NOVEL APPROACHES TO THE CONTROL OF CYATHOSTOMINS IN EQUIDS

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BY

LAURA ELIZABETH PEACHEY

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ABSTRACT

Novel approaches to the control of cyathostomins in equids

Laura Elizabeth Peachey

Cyathostomins, are clade 5 gastrointestinal (GI) nematodes infecting equids. They are associated with a range of pathologies, the most serious of which is larval cyathostominosis, a protein losing enteropathy with a 50 % mortality rate. Importantly, they are the most abundant GI nematode of equids in the developed world and, as they do not induce protective immunity, equids remain at risk of infection throughout their lives. The effective control of cyathostomins is currently threatened by the development of anthelmintic resistance (AR) to all three major classes of anthelmintic licenced for use in equids. Of primary concern is the emerging resistance to the macrocyclic lactones (MLs), which are the mainstay of cyathostomin control. It is therefore important that novel options for controlling cyathostomins are explored. In this study *in vitro* tests were used to explore a range of options for developing novel treatments against AR cyathostomins and improving the efficacy of the widely used ML, ivermectin (IVM).

First the potential use of ethnoveterinary medicines for treatment of cyathostomins in equids in Ethiopia and the UK was explored (Chapters 3, 4 and 5). A participatory rural appraisal was performed in the Oromia region of Ethiopia to identify plants currently used as anthelmintics in equids and other livestock. A total of 37 species of plant were identified, data on dosing and side effects were also recorded. These data were triangulated with a literature review to identify five plants for *in vitro* screening for anthelmintic activity against cyathostomins. A literature review was used to identify plants for *in vitro* screening in the UK. A total of 138 publications, reporting the anthelmintic activity of plant extracts against nematodes, were found. The data collected was used to design a ranking system to provide a shortlist of five plant candidates. Shortlisted plants were collected, dried and chemically extracted to give crude extracts, which were screened against cyathostomins in the egg hatch test (EHT) and larval migration inhibition test (LMIT). A total of 7/9 plants screened in this way showed anthelmintic activity in either the EHT and/or the LMIT with median effective concentrations (EC-50s) in the range 0.18-8.90 mg/ml. For some of the most efficacious extracts there were reports of *in vivo* efficacy against GI nematodes of other veterinary parasitic nematodes, making these good candidates for future *in vivo* trials. One of the plants shortlisted for the UK, for which there was extensive evidence of anthelmintic activity in the literature, was the cysteine proteinase (CP) containing *Carica papaya*. Extract from this species, papaya latex supernatant (PLS), could not be processed and tested using the same protocols as the other plant species, as the active compound is an enzyme. The EHT and LMIT were optimised for use with PLS and its efficacy evaluated in chapter 5. It was found that PLS had a potent anthelmintic effect against cyathostomins in the EHT, which was attributable to the action of CPs, with EC-50 values in repeats ranging between 0.12-0.22 µM. PLS also showed efficacy in the LMIT, although this appeared due to another active compound. To the authors knowledge this is the first report of efficacy of PLS against the free living stages of any parasitic nematode, which may indicate that cyathostomins are particularly susceptible to CPs.

In chapter 6 the role of P-glycoproteins (P-gps) in IVM resistance in cyathostomins was investigated, with the more specific aim of assessing whether P-gp inhibitors could potentially be used in combination with IVM to improve its efficacy against AR parasites. The transcription of *pgp-9*, which is putatively associated with IVM resistance in *Teladorsagia*

circumcincta and *Haemonchus contortus*, was measured after exposure to IVM in in ML resistant (AR) versus anthelmintic naïve (AN) cyathostomin third stage larvae (L3). *Pgp-9* expression was significantly increased after incubation with IVM in the AR, but not the AN, parasite population. Two *in vitro* tests, the LMIT and the larval development test (LDT), were used to assess the effect of a range of P-gp inhibitors (ketoconazole, pluronic 85 and ivermectin aglycone) on IVM efficacy in ML resistant (AR) versus anthelmintic naïve (AN) cyathostomins. In the LMIT ketoconazole and ivermectin aglycone conferred a significant increase in IVM efficacy in the AR parasites, but had less effect on the AN parasites. Pluronic 85 also had a profound effect on IVM efficacy which depended on IVM concentration, however there was no differential effect dependent on parasite population. In the LDT, ketoconazole and pluronic 85 both significantly increased IVM efficacy in both AR and AN parasites. These results suggest that there is a lifecycle stage-specific association of P-gps with AR cyathostomins, and that P-gp inhibitors could potentially be used to improve IVM efficacy in AR cyathostomin populations.

In summary in this study has identified a range of potential novel treatments for cyathostomins. The data also suggest a role for P-gps in the emerging resistance to MLs, which could potentially be exploited to improve ML efficacy in AR cyathostomin populations. Importantly, this work will underpin the financial and ethical justification for future *in vivo* trials on the extracts/compounds tested here.

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LIST OF ABBREVIATIONS

ABC- transporter	ATP-binding cassette transporter
AN	accession number
ANOVA	analysis of variance
AR	anthelmintic resistance
AWMT	adult worm motility test
BAPNA	benzoyl-arginyl-P-nitroanilide
BCRP	breast cancer resistance protein
bp	base pair
BSEP	bile salt exporter protein
BZ	benzimidazole
cDNA	complementary DNA
CDD	conserved domain database
CI	confidence interval
CP	cysteine proteinase
DA	local development agent
DEPC	diethylpyrocarbonate
dH_2O	distilled water
DNA	deoxyribonucleic acid
DS	donkey sanctuary
DL4	developing fourth stage larvae
dyf	dye filling gene
E64	L-trans-epoxysuccinyl-L-leucylamide-(4-guanidine)
EC-50	50% maximal effective concentration
EC-99	99% maximal effective concentration
EHT	egg hatch test
ELISA	enzyme-linked immunosorbent assay
EL3	early third stage larvae
ERP	egg reappearance period
FEC	faecal egg count
FECR	faecal egg count reduction
FCERT	faecal egg count reduction test

g	gram
g	acceleration of gravity
GABA	*gamma*-aminobutyric acid
GluClR	glutamate gated chloride channel receptor
GI	gastrointestinal
h	hour/s
HC110-R	latrophilic-like receptor
H_2O	water
IL3	infective third stage larvae
IVM	ivermectin
IVM-AG	ivermectin alycone
keto	ketoconazole
kg	kilogram
L	litre
L1	first stage larvae
L2	second stage larvae
L3	third stage larvae
L4	fourth stage larvae
LDT	larval development test
LL3	late third stage larvae
LMIT	larval migration inhibition test
M3-Gly	glycine residue in the third transmembrane domain of the GluClR
min	minute
ML	macrocyclic lactone
M	molar
MDR-1	multidrug resistance protein 1
mg	milligrams
ml	millilitre
mM	millimolar
mm	millimetre
MPR	mean percentage reduction in egg hatch/larval migration compared to control
MOX	moxidectin
MPTL-1	nematode nAchR

mRNA	message RNA
MRP	membrane resistance protein
nAchR	nicotinic acetylcholine receptor
NaCl	sodium chloride
NCBI	National Centre for Biotechnology Information
nM	nanomolar
OR	odds ratio
P85	pluronic 85
PCR	polymerase chain reaction
PD	pharmacodynamic
P-gp	p-glycoprotein
pH	power of hydrogen
PK	pharmacokinetic
PLS	papaya latex supernatant
ppm	parts per million
PRA	participatory rural appraisal
PROBIT	unit of probability
PVPP	polyvinylpolypyrrolidine
PYR	pyrantel
RNA	ribonucleic acid
rRNA	ribosomal RNA
SB	stem bromelain
SCO-1	cytochrome c oxidase assembly protein
SNP	single nucleotide polymorphism
THP	tetrahydropyrimidine
Th2	t helper type 2 immune response
tRNA	transfer RNA
µl	microliter
µM	micromolar
µm	micrometre
v/v	volume/volume
WAAVP	World Association for the Advancement of Veterinary Parasitology
ºC	degrees centigrade
18S	18 Svedberg

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1. INTRODUCTION

1.1. Cyathostominae

Globally equids are susceptible to a range of gastrointestinal (GI) helminths, these include the ascarid *Parascaris equorum*, tapeworms such as *Anoplocephola perfoliata*, the stomach worms *Habronema spp.* and *Trichostrongylus axei*, the threadworm *Strongloides westeri* and the strongyles (Bucknell et al., 1995; Cirak et al., 1996; Gawor, 1995; Krecek et al., 1989; Ogbourne, 1976; Reinemeyer et al., 1984; Torbert et al., 1986). The strongyles are the most prevalent and represent a complex group which are highly significant to the health and welfare of equids; they are nematodes of the order Strongylida, belonging to the Strongylidae family. They are subdivided into Strongylinea, comprising *Strongylus vulgaris, Strongylus edentatus, Strongylus equinus* and *Strongylus asinsi,* (collectively known as the large strongyles) and cyathostominae (known as the small strongyles or cyathostomins).

Naturally infected, untreated equids are usually co-infected with multiple species of strongyle nematode, although in populations receiving regular anthelmintic treatments the large strongyles are rare (Boxell et al., 2004; Herd and Coles, 1995; Kaplan, 2002). In many developed countries cyathostomins are the most prevalent, at almost 100 %, and are the focus of control regimens (Lyons et al., 1999). Cyathostomins themselves are a complex group, comprised of 52 different species belonging to 13 genera (Lichtenfels et al., 2002; Lichtenfels et al., 1998; Matthee et al., 2002a). Infections are complex mixes of species and may differ geographically, however in horses 90-99 % of infections usually consist of 5-10 common species: *Cylicostephanus longibursatus, Cyathostomum catinatum, Cylicostephanus goldi, Cylicocyclus nassatus, Cyclicocyclus brevicapsulatis, Cyathostomum coronatum, Cylicostephanus calicatus, Cyclicostephanus, minutus, Cyclicocyclus leptostomus, Cyathostomum pateratum, Cylicocyclus insigne, Cylicocyclus radiatus* and *Cylicocyclus*

ashworthi (Bucknell et al., 1995; Gawor, 1995; Love and Duncan, 1992; Ogbourne, 1976; Reinemeyer et al., 1984; Traversa et al., 2010). Only a few studies have determined species mix in donkeys and zebras, but the evidence suggests that there are some differences in relative abundance to those found infecting horses, in particular *Cyathostomum tetracanthum* is commonly found in donkeys (Eysker and Pandey, 1989; Getachew et al., 2010; Kharchenko et al., 2009; Krecek et al., 1994). In terms of species, the composition of co-infections has been shown to be relatively stable over decades within equid populations (Chapman et al., 2002a). There is some evidence of infection site specificity for cyathostomins; it has been reported that *C. longibursatus, C. goldi* and *C. insigne* are more prevalent in the dorsal colon, with *C. catinatum, C. nassatus* and *C. minutus* most abundant in the ventral colon and *C. calicatus* and *C. coronatum* showing a predilection for the caecum (Bucknell et al., 1995; Collobert-Laugier et al., 2002b; Gawor, 1995; Ogbourne, 1976). The large number of cyathostomin species present in an infected host or population of equids is an important consideration when studying their biology and epidemiology; much of what is currently known is based on mixed co-infections, rather than on an individual species basis. Historically the only way of accurately differentiating between species infecting a host was to identify adults at post-mortem (Lichtenfels et al., 2002; Lichtenfels et al., 2008), improvement to these methods has since allowed the culture of eggs from faeces to larval stages, followed by morphological identification (Kornas et al., 2009), however these methods are laborious and this has largely prohibited studies on the effect of different species in co-infections. Over the last 15 years molecular tools have been developed which can quickly differentiate between a range of cyathostomins species within samples of eggs collected from faeces. These developments include a PCR-enzyme-linked immunosorbent assay (PCR-ELISA) (Hodgkinson et al., 2005; Hodgkinson et al., 2003; Hodgkinson et al., 2001) and a reverse line blot assay (Traversa et al.,

2007a) both of which apply species specific probes to intergenic spacer DNA fragments and can identify a range of six and 13 species, respectively.

1.1.1. Life cycle of cyathostomins

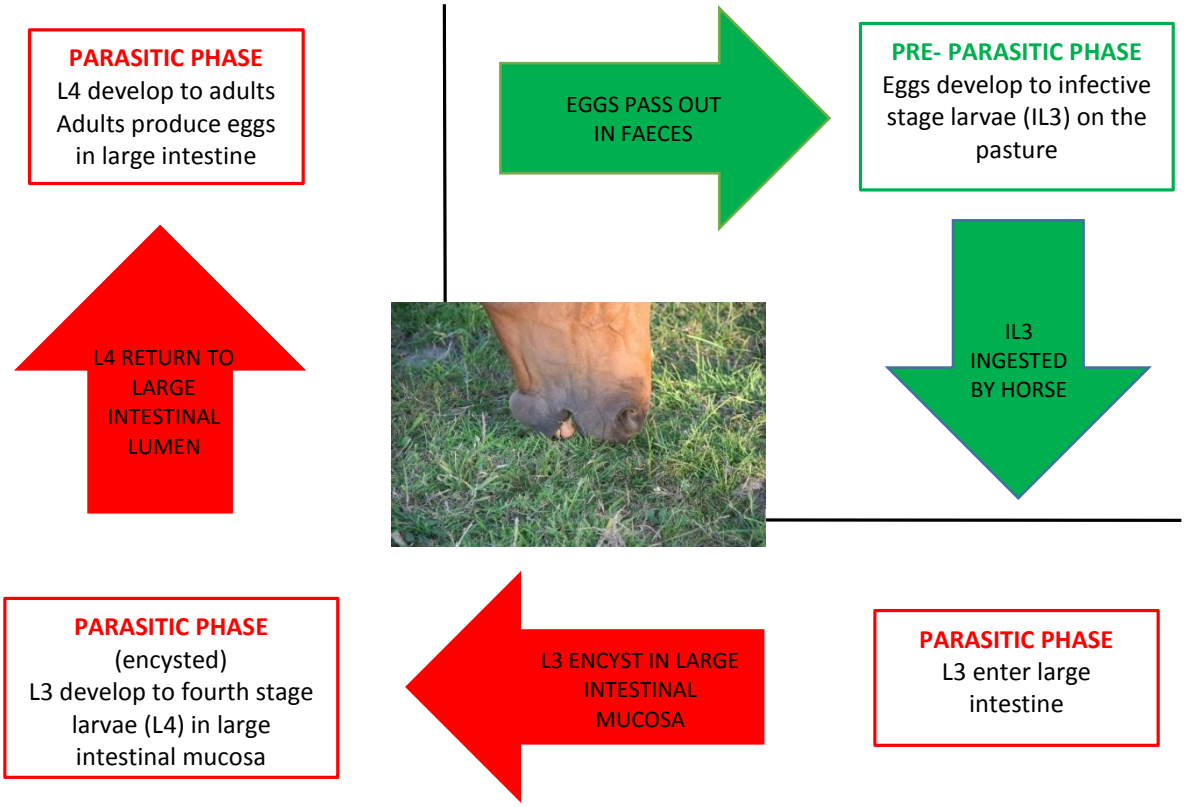

Figure 1.1. Diagrammatic representation of the direct life-cycle of cyathostomins. The life cycle can be halted for up to three years at the encysted L3 stage.

Cyathostomins have a direct life cycle, see Figure 1.1. Eggs laid by adult females in the large intestine are passed out in faeces, in which they hatch to first stage larvae (L1). Hatching requires moisture and a minimum temperature of 7.2 ºC (Ogbourne, 1972). The L1 feed on bacteria in the faecal pat and develop into second stage larvae (L2), which then moult to the infective third stage larvae (IL3). The L2 and L3 have a cuticle to help protect them against

desiccation or freezing. The time for development of IL3 from eggs depends on climatic conditions; the optimum temperatures for maturation have been shown to range from 20-33 ºC (Mfitilodze and Hutchinson, 1987). Development can take between 3-24 days at these temperatures, but can be considerably affected by desiccation (Mfitilodze and Hutchinson, 1987, 1988; Ogbourne, 1972). The L3 are not thought to move more than 15 cm horizontally, or climb higher than 10 cm from the ground on herbage (English, 1979), and hence the main mechanism of dispersal over the pasture is through rainfall (Hutchinson et al., 1989; Ogbourne, 1972). The IL3 are then ingested by the host where they are immediately ex-sheathed in the stomach and small intestine (Poynter, 1954), before passing to the caecum and large colon where they encyst in the large intestinal mucosa or sub-mucosa (Urquhart et al., 1996). The L3 undergo several stages of development within the mucosa from early L3 (EL3), to late L3 (LL3), to developing L4 (DL4) and then to fourth larval stages (L4) (Chapman et al., 1999). This process is reported to take a minimum of 1-2 months (Love and Duncan, 1992). L3 can also undergo a period of arrested development where they are thought to persist in host tissues for up to three years (Gibson, 1953). After development to the L4 stage is complete, larvae excyst and return to the gut lumen where they moult to the L5 stage and become adults. The pre-patent period of cyathostomin infections has been reported to be between 38-83 days (Love and Duncan, 1992).

The inhibition of EL3 is an important factor in the epidemiology and pathogenicity of cyathostomin infections. There are several factors thought to be involved in this process, although the exact mechanisms are not fully understood. The proportion of inhibited larvae has been shown to be higher in young horses suggesting a link with immunity (Chapman et al., 2001; Klei and Chapman, 1999; Love and Duncan, 1992). In temperate regions such as the UK, inhibited EL3 have also been shown to reach peak intensities in the winter when conditions

are unfavourable for egg development on the pasture, with resumption of larval development in spring (Chapman et al., 2001; Eysker et al., 1990; Ogbourne, 1975). This resumption of development plays a role in pathogenicity, as when this occurs simultaneously it can lead to a mass emergence of larvae from the mucosa causing serious disease (Giles et al., 1985; Love et al., 1999; Mair, 1994; Reilly et al., 1993). Inhibited EL3 are also difficult to control as they are refractory to the majority of available anthelmintics, see Section *1.1.6.2.*

1.1.2. Epidemiology and transmission of cyathostomins

The transmission of cyathostomins is dependent on climatic conditions and therefore follows a seasonal pattern, which is dependent on the regional conditions (Nielsen et al., 2007). In northern temperate regions, faecal egg outputs peak during the warmer spring and early summer months, the warm conditions favour rapid development and hence peak pasture infectivity occurs during the summer and early autumn (Herd and Coles, 1995; Kornas et al., 2010; Ramsey et al., 2004; Reinemeyer et al., 1986). Inhibition of EL3 stages occurs later in the autumn and explains the reduction in faecal egg output which is observed during this period, despite high pasture infectivity (Schankova et al., 2014). During the colder winter months egg hatch and development is slowed or inhibited, and hence transmission is reduced further (Ogbourne, 1972). There is evidence that both eggs and IL3 can overwinter at freezing temperatures, although the number depends on the specific conditions, with both surviving long freezing periods better than repeated freeze thawing (Ogbourne, 1972; Polley, 1986). With warmer spring weather eggs start to develop, and inhibited EL3 develop to adulthood within the host, thus leading to an initial rise in transmission and faecal egg output in spring. In sub-tropical and tropical climates the cycle is altered somewhat as the dry hot conditions during the summer months cause desiccation of larval stages and do not favour dispersal of IL3

on pasture (Mfitilodze and Hutchinson, 1988). Hence peak transmission and faecal egg output occurs during the warm, wet winter months, or wet seasons in tropical regions (Chapman et al., 2001; Courtney, 1999; Getachew et al., 2008). Understanding the epidemiology of cyathostomins under different climatic conditions is important when considering optimal control regimens, which interrupt parasite transmission when it is at its peak and prevent or remove inhibited EL3. A recent study has created a model for the effect of different climatic conditions on the development and survival of L3 on pasture, and will aid in future prediction of cyathostomin transmission under different conditions (Leathwick et al., 2015). There is also evidence that age of the host and history of previous exposure have an effect on egg output, with young stock having a greater egg output; this must be taken into account when considering herd management regimens (Love and Duncan, 1992).

1.1.3. Pathogenicity of cyathostomin infection

Cyathostomin infection is associated with a range of clinical syndromes, which can be life-threatening, however some individual equids have high levels of cyathostomin infection and do not appear to suffer adverse effects; this tolerance may be related to the host response to infection (Love et al., 1999). The mass emergence of inhibited larvae from the large intestinal mucosa can cause a serious protein-losing enteropathy, termed larval cyathostominosis (Love et al., 1999). This most commonly occurs in later winter and early spring in temperate regions and in younger animals, however the trigger factors for mass emergence are poorly understood. Cases typically suffer from diarrhoea, pyrexia, weight loss and subcutaneous oedema, and the prognosis is poor, with a 50% reported mortality rate (Giles et al., 1985; Mair, 1994; Reilly et al., 1993; Van Loon et al., 1995). Reports of larval cyathostominosis in equids other than horses are rare; a recent case study has confirmed that this condition also occurs in donkeys (Oryan et

al., 2015). Weight loss and poor weight gain has also been associated with cyathostomin infections in both horses and donkeys (Matthee et al., 2002b; Murphy and Love, 1997; Yoseph et al., 2005), with an improvement noted upon anthelmintic treatment. There are several epidemiological studies linking high cyathostomin burdens with certain types of colic and one clinical description of caeco-colic intussusception in a case of larval cyathostominosis (Archer and Proudman, 2006; Mair et al., 2000; Murphy and Love, 1997; Uhlinger, 1990).

1.1.4. Immunity to cyathostomin infection

There are consistent reports of lower faecal egg output in adult equids compared with foals and yearlings, which is suggestive of the development of a degree of acquired immunity to infection (Chapman et al., 2003a; Kornas et al., 2010; Love and Duncan, 1992; Sheferaw and Alemu, 2015). Numbers of arrested larval stages also vary according to age, with younger horses having higher numbers in general (Klei and Chapman, 1999; Love and Duncan, 1992). One study has also shown that numbers of EL3 are increased in young animals in their second exposure to cyathostomins *versus* their first, suggesting that a degree of immunity to infection can actually promote arrested larval development in the first instance (Chapman et al., 2002b). Cyathostomin populations exhibit a classic negative binomial distribution within a herd of co-grazing adult horses, suggesting that the majority of animals develop a reasonably effective acquired response to infection, whereas a minority remain consistently highly susceptible (Lester et al., 2013b; Relf et al., 2013). Identifying the differences between these groups could be key to identifying what constitutes an effective immune response to infection, however there have been no significant advances in this field. Studies to date have examined the local inflammatory response to mucosal larval stages and found significant positive association of mast cells, eosinophils and Th2 cytokine responses with infection levels (Collobert-Laugier et

al., 2002a; Davidson et al., 2005; du Toit et al., 2007; Pickles et al., 2010). In a quest to identify suitable antigens for a diagnostic ELISA for mucosal larval stages, a significant IgG (T) antibody response to infection has also been identified (Dowdall et al., 2004).

1.1.5. Diagnosis of cyathostomin infection

The diagnosis of patent cyathostomin infection is by faecal egg count (FEC). This technique has been used for diagnosing GI nematode species for decades and is recommended by the World Association for the Advancement of Veterinary Parasitology (WAAVP) for measurement of the efficacy of anthelmintic drugs (Coles et al., 1992). A number of different FEC methods exist, which vary in their sensitivity (Lester and Matthews, 2014), but all of which are underpinned by faecal flotation. Essentially, faeces are mixed with saturated sodium chloride, within which eggs will float to the surface and can be recovered for microscopic enumeration. In populations of equids with low levels of infection, highly sensitive methods, such as centrifugal flotation, are preferred (Bello and Allen, 2009). There are numerous drawbacks to the use of FEC alone as a measure of cyathostomin infections. Firstly, as large strongyle and cyathostomin eggs are identical, larval culture is needed to differentiate between them (Lichtenfels et al., 2008). In addition it has been shown that FEC does not correlate well with adult worm burden in the gut (Nielsen et al., 2010), and it does not determine the number of inhibited or developing larval stages within individual hosts. There are also many causes of variation in the FEC that should be taken into consideration when interpreting samples. For example, there are variations in the number of eggs being shed over time which may be independent of the parasite load, such as the immune status of the animal (Warnick, 1992). In addition there has been shown to be an uneven distribution of eggs within the faecal pat which means that the sampling method can greatly affect the outcome of the FEC (Lester et al., 2012).

There is considered to be a need for standardisation of FEC techniques and improved training across the industry (Lester and Matthews, 2014).

New and improved diagnostic tools are required for determining the burden of cyathostomin infection, particularly in relation to the pathogenic developing mucosal stages. To date, it has only been possible to determine levels of mucosal stages at post-mortem using various techniques such as transmucosal illumination, whereby mucosa is stretched over a light source and viewed with a dissecting microscope; and peptic digestion of the mucosa to allow the recovery of larvae for enumeration (Chapman et al., 1999). However, in recent years there has been considerable progress in the development of an ELISA to measure mucosal larval burden. A 25kDa antigen complex has been identified which leads to specific IgG (T) responses that correlate with larval burden (Dowdall et al., 2004; McWilliam et al., 2010). An ELISA of this sort will greatly aid the diagnosis of larval cyathostominosis cases, which often have negative FEC (Giles et al., 1985).

1.1.6. Control of cyathostomins

Control of cyathostomins is centred on effective environmental control coupled with anthelmintic treatments.

1.1.6.1. Environmental control of cyathostomins

Pasture management is key in controlling transmission of cyathostomins between equids, and thus preventing the successful completion of the cyathostomin life cycle within the host. The removal of faecal pats from the pasture before eggs hatch and develop into IL3 is the primary

mechanism of environmental control. It has been reported that twice-weekly faecal removal is an effective frequency to significantly reduce transmission and control cyathostomin infections (Proudman and Matthews, 2000). Different methods of faecal removal are employed such as manual on small holdings, and automatic suction machinery on larger establishments, and these have been shown to be equally effective (Corbett et al., 2014). Other complementary practices include reducing stocking densities, resting heavily grazed pastures, harrowing pastures in dry conditions to expose IL3 to desiccation and co-grazing/rotating pasture with ruminants (Lyons et al., 1999).

1.1.6.2. Anthelmintic treatments licensed to treat cyathostomins

Since their introduction in the 1950s anthelmintic drugs have provided a major means of cyathostomin control, especially in large herds where environmental control is practically challenging. Anthelmintics which are currently licensed for use in horses fall into three categories, the benzimidazoles (BZs), the tetrahydropyrimidines (THPs) e.g. pyrantel (PYR), and macrocyclic lactones (MLs), such as ivermectin (IVM) and moxidectin (MOX). The BZs were introduced in the 1960s as the first nematocidal anthelmintics to show broad-spectrum efficacy and low toxicity (Baker and Douglas, 1962; Turk et al., 1962). The advent of THPs followed in the 1970s (Lyons et al., 1974; Slocombe and Smart, 1975). IVM was introduced in the 1980s (Campbell et al., 1983; Slocombe and Cote, 1984), and more recently MOX has been developed (Lyons et al., 1992). The BZs work as microtubule inhibitors; THPs function as agonists of nicotinic acetylcholine receptors (nAChR) in nematode muscle, causing paralysis; and MLs are glutamate-gated chloride channel (GluClR) and gamma-aminobutyric (GABA) receptor potentiators which results in paralysis of pharyngeal pumping (Martin, 1997).

Along with a unique mode of action each class of drug differs in its efficacy against the different parasitic stages of cyathostomins. The BZs were initially found to be between 92.5-100 % efficacious against adult cyathostomins (Bello et al., 1973; Bennett, 1973; Cairns and Holmden, 1977; Colglazier et al., 1977; Drudge et al., 1974, 1975; Duncan and Reid, 1978; Kates et al., 1975; Nawalinski and Theodorides, 1976; Neave and Callear, 1973; Walker and Knight, 1972), however lower efficacies against luminal L4, of between 47-100 %, have been observed (Colglazier et al., 1977; Duncan and Reid, 1978). The efficacy of a single dose of 5 mg/kg fenbendazole against encysted larval stages is poor, however a five day course of fenbendazole at 7.5 mg/kg was shown to reduce mucosal LL3 and L4 stages by 99.4 % and EL3 by 91.5 % (Duncan et al., 1998). The faecal egg reappearance period (ERP) was initially reported to be two to four weeks for BZs (Lumsden et al., 1989). The THPs, such as PYR, have been shown to be efficacious against adult and L4 luminal stages, although they have little effect against encysted larval stages (Lyons et al., 1974). When first introduced PYR led to FEC reductions of between 99-100 % (Boersema et al., 1995; Slocombe and Smart, 1975), with a faecal ERP in the region of five weeks (Boersema et al., 1995; Lumsden et al., 1989; Piche et al., 1991). The ML IVM was marketed as having a longer ERP than either the BZs or THPs, of around eight to 13 weeks (Demeulenaere et al., 1997; Piche et al., 1991), and high efficacy against luminal adult and L4 stages, 99 % and 98 % respectively (Xiao et al., 1994). One study has reported an efficacy of 76.8 % against EL3 for IVM (Love et al., 1995), however the majority of studies showed little effect on encysted larval stages (Demeulenaere et al., 1997; Eysker et al., 1992; Xiao et al., 1994). The related MOX, was found to have an even longer ERP of 22-24 weeks, as well as a high efficacy against luminal adult and L4 stages (Demeulenaere et al., 1997; Xiao et al., 1994). Initial studies with MOX, where post-mortem was performed two weeks post-treatment, showed it to have a moderate efficacy against encysted LL3 and L4 stages (62.6-79.1 %), but no effect on EL3 hypobiosed stages (Bauer et

al., 1998; Xiao et al., 1994). More recent studies, where post-mortem was performed eight weeks post treatment, have shown that MOX actually has > 90 % efficacy against all encysted larval stages (Bairden et al., 2001; Bairden et al., 2006).

