Development of a copro-diagnostic molecular tool for the
detection and identification of amphibian infecting helminths

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Lucas Huggins

School of Biological Sciences
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List of Abbreviations

- **18S rRNA** = 18S small ribosomal subunit
- **Asc-1** = Ascaridoidea family specific primers set one (Loreille et al., 2001)
- **Asc-2** = Ascaridoidea family specific primers set two (Loreille et al., 2001)
- **Bd** = *Batrachochytrium dendrobatidis*
- **BLAST** = Basic local alignment search tool
- **BSA** = Bovine serum albumin
- **COI** = Cytochrome c oxidase gene
- **Cytb** = Cytochrome b
- **eDNA** = Environmental DNA
- **ELISA** = Enzyme-linked immunosorbent assay
- **iBOL** = International Barcode of Life project
- **ITS** = Internal transcribed spacer
- **LAMP** = Loop-mediated isothermal amplification
- **mtDNA** = Mitochondrial DNA
- **Nem27** = Nematode universal primers from this study
- **NemUni-1** = Nematode universal primers (Floyd et al., 2005)
- **NemUni-2** = Nematode universal primers (Bhadury and Austen, 2010)
- **NemUni-3** = Nematode universal primers (Prof Marx Blaxter, pers. comm. 14/03/16)
- **PCR** = Polymerase chain reaction
- **PlatUni** = Platyhelminth universal primers (Van Steenkiste et al., 2015)
- **qPCR** = Quantitative PCR
- **R1** = The PlatUni principal reverse primer (Dice11R)
- **R2** = The PlatUni alternate reverse primer (Dice14R)
- **rDNA** = Ribosomal DNA
- **Rhab** = Rhabdiasidae family specific primers (Tkach et al., 2014)
- **SSCP** = Single-strand conformation polymorphism
- **Str** = *Strongyloides* genus specific primers (Kramme et al., 2011)
- **Tmu** = *Trichuris muris* specific primers (Cutillas et al., 2002)
- **Tsp** = *Trichinella spiralis* specific primers (Golab et al., 2009)
Abstract

Lucas Huggins
The University of Manchester
Animal Biology (MPhil)
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Development of a copro-diagnostic molecular tool for the detection and identification of amphibian infecting helminths.

Worldwide at least a third of amphibians are currently categorised as being threatened with extinction, with many species experiencing population declines at an unprecedented rate. The causes of such synchronised amphibian losses are varied but recent research has begun to highlight a growing role that macroparasites are playing in this decline. However, diagnosing parasite infection in the field can be problematic and has principally relied on collection and euthanasia of hosts, followed by necropsy and morphological identification of parasites in situ. To improve upon this, the current study developed a non-invasive PCR-based methodology for sensitive detection and identification of nematode infecting amphibians by detecting environmental DNA released in faeces or shed eggs. A DNA extraction protocol optimised for liberation of DNA from resilient parasite eggs was developed alongside the design of a novel, nematode universal, degenerate primer pair. Used in conjunction this DNA extraction protocol and primer pair was tested on a wide range of faecal samples from both captive and wild amphibians, showing great promise at detecting parasitic nematode infections. New infections, such as a Railletnema nematode infestation in poison dart frogs from ZSL London Zoo were uncovered, as well as a similar infection from Mantella cowani frogs in the wild. These results demonstrate the utility of our developed protocol for revealing previously undetected parasitic infections from amphibians in both captive colonies and in situ. Furthermore, the method was proven to function equally well on reptile samples. When our new primers were compared to previously reported nematode specific primers in the literature they were demonstrated to have greater sensitivity and specificity for nematode DNA. Our developed eDNA approach mitigates problems associated with microscopic identification, such as the necessity for researcher expertise and could also provide insight into the dynamics of parasite infection in wild amphibians, underscoring links to their declines. In addition, the work within this thesis raises awareness of some of the issues relating to the diagnostic use of eDNA from faeces, for example the contamination of samples by free-living organisms.
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1. Introduction

In the last two decades scientific recognition of amphibian population losses and species extinctions has grown vastly. Akin to biodiversity losses in other taxa, the principal reasons for these declines are due to human impacts on our biosphere as well as the effects these impacts have on wildlife infecting pathogens. Now, current research is beginning to underscore an important role for macroparasite infections in amphibian extirpations. However, there has been little development in ways of detecting and identifying the parasites of amphibians past long-established methods, such as necropsy and examination of organs for parasites in situ. These methods require great expertise in morphological species identification and act to further reduce amphibian population numbers if used on an already threatened species. Therefore, this thesis sets out to develop a non-invasive, diagnostic protocol for detection of parasite DNA released in the faeces of the amphibian host. It is hoped that such a protocol could also identify the parasitic species present in the host by use of DNA barcoding and thus provide important information on the presence of helminths in wild amphibian populations.

1.1 The amphibian crisis

Amphibians worldwide are currently undergoing such dramatic population declines that many believe the Earth is in the midst of a sixth mass extinction, the likes of which have not been seen since the Cretaceous-Paleogene event that eliminated the dinosaurs (Cheng et al., 2011; Cushman, 2006; Wake and Vredenburg, 2008). A third of extant amphibian species are categorised as being threatened by extinction by the IUCN red list with many more as yet uncategorised due to insufficient data (Roedder et al., 2009). The reasons behind such alarming declines are multifactorial but principally originate from anthropogenic alteration to ecosystems via habitat alteration or degradation, climate change, pollution and introduction of novel diseases (Cheng et al., 2011; Laurance et al., 2011; Wake and Vredenburg, 2008). One pathogen in particular, the panzootic fungus _Batrachochytrium dendrobatidis_ (Bd), has been identified as responsible for a wide range of amphibian extirpations even in very isolated ecosystems that were assumed to be protected (Cheng et al., 2011; Roedder et al., 2009). More concerning still, is that in response to global climate change, the range normally occupied by Bd has been altered, bringing it into contact with naïve amphibian species and populations (Seimon et al., 2007; Zippel et al., 2011). Examples such as this highlight the complexity of predicting biological responses to anthropogenic changes in our environment and draw attention to the necessity for further study on how ecosystem perturbation can give rise to disease outbreaks in wildlife and man (Pounds et al., 1999; Puschendorf et al., 2011).

Amphibians play a central role in many ecological networks and are found in nearly all habitats that have at least a minor aquatic component (Tyler et al., 2007). Their population numbers frequently exceed those of mammals and in the tropics extensive adaptive radiation has created a plethora of different species, significantly contributing to ecological biodiversity (Beebee, 1996; Pearman, 1997; Vigle, 2008). Additionally, amphibians are frequently used as bioindicators i.e. species or...
communities that give an indication of the quality of the local environmental (Oldekop et al., 2012). Their permeable skin and biphasic life cycles bring them into closer contact with more aspects of their habitat than most species, thus making them sensitive to environmental changes such as pollution, eutrophication or introduction of new species (Beebee, 1996; Oldekop et al., 2012). From a utilitarian perspective, amphibians have provided many drugs of medical and veterinary importance. Biochemical compounds produced by amphibians as defensive skin secretions have found uses to man as a range of antibiotics, analgesics and anti-tumour agents (Tyler et al., 2007). Such utility in research and their ecological predominance underscores the importance of the amphibian crisis to mankind and provides an onus for human action to alleviate the situation.

Whilst substantial amounts of investigation have focussed on the effects of anthropogenic ecosystem alteration and Bd on amphibian die-offs there has been less interest in other, more subtle and insidious causes that may play an important role (Koprivnikar et al., 2012). An expanding literature has begun to shed light on the crucial part macroparasite infections play in amphibian declines, with particular parasites being associated with the extirpation of a host species or community from a habitat (Romansic et al., 2011; Wright and Whitaker, 2001).

1.2 Parasite infections of amphibians

The digenean trematode, *Ribeiroia ondatrae*, is now recognised as the principal causative agent for widespread outbreaks of severe limb deformities in many different North American frog populations, causing high levels of mortality (Johnson et al., 1999; Johnson et al., 2007). Other culprits include members of the trematode genera *Echinostoma* and *Echinoparyphium* that are found in wetland habitats worldwide, infecting a range of Anuran hosts. These species cause stunted growth and oedema in tadpoles, renal pathology in adult frogs and have been observed to reach infection prevalence as high as 100% in some zones (Johnson and McKenzie, 2009). In Australia, introduction of Myxozoan parasites harboured within invasive and resistant Cane toads has exacerbated endemic Anuran declines, (Harris et al., 2013) whilst many captive amphibian populations have been reported to die-off after succumbing to lungworm infections, such as the nematode *Rhabdia bufonis* (Tinsley, 1995). Often, the opportunistic spread of an ever-present or newly introduced macroparasite can be the final insult to an already weakened amphibian community that has been previously damaged by more pervasive pathogens, like Bd (Romansic et al., 2011).

A comprehensive understanding of the presence and dynamics of macroparasitic infections in amphibians is also vitally important for their ex situ care, especially if captive populations are intended for reintroduction in the future (Densmore and Green, 2007; Griffiths and Pavajeau, 2008). Many critically endangered Anuran species are currently maintained in zoos and other research institutes, as backup populations, if species are eradicated in situ (Browne et al., 2012; Zippel et al., 2011). Standard procedure upon removal from the wild is a period of quarantine and an anti-helminthic treatment, to attempt to eliminate the risk of infection into the captive population (Pessier et al., 2014). However, the long-term ramifications of such procedures are unknown. Lack
of exposure to parasites ex situ may leave populations immunologically naïve or remove selection for immunologically important genes, making future release of such populations back into the wild difficult (Browne et al., 2012; Fournie et al., 2015; Griffiths and Pavajeau, 2008). This is particularly pertinent given the frequent failures of amphibian reintroduction programs, emphasising the need for more research on amphibian macroparasites (Griffiths and Pavajeau, 2008).

A broad variety of metazoans parasite all stages of the amphibian lifecycle and these share many similarities with the taxa found in fish, with interclass transmission being a relatively frequent phenomenon (Densmore and Green, 2007; Wright and Whitaker, 2001). The most common parasites of Anurans are helminths, including nematodes, trematodes and cestodes (Figure 1), whilst other minor groups are also observed, such as monogeneans, acanthocephalans, myxozoans, leeches and arthropods (Tinsley, 1995; Wright and Whitaker, 2001). Common sites of infection by helminths are the skin, cloaca, gut, lungs and kidneys but if the infected amphibian is being occupied as an intermediate or paratenic host, then encysted larval forms may be found in virtually any organ or tissue (Densmore and Green, 2007; Fournie et al., 2015; Harris et al., 2013; Johnson and McKenzie, 2009; Wright and Whitaker, 2001).

The occupation of amphibians by helminths is so widespread that they can even become a vector for human infection (Dorny et al., 2009). Thus, human tapeworms of the genus Spirometra (Li, 1991; Wiwanitkit, 2005), nematodes of the genus Angiostrongylus (Dorny et al., 2009) and Echinostoma flukes (Fried et al., 2004; Graczyk and Fried, 1998) can all be contracted by eating raw, infected frog meat and cause serious morbidity in the Far East, where such uncooked aquatic animals can comprise a large part of local diets (Wei et al., 2015).

**Figure 1:** Phylogenetic relationships of bilaterians. Major phyla of amphibian macroparasites are highlighted in red. Platyhelminthes include trematodes and cestodes. Taken and adapted from Nielsen (2001).
Given the importance of amphibian parasites, in species declines and ecological dynamics, it is surprising that so little attention has been paid to them (Koprivnikar et al., 2012). Research attempts have primarily been hampered by difficulties in identification, which has traditionally been done by morphology (Budischak et al., 2015). Identification and quantification of different helminth life cycle stages in faeces or at necropsy has long been considered the 'gold standard' with regard to characterisation of helminth communities in nature, however, it presents many problems (Budischak et al., 2015; McManus, 2006). Egg counts using faecal smears are very susceptible to human error, whilst egg and larval stage morphology is often identical between species and many cannot be taxonomically distinguished past family level (Budischak et al., 2015; Gobert et al., 2005). Furthermore, many helminths are soft-bodied with few, stable anatomical parts and can exhibit dramatic phenotypic plasticity within a species, making accurate identification even more error prone (Moszczynska et al., 2009; Prosser et al., 2013; Rodrigues da Silva et al., 2010).

Hence, to accrue more information on helminths in a standardised way, greater attention needs to be paid to molecular techniques that mitigate challenging morphological identification under the microscope, distinguishing and recognising the presence of an organism using its DNA (Rodrigues da Silva et al., 2010).

1.3 The growing importance of molecular techniques in parasitology

With the advent of the Polymerase Chain Reaction (PCR) there has been an increasing body of research applying molecular techniques to the detection, identification and quantification of parasites (Gasser, 2006). Such techniques are beginning to supersede previous microscopy-based methods not only due to the aforementioned problems with parasite morphology but also because they are more sensitive, lack a necessity for expertise and are less time-consuming (Bhadury and Austen, 2010; Dinkel et al., 1998; Eckert, 2003; Taniuchi et al., 2011).

Molecular techniques are also recognised as being superior to serological analysis (Praet et al., 2013). Serology-based methods have been used extensively in the context of human parasite infections, such as Taeniasis and Echinococcosis, relying on the detection of antibody or parasite antigen by ELISA (Abbasi et al., 2003; Praet et al., 2013). However, they frequently demonstrate poor sensitivity in low burden human infections and an inability to discern between different species, which is of critical importance if an appropriate treatment is to be administered (Praet et al., 2013).

PCR methods have unparalleled sensitivity and specificity because they involve the selective amplification of a particular gene or DNA sequence of interest, from a background of genetic sample material (Gasser, 2006). The starting material may consist of minute quantities of DNA but enzymatic DNA synthesis allows for its rapid replication, whilst carefully selected primers ensure that only the sequence of interest is copied. By the end of a process of carefully controlled thermocycling, millions of copies of the sequence may be present, allowing for further analysis, or characterisation, via DNA sequencing (Gasser, 2006).
Information gained through molecular techniques has already revolutionised many areas of parasitology by informing us on species taxonomy, phylogenetics, gene flow and its barriers, as well as parasite population dynamics which are important for transmission (Daniel Pinacho-Pinacho et al., 2015; Gasser, 2006).

In the critically important fields of disease surveillance and discovery of zoonotic reservoirs, molecular techniques consistently prove to be invaluable. For example, a study by Wei et al. (2014) used analysis of three mitochondrial molecular markers on tissue cysts found in wild caught frogs to identify tapeworms of the *Spirometra* genus. These tapeworms pose a disease threat to human populations in some East Asian countries, where consumption of frog meat is thought to provide medicinal benefits. These researchers were able to identify the main *Spirometra* species with zoonotic potential, which frog hosts they were most common in and uncover phylogenetic relationships between the different tapeworms (Wei et al., 2015).

Moreover, research in China on roundworms of the *Ascaris* genus, which infects between 100 to 200 million people worldwide, settled a debate on the role pigs play as a potential zoonotic reservoir of the infection (Peng et al., 2003). Molecular techniques elucidated a variety of different *Ascaris* strains within a species, all of which were associated with either human or porcine hosts. Results showed strong parasite strain to host affiliations, with very limited gene flow between strains, confirming that pigs do not play an important role in *Ascaris* transmission in the region (Gasser, 2006; Peng et al., 2003). Such findings led the researchers involved to question the species status of these strains; as such a barrier to gene flow may provide evidence that these strains are actually different species (Peng et al., 2003).

1.4 DNA barcoding for species identification

Given the power of molecular techniques in parasite detection, interest has also grown in the potential use of such techniques for identifying species based purely on certain DNA sequences in the genome, namely, DNA-barcoding (Gasser, 2006; Van Steenkiste et al., 2015). Barcoding relies on molecular markers that are relatively stable over evolutionary time (Floyd et al., 2002). Tissue samples from a morphologically identified species are analysed at a particular genetic locus and a unique, diagnostic sequence of base pairs found, that identifies that species but no other (Bhadury and Austen, 2010; Rodrigues da Silva et al., 2010). Appropriate diagnostic regions must have enough interspecific sequence variation so as to distinguish between species but with minimal intraspecific variation (Floyd et al., 2002; Gasser, 2006). More variable DNA sequences can be used if different strains within a species need to be identified (Gasser, 2006). Such barcoding approaches have already been used in characterisation of meiofaunal nematode communities in different soil and marine ecosystems, accelerating discovery of cryptic species (Bhadury et al., 2006a; Bhadury and Austen, 2010; Floyd et al., 2002; Rodrigues da Silva et al., 2010).

If barcoding approaches are to be successful an appropriate genetic locus must be selected (Gasser, 2006). Most proponents of the universal barcoding approach advocate the use of the 5'
end of the cytochrome c oxidase gene (COI), encoded in the mitochondrial genome (Hadziavdic et al., 2014; Hajibabaei et al., 2007). This codes for a key evolutionarily conserved protein in the mitochondria’s respiratory complex and has a relatively high evolutionary rate, capable of good discrimination between closely related species (McManus, 2006; Prosser et al., 2013). However, within macroparasite research, commonly used barcoding targets in lieu of cox1 are sequences of the nuclear ribosomal DNA (rDNA) region (Floyd et al., 2005; Gasser, 2006). Key sequences in this region include the first and second internal transcribed spacers (ITS-1 and 2) that have proven reliable and been used in a multitude of different studies involving macroparasites, particularly nematodes (Gasser, 2006; Prosser et al., 2013). Apart from targeting of the ITS-1 and 2, the rDNA sequence encoding the 18S small ribosomal subunit (18S rRNA) has also been very successful. Using this, Floyd et al. (2005) was able to design primers that are uniquely nematode-specific and have consequently been used in a range of studies, investigating nematode diversity and abundance (Bhadury et al., 2006a; Bucklin et al., 2011; Shen et al., 2007).

Once a potential DNA barcoding sequence has been found, there is a broadening range of PCR types and further analysis tools that are employable, to help in the detection and identification of the parasite in the host of interest (Gasser, 2006).

The most commonly used PCR-based method is singleplex PCR i.e. use of just one primer pair that only amplifies one sequence of interest, followed by an electrophoretic approach, such as single-strand conformation polymorphism (SSCP) (Beveridge and Gasser, 2014; Gasser, 2006). SSCP is technically simplistic and is sensitive enough to detect just single base pair changes between different DNA sequences (Gasser, 2006; Yamasaki et al., 2002). Examples of SSCP usage include identification and diagnosis of a variety of human-infecting Taeniid cestodes using the COI and cyt b genes (Yamasaki et al., 2002) as well as for investigating the prevalence of the important human zoonosis E. granulosus in Palestinian dogs (Al-Jawabreh et al., 2015). A simple extension of the singleplex PCR format is multiplex PCR (Taniuchi et al., 2011). This constitutes having a number of different primer pairs that bind to a variety of different sequences or genes of interest (Gasser, 2006). Mixtures of primers can be designed against species-specific sequences, producing banding patterns that identify which species are present in a sample using just one reaction, saving much experimental time (Gobert et al., 2005; McManus, 2006; Prosser et al., 2013). Multiplex PCR has already been used in an extensive variety of applications, including identification of Schistosoma and Taenia species from egg and proglottid DNA (Gobert et al., 2005; Ito and Craig, 2003; Taniuchi et al., 2011).

PCR techniques when modified to include fluorescent dyes, can also give quantitative data, potentially providing information on the parasite burden of an individual or abundance in an environment (Bass et al., 2015; Gasser, 2006; Huver et al., 2015). Such, quantitative PCR methods (qPCR) provide detailed information on the quantity of template sequence that was present in a sample of interest, before amplification (Huver et al., 2015). These protocols have found function in surveys of infectious cercariae numbers in environmental sampling of wetland ecosystems (Huver
et al., 2015; Longshaw et al., 2012), as well as for gastrointestinal nematodes infecting sheep (Dong et al., 2013; McNally et al., 2013).

Additionally, fieldwork carried out to explore parasite prevalence in human and animal populations is often carried out in remote locations, where access to costly molecular tools or even electricity can be impossible (Arimatsu et al., 2015). This poses limits on the use of conventional PCR but can be overcome by loop-mediated isothermal amplification (LAMP), which does not require temperature cycling for the reaction to work (Arimatsu et al., 2015). The reaction can proceed at a single temperature with use of a transportable heating block and addition of fluorophores means a successful amplification can be observed by a colour change, detectable to the naked eye (Kang et al., 2015). The relatively simplicity and transportability of LAMP has made it become the technique of choice for many field studies investigating the epidemiology of tropical diseases in developing regions (Arimatsu et al., 2015; Gandasegui et al., 2015). Gandasegui et al. (2015) found LAMP to be highly successful in surveillance of Schistosomiasis in endemic regions of sub-Saharan Africa. They assessed LAMP as being superior to other PCR methods due to its low susceptibility to inhibitors, lack of requisite DNA extraction steps and greater sensitivity, detecting as little as 10 fg of Schistosome DNA, even if the host was not excreting eggs (Gandasegui et al., 2015).

