COI	*T.cancriformis*	Scotland
J24_COI	*T.cancriformis*	Scotland
J39_COI	*T.cancriformis*	Scotland
J47_COI	*T.cancriformis*	Scotland
J49_COI	*T.cancriformis*	Scotland
K4_COI	*T.cancriformis*	Scotland
K13_COI	*T.cancriformis*	Scotland

Table 6: Successful long amplifications on extractions from the remaining unhatched eggs from both sediment and isolation hatching. Shown are the number of unhatched eggs that had successful long amplifications and the total number of unhatched eggs for each site for sediment and isolation hatching.

Hatching method		Site											
		A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**
Sediment	Long amplifications	-	-	-	-	-	-	-	-	-	2	-	-
	Total unhatched eggs	22	10	20	4	9	5	21	4	10	21	5	7
Isolation	Long amplifications	-	-	-	-	-	-	2	-	-	8	-	-
	Total unhatched eggs	22	11	15	1	7	1	33	15	11	34	14	6

Table 7. Time expenditure and equipment cost for each of the three egg viability estimation methods used in this study. Approximated times are for processing a single dried 20 g subsample of sediment. Times are given as time to complete each process (completion time) and the maximum time a researcher must expend to execute it (staff time). Consumables costs are based upon retail values of the consumables used.

Process	**Sediment hatching**		**Isolation hatching**		**DNA barcoding**	
	Completion time	Staff time	Completion time	Staff time	Completion time	Staff time
Isolation of eggs	1 hr	1 hr	1 hr	1 hr	1 hr	1 hr
Observation periods	2 x 8 d	2 hr 40 min	2 x 8 d	2 hr 40 min	-	-
Drying periods	2 x 8 d	20 mins	8 d*	20 mins	-	-
DNA extraction	-	-	-	-	1 hr	1 hr
PCR	-	-	-	-	4 hours	1 hr
Gel electrophoresis	-	-	-	-	1 hr, 30 min	30 min
Total time taken	32 d, 1 hr, 10 min	4 hr, 10 min	24 d, 1 hr, 10 min	4 hr, 10 min	7 hr, 30 min	3 hr, 30 min
Consumables cost	Very low (0.20 GBP)		Very low (0.20 GBP)		High (30.00 GBP)	

*isolation hatching only requires a single drying period between observations for completion of the method.

Table 8: *T. cancriformis* egg bank density. Estimated egg bank densities (eggs/kg sediment) for the 12 sites of this study.

Site	Eggs/20g subsample			Eggs/kg sediment			Average eggs/ kg sediment
	Sediment	Isolation	DNA Barcoding	Sediment	Isolation	DNA Barcoding	
A	22	22	18	1100	1100	900	1033.3
B	10	11	11	500	550	550	533.3
C	20	15	23	1000	750	1150	966.7
D	4	2	5	200	100	250	183.3
E	9	7	6	450	350	300	366.7
F	7	1	10	350	50	500	300
G	49	58	60	2450	2900	3000	2783.3
H	4	15	6	200	750	300	416.7
I	14	13	17	700	650	850	733.3
J	30	49	51	1500	2450	2550	2166.7
K	5	15	13	250	750	650	550
L	7	6	6	350	300	300	316.7

Table 9: *T. cancriformis* egg bank condition at the WWT Caerlaverock reserve. Viable, degraded and totally degraded eggs per kg sediment for the 12 sites of this study from the molecular method.

Site	Egg condition/kg sediment		
	Viable	Degraded	Totally degraded
A	0	0	900
B	0	0	550
C	0	0	1150
D	50	0	200
E	0	0	300
F	50	0	450
G	1250	250	1500
H	0	0	300
I	150	50	650
J	1100	100	1350
K	100	250	300
L	0	0	300

Appendix 2: Additional information for Chapter 3

Table 1. Locations of sites and site types sampled at RSPB Mersehead and West Preston.

RSPB Mersehead (13/06/16)

Site	Lat, Long (WGS 1984)	Notes
1	54.88923, -3.68100	shallow ditch and depression on wetland
2	54.88997, -3.68035	scrape in wet grassland
3	54.88947, -3.68046	shallow drainage ditch
4	54.88921, -3.68022	dried pool with iris
5	54.89011, -3.68114	scrape in wet grassland
6	54.88994, -3.68223	shallow channel of dried mud in wetland
7	54.88857, -3.68231	low area near saltmarsh channel/river possibly salty
8	54.88865, -3.68278	shallow pool depression higher than site 7
9	54.88825, -3.68286	long pool/channel in cattle grazed field
10	54.88491, -3.68723	wheel rut in gateway to field
11	54.88164, -3.68496	wheel rut in site 10 field
12	54.88119, -3.68413	wheel rut in site 10 field
13	54.88124, -3.68355	dried pool in site 10 field
14	54.88158, -3.67666	modified pool in site 10 field
15	54.88116, -3.67534	modified pool in site 10 field

RSPB West Preston (14/06/16)

Site	Lat, Long (WGS 1984)	Notes
16	54.88086, -3.63948	large shallow drainage ditch in cattle and sheep field
17	54.88463, -3.64524	wet meadow in floodland
18	54.88293, -3.64848	cattle grazed wetland pasture
19	54.88348, -3.6498	cattle grazed wetland pasture
20	54.88479, -3.64972	large wallow in cattle grazed wetland pasture
21	54.88231, -3.65354	hawthorn tree scratch post in channel in above
22	54.88126, -3.64875	small wallow in cattle grazed field
23	54.88147, -3.64242	military pillbox in cattle grazed field
24	54.88136, -3.64174	small wallow at fence in cattle grazed field

Appendix 3: Additional information for Chapter 4

Article 1: Mu-DNA extraction protocols

Materials required

Below are listed the materials required for all Mu-DNA extraction protocols. Companies used and product codes are provided.

Chemicals

Guanidine thiocyanate (Alfa Aesar: B21250.22)
Trisodium phosphate dodecahydrate (Sigma Aldrich: 04277-1KG)
Sodium chloride (Sigma Aldrich: S7653-250G)
Tris HCl (Alfa Aesar: J67233.22)
Disodium EDTA dihydrate (Sigma Aldrich: E5134-250G)
Sodium dodecyl sulphate (Alfa Aesar: J75819.22)
Proteinase K (Thermofisher: AM2542)
Ammonium acetate (Sigma Aldrich: A1542-500G)
Aluminium ammonium sulphate dodecahydrate (Alfa Aesar: 13802.22)
Calcium chloride dihydrate (Sigma Aldrich: 1.02382.0250)
Guanidine hydrochloride (Thermofisher: 10071503)

Plastics

2 ml screw cap tubes (Starlab: E1420-2341)
7ml Bijou tubes (Sigma-Aldrich: 129A)*
1.5 ml tubes (Starlab: S1615-5510)
2 ml tubes (Starlab: E1420-2000)
Spin Columns (NBS Biologicals: SD5005)

* these tubes are the ones used in our study and fit in a TissueLyser II but do not fit in many centrifuges. Alternatively you can use tubes that fit in a Mobio Vortex Adapter and all centrifuges with 15 ml falcon tube fittings (Axygen SCT-5ML-S, Fisher Scientific: 12559107).

Garnet beads

Garnet grit was sourced from an abrasives company at the grades required (0.15 mm and 1 - 1.4 mm). Each grade was thoroughly washed with purified water through a suitable mesh sieve to remove any detritus and fine particles. After washing, the garnet grit was transferred to 250 ml conical flasks capped with aluminium foil and sterilised at 210°C for three hours. The garnet grit was left to cool before being transferred to sterile 50 ml falcon tubes. Alternatively sterile garnet bead tubes, or similar, can be purchased commercially (e.g. Qiagen).

Reagent preparation

Stock solutions

Stock solutions are given as compositions for 100 ml with the exception of ***PK***.

1 M Tris HCl (pH 8):
Dissolve 15.7 g of Tris HCl in 75 ml ddH2O. Adjust to pH 8 with 5 M NaOH. Bring to 100 ml with ddH2O.

0.5 M EDTA (pH 8):
Dissolve 18.6 g of disodium EDTA dihydrate in 75 ml ddH2O. Adjust to pH 8 with 5 M NaOH. Bring to 100 ml with ddH2O.

20% SDS:
Dissolve 20 g sodium dodecyl sulphate in 75 ml ddH2O, bring to 100 ml with ddH2O.

PK:
To 7 ml ddH2O add 0.5 ml 1 M Tris HCl (pH 8) and 100 mg Proteinase K, bring to 10 ml with ddH2O.