Traditionally anthelmintics were used on a regular basis at intervals defined by their ERPs, however due to the development of drug resistance, increasing emphasis has been placed on using selective treatment regimens targeting horses with high FECs (Nielsen et al., 2014a). This method has been shown to reduce treatment to 18-50 % of horses within a herd, which not only reduces selection pressure for anthelmintic resistance on cyathostomins, but has been shown to have economic benefits (Lester et al., 2013a; Nielsen et al., 2014a; Salle et al., 2015). These regimens are not always taken up by yards and studs, indeed the evidence suggests that many equine establishments do not delay treatment in any animal beyond the defined ERP (Relf et al., 2013; Stratford et al., 2014a). In some countries, such as Denmark, anthelmintics have been made prescription-only medications requiring a veterinary surgeon to advise treatment. This has been shown to increase surveillance for strongyles, and therefore should be considered for other countries where resistance is an issue (Nielsen et al., 2006). One issue with targeted treatment regimens is that they have not been proven to be effective in groups of foals or young horses. Additionally there have been reports of the re-emergence of large strongyle infections on yards where strict targeted regimens are in place (Nielsen et al., 2012).

1.2. Current threats to the control of cyathostomins

The major threat to controlling cyathostomins infection in equids in developed countries is anthelmintic resistance (AR), whilst in some developing countries, a lack of access to effective anthelmintic treatments also constitutes a substantial obstacle to their effective control.

1.2.1. Anthelmintic resistance in cyathostomins

Anthelmintic resistance is recognised as a global problem affecting many hosts and nematode species, and cyathostomins are no exception. AR in cyathostomins has significant impacts for future control regimens, as currently no novel anthelmintics are likely to be licensed in equids in the short-to-medium term. Concerns over AR have led to the recommendation of surveillance based treatment regimens and improved environmental management in order to reduce the frequency of anthelmintic administration and hence selection for resistance.

1.2.1.1. Detection of anthelmintic resistance in cyathostomins

Detection of AR is dependent on having accurate and sensitive diagnostics for cyathostomin infection but, as discussed above in Section *1.1.5.*, this poses a significant challenge for cyathostomins. The gold standard for the measurement of efficacy of any anthelmintic are critical tests, which measure the actual worm burden after treatment at post-mortem (Bauer et al., 1998; Drudge et al., 1975; Eysker et al., 1992), however this is not a practical approach to routinely monitor for AR in the field. The faecal egg count reduction test (FECRT), has been recommended by the WAAVP for identifying AR in sheep. The FECRT defines resistance as a faecal egg count reduction (FECR) of < 95 %, with < 90 % for the lower confidence interval, 10-14 days after treatment with an anthelmintic (Coles et al., 1992). In the absence of

standardised criteria for defining anthelmintic resistance in horses in the field, the equine research community has adopted these WAAVP guidelines. However, there is still considerable inconsistency in the study design, sensitivity of FEC methodology and statistical methods used to define resistance in the equine literature, which make it difficult to draw accurate conclusions and comparisons as to the presence or absence of resistance within a given population (Vidyashankar et al., 2012). Amongst the key factors that lack consistency in studies are: group sizes, FEC technique and FECRT values for inclusion in the study group. For example: Kaplan et al (2004) used a mean FECR of 80 % to define resistance, whilst Traversa et al (2009, 2012) used a mean FECR of 90 % and others used a mean FECR of 95 % (Craven et al., 1999; Larsen et al., 2011). Some studies looking at the efficacy of MLs also use a reduced ERP as a marker of developing AR in cyathostomins (Molento et al., 2008; Relf et al., 2014). In summary, there is a need for further clarification of guidelines for detection of AR in cyathostomins *in vivo*.

A number of *in vitro* tests for detecting anthelmintic resistance have been developed and validated in sheep but are yet to be successfully validated for cyathostomins. The egg hatch test (EHT) involves the incubation of nematode eggs with increasing concentrations of drug, followed by a measurement of egg hatch percentage (Coles et al., 1992). This test has been widely validated for the detection of BZ resistance in ruminants (von Samson-Himmelstjerna et al., 2009) and studies have reported some utility of the EHT in horses (Craven et al., 1999; von Samson-Himmelstjerna et al., 2002; Wirtherle et al., 2004). However, the unique ovicidal activity of the BZs, means that the EHT is only effective for detecting resistance to the BZ class of anthelmintics, and in equids widespread BZ resistance is already well established. An *in vitro* test that has the advantage of detecting resistance to multiple classes of anthelmintic is the larval development test (LDT). The LDT involves the incubation of nematode eggs in

increasing concentrations of drug and growth medium for a period of 6-7 days, at which point the percentage of developed larvae verses total larvae is evaluated. The LDT is sold commercially for use in ruminants as Drenchrite ® (Dr Jennifer Gill, Microbial Screening Technologies, Smithfield, Australia), and it has been used successfully for small ruminants (Ancheta et al., 2004; Howell et al., 2008; Kaplan et al., 2007). It has also shown promise in cattle GI nematodes (Demeler et al., 2010b). However results for equids so far have shown poor reproducibility, poor correlation with *in vivo* FECRT data and narrow resistance ratios (Lind et al., 2005; Pook et al., 2002; Tandon and Kaplan, 2004). The larval migration inhibition test (LMIT), is primarily designed to detect ML resistance, and measures the ability of L3 to migrate across a nylon mesh after incubation with increasing concentrations of IVM. It was originally developed and refined for sheep GI nematodes (Kotze et al., 2006) and has been shown to correlate with *in vivo* resistance in cattle nematodes (Demeler et al., 2012; Demeler et al., 2010a; Demeler et al., 2010b). In horses, a recent study has demonstrated that this test can also be correlated with *in vivo* IVM efficacy in cyathostomins (McArthur et al., 2015), highlighting that it shows promise as an *in vitro* tool for evaluating relative ML efficacy in cyathostomins. However validation of the LMIT is not currently possible due to lack of a positive control population of cyathostomins that has been defined as unequivocally resistant to MLs.

A genetic test would be ideal for detection of anthelmintic resistance within a given population of parasites, and a lot of work has been undertaken to identify molecular markers for resistance for each of the major anthelmintic classes (see Section *1.2.1.3.*). For BZ resistance this has been successful, and a pyrosequencing assay has been developed to detect mutations in the beta-tubulin gene in cyathostomins (Lake et al., 2009). This test has not been applied clinically, largely due to the fact that BZ resistance is so widespread that it is now assumed in the clinical

setting. The complexity of the genetic basis of resistance to THPs and MLs leads researchers to conclude that it will be particularly challenging to develop a simple molecular test for resistance to these drug classes (Kotze et al., 2014).

1.2.1.2. The extent of anthelmintic resistance in cyathostomins

Reports of reduced efficacy of the BZs in cyathostomins was first reported soon after their introduction (Drudge and Lyons, 1965). Currently BZ resistance is widespread and has been reported in 15 countries (Table 1.1.). Resistance to PYR was first reported in 1996, and has now been reported in 13 countries (Table 1.1.). Resistance to the MLs, IVM and MOX, has been much slower to develop and has only recently been reported. As a consequence the MLs have been the mainstay of cyathostomin control over the last three decades (Nielsen et al., 2014b). Unfortunately, there is now an increasing amount of evidence that resistance to MLs is beginning to emerge (Table 1.1.). There have been reports of IVM resistance based on the FECRT in Brazil, Finland, Italy and the UK (Canever et al., 2013; Milillo et al., 2009; Molento et al., 2008; Nareaho et al., 2011; Relf et al., 2014; Traversa et al., 2009b). There have been further reports of reduced ERPs for IVM in Brazil, Germany, Belgium, Italy, the Netherlands, the USA and the UK (Dudeney et al., 2008; Geurden et al., 2014; Little et al., 2003; Lyons et al., 2011b; Lyons et al., 2008; Molento et al., 2008; Relf et al., 2014; von Samson-Himmelstjerna et al., 2007). The ERP of IVM when it was introduced was eight weeks, but it is now being reported as being as low as two weeks (Relf et al., 2014). The significance of this change is not fully understood, however there is evidence that it is associated with the survival of L4 stages post-treatment, hence they can develop rapidly into adult stages and start producing eggs (Lyons et al., 2009). There are few reports of critical tests demonstrating reduced IVM efficacy (Lyons et al., 2009), although this is probably because these are not

routinely carried out in equine research for ethical reasons. Compared to IVM, there are fewer reports of reduced efficacy of MOX and these are primarily in the form of reduced ERPs, which have been reported in Brazil, Belgium, Italy, the Netherlands, the UK and the USA (Geurden et al., 2014; Lyons et al., 2011a; Molento et al., 2008; Rossano et al., 2010; Trawford et al., 2005), there has also been one critical study demonstrating reduced MOX efficacy (Lyons et al., 2010). In summary the evidence suggests that, albeit slowly, resistance to the MLs is now developing in cyathostomins. Alarmingly, there are now reports of some farms that appear have resistance to all three major classes of anthelmintic (Canever et al., 2013; Molento et al., 2008; Relf et al., 2014; Traversa et al., 2009b).

1.2.1.3. Mechanisms of anthelmintic resistance in cyathostomins

It is outside the scope of this thesis to extensively report the findings of studies investigating mechanisms of resistance for nematodes. Several broad mechanisms have been proposed; drug target site mutations, altered detoxification pathways, increased drug efflux and reduced drug uptake, which were comprehensively reviewed by Kotze et al., (2014), and are only briefly outlined here. To date, mechanisms of anthelmintic resistance are poorly understood for all anthelmintic classes except the BZs, in which resistance has been attributed to mutations in the drug target isotype-1 beta-tubulin gene. The first mutation to be described was a single nucleotide polymorphism (SNP), an A to a T transversion, resulting in a tyrosine for phenylalanine substitution at codon 200 (the F200Y SNP) in *Haemonchus contortus* (Kwa et al., 1994). Further SNPs have been demonstrated at codons 167 (F167Y) and 198 (E198A) in a number of nematode species (Ghisi et al., 2007; Silvestre and Cabaret, 2002).

Table 1.1. A summary of studies reporting resistance to the three licensed classes of anthelmintic in equids, benzimidazoles (BZ), tetrahydropyrimidines (THP) and macrocyclic lactones (ML). A tick indicates resistance has been reported.

Country	Publication	BZ	THP	ML
Belgium	(Geurden et al., 2014)	√	√	√
Brazil	(Pook et al., 2002)	√		
	(Molento et al., 2008)	√	√	√
	(Canever et al., 2013)	√	√	√
Canada	(Slocombe and Cote, 1977)	√		
	(Slocombe and de Gannes, 2006)		√	
Denmark	(Bjorn et al., 1991)	√		
	(Craven et al., 1998)	√	√	
	(Nielsen et al., 2013)		√	
Finland	(Nareaho et al., 2011)		√	√
France	(Traversa et al., 2012)	√	√	
	(Geurden et al., 2013)	√		
Germany	(Wirtherle et al., 2004)	√		
	(Traversa et al., 2009b)	√	√	
Italy	(Geurden et al., 2014)	√	√	√
	(Traversa et al., 2007b)	√	√	
	(Milillo et al., 2009)	√	√	√
	(Traversa et al., 2009b)	√	√	√
Netherlands	(Geurden et al., 2014)	√	√	√
Norway	(Ihler, 1995)	√	√	
Slovak Republic	(Varady et al., 2000)	√		
	(Cernanska et al., 2009)	√		
Sweden	(Nilsson et al., 1989)	√		
	(Lind et al., 2007)	√	√	
Switzerland	(Meier and Hertzberg, 2005)	√	√	
Ukraine	(Kuzmina and Kharchenko, 2008)	√		
United Kingdom	(Fisher et al., 1992)	√		
	(Comer et al., 2006)	√	√	√
	(Traversa et al., 2009b)	√	√	√
	(Lester et al., 2013b)	√		
	(Stratford et al., 2014b)	√	√	
	(Relf et al., 2014)	√	√	√
United States	(Herd et al., 1981)	√		
	(Chapman et al., 1996)		√	
	(Woods et al., 1998)	√	√	
	(Lyons et al., 2001)	√	√	
	(Tarigo-Martinie et al., 2001)	√	√	
	(Kaplan et al., 2004)	√	√	
	(Rossano et al., 2010)	√		√
	(Lyons et al., 2010)			√
	(Garcia et al., 2013)	√		

In cyathostomins it has been found that the F167Y and F200Y are responsible for resistance to BZs (Hodgkinson et al., 2008; Lake et al., 2009). Even where the BZs are not used for extended periods and subsequently re-introduced, reversion to a resistant phenotype is rapid (Borgsteede and Duyn, 1989; Lester et al., 2013b), leading to the conclusion that the development of BZ resistance in cyathostomins is irreversible, but this has yet to be confirmed with genetic studies.

Mechanisms of resistance to the THPs, such as PYR and levamisole are less well understood. The body of literature, reviewed in Kotze et al (2014), suggests that phenotypic resistance is polygenic and involves changes in expression of nAChR subunits (Kopp et al., 2009), truncated receptor subunits (Fauvin et al., 2010; Neveu et al., 2010; Williamson et al., 2011), and mutations in receptor subunits (Barrere et al., 2014), as well as a possible contribution from P-glycoprotein (P-gp) mediated increased drug efflux (Sarai et al., 2014). There has been no work looking at specific molecular mechanisms of PYR resistance in cyathostomins.

The exact molecular mechanism behind resistance to MLs in parasitic nematodes remains elusive. Initial studies indicated that target site mutations in the GluClRs may play a role (Blackhall et al., 1998; Njue et al., 2004). Blackhall et al (1998) showed an increase in frequency of an allele in the alpha-subunit of the GluClR gene of IVM/MOX resistant *H. contortus*, however resistance in the strain used in this study was generated in the laboratory. Njue et al (2004) described a mutation in the GluClR, L256F, associated with decreased response to IVM in *Cooperia oncophora.* In addition some studies have demonstrated an association between IVM sensitivity and the absence of a glycine residue in the third transmembrane domain of the GluClR (M3-Gly) (Hibbs and Gouaux, 2011; Lynagh and Lynch, 2010, 2012). Recent studies however have failed to find a link between L256F or M3-

Gly and ML resistance, and no additional polymorphisms have been associated with ML resistance (El-Abdellati et al., 2011; Williamson et al., 2011).

As no clear link has been found between the target site gene and ML resistance, attention has recently focussed on alternative mechanisms of resistance such as increased drug efflux by P-gps (Lespine et al., 2012). P-gps belong to a large group of proteins called the multidrug-resistance ATP-binding cassette (ABC) transporters, which in turn belong to a well-conserved family of ABC membrane proteins. They will be discussed in greater depth in Section 6.1., but in summary their main function is the active efflux of structurally unrelated endogenous and exogenous compounds from parasite cells. There is a large body of evidence reporting that increased transcript expression and selection of P-gp variants are associated with reduced sensitivity to MLs (Ardelli et al., 2005; Dicker et al., 2011b; James and Davey, 2009; Janssen et al., 2013; Kerboeuf et al., 2003; Williamson et al., 2011). Another alternative resistance mechanism that has recently been highlighted is the acquisition of reduction of function alleles in the so called dye filling (*dyf*) genes, which cause amphid sensory neuron deficits and reduced drug uptake. This mechanism has been described for *Caenorhabditis elegans* and *H. contortus;* and field isolates of *H. contortus* across five continents have been shown to be enriched for a resistant haplotype in the *dyf-7* gene (Dent et al., 2000; Urdaneta-Marquez et al., 2014). In summary the molecular basis of ML resistance in parasitic nematodes remains to be fully defined, but the evidence suggests it is multigenic. As ML resistance in cyathostomins is only just emerging, no investigations of the mechanisms of resistance have been published to date.

1.2.2. A lack of availability of high quality, reliable anthelmintic formulations impacts on the control of cyathostomins in developing countries

The two major issues currently limiting effective parasite control in cyathostomins in poor communities in developing countries are, a lack of access to treatment and the poor quality of available drugs. Rural communities are often far from clinics distributing anthelmintics and the poorest amongst them are unable to afford the treatment. There are also reports of poor quality and counterfeit products, which will not only be ineffective in controlling parasitism but may pose a health risk (Monteiro et al., 1998; Shakoor et al., 1997). The development of anthelmintic resistance could, in theory, be accelerated by the use of poor quality anthelmintics in these environments. To date, there have been no published reports investigating anthelmintic efficacy against cyathostomins in developing countries, however AR has been demonstrated in helminths of small ruminants, indicating that it is likely to occur under conditions of frequent anthelmintic use and under-dosing due to poor quality drugs (Eguale et al., 2009; Kumsa and Abebe, 2009; Sissay et al., 2006a, b).

1.3. Novel options for controlling cyathostomins

Due to AR and the constraints of treatment in developing countries there is a need for the development of novel treatment options and improved control strategies for cyathostomin infections. A number of management and treatment options are currently being investigated to improve control of GI nematodes.

1.3.1. Biological control of cyathostomins

There has been considerable investigation into the use of biological control on pastures to minimise transmission of cyathostomins by direct antagonism of IL3 (Gronvold et al., 1996). Many organisms act as antagonists of parasites but one group to have shown potential is the nematophagus fungi. Research has focussed on *Arthrobotrys oligospora*, *Arthrobotrys flagrans* and *Duddingtonia flagrans*, with the majority of work on the latter (Bird and Herd, 1995; Braga et al., 2010; Larsen et al., 1996). These fungi produce nematode trapping organs that anchor nematode larvae whilst hyphae penetrate the cuticle and digest the worm. They have been shown to survive the equine GI tract (Larsen et al., 1995), and produce significant reduction in cyathostomin burdens and pasture infectivity resulting in improved growth rates in horses (Braga et al., 2009; Larsen et al., 1996). Recent work in the field has focussed on the extracts of *D. flagrans* that contains proteolytic enzymes, which can have a direct effect against cyathostomin IL3 in the absence of the fungus itself (Braga et al., 2013; Braga et al., 2015). Despite the evidence however the production and use of *D. flagrans* or its extract in equids has not yet been commercialised.

1.3.2. Improving host resistance to cyathostomins

For several decades breeding programs selecting for host resistance to GI nematode infections have been explored in ruminant production systems, in order to reduce reliance on anthelmintics and improve production. There is good evidence that improved host responses can be achieved by selecting for various parameters such as FEC, worm burden, serum antibodies, peripheral eosinophilia, packed cell volume, live weight and serum albumin concentrations (Hooda et al., 1999; Saddiqi et al., 2012; Windon, 1990). Recently the focus has been on defining the genetic basis for host susceptibility in sheep and cattle so that selection

can be refined by the use of multiple quantitative trait loci (McManus et al., 2014). The advent of high-throughput sequencing technologies has facilitated genome-wide association studies to identify such markers (Kim et al., 2014; Riggio et al., 2014; Salle et al., 2012). The success of breeding programs is dependent on the heritability and genetic variability of the trait. As with many aspects of cyathostomin biology the basis of host resistance to infection has not been adequately explored. One recent study evaluated the FECs in a large cohort of Arabian horses over time, and found that the trait has a mean heritability of 0.21 (±0.04) in adults (Kornas et al., 2015), which is comparable with values for ruminants (range of heritability 0.2-0.4) (Assenza et al., 2014; Davies et al., 2006; Kemper et al., 2011; Salle et al., 2012). This suggests that similar breeding programs could be implemented in horses, however there may be other obstacles to this taking place in the equine industry. For example, there is no regulation of breeding horses in the UK, and in those establishments where selective breeding may be appropriate such as large thoroughbred studs, breeding for racing success takes priority.

1.3.3. Vaccine development

Given that AR is such a major issue globally, vaccines represent an attractive long-term option for the control of GI nematodes. A considerable amount of research has gone into the development of vaccines against parasitic nematodes, with only a few commercial vaccines delivered to date. In the 1960s a vaccine was developed for the cattle lung worm *Dictyocaulus viviparous*, which uses irradiated larvae (Jarrett et al., 1958), and a similar vaccine was developed for the dog hookworm *Ancyclostoma caninum* (Miller, 1978). Both vaccines have been made available commercially, however these vaccines are made *in vivo*, are expensive and have a short shelf life, hence there is ongoing research to develop recombinant forms of these vaccines. A recent study in cattle showed some efficacy using a recombinant paramyosin

vaccine, however the commercial vaccine, Bovilis ® Dictol, still far exceeds any recombinant preparation (Strube et al., 2015). Unfortunately vaccines based on irradiated larvae have not been successful for a number of other parasitic nematodes (Smith et al., 1982; Urquhart et al., 1966). Native extracts from dead parasites have also shown some promise in nematode vaccines, for example a commercial vaccine, Barbervax ®, made from the intestinal lining of the worm, has recently been developed against *H. contortus* (Bassetto and Amarante, 2015). One of the inhibiting factors in the use of native antigens is the need to generate large numbers of parasites, usually requiring live animals, which can be prohibitively expensive. Fortunately in the case of Barbervax® such a low dose is needed that the product is commercially viable (Bassetto and Amarante, 2015).

The gold standard to produce a commercially viable vaccine for any parasitic nematode is to use recombinant protein expression to generate large amounts of antigen with which to stimulate an effective immune response. Unfortunately despite decades of research the development of such vaccines for parasitic nematodes has proved difficult, with many recombinant vaccine trials producing poor results *in vivo* (Geldhof et al., 2007; Geldhof et al., 2008; Meyvis et al., 2007; Newton and Meeusen, 2003). The most promising recombinant vaccine to date has been produced against *Teladorsagia circumcinta*, where a multicomponent recombinant vaccine has been shown to reduce FECs and worm burdens by > 70 % (Nisbet et al., 2013). In cyathostomins little progress has been made towards vaccine development, a recombinant protein has been produced as an immunodiagnostic marker to aid in development of an ELISA for encysted larval stages, however there is no evidence that it produces protective responses if given as a vaccine (McWilliam et al., 2010). In addition it is widely accepted that horses do not develop a fully effective immunity against cyathostomins, which means that the development of an effective vaccine would be fundamentally extremely challenging.

1.3.4. Novel anthelmintics to treat cyathostomins

There is ongoing investigation into the discovery of novel anthelmintics for use in veterinary species, the extent of which will not be reviewed here, but it is important to note that anthelmintic discovery is a slow process and does not appear to keeping pace with the development of AR. Over the last decade three novel classes of anthelmintic have been brought to the market, these include emodepside in combination with praziquantel (Profender ®), monepantel (Zolvix ®) and derquantel in combination with abamectin (Startect ®). Emodepside is a cyclooctadepsipeptide, which causes inhibition of pharyngeal pumping, paralysis and death, by binding to the pre-synaptic latrophilic-like receptor (HC110-R), and through pre and post-synaptic interactions with a Ca^{2+} activated K^{+} channel (SCO-1) (Guest et al., 2007; Harder et al., 2003; Harder and von Samson-Himmelstjerna, 2001; Holden-Dye et al., 2012; Martin et al., 2012). It is currently licensed in cats and dogs in combination with praziquantel for the treatment of GI nematode and cestode infections. It is reportedly effective against small strongyles (Epe and Kaminsky, 2013; Harder and von Samson-Himmelstjerna, 2002), however there has been no indication of its development for use in equids, possibly due to formulation, cost or safety issues. Monepantel is an amino acetonitrile derivative, which acts as a positive allosteric modulator of the nematode specific nAChR MPTL-1 and causes depolarisation of muscle cells leading to paralysis (Kaminsky et al., 2008; Rufener et al., 2010; Rufener et al., 2009). It is currently licensed to treat all GI nematodes of sheep and has a good safety profile, however, there is no indication of its development for use in equids. In addition, there have already been reports of resistance to monepantel in sheep after a minimum of two years since its introduction (Scott et al., 2013; Van den Brom et al., 2015), which raises concern over its long term efficacy in the horse should it ever be introduced. Finally derquantel belongs to the spiroindol class, and is a semisynthetic compound derived from a fermentation product of *Penicillium simplicissimum* (Lee et al., 2001). It interferes with a B-subtype nAChR causing

flaccid paralysis and is effective against all major GI nematodes in sheep (Lee et al., 2001; Qian et al., 2006; Robertson et al., 2002). Unfortunately there are reports that derquantel is highly toxic to horses and therefore it is extremely unlikely to be developed as an anthelmintic for use in equids (Epe and Kaminsky, 2013; Woods, 2012).

It appears unlikely that any new anthelmintic will be introduced for equids in the short-to-medium term, and to the author's knowledge there are no groups currently working on the development of therapeutics specifically for use in equids against cyathostomins.

1.3.5. Combination therapies

Due to the difficulties of developing new anthelmintic molecules there has been increasing emphasis on extending the useful lifespan of the anthelmintics which are currently available. This area of research is likely to be of particular relevance for treating cyathostomins given the challenges of AR and lack of new drugs. One way of improving the lifespan of anthelmintics is to reduce their use by implementing targeted treatment regimens. Another method is to improve the efficacy of currently available anthelmintics through the use of drug combination therapies. There are three major types of drug combination that are currently in use or being investigated for treating veterinary parasitic nematodes. These are: the use anthelmintic combinations to expand the spectrum of efficacy; the use of anthelmintic combinations to prevent and tackle drug resistance; and the combination of anthelmintics with potentiating non-anthelmintic compounds. It is important to consider any pharmacokinetic and pharmacodynamic (PK/PD) interactions between drugs in such combinations, as there may be both positive and negative effects on the action of either compound (Lanusse et al., 2015).

1.3.5.1. Anthelmintic combinations to expand the spectrum of activity

In order to improve the spectrum of activity and reduce the need for multiple treatments, some veterinary anthelmintics, which have efficacy against different types of GI helminth, have been combined. These products are well established for equids, for example IVM and praziquantel in Eqvalan duo ®, and MOX and praziquantel in Equest Pramox ®; in order to target both nematodes and cestodes in one treatment (Grubbs et al., 2003; Rehbein et al., 2003). Such combinations are also used in ruminants, for example a combination of MOX and closantel is used in sheep for the control of GI nematodes and the trematode *Fasciola hepatica.* Interestingly it has also been shown that combination with MOX increases the half-life of closantel by 47 % (Suarez et al., 2013). Interactions could potentially also be antagonistic leading to a reduction in the efficacy of one or both of the drugs. There is no published evidence that these potential interactions have been investigated in equids.

1.3.5.2. Anthelmintic combinations to prevent and tackle drug resistance

An increasingly common practice in ruminants is to combine anthelmintics with a different mode of action on the same parasites in order to reduce the chance of resistance developing to either compound (Anderson et al., 1988; Barnes et al., 1995; Leathwick et al., 2009). This approach has also been proposed as a method to treat animals in areas where anthelmintic resistance is already widespread (Suarez et al., 2014; Sutherland and Leathwick, 2011). Such methods rely on the PK/PD interactions between drugs being either additive (total efficacy greater than either drug alone but not greater than the sum of the efficacy of both) or synergistic (total efficacy greater than the sum of both). For example derquantel has been shown to act synergistically with abamectin *in vitro* (Puttachary et al., 2013). The majority of interactions between anthelmintic combinations *in vivo* have been shown to be additive, and there have

been several studies which have shown mild to moderately improved efficacy of combinations in ruminants which harbour drug resistant GI nematodes (Anderson et al., 1988, 1991; Miller and Craig, 1996; Suarez et al., 2013; Suarez et al., 2014). There are however concerns over the sustainability of this approach on farms where there is resistance to all classes of anthelmintic (Lanusse et al., 2015; Suarez et al., 2014). In equids such combinations are not currently licensed in the UK.