Most studies using PCR to detect parasitic infection and burden have focussed on one target species, often in a human or livestock infection context where the parasite of interest causes substantial mortality or morbidity (Budischak et al., 2015). However, there are many host-parasite systems in which the common parasites of the host are virtually unknown, particularly in the growing field of wild parasitology (Budischak et al., 2015). In such systems a species specific primer for detection is inappropriate and may underestimate parasite community composition, hence more broad-spectrum primers must be used that target whole families, orders or even an entire phylum (Budischak et al., 2015). Phylum specific primers have been used substantially in the literature, specifically in the context of characterisation of nematode communities within soil or coastal sediments (Bhadury and Austen, 2010; Floyd et al., 2002; Shen et al., 2007). Budischak et al. (2015) also found such phylum wide barcoding primers ideal for the description of nematode communities of African buffalo, in which co-infection was common and some of the species found were as yet undescribed. Nonetheless, many difficulties of broad-spectrum primers have also been identified, the most challenging of which are issues with primer cross-reactivity on DNA from outside of the target group of interest (Bhadury et al., 2006b; Bhadury et al., 2011). Bhadury and Austen (2010) had to redesign previously developed phylum wide primers for the Nematoda, because they so frequently amplified fungal DNA, skewing analyses of nematode community composition.
1.5 The potential uses of environmental DNA and molecular copro-diagnosis for amphibian conservation

For any PCR-based methodology to be successful, the sampling technique and type of sample taken is of paramount importance if the molecular diagnostic system is to detect and identify the parasitic DNA effectively.

Sampling methods for detection of parasite in the wild can be characterised as either invasive or non-invasive (Huver et al., 2015). The former category encompasses tissue collection from a host e.g. toe-clipping or a skin or blood sample (Harris et al., 2013; Perera et al., 2013). Invasive sampling in parasitological research commonly kills the host, then examines the body at necropsy, to extensively characterise the number and species of macroparasites in all common infection sites (Eckert, 2003). If substantial morphological information on the common parasites of the host is known, then lethal sampling can be considered the ‘gold standard’ (Budischak et al., 2015; Eckert, 2003). Nonetheless, in wild host populations many macroparasites may as yet be uncharacterised, whilst the practise of killing the host itself for sampling may be unethical or exacerbate the problems of an already endangered population (Budischak et al., 2015). Even non-lethal, invasive sampling can present problems as the removal of a biological sample from a host may cause undue stress or increase subsequent infection risk (Fisher et al., 2013; Funk et al., 2005). For example, amphibian toe-clipping, a frequently used method of collecting disease or genetic information on wild populations has recently come into question, as some data suggests it may severely reduce subsequent Anuran survival (Campbell et al., 2009; Funk et al., 2005).

A less harmful approach is non-invasive sampling, which involves collection of DNA that is shed and left behind by the animal under investigation with no necessity to catch, handle or disturb the animal (Huver et al., 2015; Taberlet et al., 1999). The DNA is normally acquired through release of eggs, hair, skin, sloughed cells, feathers, mucus, saliva, blood, faeces and urine (Bohmann et al., 2014; Taberlet et al., 1999). Such, environmental DNA (eDNA) is often persistent in ecosystems for a long time and is much less labour intensive to find than traditional surveying methods that are very prone to human error, especially when surveying rare or cryptic species (Huver et al., 2015; Longshaw et al., 2012). Sampling of eDNA can circumvent many of the difficulties of parasite surveillance in the wild as parasites constantly shed live cells or extracellular DNA from cell death and destruction, providing signals that leave the host and can be easily detected (Bass et al., 2015).

A particularly rich source of eDNA is faeces, as not only are cells from the infecting parasite commonly excreted but so are the transmissible stages of many macroparasite, for example eggs or proglottids. Copro-diagnosis, the analysis of faeces for such parasite life cycle stages and eDNA, is a particularly attractive non-invasive technique as samples are easily found in situ and can be kept in common preservatives, such as 95% ethanol, for later analysis in the lab (McNally et al., 2013; Wang et al., 2010).
Molecular copro-diagnostic detection and identification of parasites has frequently been found to have both higher sensitivity and specificity than microscopy, in detection of hookworms (Sato et al., 2010; Traub et al., 2008), Taeniids (Praet et al., 2013), and Echinococcus species (Abbasi et al., 2003; Dinkel et al., 1998). Advantages of PCR-based techniques over microscopic examination are that they do not rely on egg morphology, which is often very uniform between helminths, thereby demanding a high level of taxonomic expertise for correct identification (Gobert et al., 2005). Moreover, microscopic examination is made difficult due to an irregular egg output by many parasites, leading to clumped, uneven distributions of eggs throughout faecal samples (Dinkel et al., 1998; Ito and Craig, 2003; Mathis and Deplazes, 2006). To overcome such problems, more faecal samples per host must be examined to get accurate results, compared to the relatively low quantity of sample needed in copro-diagnosis (Gobert et al., 2005; Mes, 2003). Therefore, in nearly all cases molecular copro-diagnosis is a faster and more sensitive technique than microscopy-based detection (Mes, 2003).

For molecular based copro-diagnosis of parasites to be most effective an egg lysis procedure is critical (Mathis and Deplazes, 2006). Helminth eggs and proglottids are protected by robust shells or resilient biological structures, such as the cestode embryophore (do Espirito-Santo et al., 2012; Dyachenko et al., 2008). Many of these parasite dispersal stages have evolved to be tenacious in their persistence in diverse environments and therefore an array of potent lysis technologies have been developed to free DNA from them (Demeler et al., 2013).

Common lysis procedures involve use of either a chemical membrane disruptor such as an alkali, detergent, chaotropic agent (urea, HCl, etc.) or physical rupture such as bead beating sonication, heating or freeze/thawing (Andersen et al., 2013; Slapeta et al., 2002; Weiss, 1995). Each method comes with its own set of advantages and disadvantages. Many of the stronger lysis techniques may also shear DNA making it unsuitable for molecular analysis, whilst weaker methods may not liberate sufficient DNA, or any whatsoever (de Lipthay et al., 2004). Often the protocol used must be adapted to the specific parasite species under investigation.

Molecular based copro-diagnostic detection of eDNA and parasite transmissible stages has been successfully used to track and discover reservoirs of potentially lethal zoonotic parasite infections, such as hydatidosis (Al-Jawabreh et al., 2015), ancylostomiasis (Traub et al., 2008), trichuriasis and echinostomiasis (Schaer et al., 2014). Given the success of molecular copro-diagnostic and eDNA methods in higher vertebrates, there is much to suggest that the same techniques could be applied to the amphibian crisis, to deliver important information on the role macroparasites might be playing.

The development of copro-diagnostic methodologies could shed light on the emergence of amphibian colony die-offs in ex situ conditions (Tinsley, 1995; Wright and Whitaker, 2001) and in regions where raw frog meat is consumed copro-diagnostic monitoring of wild amphibians could help prevent the acquisition of helminth zoonoses harboured in these hosts (Dorny et al., 2009;
Moreover, such techniques can provide insight into natural helminth ecology and the dynamics of amphibian-parasite coevolution (Johnson et al., 2007; Koprivnikar et al., 2012).

The large role macroparasites play in amphibian ecology and population declines is best exemplified by the trematode fluke, *Ribeiroia ondatrae*, in North America (Johnson and Lunde, 2005). This parasite was first identified after gross limb deformations including extra or missing hind legs were observed, in a wide range of sites and Anuran species (Hoverman et al., 2012; Johnson and Lunde, 2005). The rate of malformation in populations could exceed over 15% at some locations and after much investigation *R. ondatrae* was identified as the causative agent (Johnson and Lunde, 2005; Johnson and McKenzie, 2009). It was also noticed that different hosts had different susceptibilities to the parasite; some species were relatively immune, whilst those that showed high levels of induced developmental malformations displayed almost 100% mortality, due to predation (Blaustein et al., 2012; Johnson et al., 1999). This disparate effect *R. ondatrae* has on the spectrum of amphibian hosts it infects means that it plays a large, but difficult to predict role on the local ecology of a habitat (Johnson et al., 2010).

In response to this, Huver et al. (2015) have already developed and utilised an eDNA technique for detection of *R. ondatrae* via filtration of water in wetland ecosystems, to detect eDNA released by cercariae. Their analysis was so effective it identified the parasites presence in locations previously thought uncontaminated and in combination with qPCR could indicate a reliable estimate of cercarial abundance, in a given habitat (Huver et al., 2015).

Taking such successes into account, if eDNA and copro-diagnostic analytical approaches are further explored then we may be able to glean new ways of monitoring ecosystem health, employing parasites as bioindicators (Koprivnikar et al., 2012). The complex life cycles of many macroparasites, which depend on multiple hosts across numerous trophic levels, means that they are particularly sensitive to environmental disturbance (Koprivnikar et al., 2012). Such theory predicts that less perturbed habitats should exhibit a larger variety of parasitic species per host and that environmental stress will be translated to changes in parasite fauna, most likely a decrease in diversity (Koprivnikar et al., 2012). Hence, there is the potential to use data on parasite community composition and dynamics as a bioindicator, providing information on habitat condition (Koprivnikar et al., 2012).

### 1.6 Project hypothesis and aims

Here I hypothesise that amphibians in the wild and captivity that are infected with macroparasites will release a detectable molecular signal, either via eDNA in transmissible stages or in sloughed cells and excretions. I think that such a signal will allow for the development of a copro-diagnostic protocol for detection of parasite eDNA in faeces that could be honed to provide information on parasite species and burden as well. This data may assist in alleviation of the amphibian crisis by providing insight into the role helminths play in this crisis and into the ecological interplay between...
wild amphibian parasites and their hosts. In light of this, the following MPhil project sets out to answer the following questions:

1) Can an effective method for DNA extraction from faeces be developed, capable of liberating parasitic eDNA even from the most resilient transmissible structures?
2) Can existing broad-spectrum primers for entire parasitic phyla in the literature detect parasite eDNA in faeces and thus be able to identify infected amphibians?
3) Can bespoke primers for an entire parasitic phylum, such as the Nematoda, be designed and demonstrated to work in a copro-diagnostic protocol and can these primers improve upon existing ones in the literature?
4) Can such developed primers be used to amplify species barcodes and therefore identify unknown parasites?
5) Can any developed copro-diagnostic protocol be demonstrated to work in other model systems e.g. in the detection of reptile parasites?

2. Materials and Methods

2.1 DNA extractions from tissue

DNA was extracted from 15 mg of helminth tissue using the QIAGEN DNeasy® Blood & Tissue Kit (Manchester, UK) under aseptic conditions with only slight modifications to the manufacturer's protocol. The DNA was allowed to elute for 15 min into 200 µl of buffer AE on the spin column membrane during the final step of the extraction protocol. All samples were weighed on a high precision Denver Instrument MXX-412 scale (Epsom, UK). To ensure sterilisation of tips, pipettes and other consumables autoclaving and UV sterilisation was carried out using the UVP CL-1000 Ultraviolet Crosslinker (Cambridge, UK). During the proteinase K digestion step samples were heated in a Grant T100 water bath (Cambridge, UK) and all centrifugation steps were done in a Sigma 1-14 table top centrifuge (Dorset, UK). When not in use DNA samples were kept chilled at 4°C.

2.2 Ethical approval and licensing

All animal experiments were approved by the University of Manchester Animal Welfare and Ethical Review Board and performed under the regulation of the Home Office Scientific Procedures Act (1986). All experimentation using nematode models of infection was performed under the Home Office project licence 70/8127, whilst those that used a Platyhelminth infection model were performed under the Home Office project licence 70/07815.
2.3 DNA extractions from faeces

Standard protocol used for DNA extraction from faeces was as follows although quantities of materials used and timings of steps were experimented with throughout the project to optimise the final protocol. DNA was typically extracted from 200 mg of faeces using the QIAamp® Fast DNA Stool Mini Kit (QiaGen) under aseptic conditions with only slight modifications to the manufacturer's protocol. After addition of the InhibitEx buffer to the faecal sample, the sample was vortexed for one minute using the Vortex-Genie2 (Scientific Industries Inc., York, UK) and then incubated and shaken in an Eppendorf Thermomixer C (Stevenage, UK) at 40°C and 67 g for between 1 – 2 hours. The Proteinase K digestion was carried out for 20 min. Two elution steps were typically carried out, a first elution for 20 min in 100 µl of buffer AE with centrifugation, followed by a second elution step in 50 µl for 15 min and centrifugation. When not in use DNA samples were kept chilled at 4°C. As the project developed an additional bead-beating step was added to the faecal extraction protocol. The standard bead-beating process was the use of three 4 mm diameter borosilicate glass beads (Sigma) placed with each sample after the addition of the InhibitEx or ASL buffer in an Eppendorf Safelock 2 ml test tube. Samples were then bead-beaten in a Retsch MM400 mixer mill (Derbyshire, UK) at 30 Hz for between 5 – 10 min with regular movement of the samples between the pockets of the arm cradles to ensure a consistent beating across all samples. This step was carried out before the first incubation step. After the incubation and centrifugation steps the beads were removed and washed in Virkon, followed by a 10% HCl acid bath and then Milli-Q water (from Millipore Advantage A10, Feltham, UK) to allow for their re-use.

2.4 Analysis of DNA concentration

DNA concentration analysis was performed on a ThermoFisher Scientific NanoDrop 2000 spectrophotometer (Loughborough, UK). The platform was initially cleaned with 2 µl of Milli-Q water and then blanked with the appropriate buffer. 1 µl of DNA extract was mounted to allow for analysis of DNA concentration; the platform was cleaned and re-blanked between samples. DNA concentration, 260/230 and 260/280 ratios were all noted.

2.5 PCR amplification

PCRs were prepared in aseptic conditions with all consumables UV sterilised, mastermixes were made on ice. PCRs were typically 20 – 25 µl in volume comprising: 10.88 µl of Milli-Q water, 2.5 µl PCR buffer, 3.5 µl Mg, 0.5 µl dNTPs, 0.12 µl FastStart Taq DNA Polymerase (All Roche, Sussex, UK), 1.25 µl of both forward and reverse primers and 0.5 µl BSA (New England Biolabs Inc., Hitchin UK). 1 µl of tissue DNA extract was used, whilst between 5 and 10 µl of faecal DNA was used per reaction. Tissue DNA extracts typical contained 10 – 50 ng/µl and faecal extract from 5 – 100 ng/µl. Typically, a negative control containing 5 µl of Milli-Q water instead of faecal or tissue DNA was run alongside the other PCRs to check for contamination. All primers were synthesised by Eurofins Genomics (Wolverhampton, UK) and made up to 10 µm. The primers used, in relative order of their usage throughout the duration of this project, are listed in Table 1. The thermocycling
programs recommended by these two papers were also used. For nematode DNA amplification this was an initial denaturation at 94°C for 5 min; 35 cycles of amplification (94°C for 30 s; 54°C for 30 s; 72°C for 1 min); followed by a final extension at 72°C for 10 min. For Platyhelminth DNA amplification an initial denaturation at 94°C for 2 min; 3 cycles of 94°C for 40 s, 51°C for 40 s, 72°C for 1 min; 5 ‘touchdown’ cycles of 94°C for 40 s, 50°C to 46°C for 40 s (dropping 1°C per cycle), 72°C for 1 min; 35 cycles of 94°C for 40 s, 45°C for 40 s, 72°C for 1 min; and a final extension at 72°C for 5 min. All PCR amplifications were carried out in a Techne Prime Thermal Cycler (Staffordshire, UK) with a HYBAID touchdown compression pad (ThermoFisher). Before electrophoresis, PCR product was centrifuged in either the MyFuge C1012 mini table top centrifuge (Sigma) or a Sigma 2-6E plate centrifuge. PCR product was kept chilled at 4°C. As the project continued various other primers from the literature and developed primers were also tested, using the recommended thermocycling conditions or adapted conditions from those described previously.

**Table 1: Information on primers used in this study.** Data on primers used, in chronological order of use, throughout this study. First column refers to the primer’s name in the literature from which it was taken, whilst fourth column denotes how that primer is referred to in this study. Degeneracy refers to the number of degenerate base pairs in the primer and the percentage of degenerate bases relative to the entire sequence. Degenerate bases typically increase the number of sequences a primer can bind to. Asterisked primer had its sequence taken from http://xyala2.bio.ed.ac.uk/research/barcoding/sourhope/nemoprimers.shtml.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Taxa targeted</th>
<th>Degeneracy</th>
<th>Nomenclature</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nem_18S_F</td>
<td>Nematoda Phylum Universal</td>
<td>No</td>
<td>NemUni-1</td>
<td>Floyd et al. (2005)</td>
</tr>
<tr>
<td>Nem_18S_R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dice1F, Dice11R, Dice14R</td>
<td>Platyhelminth (Cestode and Trematode) Universal</td>
<td>Yes (9%), Yes (9%), Yes (12%)</td>
<td>PlatUni</td>
<td>Van Steenkiste et al. (2015)</td>
</tr>
<tr>
<td>ATP6-F, ATP6-R</td>
<td><em>Trichinella spiralis</em></td>
<td>No</td>
<td>Tsp</td>
<td>Golab et al. (2009)</td>
</tr>
<tr>
<td>NC5-F, NC2-R</td>
<td><em>Trichuris muris</em></td>
<td>No</td>
<td>Tmu</td>
<td>Cutillas et al. (2002)</td>
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<tr>
<td>RitF, 1500R</td>
<td>Rhabdiasidae family specific</td>
<td>No</td>
<td>Rhab</td>
<td>Tkach et al. (2014)</td>
</tr>
<tr>
<td>StroS-F, StroAS-R</td>
<td><em>Strongyloides</em> genus specific</td>
<td>No</td>
<td>Str</td>
<td>Kramme et al. (2011)</td>
</tr>
<tr>
<td>Asc6F, Asc7R, Asc10F, Asc11R</td>
<td>Ascaridoidea family specific</td>
<td>No, No, No, Yes (10%)</td>
<td>Asc-1, Asc-2</td>
<td>Loreille et al. (2001)</td>
</tr>
<tr>
<td>M18F, M18R</td>
<td>Nematoda Phylum Universal</td>
<td>Yes (10%), No</td>
<td>NemUni-2</td>
<td>Bhadury and Austen (2010)</td>
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<td>Nem_18S_F, nSSU_R, 22R*</td>
<td>Nematoda Phylum Universal</td>
<td>No, No</td>
<td>NemUni-3</td>
<td>Personal Communication Prof. Marx Blaxter (14/03/16)</td>
</tr>
<tr>
<td>Nem1217F, Nem1619R</td>
<td>Nematoda Phylum Universal</td>
<td>Yes (28%), Yes (13%)</td>
<td>Nem27</td>
<td>Primers designed in this study</td>
</tr>
</tbody>
</table>
2.6 Gel electrophoresis

PCR product was run and visualised on 1% agarose gels comprising molecular grade agarose (London, UK), TBE buffer and 0.5 – 2 µl GelGreen™ Nucleic Acid Gel Stain (Biotium, Cambridge, UK). To load gel, 3 µl of PCR product was added to 2 µl of blue loading buffer (Bioline) and pipetted into the wells alongside 1 µl Hyperladder 1kb (Bioline) to act as a size standard. Product sizes were separated using electrophoresis in a RunOne™ Electrophoresis Cell (Cheshire, UK) at 45 v for between 30 – 80 min, depending on the size of the gel. After separation, gels were drained, left to cool and then mounted on a PrepOne™ Sapphire illuminator (EmbiTec) covered by a PI-1002 PrepOne™ filter (EmbiTec) and camera hood. Gels were then photographed using a Samsung DV300F digital camera (Surrey, UK) and the results analysed.

2.7 PCR product clean-up

When required for sequencing, PCR product amplicons were cleaned using a MiniElute® PCR Purification Kit (Qiagen), with only slight modifications to the manufacturer’s protocol. Cleaned DNA was eluted in 10 µl of autoclaved Milli-Q water for 20 minutes and kept chilled at 4°C.

2.8 Preparation for Sanger sequencing

Cleaned PCR product was made up to a concentration of 10 – 40 ng/µl and added to 4 pmole of a single relevant primer. This was then added to the necessary quantity of Milli-Q water to ensure a final quantity of 10 µl. For each PCR amplicon one sample containing the forward and one the reverse primer was sent for sequencing. Samples were Sanger sequenced at the University of Manchester DNA Sequencing Facility using Big Dye 3.1 chemistry on an ABI 3100 Genetic Analyzer (Fisher Scientific).

2.9 Sequence analysis

Sequence traces were examined and regions of poor quality or low-confidence sequence were removed in the free sequence visualisation software BioEdit v7.2.5 (http://www.mbio.ncsu.edu/bioedit/page2.html). The complimentary sequence of that produced by the reverse primer was aligned next to the sequence produced by the forward primer, using the ClustalW function. This allowed for the extraction of the entire DNA sequence amplified by the primers. To identify the species from which the sequences were from they were run through the GenBank nucleotide BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) and the top matches noted.

2.10 Preparation of faecal smears

Faecal pellets were mounted on a glass slide with a few drops of Milli-Q water. The pellets were crushed and smeared over the slide, covered with a cover slip and sealed using nail varnish. Slides were then examined and photographed by light microscopy on both a Leica S8APO Microscope at
x80 magnification with a Leica MC 170HD video camera (Milton Keynes, UK) and also on a high power Zeiss AxioVision snapshot microscope (Cambridge, UK) at x200 and x1,000 magnifications.