5 M Ammonium acetate:
Dissolve 38.6 g ammonium acetate in 75 ml ddH2O, bring to 100 ml with ddH2O.

180 mM Aluminium etc.:
Dissolve 8.2 g aluminium ammonium sulphate dodecahydrate in 75 ml ddH2O, bring to 100 ml with ddH2O.

3% Calcium chloride:
Dissolve 3 g calcium chloride dihydrate in 75 ml ddH2O, bring to 100 ml with ddH2O.

5.5 M Guanidine HCl:
Dissolve 52.6 g guanidine hydrochloride in 75 ml ddH2O, bring to 100 ml with ddH2O.

Working solutions

All working solutions are composites of stock solutions. All working solution compositions are given for a 100 ml final volume. However, some working solutions may not be required in this amount. For simplistic creation some working solutions are designed to be easily combined from stock solutions with set volumes of stock solutions. For these solutions the number of volumes required per stock solution is given in brackets after the amount for 100 ml. Also note that some working solutions consist of a single stock solution.

Lysis Solution:
To 75 ml ddH2O add 6.7 ml ***1 M Tris HCl (pH 8)***, 5.3 ml ***0.5 M EDTA (pH 8)***, 1.7 g guanidine thiocyanate, 8.7 g trisodium phosphate dodecahydrate and 0.2 g sodium chloride. Stir mixture until all solids dissolve. Adjust to pH 9.0 with 5 M HCl. Bring to final 100 ml volume with ddH2O.

Soil Lysis Additive:
To 50 ml (8 volumes) ***180 mM Aluminium etc.*** add 43.75 ml (7 volumes) ddH2O and 6.25 ml (1 volume) ***20% SDS***. Vortex briefly to mix.

Tissue Lysis Additive:
20% SDS.

Water Lysis Additive:
To 93.75 ml (15 volumes) ddH2O add 6.25 ml (1 volume) ***20% SDS***. Vortex briefly to mix.

Flocculant Solution:
To 50 ml (2 volumes) ***5 M Ammonium acetate*** add 25 ml (1 volume) ***180 mM Aluminium etc***. Vortex briefly before adding 25 ml (1 volume) ***3% Calcium chloride***. Vortex briefly to mix.

Binding Solution:
5.5 M Guanidine HCl.

Tissue Binding Solution:
To 50 ml (1 volume) ***5.5 M Guanidine HCl*** add 50 ml (1 volume) 100% ethanol. Vortex briefly to mix.

Wash Solution:
To 20 ml (1 volume) ddH2O add 80 ml (4 volumes) 100% ethanol.

Elution Buffer:
To 75 ml ddH2O add 1 ml ***1 M Tris HCl (pH 8)*** and 0.2 ml ***0.5 M EDTA (pH 8)***. Bring to 100 ml with ddH2O.

Preparatory steps

Incubator

Preheat incubating apparatus to 55°C.

Samples

Defrost samples (if necessary) at room temperature. Samples stored in ethanol and other storage buffers (e.g. RNAlater) are best air dried on sterile blotting paper (or similar) to remove as much ethanol or buffer as possible before lysis.

Working solutions

Create the relevant working solutions in sufficient volumes required for the extraction method.

Important: Incubate ***SDS***, ***Soil Lysis Additive***, ***Water Lysis Additive***, ***Binding Solution*** and ***Tissue Binding Solution*** at 55°C until required. If any precipitate is present mix gently until redissolved. Use solutions while still warm.

Lysis master mix

To reduce processing time when extracting from a large number of samples it is possible to create a master mix of solution volumes used in the lysis step (Soil: ***Lysis Solution*** and ***Soil Lysis Additive***; Tissue: ***Lysis Solution***, ***Tissue Lysis Additive*** and ***PK***; Water: ***Lysis Solution*** and ***Water Lysis Additive***). Heat the master mix at 55°C until required, mix gently occasionally. This prevents the formation of precipitates that interfere with lysis. Use the master mix while still warm.

Mu-DNA: Soil extraction protocol

The entire process is scalable depending on initial sample weight or processed lysate amount. Large volumes will require extended centrifuge times under lower xg to obtain optimal results.

Important: Incubate ***Soil Lysis Additive*** and ***Binding Solution*** at 55°C until required. If any precipitate is present mix gently until redissolved. Use solutions while still warm.

1. Add 0.5 g (2 X sample weight) of 1 - 1.4 mm diameter sterile garnet beads to a 2 ml screw cap tube
2. Add up to 0.25g of sample to tube
3. Add 550 µl of ***Lysis Solution*** and vortex briefly
4. Add 200 µl of ***Soil Lysis Additive***
5. Place in TissueLyser II (or similar horizontal beating apparatus) at 30 hz for 10 mins
6. Centrifuge at 10,000 xg for 1 min at room temperature
7. Transfer supernatant to a 1.5 ml tube
8. Centrifuge at 10,000 xg for 1 min at room temperature
9. Transfer supernatant to a fresh 1.5 ml tube

   The following steps are based on 500 - 650 µl volume of lysate.

10. Add 300 µl (0.6 X volume) of ***Flocculant Solution***, vortex briefly and incubate on ice for a minimum of 10 mins
11. Centrifuge at 10,000 xg for 1 min at room temperature
12. Transfer supernatant to a 2 ml tube
13. Add 1200 µl (2 X volume) of ***Binding Solution***, vortex briefly to mix
14. Transfer 650 µl of the mixture to a spin column, centrifuge at ≥ 10,000 xg for 1 min at room temperature, discard the flow-through and repeat until all the mixture has passed through the spin column
15. Add 500 µl of ***Wash Solution***, centrifuge at 10,000 xg for 1 min at room temperature, discard the flow-through
16. Centrifuge at 10,000 xg for 2 min at room temperature, replace collection tube with a fresh 1.5 ml tube
17. Add 100 µl of ***Elution Buffer*** directly to the spin column membrane. Incubate for 1 min at room temperature
18. Centrifuge at 10,000 xg for 1 min at room temperature
19. DNA is now in the 1.5 ml tube

    Optional: For increased DNA yield repeat steps 17 to 18 a further time.

Troubleshooting

Spin column clogged: Centrifuge at higher xg for 2 mins. Alternatively, heat spin column (including collection tube) and binding mixture tube contents (previously transferred supernatant and ***Binding Solution*** mixture) at 55°C for 5 min then centrifuge at higher xg for 2 mins. Continue with protocol.

Mu-DNA: Tissue extraction protocol

The entire process is scalable depending on initial sample weight or lysate amount. Large volumes will require extended centrifuge times under lower xg to obtain optimal results.

Important: Incubate ***Tissue Lysis Additive*** and ***Tissue Binding Solution*** at 55°C until required. If any precipitate is present mix gently until redissolved. Use solutions while still warm.

1. Place up to 40 mg of tissue into a 1.5 ml tube
2. Add 260 µl of ***Lysis Solution***, 20 µl ***Tissue Lysis Additive*** and 20 µl ***PK***
3. Grind tissue with a tube pestle, vortex briefly
4. Incubate at 55°C with occasional vortexing until all tissue is dissolved (>3 hours or overnight).
5. Centrifuge at 10,000 xg for 1 min at room temperature
6. Transfer supernatant to a fresh 1.5 ml tube
7. Add 600 µl (2 X volume) ***Tissue Binding Solution***, vortex briefly to mix
8. Transfer 650 µl of the mixture to a spin column, centrifuge at ≥ 10,000 xg for 1 min at room temperature, discard the flow-through and repeat until all the mixture has passed through the spin column
9. Add 500 µl of ***Wash Solution***, centrifuge at 10,000 xg for 1 min at room temperature, discard the flow-through. Repeat a second time.
10. Centrifuge at 10,000 xg for 2 min at room temperature, replace collection tube with a 1.5 ml tube
11. Add 200 µl of ***Elution Buffer*** directly to the spin column membrane. Incubate for 1 min at room temperature
12. Centrifuge at 10,000 xg for 1 min at room temperature
13. DNA is now in the 1.5 ml tube

    Optional: For increased DNA yield repeat steps 10 to 12 a further time.

Troubleshooting

Lysate thickens and/or becomes white: Heat lysate at 55°C for 5 min, vortex briefly and proceed with protocol.

Lysate and *Tissue Binding Solution* mixture becomes cloudy: Heat mixture at 55°C for 5 min and proceed with protocol.