1.3.5.3. Combining anthelmintics with potentiating non-anthelmintic compounds

The combination of anthelmintics and non-anthelmintic compounds, which enhance efficacy by interfering with drug transformation, excretion or efflux mechanisms, is gaining increased attention. Anthelmintics are often marketed at the lowest effective dose of 95% to reduce the possibility of toxic side effects. When resistance occurs, and efficacy levels fall below 90%, there is evidence that exposure of helminths to increased levels of anthelmintic improves efficacy (Alvarez et al., 2015; Barrere et al., 2012; Lloberas et al., 2015; Moreno et al., 2004; Varady et al., 1996). Metabolic modulators such as methimazole and piperonyl butoxide, which inhibit flavin monooxigenase and cytochrome P450 mediated oxidation respectively, have been shown to increase the efficacy *in vivo* of some BZs in ruminants (Benchaoui and McKellar, 1996; Lanusse and Prichard, 1992). In horses piperonyl butoxide has been shown to increase the plasma availability and efficacy of oxifendazole (Sanchez Bruni et al., 2005).

Of particular relevance to MLs is the potential of pharmacological interference with cell transporter systems such as P-gps; which are implicated to play a role in ML resistance, see section *1.2.1.3.* P-gps are involved in the active efflux of MLs from both host and parasite tissues, thus in theory blocking this process can lead to increased plasma concentrations and

longer half-life of MLs, as well as increased ML levels in parasite tissues. *In vivo* trials examining the action of P-gp inhibitors on MLs have shown positive effects on the PK disposition of MLs, and improvements in ML efficacy against ML resistant GI nematodes (Bartley et al., 2012; Lifschitz et al., 2010a; Lifschitz et al., 2010b). These effects were not consistent in all studies. Further research is needed in this field to establish which combinations are likely to be of most clinical use in each host-parasite system. There has been no work investigating the potential of chemical modulation of P-gps in optimising ML efficacy against cyathostomins.

1.3.6. Ethnoveterinary medicines

Ethnoveterinary medicine is defined as the study of folk beliefs, knowledge, skills and practices relating to the health care of animals. Lack of availability of drugs, cost and risk of drug resistance are all barriers to the uptake of synthetic anthelmintics in developing countries. This means that many traditional plant-based treatments are still used for parasitic infections (Githiori et al., 2006). In developed countries increasing resistance to anthelmintics has led to interest in potential alternatives to anthelmintics, such as bioactive plant supplements, and the possibility of novel anthelmintic drug discovery from bioactive plant compounds. Plants are known to be a rich source of bioactive compounds that have potential beneficial pharmacological properties. This has been demonstrated with plants such as *Artemisia annua*, which is the origin of the widely used malaria treatment artemisinin (Klayman, 1985). Another example is *Salix alba*, from which the anti-inflammatory salicylic acid (aspirin) was originally isolated (Lafont, 2007). Surveys of plants used for anthelmintic activity have been performed in developing countries across the globe (Githiori et al., 2006). The majority of evidence for plant-based anthelmintics is observational rather than in controlled studies (Hammond et al.,

1997). However, as interest in this area increases, *in vitro* and *in vivo* studies are being carried out in order to validate claims of anthelmintic activity. Amongst the plants that have shown promise in *in vivo* studies against GI nematodes, are plants rich in tannins (Hoste et al., 2006) and commonly used herbs such as *Zingiber officinale* (ginger) (Iqbal et al., 2006a).

Prior to its adoption as a novel method for parasite control it is important that there is a thorough assessment of the anti-parasitic and potential toxic effects of a plant. Scientific evidence supporting the anti-parasitic properties of plants comes mostly from *in vitro* studies (Athanasiadou and Kyriazakis, 2004). The advantage of *in vitro* tests is that large numbers of plant extracts and purified compounds can be tested at relatively low cost. The most commonly used tests in the screening process are the EHT (Coles et al., 1992), the LDT (Demeler et al., 2010b; Gill et al., 1995), the LMIT (Demeler et al., 2010a) and the adult worm motility test (AWMT) (Smout et al., 2010). Using more than one type of test to screen plants is important as anthelmintic activity may be stage specific (Marie-Magdeleine et al., 2010b).

These tests were originally developed for the identification of anthelmintic resistant nematodes and to indicate the efficacy of chemically synthesised compounds. For licensed anthelmintics strong correlation has been shown between *in vitro* and *in vivo* efficacy (Coles et al., 1992). In horses the EHT and LMIT have proven the most useful when comparing *in vitro* and *in vivo* efficacy for BZs and MLs respectively, see Section *1.2.1.1*. It is not clear whether a similar relationship is applicable when testing plant extracts. Some studies show a correlation between *in vitro* and *in vivo* efficacy (Ademola et al., 2004; Bachaya et al., 2009), however Costa et al. (2008) found that an ethanol extract of *Azadirachta indica* inhibited egg hatch by 99.7% in *H. contortus* whilst a study *in vivo* showed no efficacy (Costa et al., 2008; Costa et al., 2006). This

highlights the fact that bioavailability of anthelmintic plant compounds *in vivo* cannot be assumed and the parasites may be exposed to little or none of the compounds being tested *in vitro.* Despite the possible pitfalls in extrapolating efficacy from *in vitro* to *in vivo* methods, it remains a useful preliminary screening technique, primarily due to reduced costs and the practical difficulty of screening large number of plants in controlled clinical trials. Ultimately randomised controlled trials using the same formulation of the plant extract *in vivo* as *in vitro* are an essential part of a scientific approach.

In horses there has been little investigation into the potential of bioactive plants to aid control of GI nematode infections. One *in vitro* study has demonstrated the efficacy of some Australian plants against free-living cyathostomins infections, but this has yet to be followed up by a randomised controlled *in vivo* trial (Payne et al., 2013). Given that feed supplements have a significant market in the equine industry, the value of bioactive plants in reducing reliance on anthelmintics for control of cyathostomins is worthy of further investigation.

1.4. Aims and objectives

The aim of this thesis is to explore novel therapeutic options for the control of cyathostomins in both developing and developed countries, with a particular focus on ethnoveterinary medicines and novel drug combinations. The specific objectives are:

- To identify plants with potential anthelmintic activity against cyathostomins using an evidence-based approach for equids in both developed and developing country settings.
- To test these plant candidates using *in vitro* parasitological tests for two different life-cycle stages of cyathostomins.
- To evaluate the role of the protein efflux channels, P-gps, in emerging resistance to MLs in cyathostominsTo use *in vitro* tests to explore the potential of anthelmintic/P-gp inhibitor combinations to improve efficacy of MLs.

2. MATERIALS AND METHODS

This chapter will describe the *in vitro* parasitological tests that were used throughout this thesis, and their optimisation. The details of optimisation experiments are referred to in Appendix 1. Methods specific to each chapter will be included in the specific materials and methods for that chapter.

2.1. Standard laboratory solutions

The source of all chemicals and drugs for the preparation of standard solutions and reagents are stated in the text. Standard solutions which were not made in the laboratory, but were purchased directly from a manufacturer, are referred to in the text along with their source.

Saturated sodium chloride: Sodium chloride (NaCl) granules (Sigma-Aldrich, UK) were added to 2 L of tap water (H_2O) and shaken until there was a significant amount of undissolved NaCl. The solution was left overnight, shaken again and more NaCl added, until there was a significant excess of granules. The granules were allowed to settle and the solution from the top was decanted for use in the laboratory.

L-cysteine solution: 10 mM (for the larval migration test) and 5 mM (for the egg hatch test) L-cysteine solutions were prepared by the addition of L-cysteine crystals (Sigma-Aldrich, UK) to distilled H_2O (dH_2O) (Sigma-Aldrich, UK). The pH of the solutions were adjusted to 7 and the solution was used directly in tests. Fresh solution was made at the time of each test.

Growth medium for the larval development test: Yeast solution was prepared by the addition of 1 g yeast (Sigma-Aldrich, UK) to 90 ml of 0.9 % NaCl (Sigma-Aldrich, UK) in

dH_2O (18.2 MOhms MilliQ water, Millipore, UK). This was sterilised by autoclaving and combined in a 9:1 ratio with 10 X Earles salt solution (Sigma-Aldrich, UK). This solution was combined in a 2:1 ratio with 1.5 mg/ml lyophilised *Escherichia coli* K12 (Sigma-Aldrich, UK), in dH_2O (18.2 MOhms MilliQ water, Millipore, UK), which had been previously sterilised by autoclaving.

LB solution with ampicillin for cloning: This consisted of 10 g LB powder (Sigma-Aldrich, UK) in 500 ml dH_2O (18.2 MOhms MilliQ water, Millipore, UK), which was sterilised by autoclaving. Before use 1 ml of 50 mg/ml ampicillin (Sigma-Aldrich, UK) was added.

2.2. Collection and storage of cyathostomins eggs and third stage larvae for use in *in vitro* tests

Throughout this thesis, cyathostomin samples were taken from either horses or donkeys for use in *in vitro* tests. The horse samples were obtained from a local yard in Warrington, UK, and the donkey samples were obtained from the Donkey Sanctuary (DS), Sidmouth, UK. Where there were specific criteria for selection, based on previous treatment and faecal egg counts (FEC), they are stated in the specific materials and methods of the relevant chapter.

2.2.1. Equid strongyle egg collection and extraction

Strongyle egg collection and extraction were performed in equid faeces to provide egg samples for use in the egg hatch test (EHT) and larval development test (LDT). Equid faeces were collected immediately after being passed, only the top part of the faecal pat was collected, to minimise the risk of environmental contamination. Faeces were placed in air tight polythene

bags (Fisher, UK) and the eggs extracted within 1 h of collection or, if this was not practical, then the faeces were stored anaerobically in tap H_2O at room temperature for no longer than one week before use. This storage method was optimised in preliminary experiments, see Appendix 1.1. Approximately 10 ml of tap H_2O was added per gram of faeces and thoroughly mixed. The suspension was then washed over sieves in order of decreasing aperture; 2 mm, 750 µm, 150 µm, 75 µm, 38 µm (Fisher, UK). The retentate was collected on the 38 µm sieve and washed into 13 ml polyallomer centrifuge tubes (Beckman Coulter, UK) using tap H_2O. These were centrifuged at 200 *g* for 2 min (MSE, Mistral 3000i) and the supernatant removed by suction. The pellet was re-suspended with saturated sodium chloride (NaCl) (Sigma-Aldrich, UK) solution by gentle inversion and re-centrifuged at 200 *g* for 2 min. The tubes were clamped just below the meniscus with artery forceps and the top layer decanted onto a 38 µm sieve and rinsed with dH_2O (Sigma-Aldrich, UK). The clean eggs were decanted into 50 ml plastic pots (VWR, UK) and the concentration of eggs ascertained by counting the number of eggs in 5 X 10 µl using a microscope at 100 x magnification (Leica, Stereo), and taking an average of each count X 100 to give number of eggs/1000 µl. Egg suspensions were used immediately after extraction, in the EHT or LDT. A subset of each faecal sample was cultured to larval stages, as described in Section *2.2.2*, to identify whether there were any large strongyles present. Only samples with pure cyathostomin infections were used in *in vitro* tests.

2.2.2. Larval culture

Larval culture was performed on faecal samples from horses and donkeys to harvest third stage larvae (L3) for use in the larval migration inhibition test (LMIT). Faeces were collected from the top of the faecal pat to minimise contamination, they were packaged in air tight polythene bags (Fisher, UK) and transported overnight to the University of Liverpool. The faecal samples

were divided into 100 g portions and moulded into a spherical shape. These were arranged in a plastic tray, placed in a polyethene bag (205 x 280mm resealable, Fisher, UK) with holes for ventilation. The trays were left at room temperature (approximately 20 °C) for 14 days. The trays were filled with luke warm tap H_2O and left for 4 h. The H_2O was then decanted through a 1 mm sieve and the filtrate collected on a 38 µm sieve (Fisher, UK). The retentate was poured over a three layer filter (Littlelamb Flushable Paper Nappy Liner) and the filter placed in the top of a jam jar filled with luke warm tap H_2O. After 12 h the filter was removed and the volume of H_2O reduced by suction, leaving a concentrated suspension of L3 in the jam jar which was transferred into a 25 ml culture flask (Nunc, UK). Each L3 sample was examined for the presence of large strongyles, only samples with pure cyathostomins were used in *in vitro* tests. It has previously been shown that this suspension can be stored at 4 °C and used in LMITs for up to three months, however motility is optimal before eight weeks (Pers comm, McArthur, C.). Hence only L3 that had been stored for less than eight weeks were used in the LMIT. The concentration of L3 for each sample was ascertained by counting the number of L3 in 5 X 10 µl using a microscope at 100 X magnification (Leica, Stereo), and taking an average of each count X 100 to give the number of L3/1000 µl.

2.3. The egg hatch test

This test was originally described to detect benzimidazole (BZ) resistance in ruminants (Coles et al., 1992). The Moredun Research Institute adapted this method to develop an in house EHT protocol for ruminant eggs. Here, the Moredun EHT was performed with increasing concentrations of thiabendazole (TBZ), in order to validate the test for use in cyathostomin samples. This test was then adapted for use in Chapters 4 and 5 of this thesis to evaluate the effect of plant extracts against cyathostomins. Preliminary optimisation included, evaluating cyathostomin percentage egg hatch in tap *versus* dH_2O. It was found that percentage egg hatch

was significantly better in dH_2O, see Appendix 1.2., hence this was used in all further tests. In addition the effect of the drug solvent dimethyl sulfoxide (DMSO) on egg hatch was established in preliminary tests, it was found that egg hatch significantly reduced at DMSO concentrations higher than 5 % v/v, see Appendix 1.3., hence no greater than 5 % DMSO was used in further tests.

2.3.1. The egg hatch test with thiabendazole

The EHT was carried out to measure the effect of an anthelmintic compound, TBZ, on cyathostomin egg hatch. Working solutions of TBZ were prepared using 1000 ppm TBZ (Sigma-Aldrich, UK) in DMSO (Sigma-Aldrich, UK), according to Table 2.3.1.

Table 2.3.1. Working and final concentrations of thiabendazole (TBZ) from a stock solution of 1000ppm

Working concentration (ppm)	**Final concentration TBZ (µg/ml)**
100	0.50
60	0.30
50	0.25
40	0.20
30	0.15
20	0.10
15	0.075
10	0.05
2	0.01
0	0.00

The volume of egg suspension containing 100 eggs was calculated (X µl) and 2000 µl – (X µl + 10 µl) dH_2O (Sigma-Aldrich, UK) was added to each well of a 24 multiwell plate (Nunc, UK). Ten µl of each working stock solution of TBZ was added to duplicate wells. Duplicate negative controls for dH_2O and DMSO (final concentration 0.05%) were included. The multiwell plate was placed in an incubator for 48 h at 26 °C. Upon removal, 10 µl of neat Lugol's iodine solution (Sigma-Aldrich, UK) was added to each well to stop further egg hatch. The number of first stage larvae (L1) and eggs were counted for each well using an inverted microscope at 400 X magnification (Olympus CK). Percentage egg hatch was calculated as follows: % egg hatch = (no. L1 /(no. L1 + no. unhatched eggs)) X 100. The results of preliminary experiments with TBZ on cyathostomins egg samples from horses and donkeys are shown in Appendix 1.4.

2.3.2. Adaptation of the egg hatch test, for evaluating the anthelmintic activity of plant extracts

The EHT was used to evaluate the effect of crude plant extracts from the UK and Ethiopia in Chapter 4 and 5. For this the EHT was adapted directly from the EHT with TBZ in Section *2.3.1.*, see Chapters 4 and 5 for specific materials and methods. The EHT required further optimisation for testing papaya latex supernatant (PLS) in Chapter 5. A range of L-cysteine concentrations have been suggested for incubation with PLS, from 1-16 mM (Mansur et al., 2014b; Stepek et al., 2006). Here a concentration of 10 mM was used initially, but was found to inhibit egg hatch. It was reduced to 5 mM for use in further EHT with PLS, and at this concentration it had no significant effect compared to dH_2O. The EHT with PLS, was initially performed with PLS over a 48 h period. However it was found that after 48 h there was complete destruction of all eggs and L1. The incubation time was thus reduced to 24 h, which

allowed visualisation of parasites. Examples of dose response curves for preliminary EHTs with PLS are shown in Appendix 1.5., Figure A.1.5.

2.4. The larval migration inhibition test

The LMIT for evaluating ivermectin (IVM) efficacy, has been previously optimised for cyathostomins at the Moredun Research Institute (McArthur et al., 2015). This method was validated for the cyathostomins populations used in this thesis, and adapted for testing plant extracts, in Chapters 4 and 5, and P-glycoprotein inhibitors in Chapter 6. Basic parameters that were optimised in preliminary tests, were the mesh size for distinguishing between live and dead L3, and a comparison of the LMIT with sheathed and ex-sheathed L3. A mesh size of 25 µM was found to be suitable, See Appendix 1.6. Ex-sheathed L3 were found to migrate better than sheathed, as a proportion of sheathed L3 became stuck in the mesh, hence ex-sheathed L3 were used for all further tests, see Appendix 1.7. In addition a titration of DMSO concentrations was performed to evaluate the effect of drug solvent alone on larval migration. As for the EHT, it was found that larval migration was inhibited at concentrations higher than 5 % v/v, and hence no greater than 5 % DMSO was used in further tests.

2.4.1. The larval migration inhibition test with ivermectin

The volume of L3 solution required for 4800 L3 was calculated from a stock of L3 that had been cultured and refrigerated within the last eight weeks. This volume was made up to 10 ml with tap H_2O and ex-sheathed with 7 % sodium hypochorite (Miltons) for 3.5 min. The L3 were washed three times by centrifugation at 200 *g* for 2 min, followed by removal of 9 ml of supernatant and addition of 9 ml of dH_2O (Sigma-Aldrich, UK). The L3 were baermannised

through two layers of filter paper (Littlelamb Flushable Paper Nappy Liner) at 26 ºC for 2 h to ensure that only viable motile L3 were included in the test. Working concentrations of IVM (Sigma-Aldrich, UK) were made up as outlined in Table 2.4.1. from a stock solution of 3000 µg/ml in DMSO (Sigma-Aldrich, UK). Six µl of each working concentration were added to 0.5 ml amber microtubes (Bioquote, UK), a negative control of 3 % DMSO and dH_2O were included. The L3 were centrifuged, reduced to 1 ml solution and 1.8 ml of dH_2O added. Then, 194 µl of the L3 solution were added to each microtube with 6 µl of each working concentration of IVM. The final IVM concentrations to which the L3 were exposed are shown in table 2.4.1. The mixture was incubated for 2 h at 26º C.

Table 2.4.1. Working and final concentrations of ivermectin (IVM) in the larval migration inhibition test.

Working Concentration (µg/ml)	**Final Concentration (µg/ml)**
3000	90
1500	45
600	18
300	9
60	1.8
20	0.6
5	0.15
2	0.06
1	0.03
0.5	0.015

Custom made migration sticks with 25 µm nylon mesh (HPC Gears) were placed in a 24 well plate (Nunc, UK), as previously described (Demeler et al., 2010a). To each well 1910 µl of a

solution of IVM made up to the final concentrations outlined in table 2.4.1., was added in duplicate. After incubation for 2 h, 90 µl of the incubated L3 were added to each migration well of the corresponding IVM concentration. Plates were covered with aluminium foil to prevent light degradation of IVM, and incubated at 26 ºC for 2 h. The LMIT was halted by removal of the migration sticks from the plate. The bottom of the migration chambers were rinsed into the wells of the multiwall plate containing migrated L3, using 600 µl of dH_2O. The sticks were then turned over and the contents of the migration chambers washed out in adjacent wells of the multiwell plate, using 2 ml of dH_2O. Finally 200 µl of ethanol (Sigma-Aldrich, UK) was added to each well to fix the L3 for counting. The number of L3 in each well were counted using an inverted microscope at 400 X magnification (Olympus CK inverted) and the percentage larval migration calculated as follows: % L3 migration = (L3 migrated/(L3 migrated + L3 not migrated) X 100. Examples of preliminary LMITs with cyathostomin samples from horses and donkeys are shown in Appendix 1.9.

2.4.2. Adaptation of the larval migration inhibition test for evaluating the effect of plant extracts on cyathostomins

The LMIT was used to evaluate the effect of crude plant extracts from the UK and Ethiopia in Chapters 4 and 5. For this, the LMIT was adapted directly from the LMIT with IVM in Section *2.4.1.*, see Chapter 4 and 5 for specific materials and methods. The LMIT required further optimisation for testing papaya latex supernatant (PLS) in Chapter 5. It was necessary to use L-cysteine solution in all incubations, instead of dH_2O. It was found that 10 mM L-cysteine had no effect on L3 migration in controls and hence it was used at this concentration for further experiments. Different incubation times with PLS in the LMIT were also evaluated, See appendix 1.10., and it was found that there was a measurable dose response effect at all

incubation times between 2 and 24 h, with the response improving with longer incubation times. For practical reasons the incubation time was kept at 16 h for future LMITs with PLS, so that the test could be run overnight. Examples of some preliminary LMITs with PLS are shown in Appendix 1.11.

2.4.3. Adaptations of the larval migration inhibition test for evaluating the effect of P-glycoproteins on ivermectin efficacy against cyathostomins

In Chapter 6 the LMIT with IVM was performed with the addition of three P-glycoprotein (P-gp) inhibitors pluronic 85 (P85) (BASF, USA), ketoconazole (Sigma-Aldrich, UK) and ivermectin aglycone (IVM-AG) (synthesised at Toxalim, Institut national de la recherche agronomique (INRA), Toulouse, and kindly donated by Dr Anne Lespine). The method was adapted directly from that described in Section *2.4.1.*, as detailed in Chapter 6. Preliminary optimisation included the evaluation of the effect of P85, ketoconazole and IVM-AG, on larval migration, to ensure that they were used at concentrations which had no effect alone in the LMIT. The LMIT was adapted and performed with dose titrations of all three P-gp inhibitors, see Appendix 1.12. The concentration of P85 and ketoconazole that lead to maximum P-gp inhibition have been reported previously as 22 µM and 10 µM, respectively (Bartley et al., 2009). Given that there was no effect on larval migration at these concentrations, they were used in all further tests. The concentration of IVM-AG that leads to maximum P-gp inhibition has not been previously established. Thus here an additional test was run, where increasing concentrations of IVM-AG were compared with increasing concentrations of IVM-AG plus 1 µM IVM. This was done to determine the concentration at which the addition of IVM-AG optimally improved inhibition of larval migration compared to IVM alone. The optimal

concentration was found to be 60 nM, see Appendix 1.13, and this was used in all further LMITs with IVM-AG.

2.5. The larval development test

The method for the LDT was adapted from a study (Demeler et al., 2010b) where its use was described for ruminant nematodes. It has been reported that a different growth medium is optimal for equid strongyles (Coles et al., 2006), hence the test was first optimised for growth medium, and amphotericin B concentration, see Appendix 1.14. The growth medium described in Demeler et al (2010b) was found to be optimal in the cyathostomin samples used here. An amphotericin B concentration of 0.25 µg/ml was chosen for all tests as there was no effect on development at this concentration and it is the concentration most often cited (Coles et al., 2006; Demeler et al., 2010b). Dose titrations with DMSO were also performed in preliminary tests, see Appendix 1.15. It was found that DMSO began to inhibit larval development at concentrations higher than 0.5 %, and hence no greater than 0.5 % DMSO was used in further tests.

2.5.1. The larval development test with ivermectin

The concentration of a cyathostomin egg suspension was adjusted to 100 eggs in 20 µl. To each well of a 48 well plate (Nunc, UK), the following were added in this order:

1) 20 µl of egg suspension
2) 30 µl of amphotericin B (0.25 mg/ml) (Sigma-Aldrich, UK)
3) 190 µl of dH_2O (Sigma-Aldrich, UK)
4) 30 µl of growth medium (see Section 2.1.)

5) 30 µl of increasing concentrations of IVM (Sigma-Aldrich, UK) solution, see table 2.5.1.

For each concentration of IVM, duplicate repeats were included. Duplicate negative controls of dH_2O were also included. The plates were sealed and covered with foil, before being incubated for 7 days at 26 ºC. The test was halted by adding 20 µl of lugols iodine (Sigma-Aldrich, UK) to each well. The number of first, second and third stage larvae (L1, L2 and L3) were counted and the percentage larval development calculated as follows: % larval development = (no. L3/(no. L3 + L1 + L2)) X 100. Examples of the LDT with IVM in preliminary experiments are shown in Appendix 1.16.

Table 2.5.1. Working concentrations of ivermectin (IVM) (1.5 µl of which were added to 28.5 µl of distilled water to be added to the larval development test), and final concentrations of IVM.

Working concentration of IVM in DMSO (nM)	**Final concentration in the LDT assay (nM)**
5000.00	25.00
2500.00	12.50
1250.00	6.25
625.00	3.13
312.50	1.56
156.25	0.78
78.13	0.39
39.06	0.20
19.53	0.10
9.77	0.05
0 (DMSO alone)	0.00

2.5.2. Adaption of the larval development test for use with P-glycoprotein inhibitors

In chapter 6, as for the LMIT, the LDT with IVM was performed with the addition of three P-gp inhibitors P85, ketoconazole and IVM-AG. The method was adapted directly from that described in Section *2.5.1.*, see Chapter 6 for specific materials and methods. Preliminary optimisation included the evaluation of the effect of P85, ketoconazole and IVM-AG, on larval development, to ensure that they were used at concentrations which has no effect alone in the LDT. The LDT was adapted and performed with dose titrations of all three P-gp inhibitors, see Appendix 1.17. The concentration of P85 and ketoconazole that lead to maximum P-gp inhibition have been reported previously as 22 µM and 10 µM, respectively (Bartley et al., 2009). Given that there was no effect on larval development at these concentrations, they were used in all further tests. For IVM-AG the concentration that leads to maximum P-gp inhibition has not been previously established, it was found to be 60 nM in the LMIT, however, for the LDT it was observed that at concentrations greater than 12.5 nM there was an independent effect on larval development (Appendix 1.17) and thus, IVM-AG was used at a lower concentration of 8 nM in all further LDTs.

2.6 Analysis of dose response data

There is more than one commonly used method for the statistical analysis of dose response data (Ademola and Eloff, 2011; AlGusbi et al., 2014; Bachaya et al., 2009; Bartley et al., 2009; Maciel et al., 2006; McArthur et al., 2015). In this thesis PROBIT analysis in SPSS version 21 (SPSS Inc., Chicago IL), the four parameter sigmoidal model in GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com) and a general linear mixed model in R studio (R Core Team, 2014) were used. These different models were selected based on the complexity of the dataset and the analysis required in each chapter.

The details of these methods are given where they are used within the specific materials and methods of Chapters 4, 5 and 6.

3. IDENTIFICATION OF PLANTS WITH POTENTIAL ANTHELMINTIC ACTIVITY IN ETHIOPIA AND THE UK

3.1. Introduction

The challenges of controlling equid helminth infection using conventional anthelmintics have led to a growing interest in alternative control strategies. In developed countries anthelmintic resistance is the primary challenge, however in developing countries the limiting factors are often financial or logistic, with poor rural communities worst affected. The use of bioactive plants to reduce parasite burden, is an alternative strategy that may aid effective control in both scenarios. Ethiopia is an example of a developing country which is rich in flora, and there have been numerous studies detailing the medicinal properties of native plant species (Assefa et al., 2010a; Assefa et al., 2010b; Bekalo et al., 2009; Chekole et al., 2015; Kidane et al., 2014b; Tolossa et al., 2013; Yigezu et al., 2014; Yineger et al., 2007; Yineger and Yewhalaw, 2007). Some studies have also reported plants that are believed to have efficacy against internal parasites of ruminants, chickens and/or humans (Assefa et al., 2010b; Bamikole and Ikhatua, 2009; Githiori et al., 2004; Hussain et al., 2008; Maphosa and Masika, 2010; Mwale and Masika, 2009), however none of these studies have described plant-based treatments used specifically for equids. Participatory research techniques, such as participatory rural appraisals (PRA) are well established approaches to obtaining ethnobotanical information from rural communities (Chekole et al., 2015; Kidane et al., 2014a; Kidane et al., 2014b; Njoroge and Bussmann, 2006; Yigezu et al., 2014). A PRA focuses on investigating and representing what people know and understand and initiating group-led plans for future adaptations (Chambers, 1994), it represents a flexible approach encompassing both quantitative and qualitative data.

In contrast to Ethiopia, the UK is highly developed country where general knowledge of native plant species is sparse. In recent years there has been investigation into the potential use of bioactive forages in UK livestock (Athanasiadou and Kyriazakis, 2004; Athanasiadou et al.,

2005), but these studies were not led by farmer knowledge. These circumstances necessitate a different approach in identifying suitable plant candidates with potential anthelmintic activity.