2.11 Group and individual housing of *M. betsileo* amphibians

The privately owned *M. betsileo* colony consisted of 12 individuals maintained at the University of Manchester in two 30 x 30 x 45cm ExoTerra™ glass and plastic terraria (Yorkshire, UK) with six frogs per terrarium. The colony was kept at room temperature and sprayed with water once a day. Each terrarium consisted of a 7 cm layer of soil:coir (ratio 1:1) with moss (*Sphagnaceae* sp.) and leaf litter (*Platanus* sp.) to build up a natural environment, in addition to a coconut shell to provide shelter. Animals were fed three times weekly on black crickets (*Gryllus bimaculatus*) dusted with the multivitamin supplement Nutrobal® (Vetark, Winchester, UK). All tanks were illuminated by a Philips daytime strip light (Netherlands) for general lighting at a 12:12 light:dark cycle. For the individual housing experiment *M. betsileo* amphibians were separately housed in an ExoTerra™ standard faunaria 370 x 220 x 250 mm with a substrate of blue roll instead of soil. An upturned plant pot and leaf litter were added to provide shelter and the tank sprayed once or more a day to ensure it remained moist.

2.12 *M. betsileo* dissection

Individuals were euthanized using ethically approved methods and then dissected. Dissections were carried out with all major organs removed and snap frozen in dry ice. The entire GI tract was maintained in Hanks’ Balanced Salt Solution (Sigma) at 37°C until it could be cut open and investigated by light microscopy using a Leica S8APO at x100 magnification.
3. Results

3.1 Optimisation of NemUni-1 and PlatUni primers and their application to a mouse model of infection

3.1.1 PCR amplification using helminth tissue DNA

DNA from the Platyhelminthes, *Schistosoma mansoni* and *Hymenolepis microstoma* was successfully extracted from tissue and amplified using the Platyhelminth specific PlatUni primers. A 570-580 bp region of the COI gene and an 800-820 bp region of the COI gene were both successfully amplified (Figure 2A, B). Nematode DNA from *Trichuris muris* and *Trichinella spiralis* tissue was also successfully amplified using the nematode specific NemUni-1 primers (Figure 2C). This amplified a 900 bp sequence of the 18S rRNA gene; however some non-specific products for the *T. muris* DNA template were also detected which is possibly due to degradation of tissue DNA samples due to inappropriate storage conditions.

![Figure 2](image_url) - **Figure 2** – PCR amplification products using NemUni-1 and PlatUni primers on helminth tissue DNA. The PlatUni primers successfully amplified from 5 μl of *S. mansoni* (Sm) and *H. microstoma* (Hm) tissue DNA extract. Asterisks indicate amplification by the alternate reverse primer Dice14R and arrows indicate the expected 570 bp and 810 bp products. The NemUni-1 primers successfully amplified from 5 μl *T. muris* (Tm) and *T. spiralis* (Ts) tissue DNA. Triangles indicate the expected 900 bp product. 1kb hyperladders were run (HL) and negative controls (X).

3.1.2 Primer cross-reactivity test on helminth tissue extractions

To confirm specificity of the published PlatUni primers they were tested for potential cross-reactivity on *T. spiralis* i.e. nematode DNA. These primers showed no cross-reactivity on *T. spiralis* DNA as no bands were seen (Figure 3), regardless of thermocycling program used. Hence, these primers demonstrated their specificity to Platyhelminth DNA.
In contrast, the NemUni-1 primers did demonstrate cross-reactivity on *S. mansoni* i.e. Platyhelminth DNA (Figure 3). These primers cross-reacted, producing amplification during both thermocycling programs recommended for use with these primers (Floyd et al., 2005; Van Steenkiste et al., 2015). A number of strong bands were produced by this cross-reactivity including one near 900 bp, the size expected to be produced by NemUni-1 primers on nematode DNA. Therefore, these primers did not exhibit specificity for nematode DNA alone.

![Figure 3](image_url)

**Figure 3 – Test for PlatUni and NemUni-1 cross-reactivity on helminth tissue DNA from non-target taxa.** NemUni-1 primers, designed for specific amplification of only nematode DNA, demonstrated cross-reactivity on 5 µl of *S. mansoni* (Sm) tissue DNA producing multiple bands, including a strong band at the expected 900 bp. Numbers in superscript indicates whether the nematode (') or Platyhelminth (**) thermocycling programs were used for the PCR. Neither of the PlatUni reverse primers, principal or alternate (*), amplified 5 µl of *T. spiralis* (Ts) tissue DNA and hence demonstrated Platyhelminth specificity. A 1kb hyperladder was run (HL) and a negative control (X).

3.1.3 Annealing temperature thermal gradient to regain specificity of NemUni-1 primers

The nematode specific NemUni-1 primers were found to amplify nematode DNA well (*T. muris* and *T. spiralis*) but also cross-react and amplify DNA from Platyhelminthes, *S. mansoni* and *H. microstoma*, at the annealing temperatures recommended by the authors (Floyd et al., 2005). Hence, a thermal gradient PCR was carried out using NemUni-1 primers on *H. microstoma* DNA (Figure 4A) with a range of annealing temperatures between 59.4°C and 65.6°C. *H. microstoma* DNA was amplified up to an annealing temperature of approximately 62.6°C, above which no bands were seen at the expected 900 bp size for NemUni-1 primers. To ascertain whether NemUni-1 primers could still replicate nematode DNA at the new higher annealing temperatures
they were also tested against *T. spiralis* DNA at 63.7°C, 65°C and 66°C (Figure 4B). At these higher temperatures a 900 bp fragment was still amplified on *T. spiralis* DNA but not on *H. microstoma* DNA (Figure 4B). Finally, to confirm the specificity of NemUni-1 primers, they were tested against *H. microstoma* DNA at 54°C the temperature recommended by the authors (Floyd et al., 2005), 63.3°C and the temperature I optimised of 66.5°C (Figure 4C) This data unequivocally demonstrated that the NemUni-1 primers lack specificity at lower annealing temperatures as they cross-react on Platyhelminth DNA but regain their specificity at higher temperatures, where this cross-reactivity is stopped.

**Figure 4** – **Annealing temperature** thermal gradient to regain specificity of NemUni-1 primers. **A:** Thermal gradient PCR demonstrated cross-reactive amplification of NemUni-1 primers on 5 µl of *H. microstoma* tissue DNA when the PCR was carried out at annealing temperatures up to 60.3°C (numbers above lanes indicate annealing temperature used in °C). Above 60.3°C no cross-reactivity occurred. Arrow indicates the expected 900 bp product. **B:** NemUni-1 primers were found to still amplify 5 µl of *T. spiralis* (Ts) tissue DNA at annealing temperatures of 63.7°C - 66°C but not on 5 µl of *H. microstoma* (Hm) tissue DNA. Arrows indicate the expected 900 bp product. **C:** NemUni-1 were shown to stop cross-reacting with 5 µl of *H. microstoma* tissue DNA above a 63.3°C annealing temperature, whilst these primers were still capable of amplifying 5 µl of *T. spiralis* tissue DNA at 66.5°C. Therefore, NemUni-1 primers regained their nematode specificity at this annealing temperature. Arrows indicate the expected 900 bp product. All gels were run with a 1 kb hyperladder (HL) and a negative control (X).
3.1.4 PCR amplification of parasite DNA extracted from faeces of infected mice

To develop the faecal eDNA extraction protocol a QIAamp DNA stool mini kit was used on faeces from mice infected with nematode and Platyhelminth (cestode and trematode) parasites to see if an eDNA signal could be detected. The manufacturer’s protocol was followed with the chemical lysis carried out at 95°C to liberate resilient parasite transmission stages. The protocol yielded large quantities of DNA but no amplification product was detected using either the NemUni-1 or PlatUni primers (data not shown). The protocol was thus modified to try and gain a parasite eDNA signal by exploring different chemical lysis temperatures (between 70°C and 95°C) and by increasing the lysis time by up to one hour. These modifications yielded larger quantities of extracted DNA but no parasite DNA was amplified (data not shown). Subsequent modifications to the PCR, included addition of Bovine serum albumin (BSA), HotStart Taq Polymerase (Rocher), more MgCl₂ and increased thermocycling program length to 45 replication cycles as well as increasing the template DNA (serial dilutions from 5 to 315 ng/µl) but all failed to amplify parasite DNA. This failure to was not due to inhibitors carried over from the DNA extraction, as PCR spiking experiments using 1 µl of parasite tissue DNA and 4 µl of faecal DNA were always successful in yielding an amplification product, when using the appropriate primer pair (data not shown).

3.1.5 PCR amplification using DNA from bead-beaten T. muris eggs

After unsuccessful amplification from faecal DNA following the manufacturer’s protocol from the QIAamp® DNA stool mini kit, methods for macerating faecal samples to liberate DNA from resilient helminth eggs or proglottids were investigated. T. muris eggs are extremely tough and difficult to lyse (Demeler et al., 2013) and therefore any protocol able to successfully disrupt these eggs would likely translate to the liberation of DNA from the majority of helminth transmissible stages. Previous studies had found mechanical disruption such as bead-beating (Demeler et al., 2013; do Espirito-Santo et al., 2012) to be effective at releasing parasite DNA from tough biological structures. Therefore, the disruptive capability of bead-beating was tested on solutions of pure T. muris eggs in water and assessed by microscopic examination. A bead-beating mixer mill was used to beat for 5 or 10 minutes at 30 Hz using three different types of beads; 3x Tungsten beads per tube, 3x borosilicate beads per tube and tubes containing fine silica sand. Microscopy identified only the borosilicate beads as being successful at breaking open T. muris eggs, whilst a bead-beating time of 10 minutes (Figure 5E, F) resulted in considerably more disrupted eggs than 5 minutes (Figure 5C, D). DNA was extracted from this solution of bead-beaten eggs and successfully amplified using the NemUni-1 primers. The DNA extract from the eggs bead-beaten for either 5 or 10 minutes was successfully amplified (Figure 6) with both bands being of equal brightness, suggesting 5 minutes may be a sufficient amount of bead-beating time to release parasite DNA.
Figure 5 – Time series photographs of bead-beaten *T. muris* eggs. Photographs of concentrated *T. muris* eggs x100 (A, C, E) and x200 (B, D, F) magnification, before bead-beating with borosilicate beads (A, B), after 5 minutes of bead-beating (C, D) and after 10 minutes of bead-beating (D, E). Arrows indicate examples of cracked and broken eggs.
3.1.6 PCR amplification using bead-beaten faecal DNA from infected mice

Bead-beating times of 5 and 10 minutes were then trialled on faecal samples from T. muris infected mice using borosilicate beads with the faeces suspended in ASL buffer. Subsequently, the manufacturer’s protocol for the QIAamp DNA stool mini kit was followed, however, the DNA extracted did not amplify using the NemUni-1 primers. The manufacturer’s protocol was thus modified to carry out an initial lysis at lower temperatures of 45°C or 65°C to reduce the overall concentration of DNA extracted (to between 30 to 50 ng/µl) by reducing the release of bacterial DNA, whilst retaining release of parasite DNA. After this modification when the NemUni-1 primers were used on 5 µl of faecal DNA, as opposed to 1 µl, they were able to produce amplification products (Figure 7A). Moreover, a doubling of primer concentration was found to improve the amplification signal brightness (Figure 7B). The lower lysis temperature protocol was also used on faeces taken from T. spiralis infected mice. Successful amplification of a 900 bp product was also achieved using the NemUni-1 primers; regardless of the starting amount of DNA extract used (Figure 7C).

The low temperature protocol was also tested to see if it was better at liberating and detecting DNA from the faeces of mice infected with Platyhelminthes, using the PlatUni primers. These primers were successful in amplifying eDNA from the faeces of mice infected with the Platyhelminth H. microstoma, regardless of whether 5 µl or 10 µl of faecal DNA was added to the PCR (Figure 8A, Figure 8B).
The PlatUni primers were also tested on the faeces of mice infected with the Platyhelminth fluke, *S. mansoni*, at different time points during infection. These primers detected *S. mansoni* eDNA in the faeces of heavily infected mice (180 cercariae) at 43 days post infection (Figure 8D). In contrast, PlatUni primers could not amplify from faecal DNA taken at day 31 post infection, regardless of infective dose, starting quantity of faeces, or alterations to the MgCl₂ concentration (Figure 8E).

![PCR amplification using NemUni-1 primers and Tsp primers on bead-beaten faecal DNA from *T. spiralis* and *T. muris* infected mice.](image)

**Figure 7**

A: NemUni-1 primers were successful in amplifying faecal DNA from *T. muris* infected mice after 5 minutes of bead-beating when 5 μl of DNA were used per reaction (asterisked lanes) but not 1 μl. The lysis temperature the DNA extraction was carried out at had no effect (numbers above lanes in °C). B: Amplification was stronger when the quantity of each primer was doubled to 2.5 μl per tube, giving a successful reaction even with 1 μl of faecal DNA. Unexpectedly, no amplification was observed when the thermocycling program was extended to 45 cycles regardless of other conditions. Arrows indicate the expected 900 bp product. C: The NemUni-1 and Tsp primers were both successfully used to amplify faecal DNA from *T. spiralis* infected mice after both 5 and 10 minutes of bead-beating (see ‘time’). The lysis temperature the DNA extraction was carried out at had no effect (see ‘temp’ in °C). The quantity of faecal DNA used per reaction also had no effect, either 5 μl (asterisked) or 10 μl (Δ). D: Using the recommended Tsp primer thermocycling program increased the strength of amplification. Arrow indicates the expected 900 bp product formed with NemUni-1 primers, whilst triangles indicate the expected 240 bp product with Tsp primers. Positive controls containing 1 μl of the relevant nematode tissue DNA and 4 μl of bead-beaten faecal DNA were also included (+). 1kb hyperladders were run (HL) and negative controls (X).
Figure 8 - PCR amplification using PlatUni primers on bead-beaten faecal DNA from *H. microstoma* and *S. mansoni* infected mice. 

**A - C:** PlatUni primers were successful in amplifying faecal DNA from *H. microstoma* infected mice after 10 minutes of bead-beating when 5 µl and 10 µl (Δ) of DNA were used per reaction. The lysis temperature the DNA extraction was carried out at had no effect on amplification (numbers above lanes in °C) when the principal PlatUni reverse primer was used (R1). However, amplification was only observed with the alternate reverse primer (R2) when the low lysis temperature was employed. Arrows indicate the expected 570 bp and triangle indicates expected 810 bp product. 

**D, E:** PlatUni primers were successful in amplifying faecal DNA from *S. mansoni* infected mice after 10 minutes of bead-beating only from samples collected late into the infection course (day 43) with a heavy infective dosage (L). Samples collected earlier in the infection (day 31) with either a high (E) or low (e) infective dosage failed to amplify. The principal reverse primer (R1) amplified this sample from earlier in the infection, regardless of whether 5 µl or 10 µl (Δ) of faecal DNA were used, whilst the alternate primer (R2) could only amplify this sample from 10 µl of faecal DNA. Arrows indicate the expected 570 bp and triangle indicates expected 810 bp product. Positive controls containing 1 µl of the relevant Platyhelminth tissue DNA and 4 µl of bead-beaten faecal DNA were also included (+). 1kb hyperladders were run (HL) and negative controls (X).
3.1.7 PCR amplification using bead-beaten faecal DNA from naïve mice

To confirm that the DNA extraction and amplification protocol was detecting a faecal eDNA signal from the parasitic helminths and not some other source, a negative control was carried out. The negative control used faeces taken from naive mice, followed by 12.5 minutes of bead-beating and a lysis step at both the milder 37ºC and 65ºC i.e. the protocol that had been successful in allowing for amplification of parasite eDNA from infected mice. NemUni-1 and PlatUni primers did not detect or amplify DNA from naive mice (Figure 9A, B), validating the utility of the methodology for accurate identification of the presence of helminth eDNA. Similarly, species specific Tmu and Tsp primers did not amplify from faecal DNA of naive mice (Figure 9A).

![PCR amplification results using NemUni-1, Tmu, Tsp and PlatUni primers on faecal DNA from naïve mice. A, B: None of the primer pairs amplified the faecal DNA from uninfected mice. This was used as a negative control to ensure these primers did not cross-react. NemUni-1 (Nu), Tmu (Tm), Tsp (Ts) and neither the principal (R1) or alternate (R2) reverse PlatUni primers amplified this faecal DNA, regardless of the lysis temperature the DNA extraction was carried out at (numbers above lanes in ºC). Arrow indicates the expected 570 bp and triangle indicates expected 810 bp, products. Positive controls containing 1 µl of the relevant helminth tissue DNA and 4 µl of bead-beaten faecal DNA were also included (+). 1kb hyperladders were run (HL) and negative controls (X).](image-url)

3.1.8 PCR amplification using species specific Tmu and Tsp primers on bead-beaten faecal DNA from infected mice

Primers that are able to detect a wider range of DNA sequences or that are more broad-spectrum, may be expected to be less sensitive than a species-specific primer pair that can be designed against a rarer or less universally conserved DNA sequence (Blackwood et al., 2005). Therefore, to test the sensitivity of broad-spectrum primer pairs, like NemUni-1, at detecting nematode DNA the results were compared with those gained using species specific Tmu and Tsp primers. As expected, species specific primers amplified tissue DNA and bead-beaten faecal DNA from both T. muris (Figures 10) and T. spiralis (Figures 7C) using the appropriate species primer. These species specific primers were also tested on DNA extracts that had not amplified using the
NemUni-1 primers i.e. from DNA extracted without a bead-beating step or at the higher lysis temperatures of 70°C and 95°C and as anticipated did not produce an amplicon (Figures 10). Additionally, the Tsp primers on faecal DNA from *T. spiralis* infected mice produced one, strong 240 bp band that was clearer than the three bands and smear produced by the NemUni-1 primers on the same faecal DNA (Figures 7C, D). Such a result demonstrates that although the NemUni-1 primers are sensitive to eDNA, species specific primers may still have greater efficacy at producing a clear PCR signal indicative of a parasite infection. However, a caveat to the use of species specific primers is that the common parasitic species of your host organism must be known.

Figure 10 – PCR amplification using Tmu primers on tissue, egg and faecal DNA. *Tmu* primers were successful in amplifying DNA from *T. murs* tissue DNA (Tm) and *T. murs* eggs (E) beaten for 5 and 10 minutes (numbers in superscript). Faecal DNA from *T. murs* infected mice when unbeaten (Ub) failed to amplify, as did faecal DNA that was beaten (B) but carried out at the high DNA extraction lysis temperature of 95°C (Numbers in black above lanes in °C). Bead-beaten faecal samples successfully amplified when the extraction lysis temperature was dropped to 45°C - 65°C, indicating the importance of a low lysis temperature. Arrows indicates the expected 1,000 bp product. 1kb hyperladders were run (HL) and negative controls (X).
3.2 DNA extraction and amplification of parasite DNA from amphibian faeces

3.2.1 PCR amplification using DNA from bead-beaten *M. betsileo* faeces

After successful optimisation of the copro-diagnostic system for detection of nematode and Platyhelminth parasites using faecal samples from a mouse model of infection, the same protocol was trialled on amphibian samples. The NemUni-1 primers were used to successfully amplify *M. betsileo* faecal DNA that had undergone 10 minutes of bead-beating and a lysis extraction step at 37°C, even when primer concentration, starting faecal quantity and quantity of DNA used were changed (Figure 11.1A). However, amplification did not occur when the MgCl₂ concentration was doubled (Figure 11.1A). Lots of low molecular weight products, identifiable as multiple, weaker bands and smearing on the gel were also produced due to the PCR being carried out over 45 cycles of replication.

PlatUni primers also provided some successful amplification (Figure 11.1B) with either the principal (R1) or alternate (R2) reverse primer, however, these bands were considerably fainter than those produced by the NemUni-1 primers. This might be due to the fact that only 35 cycles of amplification were used with the PlatUni primers, as opposed to the 45 cycles that had been employed with the NemUni-1 primers. However, some of the PCRs using the PlatUni primers failed to amplify, i.e. one that used 10 µl of DNA extract instead of 5 µl which did amplify. Such results indicate the importance of very particular PCR conditions and quantities when using these primers (Figure 11.1B).

The results using the *M. betsileo* faecal DNA were repeated to verify, the infection status of these individuals, however, successive experimental repeats frequently failed to amplify (data not shown). The NemUni-1 primers were tested against the *M. betsileo* faecal DNA over a 35 cycle amplification program and at a variety of different annealing temperatures (56°C, 58.3°C, 61°C, 63°C and 65°C) over four different PCRs to try and gain cleaner and clearer bands but none of these reactions produced any banding (data not shown). The original annealing temperature of 54°C that had been successful originally was retested but also failed to generate the appropriate amplicon, as did the same reaction carried out over 40 replication cycles (data not shown). Similarly, experiments using the PlatUni primers resulted in negative results (data not shown).

New, *M. betsileo* faecal samples were used for a second DNA extraction, and obtained a similar DNA concentration (4.8 ng/µl) but this did not amplify with the NemUni-1 or PlatUni primers either, irrespective of annealing temperature used (data not shown). To test for the possibility of inadequate storage and degradation of faecal extracted DNA, extracts were analysed by Nanodrop for reductions in DNA quantity, however, none were found.