Spin column clogged: Centrifuge at higher xg for 2 mins. Alternatively, heat spin column (including collection tube) and binding mixture tube contents (previously transferred supernatant and ***Tissue Binding Solution*** mixture) at 55°C for 5 min then centrifuge at higher xg for 2 mins. Continue with protocol.

Mu-DNA: Water extraction protocol

For water samples vacuum filtered through 47 mm diameter cellulose nitrate membrane filters or similar. Some steps of the following protocol can be based upon transferred supernatant volumes.

Important: Incubate ***Water Lysis Additive*** and ***Binding Solution*** at 55°C until required. If any precipitate is present mix gently until dissolved. Use solutions while still warm.

1. Add 1 g each of 0.15 mm and 1 - 1.4 mm diameter sterile garnet beads to 7 ml bijou tube.
2. Roll filter and place in tube
3. Add 750 µl of ***Lysis Solution***
4. Add 250 µl of ***Water Lysis Additive***
5. Place in TissueLyser II at 30 hz (or similar apparatus at maximum speed) for 5 mins
6. Centrifuge at 4,000 xg for 1 min at room temperature
7. Transfer supernatant to a 1.5 ml tube
8. Centrifuge at 10,000 xg for 1 min at room temperature
9. Transfer supernatant to a fresh 1.5 ml tube

   The following steps are based on a 600 - 750 µl volume of lysate.

10. Add 200 µl (0.3 X volume) of ***Flocculant Solution***, vortex briefly and incubate at 4°C or on ice for a minimum of 10 mins
11. Centrifuge at 10,000 xg for 1 min at room temperature
12. Transfer supernatant to a 2 ml tube
13. Add 1200 µl (2 X volume) of ***Binding Solution***, vortex briefly to mix
14. Transfer 650 µl of the mixture to a spin column, centrifuge at ≥ 10,000 xg for 1 min at room temperature, discard the flow-through and repeat until all the mixture has passed through the spin column
15. Add 500 µl of ***Wash Solution***, centrifuge at 10,000 xg for 1 min at room temperature, discard flow-through
16. Centrifuge at 10,000 xg for 2 min at room temperature, replace collection tube with a 1.5 ml tube
17. Add 100 µl of ***Elution Buffer*** directly to the spin column membrane. Incubate for 1 min at room temperature
18. Centrifuge at 10,000 xg for 1 min at room temperature
19. DNA is now in the 1.5 ml tube

    Optional: For increased DNA yield repeat steps 17 to 18 a further time.

Troubleshooting

Spin column clogged: Centrifuge at higher xg for 2 mins. Alternatively, heat spin column (including collection tube) and binding mixture tube contents (previously transferred supernatant and ***Binding Solution*** mixture) at 55°C for 5 min then centrifuge at higher xg for 2 mins. Continue with protocol.

Table 1. Summary statistics for planned contrast linear models between methods for samples used in this study. Shown are the outcomes for DNA yield, A_{260}/A_{280} and A_{260}/A_{230} ratios from the comparison of methods.

DNA yield

Model term	Estimate	SE	df	F	*t*	*p*
Effect of dropping treatment			23, 80	146.7		<0.0001
Soil A	-3.469	1.5365			-2.2577	0.0267
Soil B	0.789	1.5365			0.5135	0.609
Soil C	-8.9217	1.9836			-4.4977	<0.0001
Stool A	-1.899	1.5365			-1.2359	0.2201
Stool B	0.254	1.5365			0.1653	0.8691
Stool C	-0.115	1.5365			-0.0748	0.9405
Tissue A	-5.973	1.5365			-3.8874	0.0002
Tissue B	12.7333	1.9836			6.4193	<0.0001
Tissue C	0.235	1.9836			0.1185	0.906
Water A	-3.735	1.5365			-2.4309	0.0173
Water B	-15.1217	1.9836			-7.6233	<0.0001
Water C	-4.6	1.5365			-2.9938	0.0037

A260/280

Model term	Estimate	SE	df	F	*t*	*p*
Effect of dropping treatment			23, 80	41.96		<0.0001
Soil A	-0.062	0.0368			-1.6848	0.0959
Soil B	-0.042	0.0368			-1.1413	0.2571
Soil C	-0.0967	0.0475			-2.0348	0.0452
Stool A	-0.072	0.0368			-1.9566	0.0539
Stool B	0.406	0.0368			11.0328	<0.0001
Stool C	-0.001	0.0368			-0.0272	0.9784
Tissue A	0.005	0.0368			0.1359	0.8923
Tissue B	-0.05	0.0475			-1.0525	0.2958
Tissue C	0.01	0.0475			0.2105	0.8338
Water A	0.019	0.0368			0.5163	0.6071
Water B	0.025	0.0475			0.5262	0.6002
Water C	0.024	0.0368			0.6522	0.5161

A260/230

Model term	Estimate	SE	df	F	*t*	*p*
Effect of dropping treatment			23, 80	40.24		<0.0001
Soil A	-0.062	0.0634			-0.9781	0.331
Soil B	0.121	0.0634			1.9088	0.0599
Soil C	-0.405	0.0818			-4.949	<0.0001
Stool A	-0.263	0.0634			-4.149	<0.0001
Stool B	0.471	0.0634			7.4303	<0.0001
Stool C	0.118	0.0634			1.8615	0.0663
Tissue A	-0.033	0.0634			-0.5206	0.6041
Tissue B	-0.115	0.0818			-1.4053	0.1638
Tissue C	-0.1533	0.0818			-1.8737	0.0646
Water A	-0.605	0.0634			-9.5442	<0.0001
Water B	-0.2333	0.0818			-2.8513	0.0055
Water C	-0.604	0.0634			-9.5285	<0.0001

Table 2. Summary statistics for planned contrast linear models between methods for optimised Mu-DNA protocols. Shown are the DNA yield, A_{260}/A_{280} and A_{260}/A_{230} ratios for stool samples B and C.

DNA yield

Model term		Estimate	SE	df	F	*t*	*p*
Effect of dropping treatment				3, 16	123.2		<0.0001
	Stool B	-5.941	0.5398			-11.0069	<0.0001
	Stool C	-0.125	0.5398			-0.2316	0.8198

A260/280

Model term		Estimate	SE	df	F	*t*	*p*
Effect of dropping treatment				3, 16	64.8		<0.0001
	Stool B	0	0.02			0	1
	Stool C	-0.058	0.02			-2.8982	0.0105

A260/230

Model term		Estimate	SE	df	F	*t*	*p*
Effect of dropping treatment				3, 16	197.5		<0.0001
	Stool B	0.008	0.0277			0.2886	0.7766
	Stool C	-0.035	0.0277			-1.2625	0.2248

Article 2: Library preparation protocol

The final double-indexed library was prepared as described below:

1. **First amplification.** PCR reactions were performed in a final volume of 25 µl with the following reagents: 12.5 µl of Q5 Hot-Start High-Fidelity 2X Master Mix (NEB), 1.5 µl (10 µM) of each indexed primer, 8 µl of molecular grade water and 2 µl of DNA template. Every PCR was covered with a drop of mineral oil to avoid cross-contamination between samples and reactions were performed into single capped PCR strips. Thermal cycling conditions: 300 s at 98°C, 37 x (10 s at 98°C, 20 s at 58°C, 30 s at 72°C), 420 s at 72°C, 600 s at 4°C. Amplification success and correct amplicons size (approximately 150 bp) was visually checked on a 2% agarose gel. Primers were designed as described in Kitson et al. (2018). Primers contained indexes consisting of 8-nucleotide sequences for both forward and reverse as well as heterogeneity spacers of two to four random bases. PCR positives (genomic DNA) and negatives (water) were run as controls along the entire library preparation. As a positive, genomic DNA extracted from the cichlid Maylandia zebra and diluted to 0.05 ng/µl was used.

2. **Pooling.** Indexed amplicons were pooled based on gel images. 5 µl of PCR products were added for samples with higher concentrations, while 10 µl were used for samples with lower concentrations. This balanced pooling allowed the normalization of sequence reads per sample within each library.

3. **Bead clean-up.** Purification was performed applying a double-size selection protocol with Mag-Bind RxnPure Plus beads (Omega Bio-tek). Purified barcoded libraries were visualised on a 2% agarose gel to verify the absence of nonspecific products and primer dimers.