The present study was performed in collaboration with the Donkey Sanctuary (DS), and the aim was to identify plants which have potential for treating equid gastrointestinal (GI) nematodes in two countries where the DS operates, the UK and Ethiopia. This chapter describes the selection process that was used to identify suitable candidates for *in vitro* screening for anthelmintic activity. A PRA was used to identify plants which are currently used as ethnoveterinary treatments in equids in rural Ethiopian communities, in order to make recommendations for poor communities who struggle to access synthetic anthelmintics for financial or logistic reasons. In the UK a literature review was chosen to identify suitable plants for further testing, for which there was existing evidence of anthelmintic activity.

3.2. Materials and Methods

3.2.1. Identification of plants with potential anthelmintic activity in Ethiopia using a Participatory Rural Appraisal

The PRA was designed and carried out in collaboration with a research assistant, Dr Claire Scantlebury and was divided into part a) focus group discussions and part b) key informant interviews. The methods and results of the focus group discussions were led by Dr Scantlebury and have been published (Scantlebury et al., 2013), however the sections relevant to plant selection are presented here for completeness. The author visited Ethiopia with Dr Scantlebury for part b) and assisted in the key informant interviews, collected plants named in part a) and

b) for identification, and triangulated results with a literature review in order to select a shortlist of plants for *in vitro* testing.

3.2.1.1. Study area

The study area consisted of 14 kebeles (neighbourhoods) in the Oromia region of Ethiopia, five of these were Donkey Sanctuary (DS) clinic sites and nine were non-DS sites. The kebeles were selected for their topographic variation and accessibility, to maximise the range of sampled flora. Data collection was conducted over a six week period during June and July 2011.

3.2.1.2. Focus group discussions

As stated above focus group discussions were conducted by Dr Claire Scantlebury and the methods have been published (Scantlebury et al., 2013). Briefly, participants from each kebele were selected via the local development agent (DA) from each region. Each DA was briefed about the study and asked to select donkey owners of a range of ages (>16 years of age). Two groups per village were requested, with a maximum of 6-8 people per group. The group sessions were of a semi-structured format including some key questions on the use of plants to treat parasitic infections in livestock and donkeys, along with opportunities for open discussion. Additionally, each group participated in constructing two sets of matrices drawn on laminated white card. Photographs were taken of the matrices to record the data. The names of the volunteered plants were listed along one side of the matrix. For each plant, brief details of method of preparation, plant location, how owners know that this is an effective treatment and any reported side effects, were recorded. If a number of plants were named for use in donkeys,

the group were asked to arrive at a consensus of how these were ranked in terms of effectiveness. All groups were asked if they could name a local person or local traditional healer who was known to have knowledge of plant based treatments for animals and, particularly, preparations with believed anti-parasitic properties. In this way a list of names was compiled at the time of each focus group from which key informants were selected. At the end of the focus group a leaflet was given to all participants explaining practical approaches to controlling GI parasites in their donkeys.

3.2.1.3. Data handling for focus group discussions

Data analysis for focus groups was carried out by Dr. Claire Scantlebury and has been published (Scantlebury et al., 2013). The discussions were translated into English at the time and recorded on a digital Dictaphone. The English translation from the focus groups was transcribed. Audio recordings and English transcriptions are available for each group discussion. Microsoft Office Excel software was used to tabulate the photographed matrices and NVivo qualitative data analysis software (QSR International Pty Ltd. Version 8) was used to thematically code the content of the portions of open discussion occurring during the focus groups. A list of plants named for each species was generated including frequency tables of how many groups named each plant type. Where applicable, ranking data was tabulated to show the perceived effectiveness of the plants for use in donkeys. A table summarising the preparation and methods of administration and side effects of each plant species was also prepared.

3.2.1.4. Key Informant interviews

Key informant interviews were led by Dr. Claire Scantlebury, and carried out in collaboration with the Author. From the list of key informants identified during the focus group discussions, the DAs from each region contacted and recruited two people from each village to participate in interviews. At the beginning of each interview the participant was introduced, informed of the nature and aims of the study and invited to take part voluntarily. Verbal informed consent was gained and recorded from each participant. A semi-structured interview approach was used with the help of a co-facilitator/translator, (Appendix 2.1.) for the interview outline. Information gained during the interviews was written down at the time of the interview on the prepared data sheet to facilitate data entry and all interviews were recorded on a digital dictaphone. Participants were first asked to describe which plants/plant based treatments they used to treat worms in any species and to detail information relating to the description of the plant, method of preparation and administration and side effects for each plant. Additionally, some of the highly ranked plants from the focus group discussions and some key plant candidates from previous key informant interviews were named at the end of the interview, and participants were asked if they knew of their use for any species and for what conditions/disease presentations they were used for. If they reported knowing about one of these plants, further details relating to the preparation and dosage and side effects were obtained. This was one of the methods by which the data from the focus group discussions was triangulated. Data were entered into and analysed using Excel (Microsoft) and NVivo 8 (QSR), as described above.

3.2.1.5. Plant collection, identification and selection

Where possible, plants named by participants were sampled. In some cases the plant was too far from the study site or out of season. Cutting samples of each plant were taken, incorporating

the leaves, stem, flowers and seeds where possible (Forman and Bridson, 1989). These samples were pressed and dried before transportation to the National Herbarium, Addis Ababa, Ethiopia, where they were formally identified. Photographs were taken of plants to include details of the leaf/branch structure and any flowers or seed heads to aid identification. Subsequent to identification, results were triangulated with available literature for each plant to provide supportive evidence for use against GI nematodes and to identify any reports of toxicity. Four plants were shortlisted based on the following criteria: identification in the PRA, ranking in the PRA, supportive evidence of *in vitro* or *in vivo* efficacy in the literature, a lack of reported severe side effects in the literature and availability.

3.2.2. Identification of UK plant candidates using a literature review

Plants were shortlisted by means of a literature review using the PubMed search engine (www.ncbi.nlm.nih.gov>NCBI>Literature). The search criteria entered were 'plant' AND 'anthelmintic'. The data from the literature search was tabulated using Microsoft Office Word (2007) under the following headings: number of citations supporting efficacy against parasitic nematodes *in vitro*, number of citations supporting efficacy against gastrointestinal (GI) nematodes *in vivo*, reports of toxicity and local availability. Supervisors and advisors from the DS, UK and Ethiopia, independently used these data to rank each plant, and the results were collated to create a preliminary shortlist of plants. Plants were excluded if there were significant reports of toxicity in the literature. Once a preliminary shortlist of plants had been identified, a more general search, using google scholar (www.scholar.google.co.uk), was performed to identify additional references for the chosen plant extracts. The total number of citations supportive of each plant ranged between 4 and 26. Investigations into availability at the time of the study were used to make a final shortlist of five plants.

3.3. Results

3.3.1. Ethiopian plant selection using a participatory rural appraisal

A total of 50 plants/plant mixtures were identified during the focus group discussions and key informant interviews. For 37 of these, cutting samples were taken for identification. For some plants it was only possible to identify to family and genus level due to the lack of flowering parts at the time of collection. Table 3.3.1. shows the plants that were identified during the focus group discussions, including the frequency with which each plant was mentioned within groups for treatment in donkeys, other livestock species and humans. A total of 22 out of 29 groups reported using plant preparations to treat GI parasites in donkeys, whereas all groups described their use in livestock and humans. Table 3.3.2. shows the ranking of plants, named by groups, for donkeys, according to the frequency of use and opinions on efficacy. Table 3.3.3.a. shows the plants mentioned by key informants for efficacy against GI parasites in donkeys; Table 3.3.3.b. shows plants mentioned by key informants for efficacy against GI nematodes in mixed species. This data was collected as some people did not treat donkeys. The reported efficacy, side effects, method of preparation and dosing were also recorded where possible from focus groups and key informants (Table 3.3.4.a and b). It was noted for some plant species that the method of preparation of the plant before administration varied widely between participants. The reported side effects also frequently varied from none, to severe diarrhoea and death; toxicity was usually reported to be dosage dependant. This data was triangulated with a literature review and plants were shortlisted, as described in Section *3.2.1.5.*, for collection and testing in *in vitro* parasitological tests, see Table 3.3.5. A fifth plant, *Acacia nilotica* (L.), was also included, identified directly based on its availability in the region and published evidence of *in vivo* efficacy against GI nematodes (Bachaya et al., 2009).

3.3.2. UK plant selection using a literature review

The PubMed search yielded 2480 results, 138 of which reported the *in vitro* or *in vivo* effect of plants on nematodes (Appendix 2.2.). Plants were shortlisted as described in Section *3.2.2.* Table 3.3.6. shows the preliminary shortlist of plants that were chosen for UK testing after ranking by independent researchers. The final shortlist of plants was *Carica papaya* L., *Allium sativum* L. (garlic), *Artemisia absinthium* L., *Chenopodium album* L. and *Zingiber officinale* Roscoe. (ginger).

Table 3.3.1. Plants named by focus groups for use in donkeys, other livestock and humans, with the frequency of groups naming them (adapted from (Scantlebury et al., 2013)).

Named plants	Vernacular plant names	Frequency named for use in donkeys - no. of groups and % freq (out of 22 groups which used plants in donkeys)	Frequency named for use in other livestock - no. of groups and % freq (out of all 29 groups)	Frequency named for use in people - no. of groups and % freq (out of all 29 groups)	TOTAL frequency named for use in all species – no. of groups and % freq
Hagenia abyssinica J. F. G mel.	Koso	9 (40.0%)	9 (31%)	23 (79.3%)	26 (89.7%)
Unknown	Mixed roots / leaves / trad remedy / unknown	8 (36.4%)	12 (41.4%)	3 (10.3%)	22 (75.9%)
Embelia schimperi Vatke	Enkoko / Hanku	4 (18.2%)	0	18 (62.1%)	20 (67%)
Nicotiana tabacum L.	Tobacco	0	10 (34.5%)	0	19 (65.5%)
Vernonia amygdalina Delile	Grawa	7 (31.8%)	13 (44.8%)	0	15 (51.7%)
Unknown	Metere	0	0	8 (27.6%)	8 (27.6%)
Cucumis prophetarum L.	Holoto	3 (13.6%)	2 (6.9%)	2 (6.9%)	4 (13.8%)
Trigonella foenum-graecum L.	Abish	1 (4.5%)	2 (6.9%)	0	3 (10.3%)
Verbascum sinaiticum Benth.	Donkey Ear / Gurra Harre	3 (13.6%)	0	0	3 (10.3%)
Withania somnifera (L.) Dunal	Wahale / Gizawa	3 (13.6%)	1 (3.4%)	0	3 (10.3%)
Unknown	Senebecki	0	0	2 (6.9%)	2 (6.9%)
Unknown	Dodoho	0	1 (3.4%)	2 (6.9%)	2 (6.9%)

Capparis cartilaginea Decne.	Delensisa	0	2 (6.9%)	0	2 (6.9%)
Unknown	Gime kitel/Sara-aja	0	2 (6.9%)	0	2 (6.9%)
Melia azedarach L.	Milia	0	1 (3.4%)	0	1 (3.4%)
Tapinanthus globuliferus Tiegh.	Harmo	1 (4.5%)	0	0	1 (3.4%)
Phytolacca dodecandre L. Her	Endod	1 (4.5%)	0	0	1 (3.4%)
Unknown	Bean Straw	1 (4.5%)	0	0	1 (3.4%)
Unknown	Keskesco	0	1 (3.4%)	0	1 (3.4%)
Unknown	Bure (root)	1 (4.5%)	0	0	1 (3.4%)
Unknown	Feto	0	1 (3.4%)	0	1 (3.4%)
Unknown	Abdul Salim	1 (4.5%)	1 (3.4%)	1 (3.4%)	1 (3.4%)
Capparis cartilaginea Decne.	Delensisa	0	2 (6.9%)	0	2 (6.9%)
Dodonea angustifolia L. f.	Kitkita	1 (4.5%)	0	0	1 (3.5%)
Unknown	Kedida	0	0	1 (3.4%)	1 (3.5%)
Croton macrostachyus Hochst. ex. Delile	Bisana	1 (4.5%)	0	0	1 (3.5%)
Myrsine Africana L.	Kechamo	0	0	1 (3.4%)	1 (3.5%)
Unknown	Chobi	0	1 (3.4%)	1	1 (3.5%)

Table 3.3.2. Plant rankings for perceived effectiveness in donkeys (focus groups) (taken from (Scantlebury et al., 2013))

	Grawa *(Vernonia amygdalina* Delile*)*	**Koso *(Hagenia abyssinica* J. F. G Mel*)***	**Holoto *(Cucumis prophetarum* L.*)***	**Enkoko *(Embelia schimperi* Vatke*)***	**Wahale *(Withania somnifera* Dunal*)***	**Roots and leaves mixture (unknown)**	**Abdul Salim (unknown)**	**Roots of plant mix (unknown)**	**Harmo *(Tapinanthus globiferus* Tiegh.*)***	**Gurra harre *(Verbascum sinaiticum* Benth.*)***	**Abish *(Trigonella foenum-graecum* L.*)***	**Endod *(Phytolacca dodecandre* L. Her)**
Average rank score	2.2	2	3	1	2.5	1	1	2	2	2	3	3
Combined rank score	27	23	14	8	5	4	4	3	3	3	2	2
Number of groups ranked (out of 22)	6	6	4	2	2	1	1	1	1	1	1	1

Combined rank score obtained by re-assigning ranks with a score and adding together (e.g. if ranked 1st then scored 4, if 2nd scored 3 etc.)

Table 3.3.3.a. Plants named by key informants with reported efficacy against gastrointestinal helminths in donkeys

Interview code	Plant species	Vernacular plant names	Species given to
22.06.11 Dire Participant 1	*Cyphostemma adenocaule* (Steud.) Desc.	Hindirifa	Equids
22.06.11 Dire Participant 2	*Echinops kebericho* Mesfin	Balawaraanti	Equids
21.06.11 Bekejo Participant 2	Unknown	Bure	Cattle / donkeys
21.06.11 Bekejo Participant 2	Unknown	Derartu	Cattle / donkeys
21.06.11 Bekejo participant 3	*Ranunculus multifidus* Forssk.	Not Known	Cattle, people and donkeys
27.06.11 Boru Lencha Participant 2	*Verbascum sinaiticum* Benth.	Gurra hare	Donkeys
21.06.11 Insilale Participant 1	*Eucalyptus staigeriana* F. M. Bailey *Foeniculum vulgare* Mill.	Eucalyptus Insilale	Cattle / donkeys
27.06.11 Boru Lencha Participant 1	*Vernonia amygdalina* Delile	Grawa	Cattle / donkeys
28.06.11 Jawi Cilalo Participant 2	*Vernonia amygdalina* Delile *Trigonella foenum-graecum* L. Unknown	Grawa Abish Kedida	All species
22.06.11 Dire Participant 3	*Verbascum sinaiticum* Benth. *Ferula communis* L. *Satureja punctate* R. Br.	Gurra harre Haladawa Tosigni	Equids
21.06.11 Insilale Participant 1	*Solanum incanum* L. *Cucumis prophetarum* L.	Hidi Holoto	Donkeys
21.06.11 Bekejo Participant 2	*Cyphostemma adenocaule* (Steud.) Desc.	Hindirifa	Donkeys / cattle
21.06.11 Bekejo participant 3	*Cyphostemma adenocaule* (Steud.) Desc.	Hindirifa	Donkey / cattle

28.06.11 Sibu Abidire Participant 2	*Stephania abyssinica* Walp.	Kalala	All species
27.06.11 Boru Lencha Participant 1	*Hagenia abyssinica* J. F. G Mel	Koso	Cattle / donkeys
28.06.11 Sibu Abidire Participant 2	*Arisaema shimperianum* Schott	Yekib bokolo	Equids
28.06.11 Debeye Adere Participant 1	Unknown	Abdul Salim	All species

Table 3.3.3.b. Plants named by key informants with reported efficacy against gastrointestinal helminths in mixed species

Interview code	Plant species	Vernacular plant names	Species given to
29.06.11 Gasala Chicha Participant 2	*Grewia ferruginea* Hochst.	Alanquatta (Tekonu)	Cattle, sheep and goats
21.06.11 Bekejo Participant 2	Unknown	Bure	Cattle / donkeys
21.06.11 Bekejo Participant 2	Unknown	Derartu	Donkeys and cattle
21.06.11 Bekejo participant 3	*Ranunculi multifidus* Forssk	Doesn't know name	Cattle, people and donkeys
21.06.11 Insilale Participant 1	*Eucalyptus staigeriana* F. M. Bailey *Foeniculum vulgare* Mill.	Eucalyptus Insilale	Cattle / donkeys
21.06.11 Bekejo Participant 1	*Withania somnifera* (L.) Dunal	Gizawa	Cattle, sheep and goats
27.06.11 Boru Lencha Participant 1	*Vernonia amygdalina* Delile	Grawa	Cattle and donkeys
21.06.11 Insilale Participant 1	*Vernonia amygdalina* Delile *Trigonella foenum-graecum* L.	Grawa Abish	Cattle, sheep and goats
28.06.11 Jawi Cilalo Participant 2	*Vernonia amygdalina* Delile *Trigonella foenum-graecum* L. Unknown	Grawa Abish Kedida	All types of animals
29.06.11 Dawe Guticha Participant 1	*Tapinanthus globiferus* Tiegh.	Harmo	People
23.06.11 Boset Participant 1	*Gomphocarpus fruticosus* (L.) W.T.Aiton	Hatifachisa	Calves
21.06.11 Bekejo Participant 2	*Cyphostemma adenocaule* (Steud.) Desc.	Hindirifa	Donkeys and cattle

22.06.11 Dire Participant 1	Ijaji 8 plants mixed Unknown *Rumex abyssinicus* Jacq *Verbascum sinaiticum* Benth. Unknown Unknown *Zehneria scabra* Sond. *Plectranthus lanuginosus* (Benth.) Agnew *Aerva javanica* Juss. *Stephania abyssinica* Walp.	Ijaji 8 plants mixed Dergu Gurbdu Gurre harre Jage Xirarro Fiti Gurra Adala Abbayi Kalala	Cattle
28.06.11 Sibu Abidire Participant 2	*Stephania abyssinica* Walp.	Kalala	All species
27.06.11 Boru Lencha Participant 1	*Hagenia abyssinica* J. F. G mel.	Koso	Cattle and donkeys
28.06.11 Sibu Abidire Participant 2	*Hagenia abyssinica* J. F. G mel.	Koso	People
22.06.11 Chole Sonkole Participant 2	*Hagenia abyssinica* J. F. G mel.	Koso	People
22.06.11 Chole sonkole participant 1	*Verbascum sinaiticum* Benth. *Cyphostemma adenocaule* (Steud.) Desc. Un-disclosed plant	Gurra harre Hindifira	Calves
21.06.11 Bekejo Participant 1	*Foeniculum vulgare* *Calpurnia aurea* (Lam.) Benth. *Justicia schimperiana* T. Anderson *Withania somnifera* (L.) Dunal	Insilale Digita (amharic) / Cheketa (oromofa) Sensel Gizawa	
28.06.11 Debeye Adere Participant 1	Asteraceae/Solanaceae	Osole	People
28.06.11 Debeye Adere Participant 2	Unknown	Abdul Salim	All species
28.06.11 Debeye Adere Participant 3	*Cordia africana* Lam.	Wanza	Just for cattle

Table 3.3.4.a. Focus groups: summary of methods of preparation, where obtained, effectiveness and side effects of plants named for use in donkeys

Named plants	Range of methods of preparation described	Where from	Effectiveness	Range of side effects described in donkeys
Koso *Hagenia abyssinica* J. F. G mel.	-Crush and mix with water, either use straight away or keep for 1 – 3 nights and drench in morning -One group specified a dose of one handful leaves -One group crush it, sometimes mix with oil seed, keep for 1 night, and drench in the morning	Market / some find it growing wild in highland regions		-Not reported in donkeys -Will cause diarrhoea in adults if more than one glass full given -If poor body condition will cause diarrhoea -Beyond 1 coke bottle will kill -Severe diarrhoea can kill -If overdose severe diarrhoea and will die
Grawa *Vernonia amygdalina* Delile	-Crush (plus / minus mix with salt) and water and drench -One group crush with salt and keep one night before giving to animal, n.b. donkeys may drink voluntarily	Grow it : general purpose plant e.g. used as fence		-None -If overdose severe diarrhoea, give little by little -None in donkeys but if mix with lots of salt will kill animal -Severe diarrhoea and can kill
Holoto *Cucumis prophetarum* L.	-Crush, mix with water and drench -One group keep one night and drench juice part in morning -One group use root -Crush with water and drench juice part - use wood mouth gag	Wild in the lowlands, dies back in dry season.		-Severe diarrhoea and can kill -None if proper dosage depends on size of donkey if overdose will kill -Severe diarrhoea and can kill

Enkoko (seed) *Embelia schimperi* Vatke	Crush and mix with barley plus or minus salt and feed	Market	Improves body condition Tablets preferred as enkoko eliminates worms over 4 days.	-Does cause diarrhoea but not like Koso -None -Induces severe diarrhoea in people. -Overdose, causes abdominal pain can kill people. -No side effects -If beyond milk cup dose will cause severe diarrhoea
Wahale / Gizawa *Withania somnifera* (L.) Dunal	-Crush mix with water and salt and drench (may also mix with garlic)	Grows wild (in lowlands)	Starts eating again. Will be cured.	-None reported
Roots and leaves mixture	-Mix with water and drench traditional person knows dose			
Abdul Salim (root)	-Crush mix with garlic and drop in nostril of donkey.	Market		-If overdose horse / donkey can die
Gurre harre *Verbascum sinaiticum* Benth.	-Crush it mix with water (sometimes mixed with wahale sometimes grawa, or salt) and drench	Grows wild	Grawa plus donkey ear as effective as tablet	-None
Harmu	-Collect and crush, keep for 1 night	Collected from different trees: Wadesa, Bekenisa,		-None if proper dose

Tapinanthus globiferus Tiegh.		Tatesa, Efersa, Birbirsa		
Roots of plant mix	Crush just with water for donkeys no boiling -Crush mix with water and drench use mouth gag in donkeys	Traditional person	Induces diarrhoea and G.I.T. gets washed out.	-None
Abish *Trigonella foenum-graecum* L.	-Powder, mix with water and drench	Grow or buy seed but expensive	Expels worms, curative	-None
Endod *Phytolacca dodecandre* L. Her	-Crush, mix with Sensete and salt drench with water (use coke bottle)	Grows wild everywhere		-Endod is like acid so will burn inside which causes animal to shiver
Bean straw	-'Smoking' used for general disease and coughing			
Bure	-Crush with water and drench juice	Wild in lowlands		-Severe diarrhoea and can kill
Kitkita leaf *Dodonea angustifolia* L. f.	-Crush with water and drench juice	Grow it		-Severe diarrhoea and can kill
Sara-aja leaf and root	-Mix with water drench juicy part	Grows wild	Expels worms (Alelu Gasala group 1)	-None

Bisana *Croton macrostachyus* Hochst. ex. Delile	-Crush, mix with water and drench juice	Wild	Not used now	-Burning and severe diarrhoea not used now

Table 3.3.4.b. Key informant interviews: summary of preparation of plants for use in donkeys

Plant	Part used	Preparation	Dosage	How administered	How often	Effect
Cyphostemma adenocaule (Steud.) Desc.	Root part	Chop root then dry then crush then dry again. Mix with water and then sieve it and immediately drench to donkey	One finger pinch of dry root powder for donkey, sometimes mixed with other plants (arita / masarata)	Drench juice, use beer bottle	Given for three consecutive days	
Balawaraanti *Echinops kebericho* Mesfin	Root	Chop root, then dry then crush to powder and dry and keep for when want to use. People buy and mix with water at home before giving to animal. Keeps for long time dried. Boil for one night and in morning mix with cold water and sieve it then mix with chilli, salt butter then drench one beer bottle full for donkey.	Use a handful for donkey	Drench in nostril or with wooden mouth gag	Just given once a year for worms, will last all year. May repeat if other problems.	Animal will eat and become good body condition. No side effects reported
Bure	Root of plant looks like onion	Same prep as Hindirifa but less volume as very strong. Does not matter if stored.	Coffee cup full of juice sometimes mix bure and hindirifa		Just providing so owners choose	Eliminates bloating encourages urination immediately and then diarrhoea, is strong cause bad diarrhoea and may debilitate
Derartu	Root part	Same as Hindirifa sometimes mi with crushed leaf same plant	one litre at a time	Drench	When asked to supply	Diarrhoea same as others

Unknown *Ranunculi multifidus* Forssk	Roots	Crush with salt and sieve fill bottle like glass bottle (~500 ml).	About 10cm of root crushed and mixed in 500ml bottle	Drench through nostril for donkeys	Just when have problem, one off, if no relief then will repeat	Will eat after administering no side effects
Donkey ear/Gurra Harre *Verbascum sinaiticum* Benth.	Root	Chop it crush with mortar crush add water immediately sieve it with juicy part drench juice part one litre.	Six inches times three of the plants roots.	Drench		No side effects other than diarrhoea
Eucalyptus plus Insilale *Eucalyptus staigeriana* F. M. Bailey *Foeniculum vulgare* Mill.	Insilale root, and leaf part eucalyptus	Crush together and mix with water and sieve it. Mix from half to 1 litre of water and drench	Four leaves eucalyptus and one finger length root of insilale	Drench using wooden mouthguard		None donkey will start eating and come to friends will not lay down
Grawa *Vernonia amygdalina* Delile	Leaf	Crush leaf plus salt and water keep in covered container for one night in morning sieve. Let drink juice if not willing to drink then drench.	Two handfuls of leaf, donkeys doesn't matter amount of leaf as animal eats sometimes itself (~10cc salt half local coffee cup).	Leave to drink or drench. All animals will eat normally from fence	Whenever see loss body condition given for three consecutive days	No difference between collections in dry or wet season. After drench see lots worms. Improved appetite and improved. Body condition no worms in faeces days later not as many and no bad effects. Salt should be proper

						amount maybe half local coffee cup.
Grawa/Ebicha *Vernonia amygdalina* Delile / *Trigonella foenum-graecum* L.	Leaf	Crush leaf, mix with salt for drenching sieve keep for one night or use immediately		Donkeys, drenched as not willing to drink drench through nostril with glass bottle, one litre	Only once	Will be worms in faeces after drenching no negative side effects
Gurra Harre Haladawa and Tosigni mixed *Verbascum sinaiticum* Benth. *Ferula communis* L. *Satureja punctate* R. Br.	Leaves and roots	Crush roots and leaf together and make pie (about two handfuls worth) for an animal let it dry in sun keep it in closed container give whole pie for one animal (horses / donkeys)	Crush dry pie, mix with three litres of water, keep overnight drench one litre after sieving	Drench	Give 1st day and then every other day for three days	Positive reports from people using treatment. Effective and people ask for more to keep at home. Animal eats and it induces diarrhoea and the worms come out
Hidi and Holoto mixed *Solanum incanum* L. *Cucumis prophetarum* L.	Roots	Equal shares of hidi and holoto, sometimes use eucalyptus leaf to make it strong. Mix it with salt (handful) and chilli (two teaspoons of chilli) sieve one litre and use immediately		Drench, after drenching do not let animal drink after drenching as believe will make strong		Animals not willing to drink no side effects
Hindirifa	Root part, any part of root	Crush mix with salt and drench juicy part after sieving, prepare and use immediately		Use beer bottle		Induce diarrhoea wash all disease away in donkeys kills

Cyphostemma adenocaule (Steud.) Desc.		may prepare and keep for two months, storing strengthens it allows fermentation. Keep in glass bottle, use beer bottle full (300ml)				worms will see coming out in faeces
Hindirifa *Cyphostemma adenocaule* (Steud.) Desc.	Root part creeps around on floor and climbs up root has bulb use fistful	Use fistful of root, crush and salt and sieve, fill bottle like glass ~500ml	Use 500 ml water plus ~15cm root	Drench though nose of donkey use immediately	Whenever there is a problem with the animal, may repeat if necessary	Will eat after drenching / moving / stopped rolling / mix with other donkeys and feel good
Kalala *Stephania abyssinica* Walp.	Root	Root, crush in mortar, then mix with water then immediately drench sieve it and drench juice of root.	Difficult to estimate as long thin roots.	Drench	Like koso, follow faeces of donkeys / horse will drench once daily follow droppings of animals everyday for as long as need to.	No side effects but can be used for coughing as well. Effectivity known by clinical sign of animal free from worms / coughing believe that is cure.
Koso *Hagenia abyssinica* J. F. G Mel	Dried leaves	Crush mix with water leave one night in morning add water when ready drench put in bottle and drench.	One litre in donkeys.	Drench	Only once see if improves if no improvement then repeat after 15 days. Use whenever worms, bloating also as purgative and given at beginning of	Worms in donkeys maybe stronger than in cattle worms look bigger and when coming out of faeces. Better appetite, body condition. Can cause severe diarrhoea if

					rainy season when grassy.	overdose and use on consecutive days.
Yejib bokolo (hyena maize) *Arisaema shimperianum* Schott	Use root not stem	Dig it up in gorge, bring root. Cannot estimate amount used, collect root chop and crush mix with water and drench juicy part (drench root).	Take whole part of root and use one root per animal but depends on size of root very much an estimate.	Drench	Will repeat every two-three until the animal gets relief, is cured and can walk and eat.	No side effects, if used early can treat dourine, as long as animal recumbent then will not cure.
Abdul Salim	Root	Brought root and washed in water chopped single root into three. Use own interpretation, spiritual interpretation (with name of god) blessed - then ask God to help make effective. Crush it together, then mix with water and sieve. It can be used for all animals, beer bottle coca cola bottle drench through nostril for equines. Not for other animals. Bless before giving.	One coke bottle, one finger of root for one animal.	Drench through nostril	Given every morning for three consecutive days horses / donkeys half cup of coffee	Will get worms out, in case coughing lots discharging for 9 days then no more case. Might elicit gastritis burns G.I.T. Ulceration of mucous membranes if beyond proper dose. Slough of mucosa looks like fatty tissue.