In addition, faecal DNA was extracted from a variety of other amphibian colonies kept at the University of Manchester, including *Mantella aurantiaca*, *Mantella ebenaui*, *Dendrobates auratus* and *Agalychnis callidryas*. These samples were subjected to 10 minutes of bead-beating and a lysis extraction step of 37°C and all yielded good concentrations of DNA, between 15 – 120 ng/µl.
but did not amplify using the NemUni-1 or PlatUni primers when carried out using their recommended Thermocycling conditions (Floyd et al., 2005; Van Steenkiste et al., 2015).

3.2.2 Optimisation of PCR conditions and DNA extraction from *M. betsileo* faeces

The possibility that parasite eDNA was sheared due to an unnecessarily severe 10 minutes of bead-beating in the DNA extraction was investigated as such shearing could reduce the chances of amplification. New *M. betsileo* faecal samples were collected and subjugated to either 1, 2.5 or 5 minutes of bead-beating. All faecal extracts, regardless of bead-beating time were successfully amplified using the NemUni-1 primers when 10 µl of DNA template was used per reaction (Figure 11.2A). However, when only 5 µl of template DNA was used only the 2.5 minutes of beating extract amplified (Figure 11.2A). These positive results were obtained after 35 cycles of replication and produced only faint bands at the expected 900 bp size. When 45 cycles of replication were employed the bands seen were more intense (Figure 11.2B), although surprisingly the PCR carried out for 40 cycles did not amplify any of the DNA extracts (Figure 11.2B). Furthermore, a thermal gradient PCR was carried out at 52.1°C, 55.7°C, 58.4°C and 60.1°C on all the DNA extracts that had been subjugated to different bead-beating durations. However, only the 2.5 minutes of bead-beating extract at the lowest annealing temperature of 52.1°C amplified well (Figure 11.2C, D) and all other annealing temperatures failed to amplify completely.
Given the unpredictability in when a PCR would amplify, these same time series faecal DNA extracts were tested again at the normal 54°C annealing temperature on all available thermocyclers in the laboratory. This was carried out to assess if there was variation in amplification success depending on the individual thermocycler employed. All reactions from this experiment did not amplify, again highlighting an issue with repeatability (data not shown). A fourth faecal extraction was carried out using the updated QIAamp® Fast DNA stool mini kit (March, 2014), hereafter referred to as ‘new’ kit, that utilises a liquid state inhibitor extraction step. Nonetheless, this did not improve amplification success, regardless of whether 35, 40 or 45 cycles were used (data not shown).

Figure 11.2 - DNA extraction and PCR optimisation, using NemUni-1 primers on bead-beaten M. betsileo faecal DNA. A, B: NemUni-1 primers were successful in amplifying 5 μl of M. betsileo faecal DNA when it was bead-beaten for 1, 2.5 or 5 minutes (numbering in lanes) and amplified for 45 cycles. Some amplification was seen on these DNA extracts when 10 μl was used per reaction (*) and replication occurred over 35 cycles, but little was seen when 5 μl of extract was used. Unusually, the same faecal DNA failed to amplify using a 40 cycle program. Arrows indicate the expected 900 bp product. C, D: NemUni-1 primers were only successful at amplifying 5 μl of M. betsileo faecal DNA that had been bead-beaten for 2.5 minutes (numbering in lanes) at the elevated annealing temperature of 52.1°C (numbers in square brackets in °C). All the other increased annealing temperatures failed to amplify with these primers, indicating 52°C to be an optimal annealing temperature. Arrows indicate the expected 900 bp product. Positive controls containing 1 μl of tissue extracted T. mums DNA and 4 μl of extracted faecal DNA were included (+).1kb hyperladders were run (HL) and negative controls (X).

3.2.3 Light microscopy on M. betsileo faecal smears

Faecal smears from M. betsileo were investigated by microscopy to ascertain whether the colony was infected by parasitic nematodes, providing information on the accuracy of the copro-diagnostic protocol for detecting parasite DNA. A variety of different nematode life cycle stages were found,
including adult worms (Figures 12.1G and 12.2D, E), larvae (Figures 12.1F) and possible nematode eggs (Figures 12.1A, B, C, D, E and 12.2A, B, C). The nematodes observed under the microscope share some basic morphological characteristics with those of the *Strongyloides* genus which are known to be common parasites of wild animals (Baker, 2007). However, accurate identification to any taxonomic level within the nematode phylum was not possible through this crude morphology alone. The discovery of nematodes in the *M. betsileo* faeces was positive confirmation of infection. However, no Platyhelminth parasites were found in the faecal smears, thus the positive results obtained previously using the PlatUni primers were not supported by microscopy.

![Light microscopy of *M. betsileo* faecal smears](image)

**Figure 12.1 – Light microscopy of *M. betsileo* faecal smears.** Faecal smears from *M. betsileo* individuals were examined by light microscopy at x80 magnification. To obtain an idea of appropriate egg size one smear was spiked with *T. muris* eggs (A); objects of similar size and shape were regularly found from a variety of smears (B to E) as well as nematode worm larvae and adults (F and G). Arrows indicate putative nematode eggs.
3.2.4 PCR amplification using nematode family and genus specific primers

To attempt to elucidate which families and genera of parasitic nematode were infecting the *M. betsileo* colony as well as which might be infecting the other amphibian species maintained at the University of Manchester, more primers from the literature were tested. Nematode families known to commonly infect wild animals, particularly amphibians, are the Rhabdiasidae (lungworms), Ascaridoidea (roundworms) and also in the genus *Strongyloides* (threadworms) (Densmore and Green, 2007; McKenzie, 2007; Wright and Whitaker, 2001) and therefore family and genus specific primers to target these groups were selected. The Rhab (Rhabdiasidae specific) primers amplified a large range of tissue and faecal DNA extracts, including tissue DNA from *S. mansoni* a species outside of the Nematoda phylum and faecal DNA from uninfected mice (Figure 13.1). Furthermore, a variety of different sized bands were produced on the gel, including ones outside of the expected 1,500-1,600 bp, hence Rhab primers showed no ability to selectively amplify Rhabdiasidae DNA.

The Str (*Strongyloides* specific) primers showed greater selectivity amplifying almost solely from *M. betsileo* and *M. ebenaui* faecal DNA which may suggest that these amphibian hosts are indeed infected with a species of threadworm (Figure 13.2). However, these primers also amplified tissue
DNA from *T. spiralis* (**Figure 13.2**) a species from outside of the *Strongyloides* genus, suggesting they are not entirely genus specific.

The two primer sets, Asc-1 and Asc-2 (Ascaridoidea specific) in a similar manner to the Rhab primers also showed a lack of specificity to nematode DNA and amplified faecal DNA from uninfected mice as well as from *S. mansoni* tissue DNA (**Figure 13.3A, B, C**). Amplification resulted in a range of different fragment sizes, many of which were different to the 120-150 bp fragment expected for the two primer sets (**Figure 13.3A, C**). The Asc-1 and Asc-2 primers showed no selective ability to amplify from the Ascaridoidea family.

Overall both the Rhab, Asc-1 and Asc-2 primers showed no functional utility and were not employed further, whilst the Str primers were also seen to amplify products from outside of this genus and therefore were deemed to have limited use for continued investigation.

![Figure 13.1 - PCR amplification using Rhab primers on helminth tissue, egg and bead-beaten faecal DNA](image)

**Figure 13.1 - PCR amplification using Rhab primers on helminth tissue, egg and bead-beaten faecal DNA.** Rhab primers amplified *T. spiralis* (Ts) and *S. mansoni* (Sm) but not *T. muris* (Tm) tissue DNA. They also amplified faecal DNA from bead-beaten *T. muris* eggs (E) and naive, uninfected mice (N). Furthermore, they were successful in amplifying amphibian faecal DNA from *M. aurantiaca* (Ma), *A. callidryas* (Ac) and *M. betsileo* (Mb) but not *M. ebenaui* (Me). Neither the *M. betsileo* faecal DNA sample used; first or second (subscript), or DNA extraction kit used; old (∆) or new (∇) had an effect. Unbeaten (MU) and bead-beaten (M) faecal DNA from *T. muris* infected mice failed to amplify as did faecal DNA from uninfected mice (N) carried out at an extraction lysis temperature of 65°C (Numbers in superscript in °C). Overall, Rhab primers showed substantial cross-reactivity with many DNA extracts. Arrows indicate the expected 1,500 – 1,600 bp size product. 1kb hyperladders were run (HL) and negative controls (X).
Figure 13.2 - PCR amplification using Str primers on helminth tissue, egg and bead-beaten faecal DNA. Str primers amplified T. spiralis (Ts) but not T. muris (Tm) or S. mansoni (Sm) tissue DNA. These primers also amplified bead-beaten amphibian faecal DNA from M. ebenaui (Me), and M. betsileo (Mb), regardless of which sample was used (subscript) or DNA extraction kit used; old (S) or new (s). They failed to amplify faecal DNA from M. aurantiaca (Ma) and A. callidryas (Ac). Moreover, they could not amplify from T. muris egg DNA (E) or faecal DNA from T. muris infected (unbeaten; MU or bead-beaten; M) or uninfected mice (N). The lysis temperature the DNA extraction was carried out at had no effect (Numbers in superscript in °C). Overall, the Str primers showed reasonable specificity, only cross-reacting with T. spiralis DNA, whilst also indicating a potential Strongyloides infection in various amphibians. Arrows indicate the expected 180 bp size product. 1kb hyperladders were run (HL) and negative controls (X).
Figure 13.3 - PCR amplification using Asc-1 and Asc-2 primer sets on helminth tissue, egg and bead-beaten faecal DNA. A, B: Asc-1 primers amplified all helminth tissue DNA T. muris (Tm), T. spiralis (Ts) and S. mansoni (Sm) and T. muris egg DNA (E). They also amplified bead-beaten faecal DNA from nearly all sources, including from uninfected (N) and T. muris infected mice (M) as well as from M. ebaniui (Me), A. callidryas (Ac) and M. betsileo (Mb). They only failed to amplify from unbeaten infected mouse and M. aurantiaca (Ma) faecal DNA. The lysis temperature the DNA extraction was carried out at had no effect (numbers in superscript in °C) and neither did which M. betsileo sample was used (subscript) or which kit was used to extract the DNA; old (Δ) or new (○). Arrows indicate the 147 bp size product. B, C: The Asc-2 primers were equally indiscriminate in which DNA extracts they amplified, displaying a tendency to amplify the amphibian faecal DNA slightly less well than the Asc-1 primers. Overall, both primer sets showed no specificity to their target DNA. Solid triangles indicate the 123 bp size product. 1kb hyperladders were run (HL) and negative controls (X).
3.2.5 PCR amplification using alternative nematode universal primers

Due to the lack of repeatability when using the NemUni-1 primers two other sets of nematode universal primers were tested (NemUni-2 and NemUni-3) that amplify smaller fragments of the 18S rRNA gene. Primers that amplify smaller fragments were chosen as they could have greater success at detecting small quantities of potentially degraded nematode eDNA that might be present in faeces. In contrast, the NemUni-1 primers rely on large, 900 bp stretches of the 18S rRNA gene to remain intact for successful amplification. NemUni-2 primers were tested against a variety of different tissue and faecal DNA extracts but amplified products non-specifically, observed via formation of a 427 bp product when they were used on faecal DNA from uninfected mice and Platyhelminth tissue DNA (Figure 14.1); hence these primers were not used further.

The utility of the NemUni-3 primers was also tested and they produced 400 bp amplification bands from all nematode tissue DNA samples used, including; *Ascaris lumbricoides, Heligmosomoides polygyrus* and *Nippostrongylus brasiliensis* as well as from *M. betsileo* faecal DNA (Figure 14.2). Moreover, they did not amplify faecal DNA from non-infected mice (Figure 14.2), indicating some selectivity. However, this primer pair also cross-reacted with Platyhelminth tissue DNA (Figure 14.2), displaying a lack of specificity for detecting nematode DNA. Hence, to gain a better specificity with NemUni-3 primers a thermal gradient PCR was carried out at three higher annealing temperatures. At the highest annealing temperature tested (66°C) cross-reactivity of NemUni-3 primers on Platyhelminth tissue DNA was reduced, indicated by a fainter band, however at this temperature, DNA from *N. brasiliensis* and *H. polygyrus* nematodes did not amplify (Figure 14.3B). At the lower annealing temperatures tested (63.8°C and 65.1°C) Platyhelminth tissue DNA was amplified (Figure 14.3A). Therefore, it was not possible to improve the specificity of the NemUni-3 primers by altering the PCR conditions.

Overall both of these alternative nematode universal primers (NemUni-1 and NemUni-2) were not selective for just the Nematoda phylum and were not explored further. A summary of the specificity of the primers taken from the literature is shown in Table 2.
Figure 14.1 - PCR amplification using NemUni-2 primers on helminth tissue, egg and bead-beaten faecal DNA. NemUni-2 primers amplified tissue DNA from T. muris (Tm), T. spiralis (Ts), A. lumbricoides (Al), N. brasiliensis (Nb), H. polygyrus (Hp), S. mansoni (Sm), H. microstoma (Hm) and from T. muris egg DNA (E). They also amplified faecal DNA from uninfected (N) and T. muris infected mice (M) as well as from M. betsileo faecal DNA (Mb). The lysis temperature the DNA extraction was carried out at had no effect (numbers in superscript in °C) and neither did which M. betsileo sample was used (numbers in subscript) or which kit was used to extract the DNA; old (△) or new (○). The NemUni-2 primers showed no specificity for nematode DNA. Arrows indicate the expected 427 bp size product. 1kb hyperladders were run (HL) and negative controls (X).

Figure 14.2 - PCR amplification using NemUni-3 primers on helminth tissue, egg and bead-beaten faecal DNA. NemUni-3 primers amplified tissue DNA from T. muris (Tm), T. spiralis (Ts), A. lumbricoides (Al), N. brasiliensis (Nb), H. polygyrus (Hp), S. mansoni (Sm), H. microstoma (Hm) and from T. muris egg DNA (E). They also amplified faecal DNA from T. muris infected mice (M) as well as from M. betsileo faecal DNA (Mb). The lysis temperature the DNA extraction was carried out at had no effect (numbers in superscript in °C) and neither did which M. betsileo sample was used (subscript) or which kit was used to extract the DNA; old (△) or new (○). However, these primers did not amplify faecal DNA from uninfected mice (N), demonstrating some specificity. Arrows indicate the expected 300 bp size product. 1kb hyperladders were run (HL) and negative controls (X).
Figure 14.3 – Annealing temperature thermal gradient to attempt to gain nematode specificity for NemUni-3 primers. A, B: NemUni-3 primers always amplified tissue DNA from both the nematodes T. muris (Tm), T. spiralis (Ts), A. lumbricoides (Al), N. brasiliensis (Nb) and the Platyhelminthes S. mansoni (Sm) and H. microstoma (Hm) regardless of annealing temperature used (above lanes in °C). However, they never amplified tissue DNA from the nematodes N. brasiliensis (Nb) and H. polygyrus (Hp) nor from bead-beaten faecal DNA from M. betsileo (Mb) amphibians. Hence, specificity for just nematode DNA could not be gained with the NemUni-3 primers. Arrows indicate the expected 400 bp size product. 1kb hyperladders were run (HL) and negative controls (X).
Table 2: **Review of claimed and actual primer specificity.** *Primers taken from the literature were frequently found to cross-react with DNA from organisms outside of the taxonomic group that they were claimed to be specific for.*

<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>Taxa targeted</th>
<th>Cross-Reactivity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NemUni-1</td>
<td>Nematoda Phylum Universal</td>
<td>Yes: Platyhelminth and Collembola DNA</td>
<td>Floyd et al. (2005)</td>
</tr>
<tr>
<td>PlatUni</td>
<td>Platyhelminth (Cestode and Trematode) Universal</td>
<td>No: but regularly show non-specific bands/smears</td>
<td>Van Steenkiste et al. (2015)</td>
</tr>
<tr>
<td>Tsp</td>
<td><em>Trichinella spiralis</em> specific</td>
<td>No</td>
<td>Golab et al. (2009)</td>
</tr>
<tr>
<td>Tmu</td>
<td><em>Trichuris muris</em> specific</td>
<td>No</td>
<td>Cutillas et al. (2002)</td>
</tr>
<tr>
<td>Rhab</td>
<td>Rhabdiasidae family specific</td>
<td>Yes: amplify all tissue and faecal DNA, including from naïve mice</td>
<td>Tkach et al. (2014)</td>
</tr>
<tr>
<td>Str</td>
<td>Strongyloides genus specific</td>
<td>Yes: but only cross-reacted on <em>T. spiralis</em> tissue DNA</td>
<td>Kramme et al. (2011)</td>
</tr>
<tr>
<td>Asc-1</td>
<td>Ascaridoidea family specific</td>
<td>Yes: amplify all tissue and faecal DNA, including from naïve mice</td>
<td>Loreille et al. (2001)</td>
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<tr>
<td>Asc-2</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>NemUni-2</td>
<td>Nematoda Phylum Universal</td>
<td>Yes: amplify all tissue and faecal DNA, including from naïve mice</td>
<td>Bhadury and Austen (2010)</td>
</tr>
<tr>
<td>NemUni-3</td>
<td>Nematoda Phylum Universal</td>
<td>Yes: Platyhelminth DNA</td>
<td>Personal Communication Prof. Marx Blaxter (14/03/16)</td>
</tr>
</tbody>
</table>
3.3 Development of nematode universal primers

3.3.1 Primer design

A comprehensive list of common parasitic nematodes that infect wild animals, such as amphibians and reptiles, was compiled, consisting of a large range of different families and genera from the Nematoda phylum (Table 3). Various fungal species, including many from the Basidiomycota were also selected as these are known to have 18S rRNA sequences that share similarities with those of nematodes and therefore commonly cross-react with primers designed to be nematode specific (Bhadury and Austen, 2010). Furthermore, a list of amphibians which have had their 18S rRNA genes sequenced and banked was added as any designed primers must not amplify 18S sequences from the host (Table 3).

Sequences from all these lists were taken from the GenBank database and added to BioEdit (version 7.2.5), they were then aligned using the ClustalW function (Figure 15A). The alignment was examined by eye and highlighted in regions conserved within all of the nematode species but absent in the fungi and amphibian 18S rRNA sequences (Figure 15B). The loci of such conserved regions within the *T. muris* 18S rRNA gene were noted. Ideal loci were approximately 5 - 20 base pairs long with 80 - 90% of the nematode species having exactly the same sequence over the stretch. Few of these ideal candidate loci were found over the entire 2,000 bp length of the 18S rRNA gene and therefore less promising candidate loci were also considered so as to provide a larger variety of sites to which primers could be designed. In total ten ideal loci and 42 potentially useful loci were identified.