4. **Second amplification.** PCRs of pooled, barcoded and purified libraries were performed in a final reaction volume of 50 µl with the following reagents: 24 µl of Q5 Hot-Start High-Fidelity 2X Master Mix (NEB); 2.5 µl of each Illumina sequencing adapter (forward and reverse; 10 µM); 16 µl of molecular grade water and 5 µl of barcoded DNA amplicon as a template. Thermal cycling conditions: 180 s at 95°C, 8 x (20 s at 98°C, 60 s at 72°C), 300 s at 72°C, 600 s at 4°C. Libraries amplifications were visualised on 2% agarose gel and run alongside the non-tagged products to display differences in size after the addition of the Illumina sequencing adapters. Final product size was around 300 bp as expected.

5. **Second bead clean-up.** An additional double-size selection clean-up step was performed with a bead ratio of 0.7X and 0.15X. Purified, tagged libraries were checked on a 2% agarose gel as above.

6. **Library quantification.** Each library concentration was quantified with the Qubit dsDNA HS assay.

7. **Pooling and dilution.** Tagged libraries were pooled taking into account number of samples per library and libraries concentration.

8. **Library amplicon size assessment.** Correct final library amplicon size and DNA integrity were checked on an Agilent TapeStation using High Sensitivity D1000 assay.

9. **qPCR quantification assay.** Accurate quantification with qPCR with the NEBNext® (NEB) library quantification kit was undertaken. This took into account the TapeStation result which assessed the final library amplicon size as equal to 310bp. Library concentration was calculated as 5.3 nM and was further diluted to bring the library to 4nM.

10. **Denaturation and sequencing.** As a last step, the library was denatured and loaded at 15 pM concentration with 10% PhiX on an Illumina MiSeq using 600 bp V3 chemistry.

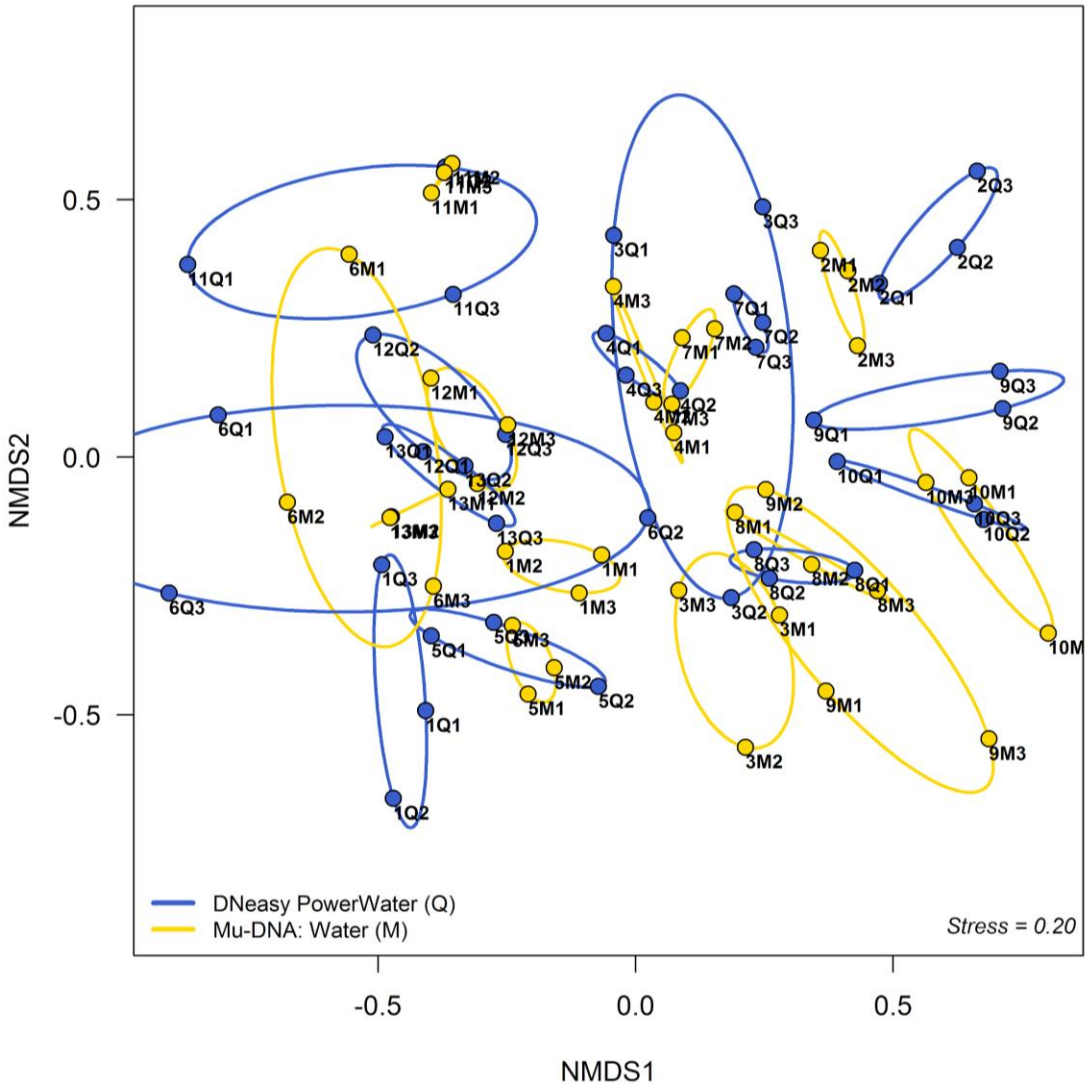

Figure 1. Non-metric multidimensional scaling (NMDS) ordination of Windermere lake water metabarcoding using the compared extraction methods of this study. NMDS generated from relative species abundance (%) of all replicates using a Bray-Curtis dissimilarity metric (stress = 0.20). Sequencing replicate ordinations for the 13 sample sites are shown for DNeasy PowerWater (blue) and Mu-DNA: Water (yellow). Points are labelled by site/ method/ sequencing replicate.