Table 3.3.5. Final short list for plant extractions and *in vitro* testing – Ethiopia

Plant Species	Vernacular Name	Ranking in PRA	Part of plant used	Evidence in literature	Reference
***Vernonia amygdalania* Delile**	Grawa	1 (3.3.2)	Leaves	*Ascaridia galli, Haemonchus contortus, Toxocara canis, Ancyclostoma caninum* and mixed GI nematodes *in vitro* and *in vivo*	(Adedapo et al., 2007) (Alawa et al., 2003) (Hördegen et al., 2006) (Iqbal et al., 2006b) (Siamba et al., 2007) (Yeap et al., 2010)
***Rumex abyssinicus* Jacq**	Gubdu	Key informant (3.3.3b)	Root	*Pheretima posthuma* and *Haemonchus contortus, in vitro*	(Raju and Yesuf, 2010) (Eguale et al., 2011)
***Cucumis prophetarum* L.**	Holoto/Hidi	3 (3.3.2)	Fruit	Anecdotal	(Njoroge and Bussmann, 2006) (Gachathi, 1989)
***Withania somnifera* (L.) Dunal**	Wahale	5 (3.3.2)	Root	Anecdotal and *Pheretima posthuma, in vitro*	(Jabbar et al., 2006) (Kirtiman, 2012) (Purwal et al., 2010)
***Acacia nilotica* (L.) Delile**	Cheba	NA	Fruit	*Trichostrongylus* spp. and mixed GI nematodes *in vivo*	(Bachaya et al., 2009)

Table 3.3.6. Preliminary short list for plant extractions and *in vitro* testing – UK

Plant species	**Part of plant used**	**Anthelmintic activity *in vivo***	**Host species**	**Reference**
***Carica papaya* L.**	Latex	*Trichuris muris* *Trichuris suis* *Haemonchus contortus* *Heligmosoides polygyrus*	Mice, sheep, pigs	(Stepek et al., 2007c) (Stepek et al., 2006) (Buttle et al., 2011) (Levecke et al., 2014)
***Allium Sativum* L.**	Rhizome	*Aspiculuris tetraptera*	Mouse	(Ayaz et al., 2008)
***Cocos nucifera* L.**	Tree bark	Gastrointestinal nematodes	Mouse	(Costa et al., 2010)
***Zingiber officinale* Roscoe.**	Rhizome	Gastrointestinal nematodes	Sheep	(Iqbal et al., 2006a)
***Fumaria parviflora* Lam.**	Whole plant	*Trichostrongylus colubiriformis*, *Haemonchus contortus*	Lambs	(Hordegen et al., 2003)
***Chenopodium album* L.**	Aerial parts	Trichostrongylids	Sheep	(Jabbar et al., 2007)
***Artemisia absinthium* L.**	Aerial parts	Gastrointestinal nematodes	Sheep	(Tariq et al., 2009)
***Cichorium intybus* L.**	Aerial parts	*Haemonchus contortus*, Gastrointestinal nematodes	Sheep	(Heckendorn et al., 2007) (Athanasiadou et al., 2007)
***Lespedeza cuneate* G. Don**	Aerial parts	*Haemonchus contortus* Gastrointestinal nematodes	Sheep, goats	(Lange et al., 2006) (Shaik et al., 2006)

3.4. Discussion

This study used evidence from a PRA and literature review to shortlist plants from Ethiopia and the UK for *in vitro* screening for anthelmintic activity.

3.4.1. Plant selection in Ethiopia

For Ethiopia, the four plants included in the final shortlist that were identified from the PRA were *Vernonia amygdalania* Delile, *Rumex abyssinicus* Jacq, *Cucumis prophetarum* L. and *Withania somnifera* (L.) Dunal. *Acacia nilotica* was not identified in the PRA but was present in abundance in the regions of interest and there are reports of good efficacy *in vivo* in the literature (Bachaya et al., 2009). The plant which was ranked highest for donkeys in the PRA, according to frequency with which it was mentioned in the interviews, and on opinions regarding its efficacy, was *V. amygdalina*; a perennial shrub that is abundant in tropical Africa including the study region in Ethiopia. It has been used for centuries by humans for the treatment of multiple ailments, and recent research has indicated that it may have a number of health benefits such as antimalarial, antimicrobial, antifungal, antitumor, anti-diabetic and anthelmintic effects (Ijeh and Ejike, 2011). One of the first indications of its medicinal potential was when ill chimpanzees were observed ingesting the leaves, which are very bitter and therefore not normally a preferred food (Huffman and Seifu, 1989). There have also been several studies demonstrating its potential as an anthelmintic for veterinary parasites (Adedapo et al., 2007; Alawa et al., 2010; Iqbal et al., 2006b). In the PRA there was mention of its potential toxicity in focus group discussions, with people describing severe diarrhoea and in worst cases death (Appendix 3.5.2 a). The method of preparation of *V. amygdalina* often included mixing with table salt, in order to improve palatability; it was noted by some participants that side effects were usually associated with excessive co-administration of salt.

In support of this there are no reports of significant adverse side effects in the literature (Amole et al., 2006), however toxicity studies and careful consideration of dosing would be necessary in any future *in vivo* trials.

Cucumis prophetarum and *W. somnifera* were ranked three and five respectively in the PRA for use in donkeys and there was some evidence for anthelmintic activity in the literature. *Withania somnifera* has been mentioned in previous surveys of ethnoveterinary medicines (Jabbar et al., 2006) as has *C. prophetarum* (Gachathi, 1989; Njoroge and Bussmann, 2006). In addition an *in vitro* study showed a significant effect of the aqueous extract of *W. somnifera* against the free living annelid *Pheretima posthuma*, although it should be noted that free living species are not necessarily good models for parasitic helminths (Kirtiman, 2012). *Rumex abyssinicus* was not ranked in group discussions in the PRA but was referred to by key informants in relation to treating parasitic diseases of livestock. It was included in the shortlist as it was available in large quantities in the study region, and there are studies reporting good anthelmintic activity *in vitro* against GI nematodes such a *H. contortus* (Eguale et al., 2011; Raju and Yesuf, 2010).

Some plant species were ranked highly in the PRA but were not shortlisted for extractions, namely *Hagenia abyssinica* J. F. G mel. (koso) and *Embelia schimperi* Vatke (enkoko). These were rejected because evidence for efficacy in the literature is restricted to anti-taenicidal activity in humans (Bogh et al., 1996; Desta, 1995; Githiori et al., 2004), and there were reports of significant toxicity in the PRA and in the literature (Arragie, 1983; Low et al., 1985). In addition, *Melia azedarach* L. (Milia), which was identified by one participant in focus groups for use in livestock, had supportive evidence for *in vitro* efficacy in the literature (Kamaraj et

al., 2010), but there were also reports of toxicity and hence this species was discounted (Cooper, 2007; Mendez et al., 2002a; Mendez et al., 2002b).

3.4.2. Plant selection in the UK

In the UK, the most important selection criteria was evidence of *in vivo* efficacy against GI nematodes in the literature and reports of a lack of significant toxicity. Once these criteria had been satisfied, the limiting factor for inclusion was the availability of the plant material in large enough quantities for extraction. The final species chosen to take forward were *C. papaya, A. sativum, A. absinthium, C. album* and *Z. officinale. Carica papaya* (papaya) is one of the earliest known medicinal anthelmintic plants. It has been shown to be effective *in vitro* and *in vivo* for a number of species of GI nematode(Behnke et al., 2008; Buttle et al., 2011; Levecke et al., 2014; Satrija et al., 1994). This anthelmintic activity is attributable to enzymes called cysteine proteinases (CPs), which occur naturally in the latex of fruits such as papaya, pineapple and figs (Stepek et al., 2004). Due to its previous reported effects against nematodes, and low toxicity in multiple host species, *C. papaya* was considered to be a good candidate for *in vitro* testing. As the active compound is an enzyme, the extract of this plant has to be prepared and handled in a specific way to preserve efficacy, hence it was sourced and tested separately to other shortlisted species (see Chapter 5).

Allium sativum (garlic) has been used as an anthelmintic for centuries and has been shown to have antibacterial, antiprotozoal and antifungal activity (Harris et al., 2001). There have been a number of *in vitro* and *in vivo* studies supporting its potential use of as an anthelmintic (Ayaz et al., 2008; Iqbal et al., 2001; Mantawy et al., 2011). One study has reported that chronic high level *A. sativum* consumption can lead to anaemia in horses (Pearson et al., 2005), but

considering its current use as an equine feed supplement (Williams and Lamprecht, 2008) and wide scale availability, it was selected for *in vitro* testing here. *Artemisia absinthium*, commonly called wormwood, is used in indigenous medicine as an anthelmintic, insecticide, antipyretic, anti-inflammatory, antispasmodic and antiseptic (Koul, 1997) and there is evidence of *in vivo* activity against GI nematodes in sheep (Tariq et al., 2009), as this plant was available at the time of the study, it was also chosen for *in vitro* testing. *Chenopodium album* is a highly abundant, common native UK plant which has been shown to have good efficacy *in vivo* against GI nematodes in sheep, with no significant toxicity (Akhtar et al., 1999; Jabbar et al., 2007). The final plant shortlisted for *in vitro* testing was *Z. officinale*, which has been used as a traditional Chinese medicine for centuries. There have been studies reporting *in vivo* efficacy against GI nematodes in sheep (Iqbal et al 2001; 2006), it was reported as 'safe' at the doses that have been tested and is currently marketed by livestock feed companies as an equine feed supplement (Williams and Lamprecht, 2008), which would facilitate any future *in vivo* trials.

Species that were on the preliminary shortlist but were not taken forward for *in vitro* testing included *Cocos nucifera* L., *Fumivaria parviflora* Lam., *Cichorium intybus* L. and *Lespedeza cuneate* G. Don. *Cocos nucifera* bark extract has been shown to be effective *in vitro* against *H. contortus*, but it was not found to be effective *in vivo* against sheep, with mixed infections with GI nematodes (Oliveira et al., 2009). Using a higher dose the butanol extract of the bark has subsequently shown good efficacy against GI nematodes in mice (Costa et al., 2010), however it was not shortlisted here due to concerns regarding sourcing sufficient bark for extraction. *Fumivaria parviflora* is used as an anthelmintic in ruminants in Pakistan and Saudi Arabia (Akhtar et al., 2000; Alyemeni et al., 2010; Jabbar et al., 2006). It also grows in the UK mainly in arable areas in chalky soils in the south east of the UK, although its abundance has decreased recently due to agricultural intensification. It has shown good *in vitro* and *in vivo* activity

(Hördegen et al., 2006; Hordegen et al., 2003), however at the time of the study it was not available in large quantities. The final two plants of interest, *C. intybus* and *L. cuneate*, are known as bioactive forages, as they have nutritional and medicinal values and can be fed as part of a balanced diet. The mode of action of *L. cuneate* due to high levels of condensed tannins and flavinol glycosides (Bahuaud et al., 2006; Barrau et al., 2005), whereas sesquiterpine lactones are thought to be the active compounds in *C. intybus* (Foster et al., 2006). When small ruminants are grazed on pastures rich in these forages a significant reduction in faecal egg count has been observed (Athanasiadou et al., 2007; Heckendorn et al., 2007; Lange et al., 2006). These forages have a relatively high nutritional value compared to poor quality hay, which may prohibit their use in UK donkeys, as obesity and its consequences are one of the primary causes of morbidity in this population (Burden, 2012). Hence, they were not selected for further testing here.

3.4.3. Methodology for selecting plants in Ethiopia and the UK

There were some drawbacks to both methods of plant selection used in this study. For Ethiopia the choice of plants was based on the findings of the PRA in combination with supportive evidence from literature. This method has been reported in the literature as a useful way of identifying plants with potential anthelmintic activity (Hussain et al., 2008; Lans et al., 2000; Nabukenya et al., 2014a; Tolossa et al., 2013). Here a total of 13 plant treatments were not identified to species level. In some cases this was due to the plant not being available nearby, and for other plants the content of the plant preparation described was not provided by participants; for example the ‘mixed root and leaf’ and ‘mixed root’ preparations mentioned in the focus groups. Some roots, for example Abdul Salim and Bure, were commonly found in markets and used medicinally, but there was no information regarding plant appearance, or

location. There was also a possibility, especially with key informants, who were often locally revered traditional healers, that information regarding the plants which they used for their medicines was being withheld. By triangulating the data collection process, it was hoped that knowledge gaps regarding the processing of specific plants would be eliminated as much as possible. Raising the possibility that information may have been deliberately retained also highlights the potential intellectual property issues that might arise should any new therapeutic be discovered based on local knowledge (Abramova and Greer, 2013). This is an issue that applies particularly to medicines that are not common knowledge, i.e. the remedies known only to key informants. In an attempt to address this, there was published evidence for all plants taken forward for *in vitro* testing. Nevertheless, should the plants identified here be taken forward for *in vivo* trials intellectual property issues may arise and these may need careful consideration.

In the literature review to identify plants for the UK, there were also potential issues which may have led to plant candidates being missed. Firstly, only one database was used for the initial search. In addition, when plant species were identified in the PubMed literature search, they were then entered into a google scholar search to find further references pertaining to their efficacy. In doing this it was possible to find additional publications of variable quality, often from non-peer reviewed journals that cannot be found on general databases. This may mean that there are reports demonstrating the anthelmintic efficacy of plant species that were not identified by this study. However it is likely that important species, for which the evidence is strong, would have been identified using the methods described here.

The major constraint in choosing plants with good reported activity in both Ethiopia and the UK, was their availability in large enough quantities for extraction at the time of the study. In particular the geographical location and abundance were important factors, but there were also restrictions due to the season of collection, as some plants and their parts were seasonal. Examples of plants which were not selected due to lack of availability include *Verbascum sinaiticum* Benth. and *Trigonella foenum-graecum* L. in Ethiopia, and *C. nucifera* and *F. parviflora* in the UK. Future work might therefore include repeating the study at different times of year to increase the spectrum of plants tested. Presumably in the UK, should a plant be found to be effective, it could be grown for purpose and distributed, whereas in developing countries, it would be sensible to recommend a plant treatment which is widely available at the times of year when treatment is recommended.

3.4.4. Considerations for future in vivo studies

The long term goal here, was to take promising candidates forward to *in vivo* studies. The PRA recorded information about potential side effects and methods of preparation and administration of plant treatments. It was hoped that this information could be used to design future *in vivo* studies based on currently used methods, eliminating some of the ethical considerations posed by this type of study (Githiori et al., 2003a). However, for a number of different treatments severe side effects were recorded, these ranged from diarrhoea, to neurological signs and even death (Table 3.3.4.a and b). It is probable that the perceived efficacy of a plant treatment is based on it having a purgative effect, which results in the passing of parasites in the faeces. Indeed some of the key informants described diarrhoea as part of the curative effect rather than a negative side effect. Potential toxicity of the plants makes calculation of the correct doses critical, however it was noted that preparation and dosing of the same plant species varied between groups and individuals. It would therefore be difficult

to choose a ‘currently used’ formula of the correct dose. Another important ethical concern was the method of administration (usually oral or nasal drenching) which can pose a serious risk of aspiration pneumonia in equids (Scarratt et al., 1998). All of these issues suggest that strict quality control procedures, alongside dosing and administration protocols, would need to be in place to prevent adverse side effects. As a consequence their distribution would best be carried out by government clinics, thus reducing availability for the most remote and impoverished people.

The PRA here revealed that plant treatments were not administered to equids with such frequency as they are to other livestock, see Table 3.3.1. As this is the first study to focus a PRA of anthelmintic practices specifically on equids, it is not known if this is also the case in other developing countries. The reason behind this discrimination may be due to the fact that equids are not used as production animals, and therefore their body condition is not of such importance from an economic point of view. In addition disease caused by intestinal parasitism in donkeys may not be as obvious to owners as with other livestock species. Interestingly body condition scores of donkeys have been shown to be highest in the rainy season when grass has a higher nutrient value, despite a concurrent rise in faecal egg count (FEC) at this time due to the abundance of food (Getachew et al., 2008; Yoseph et al., 2005). This suggests that if a donkey has enough food then parasites do not tend to cause a poor body condition. However other clinical syndromes associated with high parasite burdens which are known to occur in developing countries, such as colic and sudden death, may not be directly associated with parasitism by owners (Borji et al., 2014). In some small ruminant systems in developing countries, proxies for parasite infections based on clinical examination, have been defined to provide an indication for treatment (Nabukenya et al., 2014b; Vilela et al., 2012). This is especially useful where there are no facilities to perform FECs. Future work in working equids

to improve awareness surrounding parasitic disease might include developing similar proxies for GI parasitism in equids.

3.5. Conclusion

This study has used evidence based techniques to identify plants from Ethiopia and the UK with potential anthelmintic activity. These plant candidates were taken forward for *in vitro* testing in the next stage of the project. In addition the PRA revealed potential issues surrounding future *in vivo* trials and recommending plant based treatments for use in working equids.

4. *IN VITRO* EVALUATION OF PLANT EXTRACTS AGAINST CYATHOSTOMINS

4.1. Introduction

Scientific evidence supporting the anti-parasitic properties of plants comes mostly from *in vitro* studies (Athanasiadou and Kyriazakis, 2004), the advantage being that large numbers of plant extracts can be tested at relatively low cost. In equids the egg hatch test (EHT) and larval migration inhibition test (LMIT) have shown the best correlation with *in vivo* anthelmintic efficacy, see Section *1.2.1.1*. When this study was initiated there were no reports of the use of bioactive plants for treating cyathostomin infections, although subsequently Payne et al, (2013) demonstrated moderate to good *in vitro* efficacy for 17 crude plant extracts with the activity for 12 of them attributed to tannins. Here, the EHT and LMIT, were used to evaluate plant candidates sourced from the UK and Ethiopia in Chapter 3, against cyathostomin eggs and third stage larvae (L3). Tannin inhibition was performed on the most efficacious plant extracts and tests repeated to ascertain whether tannins were the active component.

4.2. Materials and Methods

4.2.1. Identification of plants for use in in vitro tests

Plants with potential anthelmintic activity for *in vitro* testing were identified from Ethiopia and the UK as described in Chapter 3. For Ethiopia the shortlist was: *Vernonia amygdalania* Delile (leaves), *Rumex abyssinicus* Jacq (root), *Cucumis prophetarum* L. (fruit), *Withania somnifera* (L.) Dunal. (root) *Acacia nilotica* (L.) Delile (fruit). For the UK the shortlist was: *Allium sativum* L. (garlic) (rhizome), *Artemisia absinthium* L. (aerial parts), *Chenopodium album* L. (aerial parts) and *Zingiber officinale* Roscoe. (ginger) (rhizome).

4.2.2. Preparation of crude extracts of plants for testing effects on cyathostomins

Ethiopia: Collection of fresh plant material was carried out in November 2011 in the Oromia region of Ethiopia, within a radius of 90 km of Addis Ababa. Approximately 4 kg, per plant species, of plant material were dried for five days at 50 ºC and then milled to particles of less than 1 mm. The identity of the plants was confirmed by the Ethiopian National Herbarium, Addis Ababa. Dried milled plant material was weighed and dissolved in 70 % methanol (Sigma, UK) in a 1:10 ratio of grams to millilitres. The mixture was macerated using a bench top shaker (Grant, UK) at 300 rpm for 24 h at room temperature, then vacuum filtered to remove solid plant material; the solvent was removed by rotary evaporation leaving solid crude extract (Buchi, UK). These extracts were stored at room temperature in amber vials before transportation to the University of Liverpool, UK. *UK:* Plant material was collected between November 2011 and August 2012 from the University of Liverpool Botanical Garden, Ness, UK. The plant material was dried by lyophilisation to reduce the potential for degradation of active components (Mechatech, UK). The extractions were carried out following the method described for Ethiopian plants, above. Solid crude extracts were kept at 4 ºC for no longer than four months before testing and were dissolved as required in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, UK) and dH_2O (Sigma-Aldrich, UK) at 200 mg/ml to give stock solutions for use in *in vitro* tests. These were stored at 4 ºC whilst in use, and stock solutions were kept for no longer than one week. Working solutions of crude plant extracts were made by serial dilution of the stock in either dH_2O or DMSO: two solvents were used as some extracts showed poor solubility in an aqueous solvent. Within each test, these were added to dH_2O in a 5:100 ratio to give final concentrations of 0, 0.04, 0.08, 0.16, 0.31, 0.62, 1.25, 2.5, 5, 10 mg/ml.

4.2.3. Parasite sample collection

Faecal samples were collected from donkeys at the Donkey Sanctuary UK, and strongyle eggs and third stage larvae (L3) were collected, stored and extracted/cultured as described in Section *2.2.1* and *2.2.2.*, respectively. Once extracted, eggs were used immediately in tests. Once cultured L3, were kept at 4 ºC and used within eight weeks.

4.2.4. Larval migration inhibition test

The LMIT was initially performed on L3 samples from two donkeys for each extract. If an efficacy of greater than 20 % reduction in larval migration was seen, the test was repeated on L3 samples from a further two donkeys. The protocol for testing plant extracts in the LMIT was adapted from the LMIT with IVM as described in general Section *2.4.1.* At the incubation stage of the test, a total of 10 µl of each working concentration (Section *4.2.2.*) of plant extract in dH_2O (Sigma-Aldrich, UK) or DMSO (Sigma-Aldrich, UK) was added to 190 µl of the L3 sub-samples. Negative controls of DMSO (5 %) and dH_2O and a positive control of 150 µg/ml (IVM) (Sigma-Aldrich, UK) were included, to demonstrate that the test was accurately differentiating motile from immotile L3. The L3 were incubated at 26 ºC for 24 h. During the migration stage, 100 µl of working concentration of plant extract in dH_2O and DMSO were added to dH_2O to make 1,910 µl of plant extract solution at the final concentrations described in Section *4.2.2.* This volume was added in duplicate to the migration wells and 90 µl of the L3/extract suspension from each eppendorf added in duplicate to the migration chambers of the corresponding extract concentration. The LMIT was then continued as described in Section *2.4.1.*

4.2.5. Egg hatch test

As with the LMIT, the EHT was initially performed on egg samples from two donkeys for each extract. If an efficacy of greater than 20 % reduction in egg hatch was seen, the test was repeated on egg samples from a further two donkeys. The test was adapted from the EHT with TBZ as described in Section *2.3.1*. Instead of TBZ, 100 µl of each working concentration of plant extract in dH_2O and DMSO were added to dH_2O, to make up 1900 µl of plant extract solution at the final concentrations described in Section *4.2.2*. This was added in duplicate to a 24-well plate (Nunc, UK), and 100 µl of egg suspension added. Duplicate negative controls for dH_2O and DMSO (5 %) and positive controls of thiabendazole (TBZ) at 1.5 µg/ml (Sigma-Aldrich, UK) were included. The test was completed as described Section *2.3.1*.

4.2.6. Inhibition of tannins with polyvinylpolypyrrolidine

Preliminary tannin inhibition tests were performed on four extracts from the UK and Ethiopia which had shown good efficacy in the EHT; *A. nilotica, C. prophetarum, A. sativum* and *C. album.* These tests were performed two years after initial testing (extracts had been stored at -20 ºC for this period and were thawed before use). The working concentrations of extract in dH_2O were prepared from the extract as described in Section *4.2.2.* They were mixed with polyvinylpolypyrrolidine (PVPP) (Sigma-Aldrich, UK) at a concentration of 50 mg/ml and incubated overnight at room temperature. The extracts were then centrifuged at 200 *g* for 5 min and the supernatant used for inclusion in tests. The EHT was continued according to the method in Section *4.2.5*, with untreated extract as a control.

4.2.6. Statistical analysis

A minimum threshold of 70 % egg hatch or larval migration in control wells was used to determine inclusion of data in subsequent analyses. If this was not achieved, tests were

repeated. Logarithmic dose response curves were plotted for each test using Microsoft Office Excel, 2010. The mean maximum reduction in percentage larval migration or egg hatch, compared to the negative controls (MPR), was calculated for each plant extract. Repeated measures analysis of variance (ANOVA) with the Greenhouse-Geisser correction for sphericity was carried out on data for each plant extract to detect statistically significant differences between controls and treated parasites. PROBIT regression analysis, with correction for natural mortality, was performed on data to give a median effective concentration (EC-50 value) for each plant extract. Where maximum efficacy was low, data did not accurately fit a PROBIT model and therefore an EC-50 value was not presented. All statistical data analysis was performed using SPSS version 21 (SPSS Inc., Chicago IL).

4.3. Results

4.3.1. In vitro evaluation of candidate plants

The results of *in vitro* testing are divided by country, test and solvent. The maximum percentage reduction (MPR) in egg hatch/larval migration seen at the highest concentration of extract for Ethiopian and UK plant extract is shown in Figure 4.3.1.a-d. These readings were taken between 2.5-10 mg/ml of extract, as readings were not always possible at the highest concentrations due to poor solubility of some extracts. Table 4.3.1. details the MPR in egg hatch/larval migration, the results of repeated measures ANOVA and EC-50 values calculated using PROBIT analysis for each extract. Figure 4.3.2. shows dose response curves for extracts with a maximum efficacy greater than 60 %. In all tests, there was 100 % reduction in larval migration and egg hatch in the positive control wells containing IVM and TBZ, respectively.

4.3.1.1. Ethiopian plant extracts

For the LMIT there was a statistically significant difference in migration between negative controls and L3 exposed to plant extract with *R. abyssinicus* in dH_2O and DMSO (p=<0.001:0.010), but in no other extracts (Table 4.3.1.a). The maximum percentage reduction in migration in these tests was 50.2 (±14.2) and 56.0 (±12.3) %, respectively (Figure 4.3.1.b). The EC-50 values could not be calculated, as the data did not accurately fit a PROBIT model. For the EHT there was a statistically significant difference in egg hatch between negative controls and eggs exposed to plant extract with *A. nilotica* in dH_2O and DMSO (p=<0.001:<0.001), *C. prophetarum* in dH_2O and DMSO (p=<0.001:0.036) and *R. abyssinicus* in dH_2O and DMSO (p=0.016: <0.001) (Table 4.3.1.a). The maximum percentage reduction in egg hatch in these tests was; 96.4 (±1.7), 77.7 (±14.1), 93.4 (±8.6), 49.1 (±7), 29.0 (±5.9) and 74.8 (±6.1), % respectively (Figure 4.3.1.a). The EC-50 values were 0.18 (CI 0.06-0.3) and 0.2 (CI 0-2.6) mg/ml for *A. nilotica* in dH_2O and DMSO, 1.1 (CI 0.9-1.4) mg/ml for *C. prophetarum* in dH_2O and 1.1 (CI 0.2-2.2) mg/ml for *R. abyssinicus* in DMSO (Table 4.3.1.a). It was not possible to calculate an accurate EC-50 value for *C. prophetarum* or *R. abyssinicus* in DMSO as the maximum percentage efficacy was below 60% and thus the data did not fit a PROBIT model.