### Table 3 – List of species used in primer design alignment

<table>
<thead>
<tr>
<th>Nematodes</th>
<th>Fungi</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trichuris muris</strong></td>
<td><strong>Sidera vulgaris</strong></td>
</tr>
<tr>
<td><strong>Trichurus trichiura</strong></td>
<td><strong>Sidera lenis</strong></td>
</tr>
<tr>
<td><strong>Trichinella spiralis</strong></td>
<td><strong>Herpotrichiellaceae sp.</strong></td>
</tr>
<tr>
<td><strong>Paratrichosoma sp.</strong></td>
<td><strong>Exophiala xenobiotica</strong></td>
</tr>
<tr>
<td><strong>Dicotophyme renale</strong></td>
<td><strong>Exophiala castellani</strong></td>
</tr>
<tr>
<td><strong>Eustrongylides ignotus</strong></td>
<td><strong>Onslowia edophytica</strong></td>
</tr>
<tr>
<td><strong>Rhabdias bufonis</strong></td>
<td><strong>Lulworthia fucicola</strong></td>
</tr>
<tr>
<td><strong>Rhabditis sp.</strong></td>
<td><strong>Corollospora maritima</strong></td>
</tr>
<tr>
<td><strong>Ascaris lumbricoides</strong></td>
<td><strong>Acremonium strictum</strong></td>
</tr>
<tr>
<td><strong>Ascaris suum</strong></td>
<td><strong>Acremonium asperulatum</strong></td>
</tr>
<tr>
<td><strong>Strongyloides stercoralis</strong></td>
<td><strong>Lindra obtusa</strong></td>
</tr>
<tr>
<td><strong>Strongyloides procyonis</strong></td>
<td><strong>Lindra marinera</strong></td>
</tr>
<tr>
<td><strong>Strongyloides ratti</strong></td>
<td><strong>Metarhizium anisopliae</strong></td>
</tr>
<tr>
<td><strong>Cosmocercoideae ducae</strong></td>
<td><strong>Aspergillus niger</strong></td>
</tr>
<tr>
<td><strong>Parastrongyloides trichosuri</strong></td>
<td><strong>Pleosporaceae sp.</strong></td>
</tr>
<tr>
<td><strong>Nippostrongylus brasiliensis</strong></td>
<td><strong>Torulaspora delbrueckii</strong></td>
</tr>
<tr>
<td><strong>Heligmosomoides polygyrus</strong></td>
<td><strong>Sarcoleotia turficola</strong></td>
</tr>
<tr>
<td><strong>Trichostrongylus colubriformis</strong></td>
<td><strong>Pneumocystis murina</strong></td>
</tr>
<tr>
<td><strong>Ancylostoma caninum</strong></td>
<td><strong>Amphibians</strong></td>
</tr>
<tr>
<td><strong>Dracunculus medinensis</strong></td>
<td><strong>Xenopus laevis</strong></td>
</tr>
<tr>
<td><strong>Dirofilaria immitis</strong></td>
<td><strong>Xenopus borealis</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Scinax rubra</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Phyllophthora bicolor</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Rana chensinensis</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Bufo maritiferal</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Discoglossus pictus</strong></td>
</tr>
</tbody>
</table>
Figure 15 – Species alignment used for primer design. **A:** All nematode, fungi and amphibian sequences were downloaded from the GenBank database and aligned using the ClustalW multiple alignment tool in BioEdit. **B:** Nucleotides that matched the first sequence (T. muris) were hidden by dots to assist in the identification of regions conserved within all the nematodes but that differed in the fungi and amphibian sequences. Once identified these regions were highlighted and noted to produce a list of ideal loci within the T. muris 18S rRNA sequence to which primers could be designed to allow for selective amplification of only nematode DNA.
Next, primers were designed that were complimentary to regions of the *T. muris* 18S rRNA gene that had been identified as being conserved within the nematodes. Typical primer design guidelines taken from the Thermofisher website (www.thermofisher.com/uk/en/home/products-and-services/product-types/primers-oligos-nucleotides/invitrogen-custom-dna-oligos/primer-design-tools.html) were taken into account. Primers were designed to be between 18 - 30 nucleotides long, have a GC content of between 40 - 60% (with a 3’ ending in G or C), to generally have melting temperatures of 65°C - 75°C and to avoid runs of four or more of one base, dinucleotide repeats or GC-rich and AT-rich domains. Once these guidelines were considered and a potential primer sequence selected they were analysed in the online tool OligoAnalyzer 3.1 (www.idtdna.com/calc/analyzer) to ensure the primer sequence followed the typical primer design guidelines. OligoAnalyzer 3.1 also allows for identification of potential secondary structure formation, such as hairpin loops formed by self-binding and indicates at what temperature these structures form. Hence, utilising the information provided by OligoAnalyzer 3.1 the primer sequence could be carefully adjusted with the addition or removal of particular base pairs to provide a sequence that best fulfilled as many of the primer design criteria as possible. The online tool Primer3 (version 4.0.0) was also employed to design primers against these conserved nematode loci and a few additional primer sequences were acquired.

To increase the range of nematode 18S rRNA sequences that the designed primers could detect, degenerate base pairs were added to their sequences. Many of the original primer sequences based on the *T. muris* sequence, had a single or multiple base pair positions that were different for two or more of the nematode species used in the design process. If common differences were observed then that position in the primer sequence would be changed to the relevant degenerate base so that upon primer synthesis a population of primers would be created that would be able to accurately detect a larger range of nematode sequences. For example, locus 1,403 in the *T. muris* 18S rRNA gene was always found to be either C or T within the other nematode sequences so the degenerate code Y was added to the primer sequence. Addition of degenerate bases was kept to a minimum so as to not overly augment the population of primers synthesised and therefore increase the chances of cross-reaction with sequences from outside of the Nematoda phylum. Furthermore, after replacement with some degenerate bases the primer sequence was then re-analysed to ensure it still followed as many of the criteria as possible for good primer design. In total 15 degenerate primers were designed and the reverse compliment of the NemUni-1 primer Nem_18S_R was also included (Table 4) as it could be used in conjunction with the primers designed in this study to increase the number of primers available for testing.
Table 4 – Degenerate primers designed to amplify regions of the nematode 18S rRNA gene. Loci that were well conserved within the nematodes but different in the fungi and amphibians had primers designed to bind to these regions whilst also taking into consideration primer design guidelines. Displayed are the principal loci around which primers were designed against and the full sequence the primer binds to in the T. muris 18S gene (* = denotes the locus and sequence within the C. elegans 18S gene). The final, degenerate primer sequence actually used is also shown, degenerate bases (red) increase the number of nematode sequences the primer can bind to. Important information regarding the degenerate primers mean melting temperature, melting temperature range and temperature at which it forms secondary structures such as hairpin loops is displayed as well.

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Floyd700F</td>
<td>700*</td>
<td>GGC GAT CAG ATA CGG CCC*</td>
<td>GGC GAT CAG ATA CGG CCC</td>
<td>18</td>
<td>66.7</td>
<td>58.3</td>
<td>58.3</td>
<td>53.5</td>
</tr>
<tr>
<td>Nem890F</td>
<td>890</td>
<td>TTC GTA TTG CGT CGT TAG AG</td>
<td>TTC GTA TYG CTR CGY KAG AG</td>
<td>20</td>
<td>50</td>
<td>54.5</td>
<td>49.3-59.8</td>
<td>-27</td>
</tr>
<tr>
<td>Nem890R</td>
<td>890</td>
<td>CTC TAA CGC AGC AAT ACG AA</td>
<td>CTC TMR CGY AGC RAT ACG AA</td>
<td>20</td>
<td>50</td>
<td>54.5</td>
<td>49.3-59.9</td>
<td>16.5</td>
</tr>
<tr>
<td>Nem[P3]890F</td>
<td>890</td>
<td>CGT ATT GCT GGG TTA GAG GT</td>
<td>CGT ATY SCT RCG YKA GAG GT</td>
<td>20</td>
<td>55</td>
<td>56.4</td>
<td>50.8-62.1</td>
<td>-5</td>
</tr>
<tr>
<td>Nem[P3]890R</td>
<td>890</td>
<td>ACC TCT AAC GCA GCA ATA CG</td>
<td>ACC TCT MRC GYA GSR ATA CG</td>
<td>20</td>
<td>55</td>
<td>56.4</td>
<td>50.8-62.2</td>
<td>-4</td>
</tr>
<tr>
<td>Nem996F</td>
<td>996</td>
<td>AGA GGT TCG AAG GCG ATC AGA TAC CGC</td>
<td>AGA GGT TCG AAG GCG ATY AGA TAC CGC</td>
<td>27</td>
<td>53.7</td>
<td>62.5</td>
<td>61.6-63.4</td>
<td>37-43</td>
</tr>
<tr>
<td>Nem996R</td>
<td>996</td>
<td>GCG GTA TCT GAT GCG CTG CTA ACC TCT</td>
<td>GCG GTA TCT RAT GCG CTG CGA ACC TCT</td>
<td>27</td>
<td>53.7</td>
<td>62.5</td>
<td>61.6-63.5</td>
<td>41</td>
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<tr>
<td>Nem1025F</td>
<td>1025</td>
<td>GTT GTG ACC GTA AAG GCT GCG</td>
<td>GTT STR ACC GTA AAG KAT RC</td>
<td>20</td>
<td>42.5</td>
<td>49.8</td>
<td>45.1-54.4</td>
<td>-10</td>
</tr>
<tr>
<td>Nem1025R</td>
<td>1025</td>
<td>GCA TCG TTT ACG GTC ACA AC</td>
<td>GYA TMG TTT ACG GTY ASA AC</td>
<td>20</td>
<td>42.5</td>
<td>49.8</td>
<td>45.1-54.5</td>
<td>-5</td>
</tr>
<tr>
<td>Nem[P3]1025F</td>
<td>1025</td>
<td>CGT AAA CGA TCA ACA CGG GCG</td>
<td>CGT AAA CKA TGC CWW CYA G</td>
<td>19</td>
<td>47.4</td>
<td>50.4</td>
<td>46.6-54.1</td>
<td>-13</td>
</tr>
<tr>
<td>Nem[P3]1025R</td>
<td>1025</td>
<td>CTG GTG GCG ATC GTT TAC G</td>
<td>CTR GWG GCC ATM GTT TAC G</td>
<td>19</td>
<td>47.4</td>
<td>50.4</td>
<td>47.2-54.1</td>
<td>-33</td>
</tr>
<tr>
<td>Nem1217F</td>
<td>1217</td>
<td>CGN TCC GAA CAC TGT GAG</td>
<td>CGN BCC GRA CAC YGT RAG</td>
<td>18</td>
<td>64.8</td>
<td>57.6</td>
<td>50-64.6</td>
<td>-5</td>
</tr>
<tr>
<td>Nem1217R</td>
<td>1217</td>
<td>CTG ACA GTG TTC GGA NCG</td>
<td>CTY ACR GTG TYC GGV NCG</td>
<td>18</td>
<td>64.8</td>
<td>57.6</td>
<td>50-64.7</td>
<td>-5</td>
</tr>
<tr>
<td>Nem1475F</td>
<td>1475</td>
<td>AAG CGG CAC GAG AAA GAG C</td>
<td>HAG CGG CAY GAR AWW GAG C</td>
<td>19</td>
<td>54.4</td>
<td>56.1</td>
<td>52.4-59.2</td>
<td>15.6</td>
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<tr>
<td>Nem1475R</td>
<td>1475</td>
<td>GCT TTT CCT GCG GCT T</td>
<td>GCT CWW TYT CRT GCG GCT D</td>
<td>19</td>
<td>54.4</td>
<td>56.1</td>
<td>52.4-59.3</td>
<td>16.8</td>
</tr>
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<td>Nem1619R</td>
<td>1619</td>
<td>GGA AAT AAT TTC AAT TCC CTG TCC</td>
<td>GGA AA Y AAT TDC AAT TCC CKR TCC</td>
<td>24</td>
<td>41</td>
<td>54.1</td>
<td>49-60</td>
<td>33</td>
</tr>
</tbody>
</table>
To assist in the identification of appropriate pairings of primers the relevant binding sites and direction of synthesis of the designed primers were mapped along the 18S rRNA gene of *T. muris* and *C. elegans* (Figure 16). Primer pairs were chosen based on the expected fragment size they would synthesise (predicted using the relative distance of the primer’s binding loci along the 18S rRNA gene) desired pairs would amplify fragments larger than 100 bp and smaller than 700 bp. The rationale was that if the fragment the primers amplified was large it would rely on high quality un-sheared DNA to be extracted from faeces which was thought to have been a factor in the irregular amplification of faecal DNA achieved when using the NemUni-1 primers. Primer pairs were also only selected if they had mean melting temperatures within approximately 5°C of each other. In total 28 appropriate primer pairs were identified that could go forward to testing (Table 5).

Due to the large number of primer pairs identified a workflow was designed to streamline the detection of successful pairs and remove non-functional pairs from the workflow as soon as possible (Figure 17). The workflow was separated into three stages with all pairs firstly being tested against nematode tissue DNA at stage one. If pairs failed to amplify some of these four tissue DNA extracts they were removed from the process, otherwise, they would be tested for cross-reactivity against faecal DNA from non-infected mice and Platyhelminth tissue DNA at stage two. A complete lack of amplification product at stage two would allow for progression to stage three, where the primer pair would be tested against faecal DNA from amphibians and mice infected with nematodes.
**Table 5 – Combinations of primer pairs tested.** Primers combinations that generated large fragments over 700 bp were not selected for testing.

<table>
<thead>
<tr>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Pair #</th>
<th>Fragment Size</th>
</tr>
</thead>
<tbody>
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<td>Floyd700F</td>
<td>Nem890R</td>
<td>1</td>
<td>190</td>
</tr>
<tr>
<td>Floyd700F</td>
<td>[P3]Nem890R</td>
<td>2</td>
<td>190</td>
</tr>
<tr>
<td>Floyd700F</td>
<td>Nem996R</td>
<td>3</td>
<td>296</td>
</tr>
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<td>Floyd700F</td>
<td>Nem1025R</td>
<td>4</td>
<td>325</td>
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<tr>
<td>Floyd700F</td>
<td>[P3]Nem1025R</td>
<td>5</td>
<td>325</td>
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<tr>
<td>Floyd700F</td>
<td>Nem1217R</td>
<td>6</td>
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<td>Nem996R</td>
<td>7</td>
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<td>Nem1025R</td>
<td>8</td>
<td>135</td>
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<td>9</td>
<td>135</td>
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<td>Nem1217R</td>
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<tr>
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<td>Nem1619R</td>
<td>27</td>
<td>144</td>
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**Figure 16 – Diagram of the relative, designed primer binding sites within the *T. muris* 18S rRNA gene.** Arrows indicate direction that primer permits DNA replication. * = indicates relative position in the *C. elegans* 18S rRNA gene.
Figure 17 – Workflow of primer pair testing. Due to the large number of primer pairs a workflow was developed to quickly eliminate non-functional pairs. At step (1) all pairs were positively selected for i.e. if they could amplify all nematode tissue DNA. At step (2) they were negatively selected against if they amplified when tested on faecal DNA from uninfected mice or from Platyhelminth tissue DNA. Finally, at step (3) they were positively selected for, if they could amplify faecal DNA from infected mice and amphibians.
3.3.2 Testing of designed primers

All primer pairs were put into six groups based on having similar annealing temperatures and tested against four nematode tissue DNA extracts (T. muris, T. spiralis, A. lumbricoides and H. polygyrus). As an example, the group of three primer pairs with an annealing temperature of 54.1°C is shown (Figure 18.1). All primer pairs in this group were successful in amplifying the nematode tissue DNA, however pair 19 and pair 27 formed much tighter and clearer bands than pair 28 (Figure 18.1). Pairs 19 and 27 may therefore show greater potential as accurate nematode universal primers. In total, eight of the 28 primer pairs were successful in amplifying all the nematode tissue DNA and were progressed onto stage two (Table 6A).

The eight primer pairs that had passed stage one were then tested against non-infected mouse faecal DNA that had been extracted at two different lysis temperatures (37°C and 65°C) and Platyhelminth tissue DNA (S. mansoni and H. microstoma). As an example, the results from primer pairs 13, 17, 19, 22, 27 and 28 are shown (Figure 18.2A, B). Only pair 27 amplified none of the DNA extracts and therefore demonstrated specificity via a lack of cross-reactivity with DNA from other organisms present in faeces or from parasitic Platyhelminthes (Figure 18.2B). All the other primer pairs tested in this PCR either amplified only the Platyhelminth DNA or the faecal DNA from non-infected mice as well and were therefore excluded from progression to stage three. In total, only two of the eight primer pairs at this stage progressed onto stage three (Table 6B).

The two successful primer pairs, 11 and 27, were then tested against faecal DNA from mice infected with T. muris and T. spiralis and from the amphibian species; M. betsileo, M. aurantiaca, M. ebenai, D. auratus and A. callidryas (Figure 18.3A, B). Primer pair 27 amplified all the faecal DNA extracts from T. muris infected mice and nearly all the extracts from M. betsileo amphibians, but not faecal DNA from T. spiralis infected mice (Figure 18.3A). Primer pair 11 in comparison performed poorly and did not amplify any of the faecal DNA extracts (Figure 18.3B). Primer pair 27 was re-tested on the faecal DNA from nematode infected mice, using 5 µl of DNA extract per reaction and an extended 40 cycles of replication. Under these conditions primer pair 27 was successfully used to amplify faecal DNA from both T. muris and T. spiralis infected mice and faecal DNA from the amphibian species; M. betsileo, M. aurantiaca, M. ebenai and A. callidryas (Figure 18.3C). Overall, primer pair 27 comprising Nem1217F and Nem1619R primers (hereafter referred to as Nem27 primers) was found to be the most successful for specific amplification of nematode DNA and was selected for further experimentation (Table 6C).
Figure 18.1 – Primer testing stage 1: PCR amplification on nematode tissue DNA. All of the primers pairs (subscript) tested in this set successfully amplified 1μl of tissue DNA from T. muris (Tm), T. spiralis (Ts), A. lumbricoides (Al) and H. polygyrus (Hp). However, primer pair 28 amplified poorly, producing less tight bands at the 144 bp size expected. Primer pairs 19 and 27 amplified at the expected 623 bp and 402 bp, respectively. 1kb hyperladders were run (HL).

Figure 18.2 – Primer testing stage 2: PCR amplification results on faecal DNA from uninfected mice and Platyhelminth tissue DNA. A, B: Primer pairs 22 and 17 (numbered above lanes) amplified 2 μl of faecal DNA from uninfected mice (N), carried out at a 37°C or 65°C (*) extraction lysis temperature. These primers also cross-reacted with tissue DNA from S. mansoni (Sm) and H. microstoma (Hm). B: Primer pairs 19 and 28 also cross-reacted with either one or both Platyhelminth tissue DNA extracts. B: Only primer pair 27 showed no cross-reactivity with any DNA extract. Positive controls containing 1 μl of T. muris tissue DNA were also included (+). 1kb hyperladders were run (HL) and negative controls (X).
Figure 18.3 – Primer testing stage 3: PCR amplification on faecal DNA from amphibians and nematode infected mice. A, B: Primer pair 27 successfully amplified faecal DNA from T. musri (Tm) infected mice, regardless of extraction lysis temperature (numbers in superscript) and from M. betsileo faecal DNA (Mb), whilst primer pair 11 did not amplify these samples. The second M. betsileo faecal extract amplified better (2 subscript). However, whether the old faecal extraction kit was used (Δ) or more stirring faecal sample (o) had little effect. Both primer pairs amplified the T. musri egg DNA (E), whilst neither could amplify faecal DNA from T. spiralis (Ts) infected mice, regardless of whether 5 or 10 minutes (*) of bead-beating were used in the extraction. Overall, primer pair 27 performed much better across the DNA samples tested. Positive controls containing 1μl of tissue extracted T. musri DNA were included. C: Primer pair 27 was re-tested, using an extended 40 cycle program and successfully amplified faecal DNA from mice infected with T. musri (Tm) and T. spiralis (Ts). It also amplified faecal DNA from the amphibians M. betsileo (Mb), M. aurantiaca (Ma), M. ebenaui (Me) and A. callidryas (Ac) but not D. auratus (Da). The range of faecal samples primer pair 27 was able to detect nematode DNA from, demonstrated their specificity and efficacy. 1kb hyperladders were run (HL) and negative controls (X).
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Table 6 – Performance of primer pairs through selection workflow. **A:** Performance of all 28 primer pairs at stage 1 of workflow. Each pair was tested against nematode tissue DNA. Product size, presence of amplification and percentage of nematode DNA extracts that were positive for amplification are all shown. The 8 pairs (highlighted) that amplified all DNA extracts progressed onto stage 2. **B:** Performance of eight primer pairs at stage 2. These primers were tested against faecal DNA from uninfected mice and Platyhelminth tissue DNA. Presence of amplification was selected against; hence the two pairs that showed no amplification (highlighted) progressed to stage 3. **C:** Performance of two primer pairs at stage 3. These primers were tested against faecal DNA from amphibians and experimentally infected mice. Pair 27 amplified all faecal DNA from hosts known to be infected with nematodes and hence was chosen as the optimal primer pair.
3.3.3 Confirmation of designed primer specificity

To confirm that Nem27 primers were actually amplifying nematode DNA and at the expected region of 18S rRNA gene, the amplicons produced using these primers on *M. betsileo* and *T. muris* infected mice faecal DNA were sequenced. Separate forward and reverse sequences were produced for each amplicon and their traces examined for quality. Regions that had low levels of sequencing confidence (as determined by their trace) were removed from the beginning and end of the sequence. Sequences from both the infected mice and *M. betsileo* faecal DNA were then aligned in BioEdit along with the complete *T. muris* 18S rRNA gene (Figure 19). Alignment demonstrated that both the forward primer Nem1217F (Figure 19A) and the reverse primer (Figure 19B) were binding at the same region of the 18S rRNA gene in both faecal DNA extracts. They were also seen to bind close to the region in the original *T. muris* 18S rRNA gene against which they were designed. The distance between the loci at which both primers bind was calculated to be approximately 400 bp which matched the length of the amplicon’s sequence as determined by Sanger sequencing and the size of the fragment seen when run on a gel. Overall, Nem27 primers were confirmed to bind at the expected loci in the nematode 18S rRNA gene.

The forward and reverse sequences for each amplicon were then aligned in BioEdit separately. These sequences, as expected, showed complete matching along their overlapping regions and hence the full sequence of the amplicon formed by the primer pair could be taken. Both the 427 bp fragment sequenced from the infected mice faecal DNA and the 320 bp fragment produced from the *M. betsileo* faecal DNA were analysed for potential matches using the BLAST tool in GenBank. As expected the top match for the DNA sequence from the infected mice was to *T. muris* followed by other nematode species in this genus (Table 7). This exemplifies Nem27 primers functionality at detecting and identifying parasite DNA from host faeces. The GenBank sequence matches returned using the *M. betsileo* faecal DNA identified the presence of nematode DNA from a species of the genus *Poikilolaimus*.