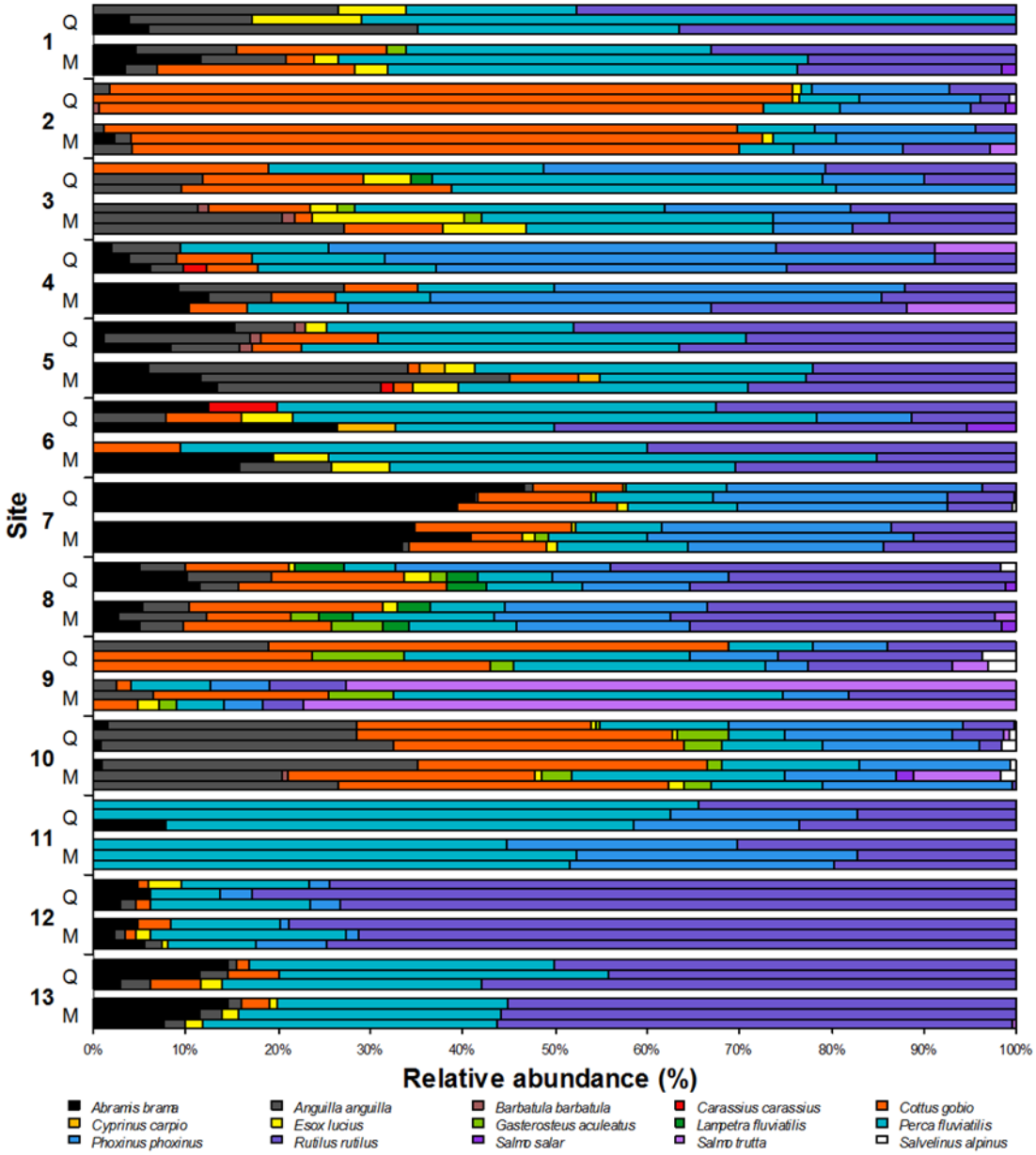

Figure 2. Species profiles of method replicates for the 13 sample sites at Windermere from metabarcoding of lake water extractions using the compared methods of this study. Relative abundance of species detected is given for each method replicate per site; DNeasy PowerWater (Q) or Mu-DNA: Water (M).

Dataset 1: Sample information and cost comparison calculations

Dataset 1: is available from https://doi.org/10.17605/osf.io/vrb4a [Mu-DNA supplementary material].

Appendix 4: Additional information for Chapter 5

Article 1: Isolation and DNA extraction of diapausing eggs from temporary pool sediments

All reagents and equipment marked with '*' are from the Mu-DNA protocol of Sellers et al. (2018). Reagent preparation is not included here for brevity. For Mu-DNA reagent preparation see: dx.doi.org/10.17504/protocols.io.nbedaje and Appendix 3: Article 1. Where possible details of additional equipment, or similar examples, used in this protocol are given in parenthesis.

Reagents

- ***8% Sucrose Solution***: Dissolve 80 g sugar in 750 ml purified water. Bring to final 1 l volume with purified water
- ***Lysis Solution****
- ***Soil Lysis Additive****
- ***Flocculant Solution****
- ***Binding Solution****
- ***Wash Solution****
- ***Elution Buffer****

Equipment

Sucrose flotation

- 50 ml falcon tubes (Starlab: E1450-0200)
- 50 µm nylon filter (cut into 45 mm x 45 mm squares) (e.g. VWR: 510-0026)
- Filter device: a modified 50 ml falcon tube. Cut of the pointed base and bore a large window in the lid
- Tweezers
- Spatulas
- Petri dishes
- Centrifuge capable of holding 50 ml falcon tubes

Sample homogenisation and DNA extraction

- TissueLyser II (Qiagen: 85300)
- Stainless steel grinding jar set (Qiagen: 69985) or Teflon grinding jar set (Qiagen: 69986)
- 5 ml tubes (Starlab: E1450-1100)
- 2 ml tubes*
- 1.5 ml tubes*
- Spin columns*
- 5 ml, 1000 µl and 200 µl pipettes and pipette tips
- Centrifuge capable of holding 2 ml microcentrifuge tubes

Protocol

The protocol is split into three distinct stages: Sucrose flotation, Sample homogenisation and DNA extraction. The distinction in the stages gives a suitable stop point after Sucrose flotation and Sample homogenisation. The final products of these stages can be stored at -20 °C until required for further processing. Short term storage in this manner has no detrimental effects on the protocol's sensitivity.

All non-disposable equipment should be sterilised prior to use and between sample processing. Equipment should be soaked in 10% bleach for 10 minutes then rinsed thoroughly with purified water. Stainless steel rusts easily in bleach so care must be taken to minimise damage to equipment.

Sucrose floatation

Secure the 50 µm nylon filter between the cap and body of the filter device.

1. Divide 20 g of dry sediment equally between four 50 ml Falcon tubes. Add ***8% Sucrose Solution*** to a final volume of 50 ml and disperse the sediment with a spatula
2. Close the tubes and shake briefly to mix. Allow to stand for 10 min to rehydrate sample
3. Vortex tubes to thoroughly mix and centrifuge at 800 rpm for 5 min
4. Pour the supernatant from all four tubes into the open end of the filter device. Rinse the filtrate thoroughly with purified water
5. Stand filter device filter end down on blotting paper to remove excess water for 1 min
6. Carefully remove the filter and filtrate with tweezers into a petri dish. Scrape any remaining organic matter from the filter device lip onto the filter with a spatula

Note: Filter and filtrate can be stored in the petri dish at -20°C until required.

Sample homogenisation

For 1.5 g combined wet weight of filter and filtrate. If the sample is more than 1.5 g, scale the amount of Lysis Solution used accordingly (to a maximum of 8 ml). Allow filter and filtrate to defrost if necessary.

1. Place filter and filtrate in the body of the grinding jar with tweezers. Place the grinding ball carefully on the sample
2. Add 3.3 ml ***Lysis Solution*** and replace the grinding jar lid
3. Secure grinding jar in TissueLyser II. Process at 30 hz for 2 min
4. Using a 5 ml wide bore pipette transfer homogenate to a 5 ml tube
5. Transfer a 1.5 ml aliquot of the homogenate to a 1.5 ml tube

Note: Homogenates and aliquots can be stored at -20°C until required.

DNA extraction

Incubate ***Soil Lysis Additive*** and ***Binding Solution*** at 55°C until required. If any precipitate is present mix gently until redissolved. Use solutions while still warm. Allow homogenate aliquot to defrost if necessary.

Lysis

1. Centrifuge homogenate aliquot at ≥ 10,000 xg for 1 min at room temperature
2. Transfer 550 µl supernatant to a fresh 1.5 ml tube
3. Add 200 µl of ***Soil Lysis Additive***. Vortex briefly to mix. Incubate at room temperature for 1 min
4. Centrifuge at ≥ 10,000 xg for 2 min at room temperature

Inhibitor removal

1. Transfer 600 µl supernatant to a fresh 1.5 ml tube
2. Add 400 µl of ***Flocculant Solution***, vortex briefly and incubate on ice for a minimum of 10 min
3. Centrifuge at ≥ 10,000 xg for 2 min at room temperature
4. Transfer supernatant to a 2 ml tube

Silica binding

1. Add 1200 µl of ***Binding Solution***, vortex briefly to mix
2. Transfer 650 µl of the mixture to a spin column, centrifuge at ≥ 10,000 xg for 10 sec at room temperature, discard the flow-through and repeat until all the mixture has passed through the spin column

Wash

1. Add 500 µl of ***Wash Solution***, centrifuge at ≥ 10,000 xg for 10 sec at room temperature, discard the flow-through. Repeat a second time.
2. Centrifuge at ≥ 10,000 xg for 2 min at room temperature, replace collection tube with a fresh 1.5 ml tube

Elution

1. Add 200 µl of ***Elution Buffer*** directly to the spin column membrane. Incubate for 1 min at room temperature
2. Centrifuge at ≥ 10,000 xg for 1 min at room temperature
3. DNA is now in the 1.5 ml tube

Table 1. Optimised sucrose flotation on replicates of all samples used in this study. Shown are the initial amount of sediment processed and the resulting filtrate mass from the 0.23 M sucrose solution.

Sample	Replicate	Sediment processed (g)	Filtrate mass (g)
EAS	1	20	0.56
EAS	2	20	1.40
EAS	3	20	1.04
EAS	4	20	0.59
KIN	1	20	0.29
KIN	2	20	0.61
KIN	3	20	0.37
KIN	4	20	0.30
MER	1	20	1.19
MER	2	20	1.48
MER	3	20	1.35
MER	4	20	1.30
MUR	1	20	0.46
MUR	2	20	0.53
MUR	3	20	0.42
MUR	4	20	0.66
CAE	1	20	0.81
CAE	2	20	0.71
CAE	3	20	0.81
CAE	4	20	0.69
KOE	1	20	0.76
KOE	2	20	1.02
KOE	3	20	1.20
KOE	4	20	0.59

Table 2. Resting eggs of non-*Triops* species isolated from optimised sucrose flotation of positive sample replicates. Resting egg presence from temporary pool invertebrate species other than *T. cancriformis* is indicated (y) for each sample replicate. All samples were processed using the 0.23 M sucrose solution.