4.3.1.2. UK plant extracts

For the LMIT there was a statistically significant difference in migration between negative controls and L3 exposed to plant extract with *A. sativum* in dH_2O and DMSO (p=0.029:<0.001), *A. absinthium* in dH_2O (p=<0.001), *C. album* in dH_2O (p=<0.001) and *Z. officinale* in dH_2O and DMSO (p=0.007: <0.001) (Table 4.3.2.b). The maximum percentage efficacy in these tests was; 23.0 (±13.3), 53.3 (±7.9), 24.5 (±7.6), 61.7 (±10.3), 30.3 (±10.3) and 28.7 (±4.5) % (Figure 4.3.1d). The EC-50 value for *C. album* was 8.91 (CI 7.06-13.21)

mg/ml (Table 4.3.1.b). It was not possible to calculate an accurate EC-50 value for the other extracts as efficacy was relatively low and the data did not fit a PROBIT model. For the EHT there was a statistically significant difference in percentage egg hatch between negative controls and eggs exposed to plant extract with *A. sativum* in dH_2O and DMSO (p=<0.001: <0.001), *A. absinthium* in dH_2O (p=0.009), *C. album* in dH_2O (p=<0.001) and *Z. officinale* in dH_2O and DMSO (p=<0.001: <0.001) (Table 4.3.1.b). The maximum percentage efficacy in these tests was; 100 (±0), 100 (±0), 17.2 (±8.3), 88.7 (±9.1), 86.9 (±10.1) and 95.9 (±6.2) %, respectively (Figure 4.3.1.c). The EC-50 values were 1.99 (CI 1.56-2.31), and 1.09 (CI 0.89-1.29) mg/ml for *A. sativum* in dH_2O and DMSO, 2.29 (CI 1.95-2.97) mg/ml for *C. album* in dH_2O and 0.9 (CI 0.55-1.26) and 0.3 (CI 0.22-0.38) mg/ml for *Z. officinale* in dH_2O and DMSO (Table 4.3.1.b). It was not possible to calculate an accurate EC-50 value for *A. absinthium* in water as the efficacy was low and hence the data did not accurately fit a PROBIT model.

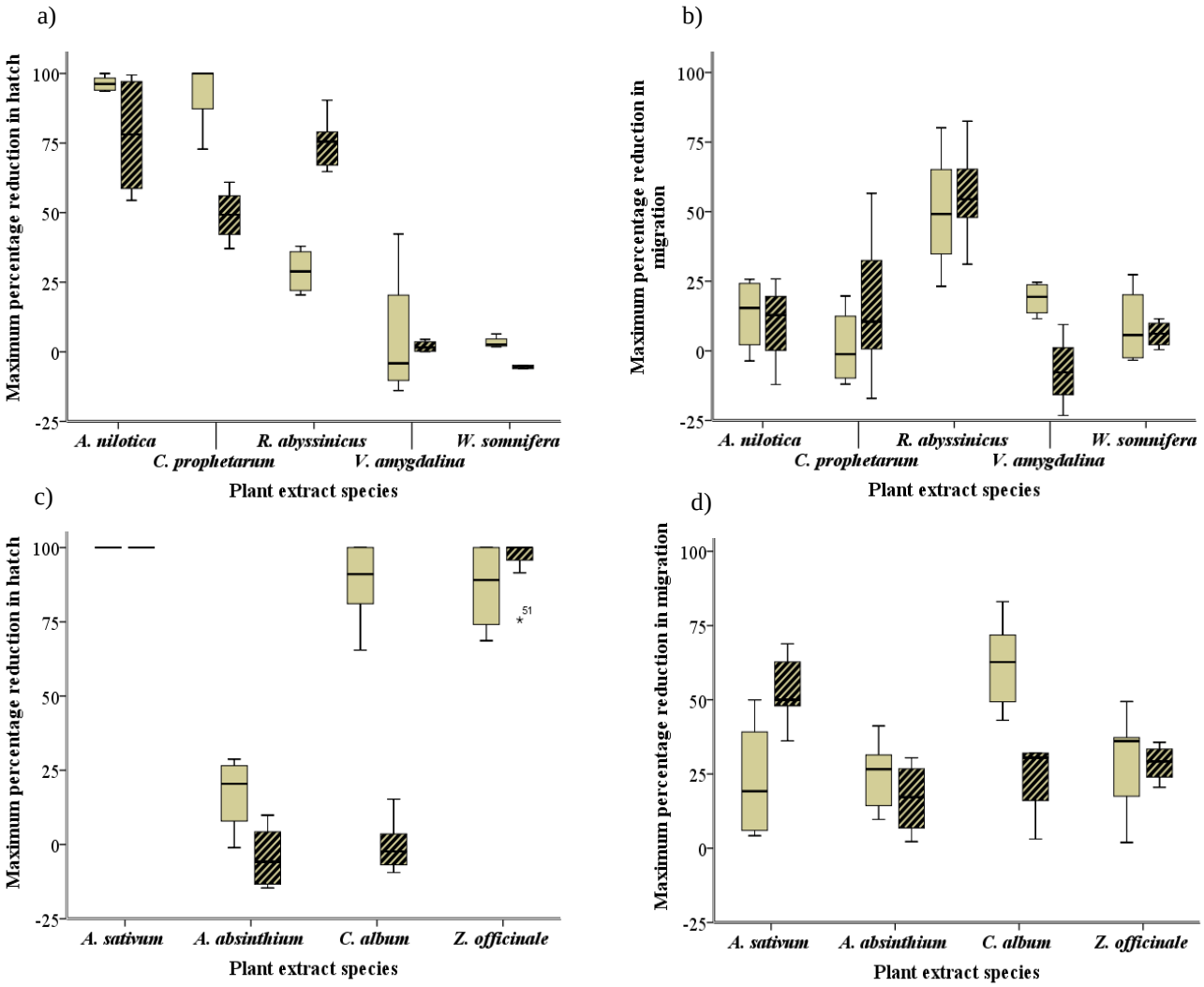

Figure 4.3.1. Graphs showing the mean maximum percentage reduction (MPR) in hatch and migration compared to the controls in the egg hatch test (EHT) (a,c) and larval migration inhibition test (LMIT) (b,d) seen at the highest concentration of extract for Ethiopian (a-b) and UK (c-d) crude plant extracts in distilled water (dH2O) (grey) and dimethyl sulfoxide (DMSO) (hatched). Each extract was tested either in two separate parasite samples (if there was less than 20% efficacy in the first test) or in four parasite samples (if there was greater than 20% efficacy in the first test).

Table 4.3.1.a. Results of repeated measures ANOVA (significant P values are highlighted bold), mean maximum percentage reduction (MPR) in larval migration or egg hatch and EC-50 value, where relevant, for Ethiopian plant extracts in distilled water (dH_2O) and dimethyl sulfoxide (DMSO) in the egg hatch test (EHT) and larval migration inhibition test (LMIT). N = number of replicate parasite samples.

Plant extract	**Assay**	**Solvent**	**Repeated measures ANOVA P value**	**Mean maximum percentage reduction in hatch or migration from controls (%) ±standard error**	**EC-50 value with confidence intervals (mg/ml)**
***Acacia nilotica* (L.) Delile.**	EHT	dH2O	**<0.001**	96.4 ±1.7 (n=4)	0.18 (0.06-0.3)
	EHT	DMSO	**<0.001**	77.8 ±14.1 (n=4)	0.2 (0-2.6)
	LMIT	dH2O	0.13	13.2 ±9.6 (n=2)	-
	LMIT	DMSO	0.22	9.9 ±11.2 (n=2)	-
***Cucumis prophetarum* L.**	EHT	dH2O	**<0.001**	93.4 ±8.6 (n=4)	1.1 (0.9-1.4)
	EHT	DMSO	**0.036**	49.1 ±7.0 (n=4)	-
	LMIT	dH2O	0.515	1.3 ±10.7 (n=2)	-
	LMIT	DMSO	0.243	15.8 ±16.9 (n=2)	-
***Rumex abyssinicus* Jacq.**	EHT	dH2O	**0.016**	29.0 ±5.9 (n=4)	-
	EHT	DMSO	**<0.001**	74.8 ±6.1 (n=4)	1.1 (0.2-2.2)
	LMIT	dH2O	**<0.001**	50.2 ±14.2 (n=4)	-
	LMIT	DMSO	**0.010**	56.0 ±12.3 (n=4)	-
***Vernonia amydalina* Delile.**	EHT	dH2O	0.285	5.0 ±17.9 (n=2)	-
	EHT	DMSO	0.696	1.9 ±1.5 (n=2)	-
	LMIT	dH2O	0.143	18.7 ±4.3 (n=2)	-
	LMIT	DMSO	0.162	-7.3 ±9.5 (n=2)	-
***Withania somnifera* (L.) Dunal.**	EHT	dH2O	0.396	3.4 ±1.5 (n=2)	-
	EHT	DMSO	0.341	-5.5 ±0.6 (n=2)	-
	LMIT	dH2O	0.480	8.8 ±10.2 (n=2)	-
	LMIT	DMSO	0.173	6.0 ±3.4 (n=2)	-

Table 4.3.1.b. Results of repeated measures ANOVA (significant P values are highlighted bold), mean maximum percentage reduction (MPR) in larval migration or egg hatch and EC-50 value, where relevant, for UK plant extracts in distilled water (dH_2O) and dimethyl sulfoxide (DMSO) in the egg hatch test (EHT) and larval migration inhibition test (LMIT). N = number of replicate parasite samples.

Plant extract	Assay	Solvent	Repeated measures ANOVA P value	Mean maximum percentage reduction in hatch or migration from controls (%) ± standard error	EC-50 value with confidence intervals (mg/ml)
***Allium sativum* L.**	EHT	dH2O	**<0.001**	100 ±0 (n=4)	2.0 (1.6-2.3)
	EHT	DMSO	**<0.001**	100 ±0 (n=4)	1.1 (0.9-1.3)
	LMIT	dH2O	**0.029**	23.0 ±13.3 (n=4)	-
	LMIT	DMSO	**<0.001**	53.3 ±7.9 (n=4)	-
***Artemisia absinthium* L.**	EHT	dH2O	**0.009**	17.2 ±8.3 (n=2)	-
	EHT	DMSO	0.157	-4.3 ±7.0 (n=2)	-
	LMIT	dH2O	**<0.001**	24.5 ±7.6 (n=4)	-
	LMIT	DMSO	0.104	16.8 ±8.8 (n=2)	-
***Chenopodium album* L.**	EHT	dH2O	**<0.001**	88.7 ±9.1 (n=4)	2.3 (1.9-2.7)
	EHT	DMSO	0.094	-0.4 ±6.3 (n=2)	-
	LMIT	dH2O	**<0.001**	61.7 ±10.3 (n=4)	8.9 (7.1-13.2)
	LMIT	DMSO	0.053	24 ±9.9 (n=2)	-
***Zingiber officinale* Roscoe.**	EHT	dH2O	**<0.001**	86.9 ±10.1 (n=4)	0.9 (0.6-1.3)
	EHT	DMSO	**<0.001**	95.9 ±6.2 (n=4)	0.3 (0.2-0.4)
	LMIT	dH2O	**0.007**	30.3 ±10.3 (n=4)	-
	LMIT	DMSO	**<0.001**	28.7 ±4.5 (n=4)	-

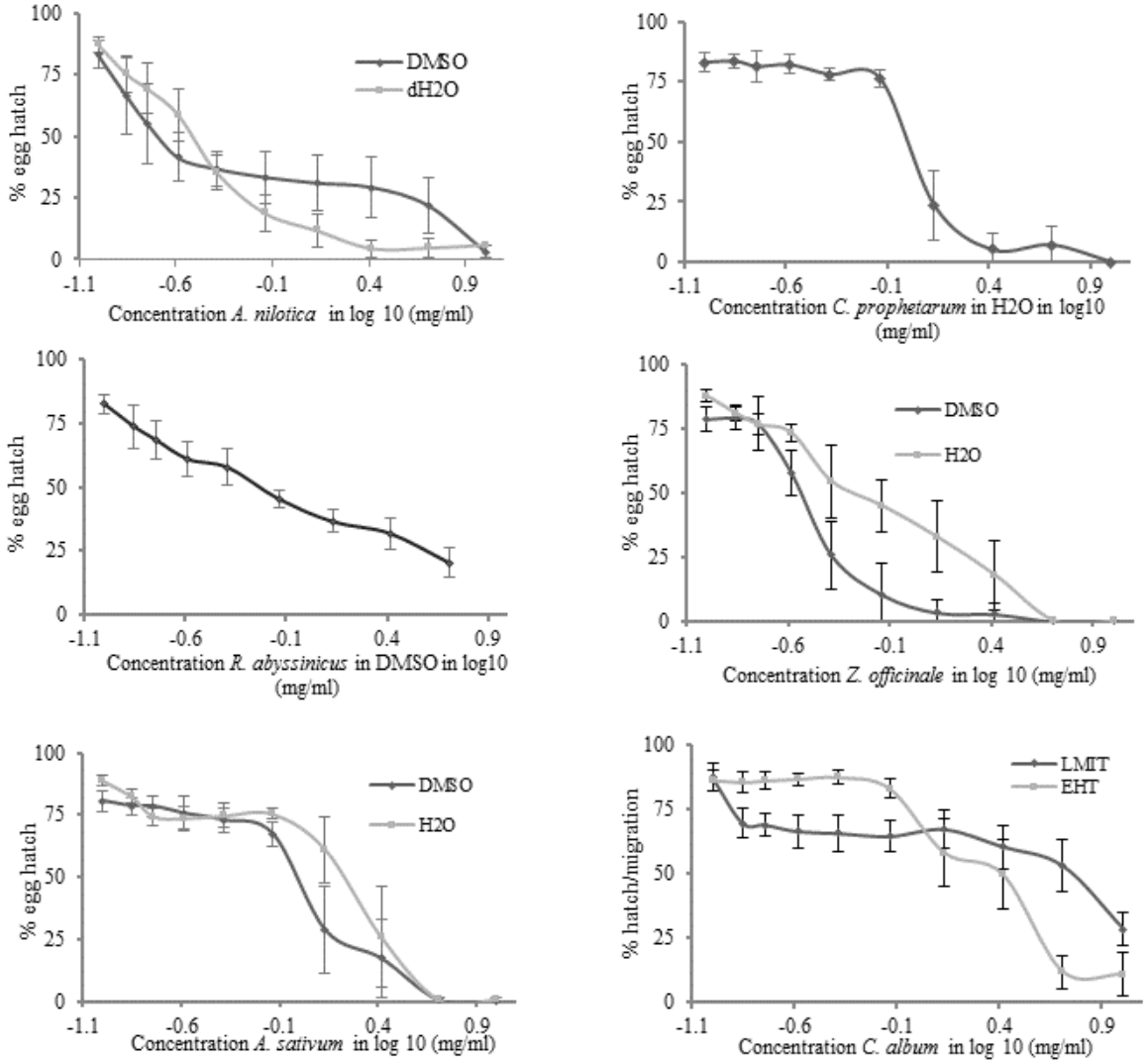

Figure 4.3.2. Dose response curves for plant extracts with a maximum percentage efficacy greater than 60 % in the egg hatch test (EHT) or larval migration inhibition test (LMIT), in either distilled water (H_2O) or dimethyl sulfoxide (DMSO).

4.3.1.3. Tannin inhibition with PVVP

Logarithmic dose response curves for tests with and without pre-incubation with PVPP are shown in Figure 4.3.3.a-d. For *A. nilotica* without PVPP the EC-50 value was 0.12 (CI 0.03-0.24) mg/ml (similar to original observation, see Table 4.3.1.a.). Controls were found to be significantly different from treated parasites using repeated measures ANOVA, p=0.001 and the maximum percentage reduction in egg hatch was 100 %. The maximum percentage reduction in egg hatch after incubation with PVPP was only 19.56 (±0.07) and the difference between controls and treated parasites was no longer significant, p=0.161. This suggests that tannins are the main active components in the *A. nilotica* extract. For *A. sativum,* the EC-50 value calculated was 4.70 (CI 3.97-5.31) mg/ml, this is higher than that previously calculated for the same extract, see Table 4.3.1.b, suggesting that the extract has lost some activity over time. The mean percentage reduction in migration at the maximum concentration was 97.53 (±0.46) %. The EC-50 increased slightly after incubation with PVPP, to 5.42 (CI 4.14-6.39) mg/ml and the MPR reduced slightly to 93.60 (±6.40) %, suggesting that, although they may have a small effect, tannins are not the main active compound in *A. sativum.* For *C. prophetarum* extract, incubation with PVPP had no impact on efficacy with EC-50 values of 0.815 (CI 0.73-0.91) and 0.892 (0.73-0.92) mg/ml before and after PVPP incubation, respectively, and MRPs of 100%. All tests with *A. sativum* and *C. prophetarum* showed a significant effect of treatment compared to controls in the repeated measures ANOVA, p=<0.001. Unfortunately, the efficacy of *C. album* was not reproducible in the EHT in the untreated control extract, presumably due to degradation of the active compound, hence results from this extract are not presented.

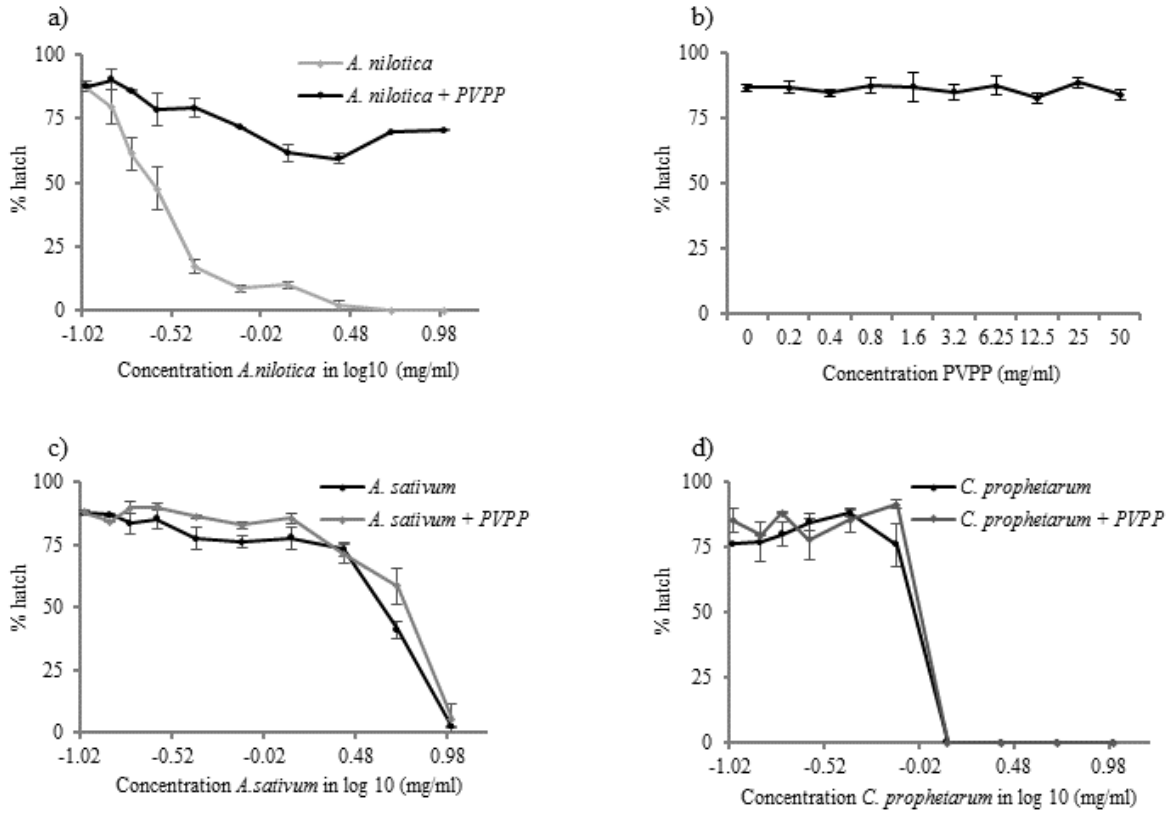

Figure 4.3.3. The results of pre-incubation with polyvinylpolypyrrolidine (PVPP) in the egg hatch test (EHT) for *Acacia nilotica* a), *Allium sativum* c) and *Cucumis prophetarum* d). In addition the effect of a PVPP titration is shown in b), to demonstrate it has no effect alone.

4.4. Discussion

This study has evaluated plants from two different regions for anthelmintic activity against cyathostomin eggs and L3. After an evidence-based shortlisting process and systematic *in vitro* testing using two techniques and two solvents, seven out of nine plant extracts selected showed anthelmintic activity *in vitro*.

4.4.1. The effect of Ethiopian plant extracts in the egg hatch test and larval migration inhibition test

Three of these were Ethiopian plant extracts; the most potent of which was *A. nilotica*. *Acacia nilotica* is a native African tree, commonly found in Ethiopia (Thulin, 1983). Its fruiting pods are distinctive and, in some regions, these are fed to ruminants as a forage supplement (Uguru et al., 2014). Here, the extract of the dried pods showed a strong inhibitory effect in the EHT in both dH_2O and DMSO, with a maximum percentage reduction in egg hatch of 96.4 (±1.7) and 77.7 (±14.1) %, respectively, and EC-50 values of 0.18 (CI 0.06-0.3) and 0.2 (CI 0-2.6) mg/ml. *Acacia nilotica* pods are high in tannins, which have been shown to have an anthelmintic effect in a number of studies (Hoste et al., 2006; Moreno et al., 2012; Williams et al., 2014). Here repeating the EHT after incubation of *A. nilotica* with PVPP to inhibit tannin activity led to a marked reduction in efficacy, suggesting that tannins are at least in part, the active component of this extract. A study on sheep gastrointestinal (GI) nematodes has shown *A. nilotica* fruit to have an EC-50 of 0.513 and 0.195 mg/ml in the EHT and larval development test (LDT), respectively, which is comparable to the findings here (Bachaya et al., 2009). The same study demonstrated that administration of the extract to sheep naturally infected with GI nematodes resulted in a mean reduction in faecal egg count (FEC) of 78 % 13 days after treatment. Therefore, given the abundance of *A. nilotica* in Ethiopia, it may be a viable option

to use as a bioactive forage in equids when there is no access to synthetic anthelmintics. However, tannins fed at high levels have been associated with anti-nutritional effects in ruminants (Hoste et al., 2006). As the effect of tannins on equids has not been investigated, *in vivo* trials to evaluate efficacy and safety in these species would be essential before any recommendations could be made.

The *C. prophetarum* crude fruit extract also showed a strong inhibitory effect in the EHT, which was greater in dH_2O than DMSO, with maximum percentage reduction in hatch of 93.4 (±8.6) and 49.1 (±7) %, respectively, and an EC-50 value of 1.1 (CI 0.9-1.4) mg/ml in dH_2O. It is a tropical plant of the family *Cuburbitaceae* native to Africa and the Arabian Peninsula. It has a small yellow/green fruit which is very bitter due to high levels of cucurbitacins, which can be toxic at high levels (Afifi et al., 1999). It was clear from the PVPP inhibition study here that tannins do not play a role in this extract's anthelmintic activity. The cucurbitacins are known to be the cause of its cytotoxic effect and this has led to investigations for its potential use as an anti-tumor agent (Ayyad et al., 2011). It is possible that these compounds are also responsible for the effects seen here, and further work would be needed to confirm this. *Cucumis prophetarum* has been identified in previous surveys of medicinal plants in developing countries (Gachathi, 1989; Njoroge and Bussmann, 2006), however, this is the first study to demonstrate its anthelmintic activity. Plants in the same family, *Cucurbitaceae,* are also used as taenicidals (Li et al., 2012), and a recent study identified a related species *Cucumis moschata* Duch. as having an anthelmintic effect against *Haemonchus contortus in vitro* (Marie-Magdeleine et al., 2009). *Cucumis prophetarum* was identified from the PRA in the Oromia region of Ethiopia (Scantlebury et al., 2013), and is currently used as an anthelmintic there, although there were some reports of toxicity at high doses. Thus it would be possible to

encourage its use in equids, however further work is necessary to evaluate its efficacy and toxicity *in vivo.*

Rumex abyssinicus root was one of the medicinal plants, identified from interviews with traditional healers, as being used in the Oromia region and was the only extract from Ethiopia to have a significant effect in both the LMIT and the EHT in dH_2O and DMSO. The efficacy measured was higher in DMSO, the maximum percentage reduction in migration and hatch being found to be 50.2 (±14.2), 56 (±12.3), 29.0 (±5.9) and 74.8 (±6.1) %, respectively, in these tests. The EC-50 for the EHT in DMSO was 1.1 (CI 0.2-2.2) mg/ml. *Rumex abyssinicus* is a perennial shrub which commonly grows in the highland regions of Eastern Africa (Hedberg, 2000). Its roots are edible and are used as a wild vegetable; it usually grows as a weed around the edge of plantations (Katende et al., 1999; Ruffo et al., 2002). There is *in vitro* evidence for its medicinal use as an antibiotic and an anti-inflammatory (Getie et al., 2003), and a study showing anthelmintic activity *in vitro* (Eguale et al., 2011), where *Rumex abyssinicus* had a significant inhibitory effect on *H. contortus* egg hatch and larval development. Indeed, in the EHT, the EC-50 value was comparable to the one measured here for cyathostomins. Further analysis needs to be done to reveal the active component of this extract against cyathostomins; previous studies have found the roots to be high in crysophanol, physcion and emodin, and so these may be of interest in future *in vitro* tests (Midiwo et al., 2002). *Rumex abyssinicus* is widely available in Ethiopia and considered safe in humans (Giday et al., 2007; Yineger et al., 2007), given the encouraging results here, further work is indicated to validate its anthelmintic activity in equids *in vivo.*

The remaining two extracts from Ethiopia, *V. amygdalina* and *W. somnifera*, did not show a significant anthelmintic effect in the LMIT or EHT. *Vernonia amydalina* was the most commonly reported plant-based anthelmintic used in livestock in one study within the Oromia region (Scantlebury et al., 2013). There have been studies demonstrating its anthelmintic effect *in vitro* against *H. contortus* (Ademola and Eloff, 2011); and *in vivo*, in dogs and calves, against mixed, natural GI helminth infections (Adedapo et al., 2007; Alawa et al., 2010). Possible reasons for the differences indicated here, could be that the active compound was denatured in the drying and extraction process, or that the extract has an effect on a different lifecycle stage to those tested. There may also be another mechanism by which this extract reduces parasite egg output *in vivo*, such as a purgative effect. Indeed, one of its reported side effects is diarrhoea, which would support this hypothesis (Scantlebury et al., 2013). *Withania somnifera* also had no effect in either test; this plant was ranked five in the PRA from the region (Scantlebury et al., 2013). It is also mentioned in surveys of ethnoveterinary plants in Pakistan (Jabbar et al., 2006), and has shown activity *in* vitro against *Pheretima posthuma* (earthworm) (Kirtiman, 2012), but there have been no studies to date evaluating its anthelmintic activity against GI nematodes.

4.4.2. The effect of UK plant extracts in the egg hatch test and larval migration inhibition test

For the UK-sourced plant extracts, all four showed significant anthelmintic activity in the LMIT and/or EHT. *Allium sativum* (garlic) showed a strong inhibitory effect in both dH_2O and DMSO in the EHT, with maximum percentage reduction in hatch of 100 (±0) % for both, and an EC-50 of 2.0 (CI 1.6-2.3) and 1.1 (CI 0.9-1.3) mg/ml, respectively. There was also a moderate effect in the LMIT in DMSO with a maximum percentage reduction in migration of 53.3 (±7.9) %. *A. sativum* has been used as a traditional anthelmintic for centuries (Rivlin,

2001) and has been shown to have a wide variety of other effects such as antiprotozoal and antifungal activity (Harris et al., 2001). There have been a number of studies supporting the use of *A. sativum* as an anthelmintic (Ahmed et al., 2013; Ayaz et al., 2008; Iqbal et al., 2001). The most convincing study described an efficacy of 91 % *in vivo* against adult *Aspiculuris tetraptera* in mice (Ayaz et al., 2008). Here, it was shown that tannins are not the main active compound in garlic, as incubation with PVPP had little effect on efficacy. Allicin is a major constituent of garlic and has been shown to have a potent antimicrobial effect (Fujisawa et al., 2009); however, there are many other potential bioactive compounds in garlic so further testing would be required to identify which has/have anthelmintic activity. Garlic supplements are already widely marketed in the equine feed industry (Williams and Lamprecht, 2008), therefore it could be relatively straightforward to test and market this extract. There is evidence, however, that chronic garlic consumption at a maximum voluntary intake of 0.25 mg/kg can lead to anaemia in horses, due to an oxidative effect on red blood cells (Pearson et al., 2005). Therefore, *in vivo* trials would be necessary to determine the dose at which an anthelmintic effect is seen against parasitic stages, and to ensure that anaemia is not induced.