Additionally, the NemUni-1 primers (nematode universal primers used at this study’s onset) were also used on *M. betsileo* faecal DNA and the amplicon produced was sequenced. A successful 571 bp sequence was taken from the trace produced and analysed for matches in GenBank. This also produced a top match with the nematode species *Poikilolaimus oxycercus*, supporting the results of the Nem27 primers that DNA from this species is present in the faeces of our *M. betsileo* colony.
Figure 19 – Sequencing and alignment results for fragments amplified by Nem27 primers. Amplicons produced by Nem27 primers on faecal DNA from M. betsileo amphibians and mice infected with T. muris were sequenced and analysed. Forward and reverse sequences were aligned along with the complete T. muris 18S rRNA gene sequence taken from GenBank. **A:** Alignment shows that forward primer Nem1217F (labelled ‘Forward’) binds at the same relevant position to nematode DNA in both the M. betsileo and mouse faecal DNA. The highlighted region also demonstrates that this primer is binding around the expected 1,217th base pair position in the T. muris 18S rRNA gene around which it was designed. **B:** Alignment shows that the reverse primer Nem1619R (labelled ‘Reverse’) binds at the same relevant position to nematode DNA sequences in both the M. betsileo and mouse faecal DNA. The highlighted region also demonstrates that this primer is binding around the expected 1,619th base pair position in the T. muris 18S rRNA gene around which it was designed. The difference between these two primer binding site loci is approximately 400 bp which is the size of the fragment produced when run on a gel using the Nem27 primers and is also the complete length of the sequence produced by sequencing of these amplicons.
Table 7 – BLAST results of nematode sequences from *M. betsileo* amphibians and mice infected with *T. muris*. Amplicons produced by Nem27 primers on faecal DNA extracts from *M. betsileo* amphibians and mice infected with *T. muris* were sequenced and matches ascertained with reference sequences in GenBank. The top five matches were noted along with accession number, query cover and identity (percentage of bases that are identical between the query sequence and the database sequence). As expected the sequence from the infected mice faecal DNA obtained a top match with the nematode *T. muris*, whilst the top matches from the *M. betsileo* faecal DNA were predominantly from the nematode genus, *Poikilolaimus*.

<table>
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3.3.4 Efficacy of developed Nem27 primers compared to NemUni-1 primers

The entire process from DNA extraction to PCR amplification and then sequencing was redone on faecal samples from the *M. betsileo* colony to ensure repeatability of previously acquired results. Both Nem27 primers from the present study and the NemUni-1 primers were successfully used to amplify *M. betsileo* faecal DNA (*Figure 20A*). One amplicon produced by each of these primer sets was sequenced.

Both the 784 bp fragment produced by the NemUni-1 primers and the 348 bp sequence produced by the Nem27 primers were analysed for matches in GenBank (*Table 8*). The top results acquired by the Nem27 primers achieved matches very similar to those acquired from previously sequenced amplicons with *P. oxy cercus* as the top match (*Table 7*). However, the NemUni-1 primers returned top matches with sequences from five species of Collembola (springtails), a subclass of hexapod arthropods commonly found in soil (Blancquaert and Mertens, 1977). The high query and identity cover of these matches to Collembola 18S rRNA sequences and defined band produced when run on a gel (*Figure 20A*) indicates that the NemUni-1 primers may be cross-reacting with DNA from outside of the Nematoda phylum. Overall, the Nem27 primers developed presently demonstrate repeatable specificity for nematode 18S rRNA sequences, whilst the NemUni-1 primers are able to cross-react with non-nematode species e.g. *Sphaeridia pumilis*. This indicates an improved efficacy
for nematode universal specificity within the primers I presently developed, over those previously reported in the literature.

**Table 8 – BLAST results of sequences from M. betsileo faecal DNA using Nem27 and NemUni-1 primers.** Amplicons produced by Nem27 and NemUni-1 primers on M. betsileo faecal DNA were sequenced and matches ascertained with reference sequences in GenBank. The top five matches were noted along with accession number, query cover and identity (percentage of bases that are identical between the query sequence and the database sequence). Matches from Nem27 primers were similar to those previously found with the *P. oxycercus* species obtaining the top match. However, the top matches found with the NemUni-1 primers belonged to species in the subclass Collembola (springtails). These are small hexapods commonly found in soil and such a result indicates cross-reactivity of these primers and therefore non-specificity to nematode DNA.
3.4 Application of copro-diagnostic protocol with Nem27 primers to amphibian samples

3.4.1 PCR amplification from individually housed *M. betsileo* amphibian samples

To investigate the potential utility of the developed copro-diagnostic protocol and Nem27 primers for identifying parasitic infection in specific amphibian individuals an isolated housing experiment was carried out. Five *M. betsileo* individuals from the entire colony were separated into different tanks so that individual faecal samples could be stockpiled and analysed. Successful PCR amplification was only achieved from one individual's samples when carried out under an extended 40 cycle program, whilst none of the other four individuals' faecal DNA produced amplification product (Figure 21). This indicated that only one of five *M. betsileo* individuals tested may have a parasitic nematode infection.

![Figure 21 - PCR amplification using Nem27 primers on faecal DNA from separately housed M. betsileo individuals. Nem27 primers failed to amplify 5 μl of faecal DNA from any of the separately housed M. betsileo individuals (lane numbers refer to amphibian individual) when 35 cycles of amplification were used. However, they amplified from individual 1 faecal DNA when 40 cycles of amplification were used (*), indicating this individual to be infected. Arrows indicate the expected 402 bp size product. Positive controls containing 1 μl of T. muris tissue DNA were included (+). 1kb hyperladders were run (HL) and negative controls (X).](image-url)
3.4.2 Dissection of infected *M. betsileo* individual and organ extracted DNA PCR

To provide confirmation of the infection status of the individual reported to be infected via the developed copro-diagnostic system a dissection was performed to attempt to find the parasitic nematodes *in situ*. Although nematodes had previously been identified in the faeces through light microscopy, a dissection experiment was needed to confirm the presence of parasitic nematodes within the host’s tissues.

The potentially infected *M. betsileo* individual was dissected with the entire GI tract removed and maintained in heated buffer solution. The GI tract was the expected site of a parasitic nematode infection (Wright and Whitaker, 2001) and was sliced open and inspected via light microscopy for the presence of nematode adults or eggs. No nematode life cycle stages, including putative nematode eggs were found (data not shown).

The liver, heart, lungs, kidney, gall bladder, ovaries, spleen and pancreas were also extracted and snap frozen on dry ice. DNA was extracted separately from all of these organs and a PCR carried out using the Nem27 primers. Organ extracted DNA was tested to check for the presence of parasites outside the GI tract. All organ extracted DNA did yield amplification products, whilst a positive control containing 1 µl of *T. muris* tissue DNA and 4 µl of *M. betsileo* organ DNA was successfully used to yield an amplification product, demonstrating an absence of PCR inhibitors (data not shown).

The original PCR on the *M. betsileo* faecal extracts from separately housed individuals was then repeated under the same PCR conditions, however no amplification was acquired and the results demonstrated previously were not repeated (data not shown). Overall, the absence of parasitic nematodes *in situ* and lack of amplification from tissue extracted DNA from commonly infected organs, suggested that the developed copro-diagnostic protocol was detecting nematode DNA from other sources than the amphibian.

3.4.3 Light microscopy on faecal smears and the dissected GI tract from a *M. betsileo* individual

To further investigate the source of the amplification from the copro-diagnostic protocol and shed light on the possibility of free-living nematodes occupying the amphibian faecal samples a second set of faecal smears were mounted. Five different *M. betsileo* individuals from the entire colony were separated into different tanks and their faecal samples mounted to create smears. Four smears per individual were then investigated using light microscopy, however only one smear showed a nematode (*Figure 22*). No other nematode life cycle stages were identified in these smears.

The *M. betsileo* individual that had produced this sample was then dissected, to ascertain whether it was infected. Upon dissection, extraction and opening of the GI tract and liver, investigation via light microscopy again did not reveal any nematode adult or other life cycle stages, nor did...
scanning of the remaining material within the gut. However, one organism believed to be an arthropod, possibly from the Acari subclass, was seen embedded within the tissue of the large intestine (Figure 22).

3.4.4 PCR amplification using Nem27 primers on soil extracted DNA from the *M. betsileo* tanks

To elucidate whether amplification from the *M. betsileo* faecal DNA was due to tank soil-dwelling nematodes moving into the faeces, two separate soil DNA extractions were carried out using tank soil from both amphibian colonies. Soil DNA was extracted according to the developed copro-diagnostic protocol along with new faecal DNA from faeces produced at the time of soil testing and from the *M. betsileo* individual dissected in section 3.4.3. Both soil DNA extracts did not amplify using the Nem27 primers (Figure 23) indicating an absence of free-living nematodes in these soil
samples. However, the faecal DNA from the *M. betsileo* individual which had, had the nematode present in its faecal smear and had been dissected did amplify (Figure 23). In contrast a faint amplification band was produced by the faecal DNA taken from the pooled faeces of all the other *M. betsileo* individuals in the colony (Figure 23). The results from this PCR were in direct disagreement with the results from the dissection as no parasitic nematodes were found upon dissection but the copro-diagnostic protocol indicated the presence of nematode DNA. Furthermore, no nematode DNA was detected in the soil from which the *M. betsileo* individuals were kept in, potentially ruling out the possibility of migration of free-living nematodes into the amphibian’s faeces.

![Figure 23 – PCR amplification using Nem27 primers on soil extracted DNA and M. betsileo faecal DNA. Nem27 primers successfully amplified M. betsileo faecal DNA from the individual dissected earlier (I) and weakly amplified from pooled M. betsileo faecal DNA from multiple individuals (P), when 35 cycles of amplification were used. When 45 cycles of amplification were employed (*) only the faecal DNA from the individual amplified (I). DNA extracted from the soil (S) present in the M. betsileo colony tanks failed to amplify, either from tank 1 or 2 (subscript). Such results indicate the presence of nematode DNA in the faeces of the individual dissected but not in the faeces of other individuals or soil. Positive controls containing 1 μl of *T. muris* tissue DNA and 4 μl of soil extracted DNA were included to check for the presence of PCR inhibitors (+). 1kb hyperladders were run (HL) and negative controls (X).](image)

### 3.4.5 Sequencing of nematode DNA from the dissected *M. betsileo* individual and its faecal smear

To enable identification of the nematode DNA that was identified by PCR in the dissected *M. betsileo* individual (Figure 23) the amplicon was sequenced and the 351 bp fragment analysed for matches in GenBank. In addition, DNA was extracted from the nematode found in the faecal smear from the dissected individual in section 3.4.3 and was amplified using the Nem27 primers. The subsequent amplicon was sequenced and the 348 bp fragment analysed for matches. Both the sequence from the faecal DNA amplicon and the faecal smear obtained identical matches with sequences in GenBank (Table 9). These results indicated that the nematode sequences were from
the species *Oscheius tipulae*, a recognised soil-dwelling nematode from the Rhabditidae family (Baille et al., 2008). Such results highlight the possibility that in at least one case, soil-dwelling nematodes from the *M. betsileo* tank environment had migrated into the amphibian faeces, thus generating a positive PCR result. Nonetheless, as previously described in section 3.4.4, soil extracted DNA had not yielded any PCR products using the Nem27 primers. These disparate results may be due to the fact that the soil DNA was extracted using the copro-diagnostic protocol but such a protocol may not be adequate for extracting DNA from a non-faecal medium, like soil. An alternative explanation is that there may not have been any nematode or nematode eDNA present in the two soil samples tested.

An al
ternative explanation is that there may not have been any nematode or nematode eDNA present in the two soil samples tested.

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3.4.6 PCR amplification and sequencing using Nem27 primers on faecal DNA from wild *Mantella cowani* amphibians

To test whether my developed copro-diagnostic protocol could have functionality investigating the infection status of wild amphibians, it was tested on faecal samples collected from various *M. cowani* individuals found in the field, in Madagascar. These samples were processed upon return to the University of Manchester and the faecal DNA amplified using the Nem27 primers. The faecal extractions that were processed using 5 minute of bead-beating, amplified best after having undergone either 40 cycles or 45 cycles of replication (Figure 24). However, those samples that had undergone 1 minute of bead-beating or had used less starting faecal material (10 mg instead of 20 mg) had fainter bands when analysed (Figure 24), suggesting damaged or insufficient high quality DNA. Results indicated the presence of nematode DNA in the *M. cowani* faeces.
To explore whether these amplification results were caused by nematode DNA from parasitic species infecting the *M. cowani* amphibians, amplicons were sequenced and the 353 bp fragments analysed for matches in GenBank (Table 10). The top match was from a nematode belonging to a genus that lies phylogenetically within the Cosmocercidae family; this family includes species known to infect amphibian hosts (Baker, 1985; Bursey and Goldberg, 2006). The other highest matches were known parasites of various arthropod species, whilst the species to obtain the fifth match, *Pseudonymus islamabadi* was a sequence deposited from a nematode found in the lizard, *Iguana iguana* (Malysheva, 2016). Such matches suggest that the developed copro-diagnostic system is detecting parasitic nematode DNA from the faeces of *M. cowani*.

![Figure 24 - PCR amplification using Nem27 primers on faecal DNA from wild M. cowani amphibians](image)

*Figure 24 – PCR amplification using Nem27 primers on faecal DNA from wild M. cowani amphibians.* DNA was successfully amplified by the Nem27 primers on bead-beaten *M. cowani* faecal DNA, regardless of whether 1 or 5 minutes of beating were employed (numbers in superscript). Whether a new or old (△) faecal extraction kit was used, also had no effect. Longer thermocycling programs were chosen due to the low DNA concentrations obtained by the extraction (~4 ng/μl) and both permitted good amplification. Such results indicate that these amphibians have nematodes in their faeces and may therefore be infected. Arrows indicate the expected 402 bp size product. Positive controls containing 1 μl of tissue extracted *T. muris* DNA and 4 μl of faecal DNA were included (+). 1kb hyperladders were run (HL) and negative controls (x).
Table 10 – BLAST results of faecal DNA from wild *M. cowani* amphibians. Amplicons produced by Nem27 primers on *M. cowani* faecal DNA were sequenced and matches obtained using GenBank. The top six matches were noted along with accession number, query cover and identity (percentage of bases that are identical between the query sequence and the database sequence). The top match belongs to a genus that lies phylogenetically within the family Cosmocercidae, in the parasitic nematode order Ascaridida (Bursey and Goldberg, 2006). The Cosmocercidae are a family containing amphibian infecting nematodes, suggesting that the 18S rRNA sequence found in the *M. cowani* faeces does belong to a parasitic nematode infecting the *M. cowani* host (Baker, 1985).

<table>
<thead>
<tr>
<th>Match</th>
<th><em>M. cowani</em> faecal DNA</th>
<th>Query Cover</th>
<th>Identity</th>
<th>Accession no.</th>
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</thead>
<tbody>
<tr>
<td>Top Match</td>
<td>Raillietnema sp.</td>
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<td>99</td>
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<tr>
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<td>Rhigonema ingens</td>
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<td>98</td>
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<td>Heth sp.</td>
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<td>98</td>
<td>JX987087.1</td>
</tr>
<tr>
<td>Fourth Match</td>
<td>Heth impalutiensis</td>
<td>99</td>
<td>98</td>
<td>KM226161.1</td>
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<tr>
<td>Fifth Match</td>
<td>Pseudonymus islamabadi</td>
<td>99</td>
<td>98</td>
<td>KJ632668.1</td>
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<tr>
<td>Sixth Match</td>
<td>Cosmocercoides pulcher</td>
<td>99</td>
<td>98</td>
<td>LC018444.1</td>
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</table>
3.5 Wider applications of copro-diagnostic protocol with Nem27 primers to captive herpetofauna in general

3.5.1 PCR amplification using Nem27 primers on faecal DNA from herpetofauna species maintained at ZSL London Zoo

Our copro-diagnostic protocol was next tested on a further array of faecal samples from both captive amphibians and reptiles, to assess whether it had utility as a tool for investigating parasite infection outside of the Amphibia class. Faecal samples from 7 different amphibian species and 17 different reptile species, representing a large variety of different orders and families were delivered from captive collections at ZSL London Zoo and processed at the University of Manchester. DNA was extracted from 30 different faecal samples and the final DNA concentrations analysed (Table 11).

All 30 samples were tested using the Nem27 primers along with positive and negative controls in three batches. Of these 30 samples, ten yielded amplification products when either 5 µl or 10 µl of faecal DNA was used. The following herpetofauna species produced an amplification signal: *P. bicolor*, *A. dumerilli*, *D. tinctorius* (Figure 25A), *G. gecko* sample 13 and 15 (Figure 26A), *S. crocodilurus*, *R. boulengeri*, *T. g. floweri*, *T. g. whitei*, *P. caeruleo* and *L. fallax* (Figure 27A, B). The results indicate the presence of nematode 18S rRNA sequences in these faecal DNA extracts and therefore a possible parasitic nematode infection.

An amplification band was also seen on the *V. crocodilurus* faecal DNA that used 5 µl of starting DNA (Figure 26A). However, because this result was not consistent with the negative result obtained when the reaction was replicated using 10 µl of DNA template, it was not investigated further (Figure 26A).

To test for the possible presence of PCR inhibitors all the faecal DNA extracts were amplified spiked with a positive control containing 1 µl of *T. muris* tissue DNA and 4 µl of the relevant herpetofauna faecal DNA. 27 of these positive controls amplified well, indicating an absence of PCR inhibitors. Three spiked samples from *G. gecko* sample 14 (Figure 26A), *S. boeleni* (Figure 26B) and *E. stokesii* (Figure 27A) faecal DNA did not yield amplification product, suggesting a carry-over of PCR inhibitors from the DNA extraction. The positive controls were repeated using spiked samples over a 40 cycle replication program instead of 35 but there was no reaction (data not shown). Hence to accurately assess the infection status of these three individuals the faecal DNA extraction would need to be repeated.
### Amphibia

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Species</th>
<th>Common name</th>
<th>Date</th>
<th>Tank code</th>
<th>Quantity (mg)</th>
<th>DNA (ng/µl)</th>
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</thead>
<tbody>
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<td>9432-9442</td>
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<tr>
<td>2</td>
<td>Alytes obstetricans</td>
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<td>60.4</td>
</tr>
<tr>
<td>3</td>
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<td>Lake Patzcuaro Salamander</td>
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<td>4</td>
<td>Dendrobates tinctorius</td>
<td>Dyeing Dart Frog</td>
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<td>5</td>
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<td>Giant Ditch Frog</td>
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<td>UMC Pen 3</td>
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<tr>
<td>6</td>
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<td>Kaiser’s Spotted Newt</td>
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<td>N/A</td>
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<tr>
<td>7</td>
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<td>Black-legged Poison Frog</td>
<td>11.06.16</td>
<td>G01026</td>
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<td>17.4</td>
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</table>

### Reptilia

<table>
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<th>Species</th>
<th>Common name</th>
<th>Date</th>
<th>Tank code</th>
<th>Quantity (mg)</th>
<th>DNA (ng/µl)</th>
</tr>
</thead>
<tbody>
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<tr>
<td>9</td>
<td>Aspidites melanocephalus</td>
<td>Black-headed Python</td>
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<td>10</td>
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<tr>
<td>11</td>
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<td>Baker’s Spinytail Iguana</td>
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<td>12</td>
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<td>Gidgee Skink</td>
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<td>Tokay Gecko</td>
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<td>G02069</td>
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<td>62.7</td>
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<td>14</td>
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<td>3251-3252</td>
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<tr>
<td>15</td>
<td>Gekko gecko</td>
<td>Tokay Gecko</td>
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<td>4188</td>
<td>80</td>
<td>173.3</td>
</tr>
<tr>
<td>16</td>
<td>Gekko gecko</td>
<td>Tokay Gecko</td>
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<td>G0270</td>
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<td>104.7</td>
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<td>34.4</td>
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<td>False Water Cobra</td>
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<td>19</td>
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<td>Australian Green Tree Frog</td>
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<td>G01013-G01024</td>
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</tr>
<tr>
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<tr>
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<td>12.6</td>
</tr>
<tr>
<td>25</td>
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<td>Boelen’s Python</td>
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<td>26</td>
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<td>Boelen’s Python</td>
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<tr>
<td>27</td>
<td>Simalia boeleni</td>
<td>Boelen’s Python</td>
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<td>7076-7077</td>
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<td>28</td>
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<tr>
<td>29</td>
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<td>C707</td>
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</tr>
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<td>Varanus prasinus</td>
<td>Emerald Tree Monitor</td>
<td>11.06.16</td>
<td>7842</td>
<td>300</td>
<td>73.1</td>
</tr>
</tbody>
</table>