Sample	Replicate	Anostracans	Cladocerans	Copepods	Ostracods
CAE	1		y	y	y
CAE	2		y	y	y
CAE	3		y	y	y
KOE	1		y	y	y
KOE	2		y	y	y
KOE	3		y	y	y
MUR	1	y	y	y	y
MUR	2	y	y	y	y
MUR	3	y	y	y	y

Appendix 5: Additional information for Chapter 6

Article 1: SPRI based DNA purification preparation

The following protocol is adapted from Rohland & Reich (2012). The protocol describes two separate SPRI bead solution preparations, one for DNA extraction and one for library preparation/DNA purification. All reagents marked with '*' are from the Mu-DNA protocol of Sellers et al. (2018). Reagent preparation is not included here for brevity. For Mu-DNA reagent preparation see: dx.doi.org/10.17504/protocols.io.nbedaje and Appendix 3: Article 1.

Materials

Sera-Mag SpeedBead Carboxylate-Modified Magnetic Particles (Hydrophobic), 15 mL: 65152105050250 (GE Healthcare)

Stock solutions

0.5 M EDTA (pH 8)*

1 M Tris HCl (pH 8)*

5 M NaCl**:** Dissolve 29.2 g sodium chloride in 75 ml ddH_2O. Bring to 100 ml final volume with ddH_20.

50% PEG 8000**:** To 50 g polyethylene glycol 8000 add ddH_20 to a final volume of 100 ml. Repeatedly invert at room temperature until dissolved (this will take a long time - use a hulamixer or similar).

10% Tween 20**:** To 900 µl ddH_2O add 100 µl Tween 20. Invert repeatedly to mix.

NOTE: With the exception of ***10% Tween 20*** all stock solutions can be UV sterilised. Stock solutions can be stored at room temperature. Store ***10% Tween 20*** in the dark.

Working solutions:

Elution Buffer*

DNA Extraction Bead Solution (for final 10 ml vol)**:** Mix 100 µl ***1 M Tris HCl (pH 8)***, 20 µl ***0.5 M EDTA (pH 8)*** and 3.2 ml ***5 M NaCl***. Add 4 ml ***50% PEG 8000*** and invert to mix. Add 2.53 ml ddH_20. Invert to mix thoroughly. Add 50 µl of ***10% Tween 20*** then add 100ul prepared ***Bead suspension***. Vortex or invert to mix thoroughly.

Library Prep Bead Solution (for final 10 ml vol)**:** Mix 100 µl ***1 M Tris HCl (pH 8)***, 20 µl ***0.5 M EDTA (pH 8)*** and 3.2 ml ***5 M NaCl***. Add 4 ml ***50% PEG 8000*** and invert to mix. Add 2.43 ml ddH_20. Invert to mix thoroughly. Add 50 µl of ***10% Tween 20*** then add 200ul prepared ***Bead suspension***. Vortex or invert to mix thoroughly.

***Bead Suspension*:**

It is simplest to take aliquots of beads from the Sera Mag SpeedBead bottle at adequate amounts for use, e.g. 100 µl for 10 ml ***DNA Extraction Bead Solution***. Allow Sera-Mag SpeedBeads bottle to reach room temperature. Vortex the bottle until the beads are completely resuspended - this may take some time but it is essential they are fully suspended. Immediately after resuspension transfer the desired volume of Sera-Mag SpeedBeads to a 1.5 ml tube. Store aliquots in the fridge ready for preparation.

Bead suspension preparation:

1. Allow Sera-Mag SpeedBeads aliquot to reach room temperature.
2. Vortex thoroughly to resuspend beads. Centrifuge briefly to remove droplets from tube lid.
3. Place on magnetic stand until supernatant is completely clear and beads are bound towards magnet. This should take approximately ten minutes but can take longer.
4. While on the stand carefully remove and discard supernatant without disturbing beads.
5. Add 500 µl ddH_2O. Vortex tube to resuspend beads. Centrifuge briefly to remove droplets from tube lid.
6. Place on magnetic stand until supernatant is completely clear and beads are bound towards magnet. This should take approximately ten minutes but can take longer.
7. While on the stand carefully remove and discard supernatant without disturbing beads.
8. Repeat steps 5 to 7 three more times.
9. Add ***Elution Buffer*** to match the starting volume of aliquot. Vortex tube to resuspend beads. Centrifuge briefly to remove droplets from tube lid.
10. ***Bead suspension*** can now be added to the bead solution

Note: For steps 5 to 7 the amount of ddH_2O added needs to be more than the starting volume of bead aliquot. If preparing 500 µl of beads adding 750 µl ddH_2O is adequate.

Store bead solutions in the fridge avoiding over exposure to light.

Article 2: SPRI based DNA extraction

The SPRI aspect of this protocol is adapted from Rohland and Reich (2012). All reagents marked with '*' are from the Mu-DNA protocol of Sellers et al. (2018). Reagent preparation is not included here for brevity. For Mu-DNA reagent preparation see: dx.doi.org/10.17504/protocols.io.nbedaje and Appendix 3: Article 1.

Reagents

Lysis Solution*

Tissue Lysis Additive:
To 600 ml (2 volumes) ddH2O add 300 ml (1 volume) ***20% SDS****. Invert to mix.

Flocculant Solution*

DNA Extraction Bead Solution: (see Appendix 5: Article 1)

Wash Solution*

Elution Buffer*

RNase A (10 mg/ml) (Thermo Fisher Scientific)

Protocol

Approximately 10 mm of a halved tail section from each individual was used for DNA extraction. Flush gut contents with 100% ethanol. Air dry samples on sterile blotting paper to remove all traces of ethanol.

Lysis

1. Create ***Lysis Master Mix***. For 1 mL: 730 µl ***Lysis Solution***, 250 µl ***Tissue Lysis Additive*** and 20 µl ***PK***. Vortex to mix
2. Place tail section in 1.5 ml eppendorf tube. Add 300 µl ***Lysis Master Mix***. Vortex to mix and centrifuge tube for 1 sec
3. Place in Thermomixer at 55°C for 3 hours at 650 rpm.
4. Add 4 µl RNase A. Vortex to mix and place in Thermomixer at 55°C for 30 min at 650 rpm
5. Centrifuge at ≥ 10,000 xg for 1 min at room temperature

Inhibitor removal

1. Transfer 300 µl supernatant to a fresh 1.5 ml tube
2. Add 150 µl of ***Flocculant Solution***, vortex briefly and incubate on ice for a minimum of 10 min
3. Centrifuge at ≥ 10,000 xg for 2 min at room temperature
4. Transfer supernatant to a fresh 1.5 ml tube

SPRI DNA binding

1. Add 600 µl of ***DNA Extraction Bead Solution***. Place on Hulamixer (continual rotation) for 10 mins
2. Place on magnetic stand until supernatant is clear and beads are bound towards magnet
3. While on the stand carefully remove and discard supernatant without disturbing beads

Wash

1. Add 1000 µl ***Wash Solution***. Incubate at room temperature for 30 secs
2. Carefully remove and discard supernatant without disturbing beads
3. Repeat steps 10 to 11 a further time
4. Centrifuge tube for 1 sec. Place back on magnetic stand ensuring beads are bound towards magnet. Remove all remaining ***Wash Solution*** with a 10 µl pipette. Air dry tube with cap open for 30 secs

Elution

1. Add 100 µl ***Elution Buffer*** (55°C) and vortex briefly to resuspend beads. Ensure all beads are resuspended with no clumps. Centrifuge tube for 1 sec
2. Place in Thermomixer at 55°C for 10 mins at 650 rpm. Centrifuge tube for 1 sec
3. Place on magnetic stand until supernatant is clear and beads are bound towards magnet
4. Carefully transfer eluate to a fresh 1.5 ml tube without disturbing beads

Article 3: ddRAD-seq library preparation

The following protocol is adapted from Kess et al. (2016). All reagents and equipment marked with '*' are from the Mu-DNA protocol of Sellers et al. (2018). Reagent preparation is not included here for brevity. For Mu-DNA reagent preparation see: dx.doi.org/10.17504/protocols.io.nbedaje and Appendix 3: Article 1.