Chenopodium album leaf and stem extract also showed a strong inhibitory effect in the EHT and moderate in the LMIT, but only in dH_2O. The maximum percentage reduction in hatch and migration were 88.7 (±9.1) and 61.7 (±10.3) %, respectively, with EC-50 values of 2.3 (CI 1.9-2.7) and 8.9 (CI 7.1-13.2) mg/ml. *Chenopodium album* is a common weedy plant native to Europe, and traditionally it has been used as food for humans and animals (Luczaj and Szymanski, 2007; Raghuvanshi et al., 2001). Before the widespread introduction of synthetic anthelmintics, oil of *Chenopodium* was listed in the British veterinary codex (1953;1965) as a veterinary anthelmintic. This oil was derived from a related species *Chenopodium*

ambrosioides L. Subsequent studies have demonstrated the anthelmintic effect of extracts of *C. ambrosioides in vitro* on *H. contortus* and *Heligmosoides bakeri* (Eguale and Giday, 2009; Pone et al., 2011); however, its use *in vivo* has been associated with toxicity attributable to the active compound, ascaridole (MacDonald et al., 2004). In contrast, studies with *C. album* have demonstrated *in vivo* anthelmintic effects with no apparent toxicity. One study demonstrated a mean reduction in FEC of 87 % in sheep naturally infected with mixed GI nematode species after a dose of 2 g/kg of aqueous extract of *C. album* (Akhtar et al., 1999). The fact no side effects have been observed and that an aqueous extract is used rather than an oil, may indicate that the active compound in aqueous extract of *C. album* is not ascaridole, but further work is needed to confirm this. A later study demonstrated a mean FEC reduction of 82 % in sheep given a hydro-alcoholic extract of this plant at 3 g/kg (Jabbar et al., 2007). Hence, there appears to be good evidence to support efficacy of *C. album in vivo.* Given its abundance in the UK and the ease with which it can be grown, further investigation into the efficacy of this plant extract against nematodes in equids is indicated.

Zingiber officinale (ginger) rhizome was shown to have an anthelmintic effect in the EHT, in both dH_2O and DMSO, with maximum percentage reductions in hatch of 86.9 (±10.1) and 95.9 (6.2) % and EC-50 values of 0.9 (CI 0.6-1.3) and 0.3 (CI 0.2-0.4) mg/ml, respectively. There was also a mild effect in the LMIT, with a mean reduction in migration of 30.3 (±10.3) and 28.7 (±4.5) %, respectively. *Zingiber officinale* has been used as a Chinese traditional medicine for over 2500 years (Wang and Wang, 2005). Studies have demonstrated that it possesses antimicrobial, anti-oxidant, anti-tumour, anti-emetic and anthelmintic activity and it has been shown to be relatively safe toxicologically (Ali et al., 2008). Iqbal et al. (2001) showed 100 % efficacy of methanol extracts *in vitro* against *H. contortus.* Subsequently, the same group

demonstrated a mean reduction in FEC of 66.6 % in sheep naturally infected with mixed GI nematode species infections, using 1-3 mg/kg of the crude aqueous extract of dried ginger (Iqbal et al., 2006a). The main bioactive constituents of *Z. officinale* rhizome appear to be 6-gingerol and 6-shogaol (Connell and McLachlan, 1972), but further work would be needed to elucidate which was the active compound here. Given its wide availability and palatability, it could be considered for further trials in equids.

The final UK extract, *A. absinthium*, had a statistically significant inhibitory effect on hatch in the EHT and on migration in the LMIT in dH_2O, but maximal efficacy was only 17.2 (±8.3) and 24.5 (±7.6) %, respectively. It was noted in the EHT that there was death of L1 shortly after hatching, but this effect was not quantifiable within the context of the tests as there was no control for larval development. A larval development test (LDT) would be indicated to investigate this further. *Artemisia* spp. are an important group of medicinal plants; *Artemisia annua* L. has given rise to a treatment for malaria based on the compound, artemisinin (Klayman, 1985). Many species have been shown to have anthelmintic activity (Ferreira, 2009), for example, a mean reduction in FEC of 90 % was demonstrated when sheep naturally infected with mixed GI nematode species were given a crude ethanolic extract of *A. absinthium* at a dose rate of 2.0 g/kg (Tariq et al., 2009). The reason for the low efficacy seen here, may be that it does not act on the stages of parasite tested or it may reduce FEC by another mechanism, such as reducing fecundity of adult female worms. It is unlikely that the active compounds had been deactivated in the drying and extraction process as the UK-sourced plants were dried by lyophilisation.

4.4.3. The use of in vitro tests to evaluate the anthelmintic activity of plant extracts

The range of EC-50 values calculated for extracts here was 0.18-8.9mg/ml. These are higher than the EC-50 values for thiabendazole in the EHT (0.052, 0.043 µg/ml) and ivermectin (0.472-8.391 µg/ml) in the LMIT, which have been previously calculated for the populations used in this study, see Appendix 1.4. and 1.9. This is expected when working with crude extracts as they contain many components, the majority of which have no effect. The actual EC-50 value of the active compound is likely to be much lower. Other studies looking at the efficacy of crude extracts in bioassays often report EC-50 values in the same range (Ademola et al., 2004; Alawa et al., 2003; Assis et al., 2003; Maciel et al., 2006; Marie-Magdeleine et al., 2010b), and some go on to demonstrate *in vivo* efficacy (Bachaya et al., 2009). This highlights that EC-50 values in relation to crude plant extracts can only be used with confidence to compare the relative effects of extracts within similar experiments, rather than drawing conclusions about efficacy compared to synthetic drugs.

In addition to identifying plants with *in vitro* anthelmintic activity, these data have highlighted that the results of *in vitro* testing of extracts can be dependent on the life cycle stage of the parasite in the test and the solvents used to deliver the extracts. In the present study, the EHT showed a higher sensitivity in detecting anti-parasitic activity, with seven out of nine extracts showing activity compared with five for the LMIT, the maximum percentage effects were also higher in the EHT. This might indicate that the EHT is a more useful technique for screening bioactive plant extracts; however, the results should be interpreted with caution as this effect may not translate *in vivo*. For example, if an extract shows significant activity in the LMIT and the EHT, such as *R. abyssinicus* and *C. album,* it is probably more likely to be efficacious *in vivo*. In the EHT with *A. absinthium* and *Z. officinale,* there was evidence of death of the L1

after hatching in exposed samples, but it was not possible to measure this effect within the test. This emphasises the need to test every life cycle stage. For two extracts, the delivery solvent had a differential effect on efficacy, with *C. album* performing better in an aqueous solvent and *R. abyssinicus* in DMSO. This suggests that the active compounds in the two extracts dissolve better in aqueous and DMSO solvent, respectively. Most studies evaluating crude plant extracts *in vitro* use only an aqueous solvent, though some use DMSO (Alawa et al., 2003; Assis et al., 2003). The results here highlight that multiple solvents with varied polarity should be used to maximise the chance of detecting anthelmintic activity.

4.4.4. Implications of in vitro findings for control of cyathostomins and future work

Here, plant extracts were tested for their effect *in vitro* against cyathostomins from donkeys. The relative prevalence of different cyathostomins species in samples from the same donkey population have previously been shown to be 80 % *C. tetracanthum*, *C. catinatum* and *C. pateratum*, with some *C. longibursatus* and *C. nassatus* (Cwiklinski et al., unpublished data). Studies on horses have shown a different spectrum of species, however there are some species in common (Chapman et al., 2003b; Ogbourne, 1976; Reinemeyer et al., 1984). Given the similarity in biology of all cyathostomins, it is likely that there will be a similar susceptibility to plant extracts in samples from horses. However there have been studies demonstrating differential drug susceptibility for different cyathostomin species (Traversa et al., 2009a), and tests would need to be repeated on samples from horses to confirm susceptibility. In Ethiopia it is also likely that there will be a high prevalence of other GI nematodes such as the large strongyles and a different range of cyathostomin species (Getachew et al., 2010), hence testing on parasite samples from populations in Ethiopia would be necessary to confirm sensitivity before recommending *in vivo* trials in these regions.

When designing experiments to extract plant compounds and testing them *in vitro,* the future practical application of the findings should be taken into account. In a developed region, technology exists to produce extracts on an industrial scale and it is useful to refine extraction techniques to achieve optimal results. However, if the plant needs to be available for use by farmers in a developing region, it is important to use techniques that can be replicated in that environment. If the aim is to develop a novel anthelmintic, rather than feeding the whole plant as forage, the active compound in the crude extract must be identified. This can be done by bioassay-guided fractionation of extracts, followed by high performance liquid chromatography and mass spectrometry or nuclear magnetic resonance analysis (Shi et al., 2009). Another method, if the index of suspicion is high, is to use inhibition assays for plant secondary compounds known to harbour anthelmintic activity, such as tannins (Manolaraki et al., 2010; Payne et al., 2013). Here, preliminary tests were conducted to evaluate the role of tannins in some of the extracts, but further work is needed to elucidate the active compounds. In every scenario, regardless of the active compound, toxicology and efficacy trials *in vivo* are essential before the plant or its purified, active compound(s) can be recommended as an anthelmintic treatment (Githiori et al., 2003a).

4.5. Conclusion

This study has identified bioactive plant extracts from the UK and Ethiopia that have potential as anthelmintic feed supplements in equids. It has highlighted the need for a high throughput systematic approach in the evaluation of such extracts for use as anthelmintics. In light of the ever-increasing prevalence of anthelmintic resistance, it is important to continue the search for novel compounds, and plants are a rich source which should continue to be explored.

5. *IN VITRO* EVALUATION OF THE EFFECT OF PAPAYA LATEX SUPERNATANT AGAINST CYATHOSTOMINS

5.1. Introduction

The threat drug resistance poses to effective parasite control has led to increased efforts in the search for novel anthelmintic compounds. In addition to screening large libraries of synthetic compounds, there has been increasing interest in natural products, historically used as traditional ethnomedical/veterinary treatments. One particularly promising group of compounds are the cysteine proteinases (CPs), derived from the fruits of plants such as papaya (*Carica papaya*), pineapple (*Ananas comosus*) and figs (*Ficus* spp.). Used for centuries for the treatment of human ascarid and hookworm infections (Berger and Asenjo, 1939, 1940; Caldwell and Caldwell, 1929; Fernan-Nunez, 1927; Mueller and Mechler, 2005; Robbins, 1930), in recent years their efficacy against a number of veterinary parasitic nematodes and cestodes has been validated both *in vitro* and *in vivo* (Buttle et al., 2011; Levecke et al., 2014; Mansur et al., 2014a, b; Satrija et al., 1994; Satrija et al., 1995; Stepek et al., 2005; Stepek et al., 2006, 2007b, c, d). They appear to be widely effective and hold promise as novel broad spectrum anthelmintics, however CPs have not yet been tested for efficacy against cyathostomins. The aim of this chapter was to evaluate the efficacy of papaya latex supernatant (PLS), a crude preparation from *C. papaya* containing CPs, against free living stages of cyathostomins, using the egg hatch test (EHT) and larval migration inhibition test (LMIT). The EHT and LMIT were first optimised for testing PLS (Sections *2.3.2.* and *2.4.2*). Next measurement of exact concentrations of active CPs in the PLS were ascertained using enzyme active site titration. The optimised LMIT and EHT tests were repeated with four samples of cyathostomins. Finally the role of CPs in the observed anthelmintic activity of PLS, in both the EHT and LMIT, was evaluated by pre-incubation of PLS with a CP inhibitor.

5.2. Materials and methods

5.2.1. Parasite sample collection and storage

Freshly voided faecal samples were collected from donkeys at the Donkey Sanctuary (DS) UK and horses at a yard in North West England. Strongyle eggs and third stage larvae (L3) were collected, stored and extracted/cultured as described in Section *2.2.1* and *2.2.2*. Once extracted, eggs were used immediately. Once cultured L3 samples were stored at 4 ºC and used within eight weeks.

5.2.2. Preparation of papaya latex supernatant and measurement of its cysteine proteinase concentration

The preparation of PLS used in this study was kindly donated by Professor Behnke at the University of Nottingham. PLS contains several CPs: chymopapain, glycyl endopeptidase, caricain and papain in order of relative abundance (Buttle et al., 1990). It was prepared as previously described by Buttle et al (2011). The extract was sent on dry ice to the University of Liverpool, where it was aliquoted into individual vials and immediately stored at -80 ºC. The extract was thawed before use and the molar active CP concentration measured using enzyme active site titration adapted from Barrett et al (1981) and Zucker et al (1985). In brief PLS was incubated with increasing concentrations of the CP specific inhibitor, L-*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino butane) (E64) (Sigma-Aldrich, UK), with 4 mMol L-cysteine (Sigma-Aldrich, UK) as a reducing agent and N-α-benzoyl-arginyl-*p*-nitroanilide (BAPNA) (Bachem Ltd, UK) as a substrate (Barrett et al., 1981; Zucker et al., 1985). As CPs and E64 bind in a 1:1 molar ratio, the concentration of E64 which completely inhibited CP activity was taken to be equal to the concentration of PLS in the assay, thus allowing calculation of the concentration in the stock PLS solution. Active enzyme site titration was performed on

each aliquot of PLS before use. An example showing the titration of enzyme activity with increasing concentrations of E64 can be seen in Figure 5.2.1. A simple linear trend line was fitted to data in each assay using Graphpad prism 6.00 (GraphPad Software, La Jolla California USA, www.graphpad.com). The regression equation describing the line, which in the example in Figure 5.2.1 was y = -1.131x + 0.215, was used to calculate the value of X when Y = 0 (i.e. the E64 concentration when the enzyme activity is 0), in this case 0.19 µM.

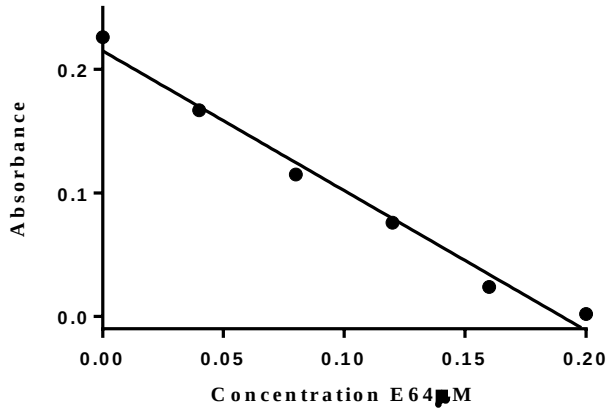

Figure 5.2.1. Graphical representation of active enzyme site titration to measure papaya latex supernatant concentration. Absorbance, representing cysteine proteinase activity, is plotted against increasing L trans-epoxysuccinyl-L-leucylamide-(4-guanidine) (E64) concentration. The concentration of E64 at which absorbance is 0, represents the concentration of cysteine proteinase (CP) within the assay.

For use in *in vitro* tests the stock PLS solution was made up to 12 mM, serially diluted in 5 mM (EHT) or 10 mM (LMIT) L-cysteine (Sigma-Aldrich, UK) (See section 2.1) at pH 7 to give working concentrations which were added in a 3.3 % ratio to 5 mM/10 mM L-cysteine (pH 7) in tests. The final concentrations in the tests were; 0.10, 0.20, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5 and 25 µM for the EHT and 1.56, 3.13, 6.25, 12.5, 25, 50, 100, 200 and 400 µM in the LMIT.

5.2.3. Evaluation of the effect of papaya latex supernatant on cyathostomins in the egg hatch test

The EHT was repeated in samples from four equids (two horses and two donkeys). This method was adapted from the egg hatch test with thiabendazole (TBZ) described in Section *2.3.1*. The test was optimised for L-cysteine concentration and incubation time (Section *2.3.2*). Instead of TBZ solutions, working and final solutions of PLS were made as described above in Section *5.2.2*. Approximately 100 eggs were incubated with each PLS concentration in duplicate for 24 h and the test continued as described in Section *2.3.1*. Duplicate negative controls for 5 mM L-cysteine and dH_2O, and positive controls of TBZ at 1.5 µg/ml (Sigma-Aldrich, UK) were included, to ensure the test accurately differentiated between live and dead eggs. In order to assess whether the active compound was a CP the test was repeated in one sample with PLS concentration constant at EC-99 (0.41 µM), measured as described in Section *5.2.5*. For this, prior to the addition of egg suspension, the PLS was incubated for 15 min at 40 ºC with E64 at increasing concentrations; 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, 2.1, 2.4, 2.7, and 3 µM. The EHT was then performed as described above. A negative control of 5 mM L-cysteine, and 3 µM E64 and positive control of PLS at EC-99 were included in the test.

5.2.4. Evaluation of the effect of papaya latex supernatant on cyathostomins in the larval migration inhibition test

The LMIT with PLS was repeated in samples from four equids (two donkeys and two horses). The method for evaluation of PLS was adapted from the LMIT with IVM described in Section *2.4.1*. The LMIT was first optimised for L-cysteine concentration and incubation time (Section *2.4.2.*). Instead of IVM solutions, working concentrations of PLS were prepared as described above (Section *5.2.2*). These were added in a 3.3 % dilution at the incubation and migration

steps of the LMIT. The L3 were ex-sheathed and washed as described in Section *2.4.1.*, but immediately prior to the incubation period they were re-suspended in 10 mM L-cysteine in dH_2O, pH7. The incubation phase of the LMIT was extended to 16 h. The test was then completed as described in Section *2.4.1*. Duplicate negative controls of 10 mM L-cysteine pH 7 and dH2O, and a positive control of 150 µg/ml ivermectin (IVM) (Sigma-Aldrich, UK) were included, to demonstrate that the test was accurately differentiating between motile and immotile L3. To ascertain whether CPs were the active component of PLS in the LMIT the test was repeated in one sample with PLS concentration constant at EC-99 (225 µM), measured as described in Section *5.2.5*. For this, the PLS was incubated for 15 min at 40 ºC with the CP inhibitor E64 at increasing concentrations; 50, 100, 150 and 200 µM, prior to its addition in the test at both the incubation and migration stage. As these concentrations of E64 did not restore L3 migration, the concentrations were subsequently increased to 500, 100, 1500, 2000 µM E64, to ensure that all CPs were inhibited. A negative control of 10 mM L-cysteine and 200/2000 µM E64 and positive control of PLS at EC-99 were included in these tests.

5.2.5. Analysis of dose response data from in vitro tests

Percentage egg hatch or larval migration at each concentration and in control reactions was calculated. The percentage egg hatch or larval migration in negative controls in each test was defined as the 'natural mortality' and the raw data were corrected for 'natural mortality' at each concentration and the percentage egg hatch or larval migration calculated. For each individual test repeated measures analysis of variance (ANOVA), in SPSS version 21 (SPSS Inc., Chicago IL), was used to compare untreated controls with treated parasites, where $p<0.05$ it was concluded that treatment had a significant effect. The corrected data were used to calculate logistic sigmoid dose response curves in Graphpad Prism 6.00 (GraphPad Software, La Jolla

California USA, www.graphpad.com). For each test four parameters were defined in a model according to the equation: $y=bottom+(top-bottom)/(1+10^{(logED-50-x)*hillslope})$, where x = log concentration, y = percentage hatch/migration, bottom is the y value at the bottom plateau, top is the y value at the top plateau, and hillslope describes the gradient of the curve. This enabled calculation of the median effective concentration (EC-50) and the 99 % effective concentration (EC-99) for comparison of efficacy between tests. Goodness of fit for each model was evaluated by the R^2 value and the confidence intervals. Where appropriate parameters (top, bottom, and/or hill slope) were constrained to allow statistical comparison between repeats using the sum of squares F test, a p value <0.05 meant that there was a significant difference between models.

5.3. Results

5.3.1. Papaya latex supernatant in the egg hatch test

Papaya latex supernatant had a potent effect in the EHT. Repeated measures ANOVA demonstrated highly significant differences between untreated controls and PLS treated eggs in all repeat samples ($p<0.001$) and EC-50s were in the range of 0.12-0.20 µM, see Table 5.3.1. Logarithmic dose response curves representing the models from which EC-50 values were calculated are shown in Figure 5.3.1a. R^2 values for the curves were all high and confidence intervals for EC-50s were small indicating the models describing the data were accurate, see Table 5.3.1. A statistically significant difference between repeats was found using the sum of squares F test, $p<0.001$. It was observed that at concentrations of PLS up to 6.25 µM, the first stage larvae (L1) developed inside the eggs but disintegrated suddenly at around 22 h when the eggs in control wells were hatching, this gave the appearance of an ‘exploded egg’, see Figure 5.3.2. At the higher concentrations, between 6.25-25 µM, there was no development of larvae

inside the egg, suggesting the PLS was preventing egg development. The EHT was repeated in sample 4 keeping PLS concentration constant at EC-99 (0.41 µM), and adding increasing concentrations of the CP inhibitor E64 to the PLS for 15 minutes at 40 ºC before inclusion in the test. Successful egg hatch was completely restored at 2.1 µM E64, (Figure 5.3.1.c.). This suggested that CPs are the active compound inhibiting egg hatch in the PLS extract. All positive control wells had 0 % egg hatch and negative controls had 83.8 ± 3.7 % average percentage hatch, this was corrected for during analysis.

5.3.2. Papaya latex supernatant in the larval migration inhibition test

Papaya latex supernatant inhibited larval migration in the LMIT after a 16 h incubation with PLS. Repeated measures ANOVA demonstrated highly significant differences between untreated controls and PLS treated samples in all repeats, $p<0.001$ and EC-50s were in the range of 67.35-106.31 µM, see Table 5.3.1. Logarithmic dose response curves representing the models from which EC-50 values were calculated are shown in Figure 5.3.1.b. R^2 values for were all high indicating the models describing the data were accurate, see Table 5.3.1. There was a statistically significant difference between repeats measured using the sum of squares F test, $p<0.001$. The larvae remained intact at all concentrations, although non-migrated L3 were visibly immotile. When the LMIT was repeated in sample 4 with PLS at EC-99 (225.0 µM) and incubated with increasing concentrations of E64 there was no effect on inhibition of migration, (Figure 5.3.1.d.). The concentration of E64 used in the LMIT was increased to ensure CPs were inhibited, however there remained no inhibition of the effect of PLS on larval migration. This suggests that CPs were not the active compound leading to immobility and death of L3. All positive control wells had 0 % larval migration and negative controls had 90.4 ± 1.2 % average percentage migration, this was corrected for during analysis.

Table 5.3.1. Summary of the results of statistical analysis on dose response data from the egg hatch test (EHT) and larval migration inhibition test (LMIT) with papaya latex supernatant. Specifically detailed are: results of repeated measure ANOVA to compare control and treated parasites within individual tests, EC-50 values calculated from the logistic sigmoid response curve for each repeat sample in the EHT and LMIT, R^2 values as indicators of model fit for the dose response curves and sum of squares F test results for evaluation of statistical significance between repeat tests.

Test	**Repeat D = donkey H= horse**	**Repeated measures ANOVA ***	**EC-50 (95% confidence intervals)**	**R^2 value for model fit**	**Sum of squares F test between test repeats****
EHT PLS repeats	1 (H)	p<0.001	0.20 (0.20-0.21)	0.99	p<0.001
	2 (H)	p<0.001	0.22 (0.21-0.23)	0.99	
	3 (D)	p<0.001	0.12 (0.12-0.13)	1.00	
	4 (D)	p<0.001	0.13 (0.12-0.13)	0.99	
LMIT PLS repeats	1 (H)	p<0.001	106.31 (93.23-121.52)	0.90	P<0.001
	2 (H)	p<0.001	68.77 (59.19-79.88)	0.91	
	3 (D)	p<0.001	67.35 (58.11-78.24)	0.97	
	4 (D)	p<0.001	70.21 (60.57-81.56)	0.97	

* in comparison to control group, p value significant if < 0.05

** p value significant if <0.05

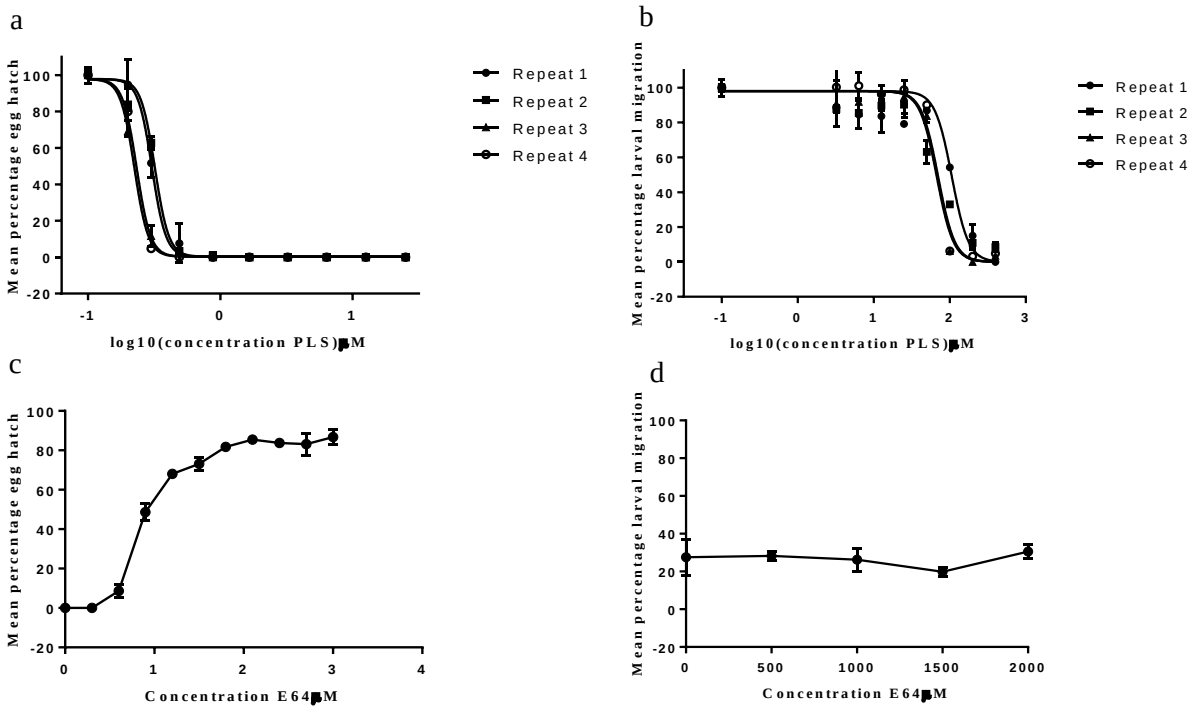

Figure 5.3.1. Graphical representation of the dose response relationship between papaya latex supernatant (PLS) and: a) percentage egg hatch in the egg hatch test (EHT); and b) percentage larval migration in the larval migration inhibition test (LMIT) in repeats 1-4. The effect of incubation with increasing concentrations of L trans-epoxysuccinyl-L-leucylamide-(4-guanidine) (E64) on the activity PLS at concentration EC-99 (0.41 µM in EHT and 225 µM in LMIT) is shown in graph (c) for the EHT and graph (d) for the LMIT.

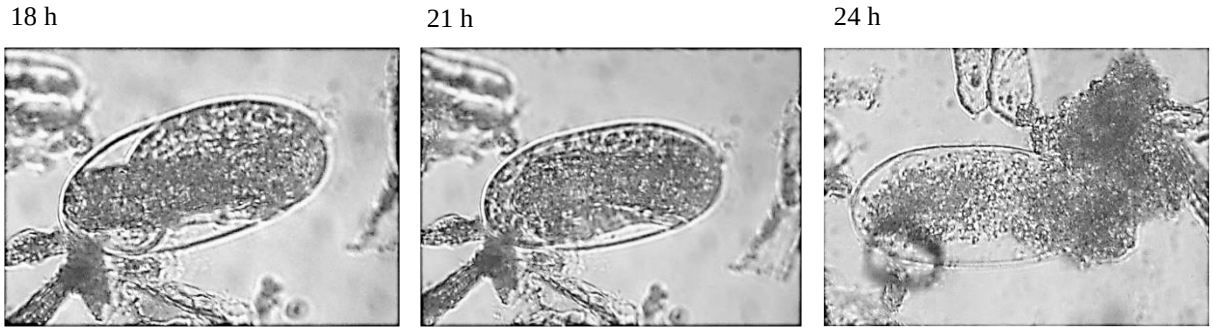

Figure 5.3.2. Digital photographic images demonstrating the effect of papaya latex supernatant (PLS) on cyathostomin eggs and first stage larvae (L1) around the time of hatching. After incubation for 18 h the L1 has developed inside the egg and is motile, at 21 h the L1 starts to lose definition and becomes immotile and at 24 h the decomposed material from the dead L1 emerges from the egg shell.

5.4. Discussion

These data demonstrate that CPs have a potent effect on cyathostomin eggs and L1 stages, and that PLS has a moderate effect on L3 in the LMIT. This is the first report of susceptibility of the free living stages of helminth parasites to CPs or PLS, and suggests that cyathostomins are highly sensitive to the effects of these enzymes. Adult stages were not tested here, due to the challenge of obtaining large numbers of adult cyathostomins, and maintaining their viability *in vitro*.