Table 11 – ZSL London Zoo faecal sample and DNA extract information.
All 30 samples provided by ZSL London Zoo were numbered based on the class of animal they belonged to and their species name. Seven amphibian species and a wide range of reptile species from different orders were provided. Details of sample collection date and tank code (to distinguish samples from different individuals of the same species) were logged. Information on starting quantity of faecal material used and the final concentration of DNA in each extract are also provided.
Figure 25 – PCR amplification using Nem27 primers on faecal DNA from London Zoo herpetofauna: batch one. **A, B:** The herpetofauna sample number that was tested is indicated at the top of lanes. Nem27 primers successfully amplified both 5 µl and 10 µl (*) of faecal DNA from samples 7 (P. bicolor), 3 (A. dumerillii) and 4 (D. tinctorius), indicating a likely nematode infection in these three species. Arrows indicate the expected 402 bp size product. Positive controls containing 1 µl of tissue extracted T. muris DNA and 4 µl of the relevant herpetofauna faecal DNA were included (+ superscript), demonstrating an absence of PCR inhibitors in these DNA extracts. 1kb hyperladders were run (HL) and negative controls (X).
Figure 26 – PCR amplification using Nem27 primers on faecal DNA from London Zoo herpetofauna: batch two. A, B: The herpetofauna sample number that was tested is indicated at the top of lanes. Nem27 primers successfully amplified both 5 μl and 10 μl (*) of faecal DNA from samples 13 and 15 (both G. gecko), indicating a likely nematode infection in two individuals from this species. Some amplification was seen in sample 30 (V. crocodilurus) when 5 μl of DNA was used but the absence of amplification when 10 μl of extract was used meant this was not explored further. Arrows indicate the expected 402 bp size product. Positive controls containing 1 μl of tissue extracted T. mus mus DNA and 4 μl of the relevant herpetofauna faecal DNA were included (+ superscript), demonstrating an absence of PCR inhibitors in most DNA extracts. However, positive controls failed to amplify from samples 14 and 26, indicating carry-over of some PCR inhibitors from the DNA extraction. Furthermore, some hyperladder can be seen to have leaked out of the gel wells in the first three lanes and this is not indicative of amplification at the band sizes seen. 1kb hyperladders were run (HL) and negative controls (X).
Figure 27 – PCR amplification using Nem27 primers on faecal DNA from London Zoo herpetofauna: batch three. A, B: The herpetofauna sample number that was tested is indicated at the top of lanes. Nem27 primers successfully amplified both 5 µl and 10 µl (*) of faecal DNA from samples 23 (S. crocodilurus), 22 (R. boulengeri), 27 (T. g. floweri), 28 (T. g. whitei), 20 (P. caeruleo) and 5 (L. fallax), indicating a likely nematode infection in these amphibian species. Arrows indicate the expected 402 bp size product. Positive controls containing 1 µl of tissue extracted T. muris DNA and 4 µl of the relevant herpetofauna faecal DNA were included (+ superscript), demonstrating an absence of PCR inhibitors in most DNA extracts. However, positive controls failed to amplify from sample 12, indicating carry-over of some PCR inhibitors from the DNA extraction. 1kb hyperladders were run (HL) and negative controls (X).
3.5.2 Sequencing results from faecal DNA of herpetofauna species maintained at ZSL London Zoo

To identify the source of the sequences that produced the ten positive amplification results in the herpetofauna sample PCRs, the amplicons were sequenced and results analysed for matches in GenBank (Table 12). The sequenced amplicons from the D. tinctorius, S. crocodilurus, T. g. whitei and T. g. floweri hosts all returned top matches from nematode species known to be parasitic or to be from nematode genera that are parasitic. The top match for the two tortoise species, T. g. whitei and T. g. floweri, was a pinworm species known to infect laboratory mice, alongside other vertebrates (Goswami et al., 2015; Pan et al., 2015). The top nematode sequence match for the amphibian host D. tinctorius, was from a genus that lies phylogenetically within the Cosmocercidae family, which contains many amphibian infecting species (Bursey et al., 2006; Bursey and Goldberg, 2006). The host lizard, S. crocodilurus, obtained top matches with the nematode genus Diploscapter a genus that contains both parasitic and free-living species (Shah and Vaid, 2015; Steel et al., 2012). Hence, with these four faecal extracts, the developed copro-diagnostic protocol was likely detecting eDNA signals from nematodes parasitising these herpetofauna species.

The sequenced amplicons from P. bicolor and R. boulengeri both returned top matches with sequences from common soil-dwelling nematodes (Table 12) (Baille et al., 2008; Hong et al., 2005). Such matches are identical to ones previously discovered throughout this study and likely represent the presence of common free-living nematode species, migrating from the soil environment into faeces.

In contrast to the nematode matches obtained in the other extracts, the sequenced amplicons from L. fallax, A. dumerili and two G. gecko samples obtained matches with sequences of organisms from outside of the Nematoda phylum (Table 12). These sequences all matched species in the mite family Acaridae, some of which inhabit the soil, whilst others are parasitic (Abou El-Atta et al., 2014; Principato et al., 2005). The amplification of mite sequences by the developed Nem27 primers demonstrates that they may be cross-reacting with sequences from outside of the Nematoda phylum and are therefore not specific to this group.

To investigate this cross-reactivity, the top three mite sequences identified by sequencing and BLAST analysis had their 18S rRNA sequences taken from GenBank. They were then aligned and checked against the Nem27 primer sequences. This allowed for identification of sites where the Nem27 primers could potentially bind to the mite sequences. The forward primer, Nem1217F has homology with a region 1,205 base pairs downstream of the beginning of the mite 18S rRNA gene with just five uncomplimentary base pairs. However, the reverse primer Nem1619R would only be able to bind upstream of the forward primer with 14 mismatched base pairs of a total 24 (data not shown). Hence, the mechanism of cross-reactivity by the Nem27 primers on possible mite DNA could not be elucidated.
Table 12 – BLAST results of faecal DNA from ZSL London Zoo herpetofauna. The ten herpetofauna faecal samples that produced amplicons with the Nem27 primers were sequenced and matches obtained using GenBank. The top five matches were noted along with accession number, query cover and the identity (percentage of bases that are identical between the query sequence and the database sequence). The top matches for the D. tinctorius, S. crocodilurus, T. g. whitei and T. g. flowreri hosts were all nematode species known to be parasitic or to be from nematode genera that are parasitic (Bursey and Goldberg, 2006; Goswami et al., 2015). The top matches for the hosts P. bicolor and R. boulengeri are common soil-dwelling nematode species (Baille et al., 2008; Hong et al., 2005). This likely represents an absence of infection in these species. The top matches for the L. fallax, A. dumerilii and two G. gecko samples are all with soil-dwelling mites of the subclass Acari (Abou El-Atta et al., 2014). Asterisks denote matching species from outside of the Nematoda phylum. Such results demonstrate cross-reactivity of Nem27 primers on sequences from non-target organisms, indicating a lack of specificity to nematodes.

<table>
<thead>
<tr>
<th>P. bicolor faecal DNA</th>
<th>Query Cover</th>
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<th>Accession no.</th>
</tr>
</thead>
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<table>
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<th>Identity</th>
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<td>Fifth Match</td>
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<tr>
<td>Fifth Match</td>
<td>Hammerschmidtiella diesingi</td>
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</tbody>
</table>
3.5.3 Annealing temperature optimisation to regain specificity of Nem27 primers and prevent cross-reactivity

Given the cross-reactivity of the Nem27 primers with mite DNA as indicated by sequencing, it was expected that primer specificity could be regained via optimisation of the PCR conditions. Two faecal DNA extracts were selected; *T. g. whitei* which the sequencing data indicated was from a pinworm infection and had produced a defined amplification band (Figure 25.3) and faecal DNA from *G. gecko* sample 13 which had matches to mite DNA (Figure 25.2). This *G. gecko* faecal sample was chosen due to it having produced the most defined band on a gel of any of the amplicons produced by cross-reaction with mite DNA, hence it may be the hardest sample to prevent cross-reacting.

A thermal gradient PCR was carried out on these two faecal samples, investigating 1°C increments of annealing temperature from 55°C - 61°C (Figure 28A, B). None of these annealing temperatures showed improved specificity of the Nem27 primers as both *T. g. whitei* and *G. gecko* samples amplified over these temperatures (Figure 28A, B). Only the 55.7°C and 57.2°C temperatures tested did not amplify due to the gel being damaged and allowing escape of PCR product (Figure 28A). A higher range of annealing temperatures from 61.3°C – 63.9°C was also tested (Figure 28C, D). Over these temperatures the specificity of the Nem27 primers was restored, as at an annealing temperature of 63.9°C the *T. g. whitei* sample containing pinworm DNA was amplified, whilst the *G. gecko* sample containing mite DNA was not (Figure 28D). Some amplification was also observed with the *T. g. whitei* sample but not the *G. gecko* sample at the 62.3°C and 63.2°C annealing temperatures (Figure 28C), suggesting that these temperatures may also be beneficial for the restoration of the Nem27 primer specificity. Some amplification was seen in both of these faecal DNA extracts at the 61.3°C annealing temperature, highlighting this temperature as the cut-off point for Nem27 primer specificity. At this annealing temperature the band produced by the mite DNA in the *G. gecko* sample was much weaker than that produced by the pinworm DNA in the *T. g. whitei* sample (Figure 28C). The results from this annealing temperature optimisation experiment indicate that the developed copro-diagnostic protocol and Nem27 primers should use a PCR with a 62°C – 64°C annealing temperature to ensure primer specificity to nematode DNA.

3.5.4 PCR amplification using PlatUni primers on faecal DNA from herpetofauna species maintained at ZSL London Zoo

The PlatUni (Platyhelminth universal) primers were also tested on the 30 faecal DNA extracts from various herpetofauna species, producing eight successful amplification results (data not shown). However, upon sequencing of these amplicons, the sequences returned were much shorter than the expected 570 and 800 bp fragments that the PlatUni primers amplify. Only a couple of the traces produced by these sequences were of a good quality and when all the sequences were analysed in GenBank, no matches were returned. Even short stretches (30 - 50 bp) of the highest quality sequence in these amplicons failed to return any matches in GenBank, hence the source of
these amplicons remained unknown and these results were not examined further. No conclusions can be made about whether these herpetofauna are infected with Platyhelminthes.

Figure 28 – Thermal-gradient PCR amplification using Nem27 primers on faecal DNA from London Zoo T. g. whitei and G. gecko. A, B: Nem27 primers successfully amplified 5 µl of faecal DNA from T. g. whitei known to have DNA from a parasitic nematode (N) and G. gecko, known to have mite DNA (M) which the Nem27 primers can cross-react to. Nem27 primers amplified both samples across the range of annealing temperatures tested (numbers above lanes in °C). Hence, nematode specificity was not regained across these annealing temperatures. No banding was seen in lanes four to eight due to damage to the gel and escape of PCR product. C, D: Nem27 primers successfully amplified faecal DNA from T. g. whitei (N) but not G. gecko (M) at 63.9°C; hence the Nem27 primer specificity for nematode DNA alone was regained at this annealing temperature. Between 62.3°C to 63.2°C some faint amplification was seen on the T. g. whitei but not the G. gecko faecal DNA, again indicating useful annealing temperatures for Nem27 primer specificity. Arrows indicate strong banding produced by the expected 402 bp size, whilst white triangles indicate some weak amplification of the expected 402 bp size product. 1kb hyperladders were run (HL) and negative controls (X).
4. Discussion

The amphibian crisis is a phenomenon that has led to the extinction of many previously common species and threatens many more, yet elucidation of the factors responsible for such declines is a relatively nascent area of research (Cheng et al., 2011; Wake and Vredenburg, 2008). Now, studies are beginning to shed light on the role metazoan parasites are playing in this crisis, weakening already susceptible populations in the wild or causing die-offs in ex situ colonies intended for species conservation (Johnson and Lunde, 2005; Johnson et al., 2007; Pessier et al., 2014). Hence, effective techniques are needed for detecting parasitic infection that are non-damaging to amphibian populations, unlike necropsy, or that are more sensitive than common non-invasive methods, e.g. microscopy on faecal smears (Giangaspero et al., 2004; Huver et al., 2015). An effective alternative is the detection of parasite eDNA. This can be detected in the faeces of the host and can be teamed with primers that target diagnostic barcoding regions of a parasite’s genome to identify the species present (Bucklin et al., 2011). Such a copro-diagnostic method, based around molecular detection and identification, has the potential to be modified for use in the field, where data on amphibian population health and the impact wild parasites play is most important (Arimatsu et al., 2015; Bass et al., 2015; Hoverman et al., 2012). Amphibian fitness and whether certain parasite infections have a disproportionate impact on this, as well as the dynamics and changes in populations of wild parasites, are all vital areas that molecular copro-diagnosis could inform on (McKenzie, 2007; Orlofske et al., 2012).

The present study set out to determine whether parasite eDNA from such sources as shed cells and transmissible stages in amphibian faeces could be detected by PCR, using phyla specific primers for the parasite groups Nematoda, Trematoda and Cestoda. My copro-diagnostic method was successful in achieving this with regard to detection of nematodes and therefore provides a strong framework that future studies can build on. Thus one overarching future aim is to use this molecular copro-diagnosis in the context of conservation to assist in alleviation of the amphibian crisis.

One of the barriers to molecular detection of parasite infective stages is the release of their DNA, which is normally strongly encapsulated (Demeler et al., 2013). This has been recognised throughout many studies which have required a rigorous disruption step in the DNA extraction protocol in order to liberate the DNA for subsequent detection (Demeler et al., 2013; do Espirito-Santo et al., 2012; Dyachenko et al., 2008). Therefore, in aim one of this study I set out to effectively release DNA from parasite transmissible stages and this was accomplished by use of a modified faecal DNA extraction protocol that included a lengthy bead-beating step. This was demonstrated to function well even on parasite species that have extremely resilient eggs, such as nematodes of the genus Trichuris (Demeler et al., 2013). Moreover, it was shown that extraction of faecal DNA also needs a low lysis temperature of between 35°C - 45°C for subsequent PCR amplification to work, as opposed to the high lysis temperatures recommended in conventional faecal extraction kits.
Initially, NemUni-1 and PlatUni primers taken from the literature (Floyd et al., 2005; Van Steenkiste et al., 2015) where utilised to try to selectively amplify parasite eDNA in faeces. However, the NemUni-1 primers produced results with poor repeatability and specificity, frequently cross-reacting with DNA from organisms outside of the target group. In contrast, the PlatUni primers produced amplicons that could not be sequenced effectively and any sequence that was detected failed to return any matches with references in GenBank. The NemUni-1 primers had been widely used within the literature (Bhadury et al., 2006a; Harris et al., 2013; Perera et al., 2013), whilst the PlatUni primers were tested and found effective on 32 different Platyhelminth DNA samples (Van Steenkiste et al., 2015). However, other studies have highlighted issues with published primers either being non-specific or demonstrating an inability to detect DNA from a target taxa (Baker et al., 2003; McLain et al., 2009). These general problems with primers in the literature have been found in fields ranging from detection of human waterborne pathogens to gene expression profiling via qPCR (McLain et al., 2009; Spandidos et al., 2010).

Taking this into consideration, in aim two of the current study, I set out to design and improve upon existing nematode specific primers in the literature. Aim two was achieved by identification of 18S rRNA gene regions that were conserved amongst a variety of different parasitic nematodes of wild animals. The developed Nem27 primer pair was used alongside the faecal DNA extraction protocol to successfully amplify nematode eDNA from samples produced by both captive and wild amphibians as well as reptiles. Sequencing of amplicons generated by these primers confirmed that they were 18S rRNA sequences from nematodes.

Having successfully developed and rigorously quality-assured the Nem27 primers, they were applied to the analyses of faecal samples from a variety of herpetofauna at ZSL London Zoo, thus tackling aim three of the present study. Key findings made included the identification of a potential pinworm infection by Aspiculuris tetraptera or a close relative, in two tortoise species from samples provided by ZSL London Zoo. These results corroborated separate findings made by staff at ZSL London Zoo that had previously identified a nematode infection in these tortoises by a member of the genus Tachygonetria, a near relative of A. tetraptera also in the Oxyuridae family (Chris Michaels, pers. comm., August 25, 2016). In addition, faecal samples from the Madagascan frog, M. cowani were demonstrated to be infected by a nematode of the genus, Railletnema. This genus is known to contain at least 22 species of amphibian parasite (Baker, 1985; Bursey and Goldberg, 2006). D. tinctorius dart frogs from ZSL London Zoo were also infected with nematodes from this genus.

In addition to applications in the field, my copro-diagnostic method may be useful to test the success of experimental infections in a research setting. The Nem27 primers successfully amplified parasite DNA when they were tested on faecal DNA from mice experimentally infected with T. spiralis and T. muris, as confirmed by sequencing and matching in GenBank. Current screening to evaluate the success of an experimental infection relies on screening of eggs in the faeces, or dissection to find larval or adult worms in situ, techniques only possible when later stages in a parasite’s life cycle have been reached (Boes and Helwigh, 2000; Keiser et al., 2016). Therefore,
my copro-diagnostic method could improve existing protocols for evaluating experimental infections, particularly if it is tailored to provide information on parasite burden in laboratory models.

Such a range of potential applications clearly demonstrates the value of my copro-diagnostic protocol as a tool for detecting and identifying the macroparasites of amphibians as well as other animal groups. Nonetheless, a number of unexpected caveats within the copro-diagnostic method were also revealed, which are discussed in detail below. A principal problem was the detection of eDNA from common free-living bacterivorous nematodes that the Nem27 primers and those in the literature amplified, due to their nematode universality. Most studies amplifying eDNA from faeces or the soil are typically looking for one species of interest and can therefore use a specific primer, avoiding this problem (Bohmann et al., 2014; Ficetola et al., 2008). However, I needed to maintain a broad-spectrum capability for detecting nematodes due to the paucity of information on amphibian parasites (McAllister et al., 2010; McKenzie, 2007; Wright and Whitaker, 2001). Primers designed to detect DNA from just one clade or family could miss a significant portion of the present nematode diversity. In addition, the Nem27 primers also demonstrated some ability to cross-react with 18S rRNA sequences from soil-inhabiting mites. However, the specificity of these primers could be regained when the PCR was performed at higher annealing temperatures, providing key information on the optimal PCR conditions for the developed methodology.

4.1 Barriers to the employment of generic, broad-spectrum primers for detection and barcoding of parasite eDNA in faeces

4.1.1 Amplification of DNA from free-living nematodes

The Nematoda phylum is thought to contain an estimated 1 – 10 million species characterised by ecological omnipresence, particularly in environments such as terrestrial soil or marine sediments (Dieterich and Sommer, 2009; Groombridge, 1992). Nematodes play a key role in nutrient cycling and decomposition processes in the soil and can regularly be found at densities of up to 29,800,000 per m$^2$ (Pan et al., 2015; Rahman et al., 2014).

Given the ubiquitous nature of nematodes it is perhaps unsurprising that individuals may have migrated from the soil compartment of the terraria into the amphibian faeces. Furthermore, the amplification of DNA from free-living nematode species persisted, even when the amphibians were separated into individual tanks that had a substrate of moistened paper instead of soil. However, the pervasiveness in soils of the two species that obtained the top matches in GenBank, *P. oxy cercus* and *O. tipulae*, could mean that they were carried over on the amphibians themselves or within clumps of dirt stuck to their skin (Baille et al., 2008; Hong et al., 2005). Additionally, the sensitivity of the copro-diagnostic protocol could mean that just cellular debris from these nematodes was all that was needed to be transferred, making contamination of faeces relatively simplistic.
Studies investigating the effects of organic soil amendments have found that addition of manure to soils causes distinct increases in the number of bacterivorous free-living nematodes present (Renco and Kovacik, 2012). This is thought to arise due to manure increasing the bacterial content of the soil, followed by heightened predation and proliferation by bacterivorous nematodes (Rahman et al., 2014). Moreover, the common bacterivore, *C. elegans* has been observed to display preferences for different manure types, migrating into faeces following trails of faecal compounds released into the soil (Kenney et al., 2006). Thus, the issue of detection of DNA from free-living nematodes in faeces may reflect the fact that these species exhibit a preference for the faecal microhabitat.

The species *O. tipulae* identified from *M. betsileo* and *P. bicolor* faecal samples is known to be most common as a hardy, dormant (dauer) stage in soils, which can quickly emerge once it detects ideal environmental conditions (Steel et al., 2013). The prevalence of this nematode in amphibian faecal samples could be due to dormant individuals sensing the release of faecal compounds in their local environs and therefore hatching to colonise such microhabitats.

This contamination problem by non-parasitic nematodes is relatively unique to the current study, owing to the fact that the molecular detection system has had to be developed to detect all nematodes given the dearth of information regarding the parasites of amphibians. Many studies using copro-diagnosis are interested in one or a few, select parasites and therefore use appropriate primers that solely identify DNA from the subject/s of interest (Al-Sabi et al., 2007; Golab et al., 2009; Perera et al., 2013). Nonetheless, contamination issues in other eDNA based studies exist, for example Gillespie (2006) highlighted the necessity for rapid sample collection after defecation in primate studies this was to avoid contamination of the sample from other individuals or the environment. The use of nematode universal primers in the current study exacerbates this problem further given the ease at which they can amplify DNA from non-parasitic and therefore non-target species. In addition, parasitism has evolved independently in the Nematoda phylum many times in different clades, making the identification of conserved DNA sequences in parasitic groups that are absent in non-parasitic ones unlikely (Dorris et al., 1999).