Reagents:

10 x CutSmart Buffer (NEB)
MseI (10 units/µl) (NEB)
SbfI HF (20 units/µl) (NEB)
100 mM rATP (Promega)
T4 DNA ligase (400 units/µl) (NEB)
2 x Phusion High-Fidelity PCR Master Mix with HF Buffer (NEB)
Wash Solution*
Elution Buffer*
Library Prep Bead Solution (see Appendix 5: Article 1)

Oligos

Adapter sequences (5' to 3')

SbfI adapter 1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGCA
SbfI adapter 2	/5Phos/CTGTCTCTTATACACATCTGACGCTGCCGACGA
MseI adapter 1	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG
MseI adapter 2y	/5Phos/TACTGTCTCTTATACGAGAACAA

Indexing primers (5' to 3')

Index primer F

CAAGCAGAAGACGGCATACGAGATxxxi7xxxGTCTCGTGGGCTCGG

Index primer R

AATGATACGGCGACCACCGAGATCTACACxxxi5xxxTCGTCGGCAGCGTC

Custom sequencing primers (5' to 3')

Custom Read 1 primer	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGCAGG
Custom Read 2 primer	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTAA
Custom Index Read primer	TTACTGTCTCTTATACACATCTCCGAGCCCACGAGAC

Notes: Oligos beginning with /5Phos/ indicate a 5' phosphorylation.
Indexing primers have a corresponding i7 or i5 index (marked by xxxi7xxx or xxxi5xxx).

Adapter annealing

SbfI adapter (0.1 µM)

1. Add 1 µl 100 µM of *Sbfl* adapter 1 and *Sbfl* adapter 2 stock oligos to 98 µl ddH_2O, vortex briefly and spin down
2. Heat at 95°C for 5 min and slowly cool to room temp (thermocycle -2°C per min)
3. Add 900 µl ddH_2O for final concentration of 0.1 µM

MseI adapter (10 µM)

1. Add 10 µl 100 µM *Msel* adapter 1 and *Msel* adapter 2y stock oligos to 80 µl ddH_2O, vortex briefly and spin down
2. Heat at 95°C for 5 min and slowly cool to room temp (thermocycle -2°C per min)

Restriction digest

Create ***Restriction digest mastermix*** (below) on ice. Allow 20% extra per sample for pipetting error. Ensure there is a minimum of 2 units of each restriction enzyme per sample.

Restriction digest mastermix

	1x (µl)
ddH_2O	3.2
10 x CutSmart	1.5
MseI (10 units/µl)	0.2
SbfI HF (20 units/µl)	0.1

Add 5 µl of ***Restriction digest mastermix*** to 10 µl of DNA (final volume of 15 µl). Briefly vortex sample and spin down. Incubate at 37°C for 3 hours. Inactivate restriction enzymes with 20 minutes at 65°C. Samples can be stored at -4°C overnight or -20°C for long term.

Adapter ligation

Create ***Adapter ligation mastermix*** (below) on ice. Allow 20% extra per sample for pipetting error. Add in T4 DNA ligase last of all.

Adapter ligation mastermix

	1x (µl)
ddH_2O	1.1
10 x CutSmart	0.5
SbfI adapter	1.5
MseI adapter	1.5
100 mM rATP	0.2
T4 DNA ligase (400 units/µl)	0.2

Add 5 µl of ***Adapter ligation mastermix*** to digested sample (final volume of 20µl). Briefly vortex sample and spin down. Incubate at 16°C for 3 hours. Inactivate restriction enzymes with 20 minutes at 65°C. Samples can be stored at -4°C overnight or -20°C for long term.

Purification

This is a 0.8 x volume size selection using ***Library Prep Bead Solution*** to reduce fragments <300 bp. Create ***Size selection mastermix*** (below). Allow 20% extra per sample for pipetting error. ***Elution Buffer*** is added to effectively increase sample volumes to manageable amounts for bead purification and makes the bead solution easier to pipette.

Size selection mastermix

	1x (µl)
Elution Buffer	20
Library Prep Bead Solution	32

Add 52 µl of ***Size selection mastermix*** to each ligated sample. Follow purification protocol (below).

1. Place on Hulamixer or similar (continual rotation) for 10 mins. Centrifuge tube for 1 sec
2. Place on magnetic stand until supernatant is clear and beads are bound towards magnet
3. While on the stand carefully remove and discard supernatant without disturbing beads
4. Add 100 µl ***Wash Solution***. Incubate at room temperature for 30 secs
5. Carefully remove and discard supernatant without disturbing beads
6. Repeat steps 10 to 11 a further time
7. Centrifuge tube for 1 sec. Place back on magnetic stand ensuring beads are bound towards magnet. Remove all remaining ***Wash Solution*** with a 10 µl pipette. Air dry tube with cap open for 30 secs
8. Add 40 µl ***Elution Buffer*** and vortex briefly to resuspend beads. Ensure all beads are resuspended with no clumps. Centrifuge tube for 1 sec
9. Place on Hulamixer or similar (continual rotation) for 10 mins. Centrifuge tubes for 1 sec
10. Place on magnetic stand until supernatant is clear and beads are bound towards magnet
11. Carefully transfer eluate to a fresh tube without disturbing beads

Sample indexing

Create a primer master plate. Add 10 µl of each 10 mM indexing primer to the relevant wells of a 96 well PCR plate or strip tubes (see example below). Vortex plate or strips to mix primers thoroughly and spin down. Store indexing primer master plate at -20°C until required.

	N701	**N702**	**N703**	**N704**	**N705**	**N706**	**N707**	**N708**	**N709**	**N710**	**N711**	**N712**
N501	*A1*	*A2*	*A3*	*A4*	*A5*	*A6*	*A7*	*A8*	*A9*	*A10*	*A11*	*A12*
N502	*B1*	*B2*	*B3*	*B4*	*B5*	*B6*	*B7*	*B8*	*B9*	*B10*	*B11*	*B12*
N503	*C1*	*C2*	*C3*	*C4*	*C5*	*C6*	*C7*	*C8*	*C9*	*C10*	*C11*	*C12*
N504	*D1*	*D2*	*D3*	*D4*	*D5*	*D6*	*D7*	*D8*	*D9*	*D10*	*D11*	*D12*
N505	*E1*	*E2*	*E3*	*E4*	*E5*	*E6*	*E7*	*E8*	*E9*	*E10*	*E11*	*E12*
N506	*F1*	*F2*	*F3*	*F4*	*F5*	*F6*	*F7*	*F8*	*F9*	*F10*	*F11*	*F12*
N507	*G1*	*G2*	*G3*	*G4*	*G5*	*G6*	*G7*	*G8*	*G9*	*G10*	*G11*	*G12*
N508	*H1*	*H2*	*H3*	*H4*	*H5*	*H6*	*H7*	*H8*	*H9*	*H10*	*H11*	*H12*

Sample indexing PCR

Create ***Indexing PCR mastermix*** (below) on ice. Allow 20% extra per sample for pipetting error. Perform 4 replicates per sample.

Indexing PCR mastermix

	1x (µl)
2 x Phusion HF mastermix	5
ddH_2O	1

Transfer 1 µl of indexing primer mix from the primer master plate to the corresponding well of a PCR plate. Add 6 µl of ***Indexing PCR mastermix*** to each well. Finally add 3 µl of purified adapter ligated DNA template.

Thermocycling conditions:
60 s at 98°C, 30 x (30 s at 98°C, 30 s at 55°C, 40 s a 72°C), 600 s at 72°C, 600 s at 4°C.

Gel size selection and purification

Pool all indexed PCR products. Concentrate pooled products with 0.8 x volume ***Library Prep Bead Solution*** (see above), elute in half starting volume. Make a 1.5% agarose TBE gel prestained with GelRed (Biotium). Wells should each hold approximately 50 µl of product. Use a suitable ladder at either side. Run gel at 70 volts for 2 hours. Cut out the region between 300 to 700 bp for each lane. Purify gel slices with QIAquick Gel Extraction kit (Qiagen), one gel slice per spin column. Pool all gel extraction products and quantify on a Qubit 3.0 fluorometer using a high sensitivity (HS) dsDNA assay (Invitrogen). Concentrate product with 0.8 x volume ***Library Prep Bead Solution*** (see above), elute in appropriate volume to result in a final concentration of 20 ng/µl.

Library quantification and Miseq loading

Fragment size distribution was measured with a 2200 Tapestation using a High Sensitivity D1000 screentape kit (Agilent). Each library was diluted to 4 mM before being loaded at 10 pM on an Illumina MiSeq using 600 bp V3 chemistry. Custom sequencing primers were loading following Illumina guidelines.

Table 1. Information for sampled individuals. For each individual the sample ID, sex (male: 'm', female: 'f' and hermaphrodite: 'h') and the year the sediment sample was collected are shown. Also provided are the Illumina index ID and index barcodes used for each individual for sequencing.

Sample ID	Sex	Location	Collected	i7 Index ID	i7 Index	i5 Index ID	i5 Index
CAE01	h	Caerlaverock, Scotland	2015	N701	TCGCCTTA	N501	TAGATCGC
CAE02	h	Caerlaverock, Scotland	2015	N701	TCGCCTTA	N502	CTCTCTAT
CAE03	h	Caerlaverock, Scotland	2015	N701	TCGCCTTA	N503	TATCCTCT
CAE04	h	Caerlaverock, Scotland	2015	N701	TCGCCTTA	N504	AGAGTAGA
CAE05	h	Caerlaverock, Scotland	2015	N701	TCGCCTTA	N505	GTAAGGAG
CAE06	h	Caerlaverock, Scotland	2015	N701	TCGCCTTA	N506	ACTGCATA
CAE07	h	Caerlaverock, Scotland	2015	N701	TCGCCTTA	N507	AAGGAGTA
CAE08	h	Caerlaverock, Scotland	2015	N701	TCGCCTTA	N508	CTAAGCCT
CAE09	h	Caerlaverock, Scotland	2015	N702	CTAGTACG	N501	TAGATCGC
CAE10	h	Caerlaverock, Scotland	2015	N702	CTAGTACG	N502	CTCTCTAT
CAE11	h	Caerlaverock, Scotland	2015	N702	CTAGTACG	N503	TATCCTCT
CAE12	h	Caerlaverock, Scotland	2015	N702	CTAGTACG	N504	AGAGTAGA
CAE13	h	Caerlaverock, Scotland	2015	N702	CTAGTACG	N505	GTAAGGAG
CAE14	h	Caerlaverock, Scotland	2015	N702	CTAGTACG	N506	ACTGCATA
CAE15	h	Caerlaverock, Scotland	2015	N702	CTAGTACG	N507	AAGGAGTA
CAE16	h	Caerlaverock, Scotland	2015	N702	CTAGTACG	N508	CTAAGCCT
CAE17	h	Caerlaverock, Scotland	2015	N703	TTCTGCCT	N501	TAGATCGC
CAE18	h	Caerlaverock, Scotland	2015	N703	TTCTGCCT	N502	CTCTCTAT
CAE19	h	Caerlaverock, Scotland	2015	N703	TTCTGCCT	N503	TATCCTCT
CAE20	h	Caerlaverock, Scotland	2015	N703	TTCTGCCT	N504	AGAGTAGA
GOD01	h	Godshill, England	2016	N703	TTCTGCCT	N505	GTAAGGAG
GOD02	h	Godshill, England	2016	N703	TTCTGCCT	N506	ACTGCATA
GOD03	h	Godshill, England	2016	N703	TTCTGCCT	N507	AAGGAGTA
GOD04	h	Godshill, England	2016	N703	TTCTGCCT	N508	CTAAGCCT
GOD05	h	Godshill, England	2016	N704	GCTCAGGA	N501	TAGATCGC
GOD06	h	Godshill, England	2016	N704	GCTCAGGA	N502	CTCTCTAT
GOD07	h	Godshill, England	2016	N704	GCTCAGGA	N503	TATCCTCT
GOD08	h	Godshill, England	2016	N704	GCTCAGGA	N504	AGAGTAGA
GOD09	h	Godshill, England	2016	N704	GCTCAGGA	N505	GTAAGGAG
GOD10	h	Godshill, England	2016	N704	GCTCAGGA	N506	ACTGCATA
GOD11	h	Godshill, England	2016	N704	GCTCAGGA	N507	AAGGAGTA
GOD12	h	Godshill, England	2016	N704	GCTCAGGA	N508	CTAAGCCT
GOD13	h	Godshill, England	2016	N705	AGGAGTCC	N501	TAGATCGC
GOD14	h	Godshill, England	2016	N705	AGGAGTCC	N502	CTCTCTAT
GOD15	h	Godshill, England	2016	N706	AGGAGTCC	N503	TATCCTCT
GOD16	h	Godshill, England	2016	N707	AGGAGTCC	N504	AGAGTAGA
GOD17	h	Godshill, England	2016	N708	AGGAGTCC	N505	GTAAGGAG
GOD18	h	Godshill, England	2016	N709	AGGAGTCC	N506	ACTGCATA

GOD19	h	Godshill, England	2016	N710	AGGAGTCC	N507	AAGGAGTA
GOD20	h	Godshill, England	2016	N711	AGGAGTCC	N508	CTAAGCCT
KOE01	h	Königswartha, Germany	2017	N706	CATGCCTA	N501	TAGATCGC
KOE02	m	Königswartha, Germany	2017	N706	CATGCCTA	N502	CTCTCTAT
KOE03	h	Königswartha, Germany	2017	N706	CATGCCTA	N503	TATCCTCT
KOE04	h	Königswartha, Germany	2017	N706	CATGCCTA	N504	AGAGTAGA
KOE05	h	Königswartha, Germany	2017	N706	CATGCCTA	N505	GTAAGGAG
KOE06	h	Königswartha, Germany	2017	N706	CATGCCTA	N506	ACTGCATA
KOE07	h	Königswartha, Germany	2017	N706	CATGCCTA	N507	AAGGAGTA
KOE08	h	Königswartha, Germany	2017	N706	CATGCCTA	N508	CTAAGCCT
KOE09	h	Königswartha, Germany	2017	N707	GTAGAGAG	N501	TAGATCGC
KOE10	h	Königswartha, Germany	2017	N707	GTAGAGAG	N502	CTCTCTAT
KOE11	h	Königswartha, Germany	2017	N707	GTAGAGAG	N503	TATCCTCT
KOE12	h	Königswartha, Germany	2017	N707	GTAGAGAG	N504	AGAGTAGA
KOE13	m	Königswartha, Germany	2017	N707	GTAGAGAG	N505	GTAAGGAG
KOE14	m	Königswartha, Germany	2017	N707	GTAGAGAG	N506	ACTGCATA
KOE15	m	Königswartha, Germany	2017	N707	GTAGAGAG	N507	AAGGAGTA
KOE16	h	Königswartha, Germany	2017	N707	GTAGAGAG	N508	CTAAGCCT
KOE17	h	Königswartha, Germany	2017	N708	CCTCTCTG	N501	TAGATCGC
KOE18	h	Königswartha, Germany	2017	N708	CCTCTCTG	N502	CTCTCTAT
KOE19	h	Königswartha, Germany	2017	N708	CCTCTCTG	N503	TATCCTCT
KOE20	h	Königswartha, Germany	2017	N708	CCTCTCTG	N504	AGAGTAGA
ESP01	f	Espolla, Spain	2017	N708	CCTCTCTG	N505	GTAAGGAG
ESP02	m	Espolla, Spain	2017	N708	CCTCTCTG	N506	ACTGCATA
ESP03	m	Espolla, Spain	2017	N708	CCTCTCTG	N507	AAGGAGTA
ESP04	f	Espolla, Spain	2017	N708	CCTCTCTG	N508	CTAAGCCT
ESP05	f	Espolla, Spain	2017	N709	AGCGTAGC	N501	TAGATCGC
ESP06	f	Espolla, Spain	2017	N709	AGCGTAGC	N502	CTCTCTAT
ESP07	f	Espolla, Spain	2017	N709	AGCGTAGC	N503	TATCCTCT
ESP08	m	Espolla, Spain	2017	N709	AGCGTAGC	N504	AGAGTAGA
ESP09	m	Espolla, Spain	2017	N709	AGCGTAGC	N505	GTAAGGAG
ESP10	m	Espolla, Spain	2017	N709	AGCGTAGC	N506	ACTGCATA
ESP11	f	Espolla, Spain	2017	N709	AGCGTAGC	N507	AAGGAGTA
ESP12	f	Espolla, Spain	2017	N709	AGCGTAGC	N508	CTAAGCCT
ESP13	m	Espolla, Spain	2017	N710	CAGCCTCG	N501	TAGATCGC
ESP14	m	Espolla, Spain	2017	N710	CAGCCTCG	N502	CTCTCTAT
ESP15	m	Espolla, Spain	2017	N710	CAGCCTCG	N503	TATCCTCT
ESP16	m	Espolla, Spain	2017	N710	CAGCCTCG	N504	AGAGTAGA
ESP17	m	Espolla, Spain	2017	N710	CAGCCTCG	N505	GTAAGGAG
ESP18	f	Espolla, Spain	2017	N710	CAGCCTCG	N506	ACTGCATA