However, although contamination by free-living nematode DNA is likely to be a recurring issue within the developed copro-diagnostic protocol it is not one that renders the protocol un-informative. The developed methodology has demonstrated a capability for detecting eDNA from parasitic and non-parasitic species and hence sequencing of the amplicons produced can quickly identify which positive results are from true infections. Moreover, PCR tends to amplify the more abundant sequences in a DNA extract (Blackwood et al., 2005). Hence, in faecal samples from a heavily infected host the eDNA signal in the faeces is likely to be stronger and outcompete any potential contaminant DNA from free-living nematodes (Blackwood et al., 2005). Bearing this in mind, parasite load in an infected individual is likely to affect the sensitivity of the copro-diagnostic assay in different ways to a low-level or trickle dose infection, making this an avenue for important further study.
In addition, the possibility to add an extra suite of primer testing after the initial PCR with the Nem27 primers also exists. Once more information on the common nematode parasites of amphibians is accrued then a two-step copro-diagnosis method could be used. This would entail a first step using the Nem27 primers, followed by a second step whereby primers for common families of amphibian infecting nematodes are run against faecal extracts that have shown amplification previously.

4.1.2 Cross-reactivity of the developed Nem27 primers on mite 18S rRNA sequences

Our developed Nem27 primers initially displayed good specificity to nematode 18S rRNA sequences alone. During the primer design process they were tested against a variety of different negative control faecal extracts, such as DNA from the faeces of various mice known to be uninfected as well as checked for cross-reactivity against tissue DNA from Platyhelmintes. Further to this, they were tested on a range of faecal DNA from captive amphibians at the University of Manchester and wild amphibians, continuing to demonstrate a specificity to solely amplify nematode 18S rRNA DNA, as demonstrated by sequencing. Nonetheless, when tested on the larger suite of faecal samples from ZSL London Zoo they exhibited cross-reactivity with mite DNA, as 40% of the samples that produced amplification were shown to be from the same mite 18S rRNA sequence.

Issues regarding a lack of developed primer specificity are very common (Blackwood et al., 2005) and this topic has been addressed in the context of the design of nematode universal primers before (Bhadury et al., 2011; Bhadury and Austen, 2010). Co-amplification of fungal species, particularly from the Basidiomycota and Ascomycota has been regularly observed in primers initially believed to be nematode specific and has led to the development of a new primer pair, specifically designed to avoid this fungal co-interference (Bhadury et al., 2006b; Bhadury and Austen, 2010). The authors postulated that close fungal-nematode associations in marine species, either on the nematodes cuticle or within the gut were facilitating such co-amplification of sequences (Bhadury et al., 2011). These results influenced the development of my Nem27 primers, as Basidiomycota and Ascomycota sequences were used in the design alignment and consequently no amplification of fungal DNA was seen.

Mites are a very common group of soil inhabiting organisms and so cross-reactivity of the Nem27 primers on their DNA presents a problem as they will be impossible to exclude from faecal samples. Moreover, the mite species that obtained the highest BLAST match, Sancassania berlesi is known to prefer microhabitats such as decaying leaf litter and manure (Abou El-Atta et al., 2014). Interestingly, during the dissection of one of the M. betsileo individuals an organism was found embedded in the intestinal wall with mite morphology. Mites are able to infect the intestinal and urinary tract of animals and they are known to be a common parasite of amphibians (Densmore and Green, 2007; Li et al., 2003). Therefore, whether this was a parasitising species or just present due to consumption by the amphibian is difficult to ascertain. However, it does demonstrate a
manner by which substantial quantities of mite DNA could arise in amphibian faeces, thus increasing the chances of co-amplification by the Nem27 primers.

The degenerate nature of the Nem27 primers could provide an explanation for their cross-reactivity. Degenerate bases substantially increase the number of potential sequences that a primer is capable of binding with. However, the inclusion of degenerate bases was a necessary trade-off for them to maintain a good breadth of detection sensitivity across the Nematoda phylum (Forney et al., 2004).

Problems of primer cross-reactivity on non-target DNA were not only observed in the developed Nem27 primers but also in nearly all the other primers taken from the literature. The NemUni-1 primers were found to amplify tissue DNA from Platyhelminthes and from the common Collembola (springtail) species, *S. pumilis* (Blancquaert and Mertens, 1977). In addition, the NemUni-2, NemUni-3 and the family and genera specific primers tested either cross-reacted with Platyhelminth tissue DNA or from faecal DNA from non-infected mice. Such findings prevented the use of these primers in the developed copro-diagnostic system. Similar issues with poor primer specificity have been reported in a number of papers exploring a wide range of different systems. McLain et al. (2009) demonstrated how primers designed for target of human infecting *Bacteroides* species in aquatic ecosystems also had a high efficiency at amplifying DNA from common fish infecting species. They highlighted how only one primer set of five stated in the literature exclusively amplified human *Bacteroides* sequences (McLain et al., 2009). Within human research the problem of poor primer specificity was identified to be so pervasive that it led to the formation of a quality assured primer database (Spandidos et al., 2010). PrimerBank is a free database of cross-checked primers designed for amplification of human and mouse DNA sequences that have been experimentally validated and had their PCR conditions made uniform (Spandidos et al., 2010).

Even though my designed Nem27 showed some cross-reactivity with mite DNA this problem was overcome by the optimisation of the PCR annealing temperature. The initial annealing temperature used with these primers was 54.1°C as recommended by the primer analysis software employed to help in their design. However, when higher annealing temperatures were explored up to the 61°C – 63°C range then mite DNA amplification was lost, whilst amplification of nematode DNA persisted. This is a common technique used to increase primer specificity (Spandidos et al., 2010) and reveals the potential for future studies to explore more optimisation of the PCR conditions in the copro-diagnostic protocol i.e. by experimenting with concentrations of starting faecal DNA, MgCl₂, and BSA.

4.1.3 Use of the 18S rRNA gene for species barcoding of nematodes

Sequenced amplicons generated using the Nem27 primers frequently returned very high matches with existing sequences in the GenBank database; however, these were often from nematodes of
differing families and genera. This range of results from some BLAST searches provided an unclear picture as to which nematode species’ sequence had been detected.

The top nematode matches from the ratsnake, *R. boulengeri* were predominantly from the free-living genus *Poikilolaimus* (Hong et al., 2005). Nonetheless, the fourth match which had an equivalent query cover and sequence identity belonged to the genus, *Krefftascaris* known to be common parasites of turtles and relatives of *Toxocara* nematodes (Tkach et al., 2010). The results for the crocodile lizard, *S. crocodilurus* were similar in that the top three matches belonged to bacterivorous genera (Abolafia and Pena-Santiago, 2007; Steel et al., 2012), whilst the fourth match was from the South American rodent parasite *Angiostrongylus costaricensis* (Rebello et al., 2012; Yong et al., 2015). The faecal amplification from the dart frog, *D. tinctorius*, also produced incongruent results. The top match was a sequence from the genus *Raillietnema*, a widely recognised group of herpetofauna infecting nematodes (Baker, 1985; Bursey et al., 2006; Espinoza-Jimenez et al., 2007). However, other matches with sequences from the genus *Rhigonema* are recognised millipede gut parasites from a relatively distant clade of nematodes (Malysheva et al., 2015).

These results underscore a potential issue with the use of the 18S rRNA for nematode barcoding and identification. Prosser et al. (2012) suggested that the 18S rRNA gene was appropriate for family-level identification of nematodes but not species barcoding which they found the mitochondrial COI gene to be better for. The 18S rRNA gene codes for the small ribosomal subunit a key evolutionarily conserved protein required for assembly of other proteins (Rodrigues da Silva et al., 2010). The selective pressure to retain the function of this ribosomal subunit means that some regions of the gene are very tightly conserved, showing little utilisable variation for interspecies identification (Prosser et al., 2013; Rodrigues da Silva et al., 2010). On the other hand, the 18S rRNA gene also contains some highly variable regions, such as the non-coding internal transcribed spacers 1 and 2 (ITS) which can be used to discriminate between and even within species (Floyd et al., 2002; Giangaspero et al., 2004). Therefore, it is possible that my Nem27 primers are not amplifying a region of the 18S rRNA gene that contains an appropriate barcoding region, such as ITS1 or 2. Thus, they repeatedly return top matches in GenBank from a host of different nematode families and genera. An improved set of primers would be able to amplify over a hypervariable region for barcoding but also still bind to highly conserved regions of the nematode 18s rRNA gene, to maintain their broad range of specificity across the phylum.

Another problem encountered when using the GenBank BLAST tool to identify 18S rRNA sequences was that results frequently returned multiple matches with sequences named ‘Uncultured Eukaryote clone’. These matches were omitted from the results in the present study because they provided no informative data on the taxonomy or identity of the matching sequence and therefore did not help in the identification of the query sequence. Nilsson et al. (2016) encountered similar issues when attempting to use BLAST for identification of fungal barcoding sequences, returning results dominated with sequences from unidentified species. In fact, a number of studies have found fault with the quality of sequence metadata in GenBank, highlighting
the prevalence of absent or poor taxonomic resolution provided with sequences, alongside a lack of country of origin and ecology data (Buhay, 2009; Nilsson et al., 2016). Furthermore, even if a taxonomic identification based on morphology is provided there is no way of guaranteeing its accuracy (Buhay, 2009; Shen et al., 2013). This is a particularly pertinent issue within phyla such as the Nematoda because they show great interspecific overlap of morphological characters, they vary in form depending on life cycle stage and have many as yet undescribed cryptic species (Buhay, 2009; Van Steenkiste et al., 2015).

Not only have problems in the sequence metadata been identified but also the quality of the sequences themselves. One paper, from 2001, identified that as many as half of all human mitochondrial DNA (mtDNA) sequences in sequence depositories contained errors (Forster, 2003), blaming poor editing of sequence data before submission to databases like GenBank (Benson et al., 1997). The incorporation of these errors led to the publication of erroneous phylogenetic networks which were later retracted, followed by remediation of the incorrect sequences (Forster, 2003; Harris, 2003). Studholme (2015) checked for sequencing errors within the many Streptomyces genomes uploaded to GenBank. This author checked genome completeness by searching for the DNA sequences of core house-keeping genes that all of the genomes should contain (Studholme, 2016). The results found that of the 653 available genomes only 63% contained all the sequences for these essential genes, again underscoring the prevalence of poor quality sequences in GenBank.

Moreover, multiple studies investigating the quality of GenBank sequences have concluded that there should be a requirement for additional key information, such as the original chromatogram from which the sequence was generated (Buhay, 2009; Forster, 2003; Harris, 2003; Nilsson et al., 2016). This would allow users and GenBank curators to quickly identify low quality sequences from messy trace data and allow them to omit poor data from their studies.

Evidently, the ramifications of such poor quality sequence data for the accuracy of published data are potentially great. GenBank administrators do some verification of submitted sequence quality by translating and checking common sequences, such as COI, for the presence of stop codons which should be absent (Benson et al., 1997; Buhay, 2009). However, even if easily detected errors such as these are found, they are not necessarily rectified and the sequences are still added to the database, flagged by the annotation ‘COI-like’ (Buhay, 2009).

Given the discussed issues regarding sequence and metadata quality within GenBank and the low level of intervention by GenBank administrators there are many proponents of other, more regulated databases (Buhay, 2009; Nilsson et al., 2016). The International Barcode of Life (iBOL) project, started in 2004, sets out to use a 650 bp region of the COI gene to barcode all animal life, whilst also using alternative barcodes for plants and fungi (Costa and Carvalho, 2010). The project’s database is now well established and is compiled of standardised DNA sequences that have come from museum and voucher specimens with thorough taxonomic identification (Buhay, 2009; Costa and Carvalho, 2010). The quality of such data is rigorously checked, permitting
effective comparison of sequences between species and clades for more accurate phylogenetic investigation (Buhay, 2009). A parallel and equally well verified sequence bank is the UNITE database for fungal ITS sequences (Nilsson et al., 2016). This database allows all users to curate and annotate sequences, highlighting those that appear to be poor quality and adding or amending the sequences that derive from poorly identified fungi (Nilsson et al., 2016). Hence, the database is flexible and easily updated, remaining a valuable resource to its users.

Taking this into account, to resolve the uncertainty of the identity of sequences amplified by the Nem27 primers, future studies could look at using other barcoding targets, such as the COI gene. This gene has already been used to design a multiplex PCR for a broad range of vertebrate-parasitising nematodes and the amplicons produced in this system were shown to produce valuable species barcodes (Prosser et al., 2013). If my copro-diagnostic protocol used these primers or newly developed nematode universal COI primers in conjunction with the Nem27 ones, then a more accurate picture of the identity of nematode DNA could be built up. The GenBank matches from both primer sets tested on a faecal DNA extract could be compared, either supporting each other or emphasising a need for further investigation. In addition, barcode sequences produced by nematode COI amplifying primers could be run through higher quality databases, such as that provided by iBOL, that consists of COI sequences uniquely (Costa and Carvalho, 2010). More confidence could be placed in matches obtained in this database due to the rigorous taxonomic identification of the source material from which sequences are derived (Nilsson et al., 2016).

4.2 What are the future applications and directions of the developed copro-diagnostic protocol?

The overarching goal of this study was to develop a copro-diagnostic protocol that could be used to help elucidate the role macroparasites are playing in amphibian declines and therefore help in efforts for their conservation. LAMP technology is now recognised as being one of the methods at the forefront of molecular diagnostic techniques amenable for use in the field, due to its use needing little expertise and minimal infrastructure (Biswal, 2016). These benefits allow LAMP assays to be taken out into remote locations to track the spread of a pathogen during an epidemic, uncover previously unknown latent reservoirs of a parasite or identify the emergence of a novel strain of crop pest (Biswal, 2016; Gandasegui et al., 2015; Kang et al., 2015).

Advantages of LAMP assays over conventional PCR are that the reaction can be carried out at a single temperature; hence all that is needed in the field is a heat block, whilst a positive result can be monitored by the presence of a colour changing fluorophore (Biswal, 2016; Gandasegui et al., 2015). Being able to detect parasites in situ also removes the necessity for freezing and transport of faecal samples, whilst also mitigating the problems sometimes associated with getting permits for collection of samples. Furthermore, many LAMP assays have been demonstrated to be more
resistant to the presence of PCR inhibitors than conventional or qPCR reactions and in these cases have also been proven to be more sensitive at detecting target DNA (Blomstrom et al., 2008; Xu et al., 2010). This is of particular importance to the current study given that when the copro-diagnostic protocol was tested on the faecal DNA extracts from ZSL London Zoo, four positive controls failed to amplify, indicating the carry-over of PCR inhibitors. Therefore, modification of the current protocol into a LAMP assay could not only improve the system for use in monitoring of amphibian parasites in situ but also more generally improve its sensitivity. Nonetheless, a positive LAMP assay result using my broad-spectrum Nem27 primers would not indicate which nematode species was present and so primers specific to a lower taxonomic rank would also have to be included for identification purposes.

Another area to which the developed copro-diagnostic protocol could be applied is in laboratory research. Infection experiments using mouse models to explore the immunology of a relevant human parasite often rely on culling and dissection to verify the number of parasites that have established an infection in an individual (Boes and Helwigh, 2000). If immunological changes along the course of an infection are being investigated then more mice are needed at the experiment’s onset for data to be meaningful at each time point investigated. However, the copro-diagnostic protocol currently developed could provide valuable information on whether an infection experiment had worked without the need for sacrificing. Moreover, if adapted to provide quantitative information on the amount of starting nematode DNA it could be used as a qPCR methodology and thereby provide insight into the parasite load in an experimentally infected individual. This could significantly reduce the amount of host organisms used in parasitological research and would not suffer from the aforementioned problems of amplification of free-living nematode DNA, given the controlled, soil-free environment of the host enclosure.

Interestingly, parasite eDNA signals were detected even from mouse models of nematode infections that do not release transmissible stages, such as *T. spiralis* (Ortega-Pierres et al., 2015). In this model system eDNA is likely detected due to the release of worms and sloughed cells in the faeces. These eDNA sources will be most prevalent at the stage of the *T. spiralis* life cycle when newly born larvae move through the intestine and penetrate the gut, migrating to striated muscle where they form nurse cell complexes (Ortega-Pierres et al., 2015). Given the protocol’s efficacy at detecting nematode infections in species that do not release eggs in the faeces, its employment in a laboratory setting could provide an easy method for validating an experimental infection in species like *T. spiralis*.

When the copro-diagnostic protocol was used with the PlatUni primers on faecal DNA from mice infected with *S. mansoni* it also provided insight as to what stage in the parasite’s life cycle had been reached. No eDNA was detected until day 43 post infection, whilst time points from as early as 28 days post infection were checked. Such results agree with the recognised life cycle for this species, which expects adult flukes to have reached and established in the circulatory system and begun to lay eggs by 6 – 8 weeks post infection (Nascimento-Carvalho and Moreno-Carvalho, 2005). This tracking, through detection of eDNA signals could allow the copro-diagnostic protocol
to be used to monitor mice experimentally infected with *S. mansoni*, elucidating what stage in the life cycle had been reached at a given time point.

After initial experimentation with the PlatUni primers they were not investigated further, due to problems with multiple amplification bands on gels and an inability to be accurately sequenced. Traces produced from amplification of these primers on faecal samples from ZSL London Zoo were normally low quality with a full sequence fragment length significantly smaller than what was expected. Nevertheless, some high quality sequences of between 50 – 400 bp were identified but when these were compared to sequences in GenBank they returned no matches. It was speculated that this could be due to non-specific primer binding and amplification of sequences that are completely unknown. Such phenomena have been reported by other researchers. For example, Illumina sequencing on archaeological bone extracts frequently returns sequences of which over 50% cannot be assigned to any known sequence in GenBank. This is thought to arise because as you move away from sampling of well characterised environments, the portion of microbes that are completely unknown and have no sequence data increases greatly (Prof Terry Brown, pers. comm., September 7, 2016). Therefore, to get an accurate idea of whether the PlatUni primers were detecting Platyhelminth DNA, future study could explore the use of other primers that are specific for lower taxonomic ranks e.g. for cestode parasites, to either support or contradict the data from the PlatUni primers.

The novel design of primers designed to target trematodes and cestodes was not attempted in this study due to the huge diversity within and between such groups, making it unlikely to be feasible over the project’s limited duration (Van Steenkiste et al., 2015). These parasitic groups frequently have complex life cycles involving multiple hosts (Densmore and Green, 2007; McKenzie, 2007). Due to this, they are less commonly observed parasitising captive amphibians and it is therefore more challenging to test out any developed primers because a good amphibian-parasite model system is lacking (Densmore and Green, 2007; Wright and Whitaker, 2001). Future investigation could attempt to identify an appropriate model of Platyhelminth parasitism in amphibians that can be maintained in the lab. Such a model could be used to develop and test *de novo* Platyhelminth primers, checking their effectiveness in my copro-diagnostic protocol.

### 4.3 Conclusion

The present study set out to determine whether eDNA released from amphibian infecting helminths could be detected in the faeces of their hosts and thereby provide important information for the conservation of this group. I have shown that not only is this possible but also that my copro-diagnostic methodology works in other host groups, such as reptiles and mice. Furthermore, the progress realised through this study lays down the groundwork for my methodology to be modified into other applied protocols. The future development of a LAMP assay that could be used to detect
amphibian infecting nematodes in the field or creation of a novel qPCR to ascertain worm burden in experimentally infected hosts, are both possible.

Whilst much progress was made into the formation and optimisation of an effective copro-diagnostic system, so too was the exposition of many challenges that needed to be overcome. The Nem27 primers were designed so as to detect DNA from any nematode and therefore frequently detected contaminant DNA from free-living species common in soil. They were also demonstrated to cross-react with DNA from mites and were therefore not as nematode specific as originally thought. Moreover, it was difficult to establish whether the region of the 18S rRNA gene amplified by the Nem27 primers was as an effective barcoding sequence, with some BLAST results indicating that it may not be.

Nonetheless, many of these challenges were either resolvable or did not eliminate the copro-diagnostic protocol’s capability for providing meaningful information on the infection status of a host. Sequencing of amplicons could determine the species from which the nematode DNA was derived, whilst optimising the annealing temperature of the PCR could prevent cross-reactivity on non-target DNA. In addition, the challenges recognised present avenues for exploration in future studies, to improve and develop the methodology. Other primer sets, specific to common parasitic taxa, could be designed to target alternative barcoding genes, such as COI. These could be used alongside the Nem27 primers to create a two-step protocol in which faecal DNA detected as having nematode DNA in a first round of testing could then have the DNA identified to a lower taxonomic level by a secondary suite of primers. Data from sequencing of both amplicons, from primer sets targeting different genes, could be used in conjunction to provide accurate identification of the infective species.

It is hoped that this preliminary work is advanced further, to hone a non-invasive copro-diagnostic protocol applicable to research in the field of wild host-parasite systems. The scientific understanding of such parasitological systems is still very much in its infancy, with little known about the dynamics of parasite populations, how this affects their hosts and how parasites swap between hosts in different species, families and also higher taxonomic divisions (Blaustein et al., 2012). More importantly, helminths are now recognised as playing an important role in the population crashes of amphibians observed globally (Kiesecker, 2011; Lips and Donnelly, 2005; Tinsley, 1995). Hence, the work developed here could be used to shed light on key parasite species responsible for exacerbation of such declines as well as highlight the negative synergies formed by helminth infection in the context of Bd, pollution and climate change.
5. Reference List


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