5.4.1. The effect of papaya latex supernatant in the egg hatch test and larval migration inhibition test against cyathostomins

In the EHT the EC-50 values calculated were in the range of 0.12-0.20 µM, which are low in comparison to a previous study by Stepek et al. (2006), who demonstrated an EC-50 of 5 µM against the adult stages of the rodent nematode *Trichuris muris.* In addition, previous studies looking at the effect of CPs over time against adult nematodes, consistently use 25 µM CP to demonstrate good effects (Stepek et al., 2007d). The low values seen here, suggest that cyathostomin eggs and L1 are highly sensitive to CPs in comparison to adult parasitic nematodes. The PLS preparation also had a significant effect on the migratory ability of cyathostomin L3, however it was not possible to attribute this to the activity of CPs, as their inactivation with E64 prior to use in the test, did not affect activity. The range of EC-50 values seen here, 67.35-106.31 µM after incubation for 16 h, was relatively high in comparison to concentrations used in previous studies on adult worms, suggesting that here, either different mechanism of action, or a lower sensitivity to CPs is present (Stepek et al., 2007b, d). In adult nematodes of other species it has been shown that CPs target the worm cuticle, which blisters, and then is weakened sufficiently to enable the internal hydrostatic pressure to rupture the body

wall resulting in the disintegration of the worm and death (Behnke et al., 2008; Phiri et al., 2014; Stepek et al., 2007c, d). Here, the L3 remained visibly intact at 400 times magnification after 16 h at 400 µM PLS, suggesting that the cuticle was not significantly damaged. Future work in cyathostomins might include the use of electron microscopy to examine the cuticle at higher resolution and look for signs of microscopic damage. In summary, the results here indicate that there is another active compound in PLS which affects L3 motility.

This is the first study to date to demonstrate the effect of CPs or PLS on the free living stages of a parasitic GI nematode. Previous studies have stated that no effect was seen on egg development, L1, L2 or L3 stages, except in the case of *T. muris*, for which egg hatch and larval development take place within the host (Stepek et al., 2006, 2007b, d). Hence, it is possible that the free living stages of cyathostomins differ to other parasitic nematodes in their structural biology making them more susceptible to CPs. The hypothesis behind tolerance of CPs in free living stages is that the cuticle differs in composition, or secretes inhibitors to CPs (Behnke et al., 2008). This theory is supported by the fact that some soil dwelling bacteria secrete CPs into the environment, which could have driven the evolution of protective mechanisms in free living stages (Oh et al., 1999). In addition it has been shown that the CP inhibitors, cystatins, are responsible for the resistance of *Caenorhabditis elegans* to the effects of CPs *in vitro* (Phiri et al., 2014). The possibility that cyathostomins may differ in their structural biology from other GI nematodes warrants further investigation, as it may be possible to exploit this apparent weakness in the development of other novel treatments.

5.4.2. Supportive evidence for the use of cysteine proteinases against cyathostomins in vivo

In this study we have demonstrated the efficacy of CPs against cyathostomins sampled from both horses and donkeys. The individual species in each group were not identified, however it is known from previous studies that differences in the spectrum and relative abundance of cyathostomin species are seen between the two equid hosts (Eysker and Pandey, 1989; Getachew et al., 2010; Ogbourne, 1976; Reinemeyer et al., 1984). Hence it can concluded that CPs/PLS show activity against a wide range of cyathostomin species that infect equids. A recent study has also found that PLS has excellent *in vitro* efficacy against the equid tapeworm *Anoplocephala perfoliata* (Mansur et al., 2015). As for the cyathostomins, *A. perfoliata*, was found to be highly sensitive to the effects of CPs in comparison to murine cestodes. This finding supports the potential use of CPs as novel broad spectrum anthelmintics in equids. Additionally there have been a number of studies demonstrating their efficacy *in vivo* for other veterinary GI nematodes. Potent effects have been demonstrated in murine models against *Heligmosomoides bakeri (polygyrus), T. muris* and *Protospirura muricola* (Stepek et al., 2006, 2007b, c). Furthermore efficacy against *Haemonchus contortus* in sheep, and *Ascaris suum* and *Trichuris suis* in pigs show that good effects can be achieved in larger hosts against both ruminant and mono-gastric species (Buttle et al., 2011; Levecke et al., 2014; Satrija et al., 1994). In the mouse stomach worm, *P. muricola*, the pre-administration of cimetidine was necessary for efficacy to raise stomach pH, as acidic conditions can inactivate the enzymic activity of CPs (Huet et al., 2006; Stepek et al., 2007b). This was not necessary in sheep for treatment of *H. contortus* as infected sheep have a raised abomasal pH (Buttle et al., 2011). There is also good evidence that CPs show activity against cestodes by causing degradation of the outer tegument (Mansur et al., 2014a, b). In mouse models measurement of CP activity throughout the GI tract after oral dosing shows that, although CP activity in the stomach is reduced due to low pH, its activity is restored when it reaches the intestines (Hale, 2004; Stepek

et al., 2007b). Of particular relevance to cyathostomins, is that CPs actually concentrate and increase in relative activity in the large intestines of mice, where adult cyathostomins are found in equids (Stepek et al., 2007b). This is thought to be due to absorption of water from the gut contents as they move through the GI tract. CPs have proven not to be effective against mucosal stages in *H. bakeri* and *T. muris* (Stepek et al., 2006, 2007c), hence they are unlikely to be effective against encysted L4 stages of cyathostomins. In the majority of studies with CPs no significant toxicity has been seen at the doses used, however toxicity has previously been described to traditional anthelmintics containing the CP ficin, in both mouse models and humans (de Amorin et al., 1999; Hansson et al., 2005). In both cases enzyme preparations were from *Ficus insipida*, it is likely that this species is significantly more toxic that other CP rich fruits, however the possibility of toxicity in all CP preparations is possible and should be evaluated.

5.4.3. Considerations for taking cysteine proteinases forward for in vivo testing in equids

The broad spectrum of activity of CPs mean that it could hold great promise as a novel anthelmintic for use in equids. It would first be necessary to evaluate efficacy against cyathostomins *in vivo* and also against other pathogenic GI helminths of equids, such as the large strongyles, *Parascaris equorum* and *A. perfoliata*. An important point when considering their use in equids, is that previous studies have used large volumes of CP preparations to achieve *in vivo* efficacy (Levecke et al., 2014), which may make dosing practically challenging in equids. However, given the sensitivity of cyathostomin eggs to CPs demonstrated in this study, it is possible that relatively lower doses would be needed in equids. Currently investigations are underway to improve CP formulation (Buttle, personal communication), the possibility of its use in equids will most likely depend on the outcome of this work.

As CPs are proteins, one potential issue regarding their use *in vivo* which has been raised, is the possibility for development of host immunity. CPs are known to be highly immunogenic in some contexts (Dando et al., 1995). Antibodies to bromelain have been detected in mice treated for a period of 18 weeks, although no reduction in enzyme activity was noted as result (Hale, 2004). With any novel anthelmintic the possibility of development of resistance should also be considered. There is suggestion that resistance would be slow to develop to CPs as the enzymes target several different constituent proteins that maintain cuticular structure (Stepek et al., 2004). However the evolution of broader mechanisms of resistance, such as the secretion of protease inhibitors, is also a possibility. In order to evaluate the risk of immunity and resistance in equids, *in vivo* trials involving repeated CP administration and re-infection with surviving parasites would be necessary.

5.5. Conclusion

In conclusion, this study has demonstrated that PLS has a potent effect against free living stages of cyathostomins and that, for eggs and L1, this effect is due to the action of CPs. A novel active compound in PLS affecting L3 has also been found, and requires further identification. This is the first example of sensitivity of the free living stages of a parasitic nematode to CPs, suggesting that cyathostomins are particularly sensitive to these enzymes. Given their proven efficacy *in vivo* and broad spectrum of action in other host species, the CPs are a good candidate for *in vivo* testing and potential development as a novel anthelmintic in equids.

6. INVESTIGATION INTO THE POTENTIAL FOR P-GLYCOPROTEIN INHIBITORS TO IMPROVE CONTROL OF MACROCYCLIC LACTONE RESISTANT CYATHOSTOMINS

6.1. Introduction

There is increasing evidence that cyathostomins are developing resistance to the macrocyclic lactone (ML) anthelmintics, ivermectin (IVM) and moxidectin (MOX) (Canever et al., 2013; Milillo et al., 2009; Molento et al., 2008; Nareaho et al., 2011; Relf et al., 2014; Traversa et al., 2009b; Trawford et al., 2005; Trawford, 2009). The evidence for emerging resistance has come primarily from reduced egg reappearance periods (ERPs) after treatment. There has been extensive investigation into the mechanisms underlying ML resistance in other parasitic nematodes such as *Haemonchus contortus*, *Teladorsagia circumcincta* and *Onchocerca volvulus*, and a simple mechanism, for example a target site mutation in a glutamate gated chloride channel, has yet to be identified (El-Abdellati et al., 2011; Williamson et al., 2011). However, there is one group of genes that has been strongly implicated in ML resistance in several nematode species; these genes encode ATP-binding cassette (ABC) transporters. ABC transporters are found in the cell membrane and are responsible for the ATP-dependant efflux of xenobiotic compounds from the cell, they play an important role in protection against toxic environmental compounds (Dassa, 2003). ABC transporters are present in many organisms, including humans. Indeed, awareness of their role in drug resistance comes from the involvement of the ABCB transporter MDR-1, in multidrug resistance of cancer cells to chemotherapy (Dano, 1973). In mammals, through their expression at epithelial barriers such as the blood brain barrier and enterocytes, ABC transporters are important mediators of the pharmacokinetics of drugs such as MLs (Ballent et al., 2006; Kiki-Mvouaka et al., 2010; Laffont et al., 2002; Schinkel et al., 1994). Amongst the best characterised are the ABCB P-glycoprotein (P-gp) sub-family; others include membrane resistance proteins (MPRs), the bile salt exporter protein (BSEP) and the breast cancer resistance protein (BCRP) (Higgins, 2007).

A relatively large number of ABC transporters have been shown to be expressed in nematodes, for example *Caenorhabditis elegans* has 15 P-gp and 8 MPR genes (Ardelli, 2013). In *T. circumcinta* P-gp genes are transcribed by all stages of the life cycle (Dicker et al., 2011b). In the free living stages of *H. contortus* they have been found to be expressed in the egg shell and larval cuticle (Riou et al., 2005). In adult worms they have been shown to predominate in the gastrointestinal (GI) tract, particularly the pharynx and anterior intestine (Smith and Prichard, 2002). This has led to the theory that, as in mammals, ABC transporters may play an important role in drug metabolism. In support of this it has been demonstrated that MLs are good substrates for ABC transporters (Dupuy et al., 2010; Dupuy et al., 2006; Kiki-Mvouaka et al., 2010; Lespine et al., 2009; Lespine et al., 2006; Lespine et al., 2007). In addition, gene knockout studies in *C. elegans* have shown that various MRP knockout combinations confer increased sensitivity to MLs (Ardelli and Prichard, 2008; Ardelli and Prichard, 2013). For example, knocking out MRPs 4 and 8, *versus* MRPs 6 and 8 in *C. elegans* confers increased sensitivity to IVM and MOX, respectively (Ardelli and Prichard, 2008). These data demonstrate the importance of ABC transporters in ML metabolism in nematodes, and supports the theory that they play a key role ML resistance.

There is a growing body of evidence that ABC transporters, in particular P-gps, are involved in ML resistance. It has been shown that substances that interfere with the action of P-gps, can lead to an increased sensitivity of ML resistant parasitic nematodes to IVM *in vitro* (AlGusbi et al., 2014; Ardelli and Prichard, 2013; Bartley et al., 2009; Demeler et al., 2013; Raza et al., 2015). Many drugs function as P-gp inhibitors, they do so either directly, or through competitive inhibition, by nature of the fact that they are also substrates. In support of these *in vitro* studies, there have been studies demonstrating that the efficacy of IVM against ML resistant nematodes *in vivo* can be improved, when given in combination with loperamide (an

opiod derivative that has been classified as a P-gp inhibitor) (Lifschitz et al., 2010a; Lifschitz et al., 2010b). This effect is thought to be due, in part, to increased bioavailability of IVM in the host due to modulation of host P-gps, but there may also be a direct effect on the parasite's drug metabolism. Importantly these *in vivo* studies suggest that MLs in combination with P-gp inhibitors, are a potential novel control option in ML resistant nematode populations. There is also increasing evidence from genetic studies for the role of ABC transporters in ML resistance. For example, there is a reduction in P-gp gene heterozygosity after IVM exposure in *O. volvulus* and *H. contortus,* which indicates that certain P-gp genotypes confer an advantage for nematodes in the presence of IVM (Ardelli et al., 2005; Ardelli and Prichard, 2007; Blackhall et al., 2008). In addition up-regulation in P-gp and MRP ribonucleic acid (RNA) transcription has been observed in ML resistant strains of *C. elegans* (MRP-1, MRP-6, *pgp-1 and pgp-2*), *H. contortus* (*pgp-2, pgp-9)* and *T. circumcincta* (*pgp-9)* (Ardelli and Prichard, 2008; Ardelli and Prichard, 2013; Dicker et al., 2011b; Williamson et al., 2011), which indicates that increased drug efflux by these channels plays a role in resistance.

There is little data regarding ABC transporters in cyathostomin parasites, and identifying them in these worms is made complex by the number of species that infect equids (Bucknell et al., 1995; Gawor, 1995; Love and Duncan, 1992; Ogbourne, 1976; Reinemeyer et al., 1984; Traversa et al., 2010). One publication reported the deoxyribonucleic acid (DNA) sequence of two P-gp nucleotide binding domains in adult cyathostomins (Drogemuller et al., 2004). P-gp gene sequence from *Cyathostomum. radiatus, Cylicocyclus insigne, Cylicocyclus nassatus, Cylicocyclus elongatus, Cylicostephanus hybridus, Cylicostephanus goldi,* and *Cyathostomum coronatum* grouped by nucleotide identity as one cluster, and a second cluster comprised *Cylicostephanus hybridus, Cyathostomum pateratum*, and *Cyathostomum catinatum*. This suggested the possibility of at least two P-gps in the study samples. Subsequently, the DNA

sequence of the *pgp-9* gene in *C. elongatus* was published and the data showed that IVM acts as a substrate for this protein and can competitively inhibit other substrates (Kaschny et al., 2015). These data demonstrate that P-gps are expressed in cyathostomins and interact directly with IVM, therefore given the evidence in other closely-related nematodes (Dicker et al., 2011b; Lifschitz et al., 2010a; Williamson et al., 2011), it is reasonable to assume that P-gps will also play a role in MLs resistance in cyathostomins.

The first aim of the work within this chapter was to identify whether upregulation of *pgp-9* plays a role in ML resistance in cyathostomins. This was determined by comparing the transcription profile of *pgp-9* in response to IVM exposure for free living infective stages of cyathostomins from: a) a population of equids with a long history of ML use and reduced egg reappearance periods (ERPs) and b) a population which have never been exposed to anthelmintics. The second aim was to examine whether interference with P-gp function could potentially be used to improve ML efficacy against cyathostomins. To assess this, the effect of a range of P-gp inhibitors on IVM efficacy was compared in cyathostomin populations a) and b) above, using the larval development test (LDT) and the larval migration inhibition test (LMIT).

6.2. Materials and Methods

6.2.1. Parasite populations

Cyathostomin samples were sourced from two equid populations to represent parasites of differing sensitivity to MLs, both of which were used for a number of specific applications throughout this Chapter. Population 1 (Pop 1, IVM-resistant), harbouring 'resistant' cyathostomins, were donkeys at the Donkey Sanctuary in Sidmouth, Devon, where there is a

history of resistance to MLs, as determined by faecal egg count reduction tests (FECRT) (Trawford et al., 2005; Trawford, 2009). Criteria for inclusion of animals in the study was a FEC of ≥ 500 eggs per gram (epg) within eight weeks of administration of IVM or moxidectin (MOX). Population 2 (Pop 2, IVM-naive), was deemed to harbour 'sensitive' cyathostomins, and was a herd of Konik horses used for conservation purposes by the National Trust at their site at Wicken Fen, East Anglia. Sensitivity to anthelmintics in Pop 2 was assumed on the basis that this was a closed herd within which animals had never been treated with an anthelmintic. It was not possible to perform a faecal egg count reduction test (FECRT) to confirm sensitivity in Pop 2 as treatment of these horses was prohibited.

In addition an archive of adult cyathostomins collected from horses and donkeys post-mortem and held at the University of Liverpool in ethanol at -20 ºC, were used for isolation of *pgp-9* and design of a real time polymerase chain reaction (PCR) (Hodgkinson et al., 2008).

6.2.2. Parasite sample collection

Faecal samples were taken from animals in Pops 1 and 2, from which eggs and third stage larvae (L3) were collected and harvested as described in Section *2.2.1* and *2.2.2* respectively.

6.2.3 Design of real time PCR for cyathostomins pgp-9, and measurement of its transcription in populations of differing ivermectin sensitivity

6.2.3.1. Production of cDNA from adult cyathostomins for isolation of pgp-9 DNA sequence

RNA was extracted from groups of 10 adult worms from a number of cyathostomin species within the archive of adult cyathostomins: *Cylicocyclus nassatus, Cyathostomum pateratum,*

Cyathostomum catinatum, and from a mixed adult cyathostomin sample taken from a donkey, W, at the Donkey Sanctuary (DSW). RNA was extracted using the QIAGEN RNeasy Mini kit according to the manufacturer's protocol with the following adaptations. Each sample was homogenised in 600 µl of RNeasy lysis buffer using a bead beater at speed 48 for 2 x 1 min, and then placed on ice. The resultant lysate was pipetted into a fresh eppendorf, mixed with 700 µl of ethanol and the protocol continued as per the manufacturer's instructions. In the final stage, RNA was eluted from the RNeasy spin column in 40 µl of RNase-free diethylpyrocarbonate (DEPC) treated dH_2O (Sigma-Aldrich, UK). RNA was used immediately or stored neat at -80 ºC. As RNA concentrations were likely to be low, quantification was not performed at this stage, and the maximum volume of RNA (12 µl) was used for cDNA synthesis. cDNA synthesis was performed using the QIAGEN Quantitect Reverse Transcriptase kit according to the manufacturer's instructions, including the genomic DNA wipeout step. All cDNA samples were stored at -20 ºC. In order to assess integrity of cDNA, PCR for beta-tubulin was performed. using the following primer combinations: 200_F, 5'-TCYGAYACHGTTGTGGAGCC-3' and 200_RS, 5'-AAGATGATTYAVATCWCCRTA-3' which amplified a 160 base pair (bp) fragment of beta-tubulin isotype-1 gene (Lake et al., 2009), and CN23F, 5'-AAGTTCTCTACTGCAATAATGCGTG-3' and CN31R 5'-AACGCAATCAATGTGTATTTCGC-3', which amplified a 1100 bp fragment of beta-tubulin isotype-1 gene (Pape et al., 2002; Pape et al., 1999). The PCR was performed using QIAGEN Taq PCR Master Mix Kit. To each reaction, 25 µl of Master Mix, 2.5 µl of forward and reverse primer (final concentration 0.5 µM), 18 µl of DNase free water, and 2 µl of template cDNA were added, to give a total volume of 50 µl. The PCR reaction was run on the Biometra T3 Thermocycler. The reaction conditions were 3 min at 94 ºC, followed by 34 cycles of 1 min denaturation at 94 ºC, 1 min at annealing temperature and 1 min extension at 72 ºC, followed by a final extension phase of 10 min at 72 ºC. The annealing temperature was 50 ºC for the

primer pair, 200_F/200_RS, and 55 ºC for the primer pair, CN23F/CN31R. The PCR products were subjected to electrophoresis on 1 % agarose gels at 100 V for 30 min and the products stained for UV visualisation using the 'SYBR safe' stain (Life Technologies, UK), alongside a 100 bp or 1000 bp ladder (Invitrogen, UK) for size estimation.

6.2.3.2. Primer design for identification of pgp-9 from cyathostomin cDNA samples

Full-length *pgp-9* DNA sequence from the cyathostomin *C. elongatus* (Accession number (AN): KJ701410.1) was kindly donated by Professor Georg von Samson-Himmelstjerna and Dr Jürgen Krücken of Freie Universität Berlin to aid in primer design. Partial *pgp-9* translated amino acid sequence from *T. circumcincta* (AN:CBX21126.1) and *H. contortus* (AN:AFX93750.1), and full *pgp-9* translated amino acid sequence from *C. elegans* (AN:CAB03973) and *C. elongatus* (AN: KJ701410NA .1) were aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) to identify regions which are conserved in nematode *pgp-9.* A region was identified, which was analysed using NCBI protein blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) to ensure that it was specific to *pgp-9* and did not align with other nematode P-gp amino acid sequences. NCBI primer design (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to identify the forward primer site in the *C. elongatus* nucleotide sequence, which corresponded to this highly conserved region. The reverse primer, also designed using NCBI, was located in the highly conserved first nucleotide binding domain of the *C. elongatus* sequence, which is found between 5' 1150-1740 3' bp (Kaschny et al., 2015). The primers with the best thermo-kinetic parameters were chosen and aligned, using Clustal Omega (as above), with *C. elongatus* and *H. contortus* (A.N. JX430937.1) nucleotide sequence to allow the design of degenerate primers which would amplify *pgp-9* from both species, thus maximising the likelihood that the primers would

amplify *pgp-9* from multiple different cyathostomins species. The primers were as follows: forward, PGP9F 5'-CKGCMACRATACAGGCAATR-3' and reverse PGP9R 5'-GTCCTCACGTCCMGAMARTTG-3, and they amplified a 1100 bp fragment of *pgp-9* cDNA.

6.2.3.3. Amplification and sequencing of the first transmembrane and nucleotide binding domain of pgp-9 from cyathostomin cDNA

PCR (see section 6.*2.3.1*) was performed on cDNA synthesised from, *C. nassatus, C. pateratum, C. catinatum* and the sample of unknown species 'DSW' with primers PGP9F and PGP9R using an annealing temperature of 52 ºC. Only the DSW template gave a product but due to non-specific products it required gel purification of the product and re-PCR with the same primers (PGP9F and PGP9R) to give a product of the correct size (1100 bp) on the agarose gel. The product was purified using the QIAGEN QIAquick Gel extraction kit and quantified using the Invitrogen Qubit Assay, as per the manufacturer's instructions. The PCR product was cloned for sequencing using the Pgem-T Easy Vector System (Promega) as per manufacturer's instructions. Following ligation and transformation, 10 white colonies were picked from plates and placed separately in 5 ml of LB broth with ampicillin (Section 2.1). Plasmid DNA was purified from the cells using the Promega Wizard Plus SV Minipreps DNA Purification System, as per manufacturer's instructions. The plasmid DNA was quantified using the Qubit assay (Invitrogen) and 100 ng/µl sent for sequencing (Source Biosciences, UK) using forward and reverse Pgem-T plasmid primers M13F/M13R. The ten resultant sequences (designated DSW A1-5 and B1-5) were entered into NCBI nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) to evaluate their similarity with other nematode nucleotide sequences and confirm that *pgp-9* sequences had been amplified. Alignments between cDNA and amino acid sequences of all clones and *C.*

elongatus pgp-9 (A.N. KJ701410.1) were created using Clustal Omega, and the percentage identity between them calculated. In addition, a phylogenetic tree comparing the relationship between the nucleotide sequence of DW1 A1-5 and B1-5, *C. elongatus pgp-9* (A.N. KJ701410.1), *H. contortus pgp-9* (A.N. JX430937.1), *T. circumcincta pgp-9* (A.N. FR691848.1), *C. elegans pgp-9* (C47A10.1 wormbase i.d.) and *Caenorhabditis briggsae pgp-9* (XM_002638567.1) was created using MEGA version 6.06 software. Specifically, a maximum likelihood tree with 500 bootstraps using a kimura 2-parameter model, was constructed.

6.2.3.4. Design of primers and standards for real time quantitative PCR for cyathostomin pgp-9 transcription

A SYBR® green (Life Technologies) real time quantitative PCR assay was designed to determine *pgp-9* transcript levels, relative to a housekeeping 18S rRNA gene, in cyathostomin cDNA samples. The 18S assay was designed using a consensus sequence of 99 bp produced from the alignment of 63 bp fragments of the 18S rRNA from six species of cyathostomins, and from a range of other organisms as follows: *C. labiatum* (A.N. AJ223727); *C. ashworthi* (A.N. AJ223346); *C. nassatus*, (A.N. AJ223348); *C. elegans*, (A.N. X03680); *Ascaris suum*, (A.N. U9436); *Toxoplasma gondii*, (A.N. L37415); *Schizosaccharomyces pombe*, (A.N. AY251644); *Dirofilaria immitis*, (A.N. AF036638); *Xenopus laevis*, (A.N. X02995); *Drosophila melanogaster*, (A.N. M21017); *Strongyloides stercoralis*, (A.N. M84229); and *Enoplus brevis*, (A.N. U88336). Primers were as follows; 18SRTf 5' GATTGATTCTGTCAGCGCTATA 3' and 18SRTr 5' TAATGAGCCGTTCGCAGT 3' and were confirmed to lack sequence identify with 18S rRNA of equids or bacteria using NCBI BLAST. A standard synthetic 99 bp 18S template was manufactured by Sigma-Aldrich, UK.

For *pgp-9*, primers were designed using Primer 3 version 4.0.0 (http://primer3.ut.ee/) based on the sequence data from DSW A1. The primers with the best thermokinetic parameters were chosen and aligned with all 10 sequenced *pgp-9* sequences and *C. elongatus pgp-9* (A.N. KJ701410.1). Due to the presence of three nucleotide polymorphisms in the sequence aligning to these primers, degenerate primers were designed to maximise the amplification of *pgp-9* from multiple cyathostomin species. The degenerate primers for *Pgp-9* were PGP9RTF 5' AYATTGGGCTYGGTCTTGCT 3' and PGP9RTR 5' ACCGTTCCYCCTTTCATCGT 3'. The 112 bp sequence flanked by the two primers was synthesised by Sigma-Aldrich, UK, for use as a standard with the most commonly occurring base incorporated at each of the 12 sites of nucleotide variation.

6.2.3.5. Optimisation of real time PCR assay for pgp-9: standard curve and optimisation of cDNA concentrations

A 10-fold dilution series of the 18S and *pgp-9* standard oligonucleotides was made in 100 µg/ml yeast tRNA (Invitrogen, UK). The final theoretical copy numbers for each dilution were $5x10^{-1}$, $5x10^{0}$, $5x10^{1}$, $5x10^{2}$, $5x10^{3}$, $5x10^{4}$, $5x10^{5}$, $5x10^{6}$, $5x10^{7}$, $5x10^{8}$. The primers 18SRTf, 18SRTr, PGP9RTF and PGP9RTR were diluted to 100 µM. A master mix was made up with 10µl 2x SensiMix (dT) (Bioline, UK), 7 µl DNase/RNase free dH_2O (Sigma-Aldrich, UK), 1 µl of 4µM forward and reverse primer (either for 18S or *pgp-9*) per sample. To each well of a 96-well plate, 19 µl of mastermix were added, followed by 1 µl of each standard concentration. Duplicates were run for each standard concentration and a non-template control (dH_2O) was also included. The plate was run on DNA Engine Opticon 2 Continuous Fluorescence detector, with the following reaction conditions: 95º C for 10 min, followed by 35 cycles of 95 ºC for 15 s, 53 ºC for 30 s and 72 ºC for 15 s. The standard curve was repeated as described above with

the addition of serial dilutions of cDNA from DSW, starting at 4.1 ng/µl of neat cDNA. Analysis of cDNA concentration, showed that *pgp-9* could be detected optimally at the concentration of 4.1 ng/µl.

6.2.3.6. Evaluation of pgp-9 transcription after ivermectin exposure in third stage larvae from mixed species cyathostomin populations of differing ivermectin sensitivity

A total of ~800,000 and ~1.1 m L3 were cultured from the faeces of 10 animals from Pop 1 and 2, respectively. These L3 were pooled for each population and suspended in 450 ml of distilled water, (dH_2O) (Sigma-Aldrich). They were mixed and, for each population, divided into three aliquots of 50 ml (negative controls), and 6 aliquots of 48.5 ml (test samples) and placed into 50 ml centrifuge tubes. The L3 in each aliquot were ex-sheathed, as described for the LMIT in Section *2.4.1*. To three of the 48.5 ml test samples, 1.5 ml of DMSO (Sigma-Aldrich, UK) was added to serve as a control for the drug solvent. To the remaining three test samples, 1.5 ml of 5 µg/ml IVM (Sigma-Aldrich, UK) in DMSO were added, giving a final IVM concentration of 0.15 µg/ml. This concentration was chosen as a 'sub-lethal' dose (i.e. where percentage larval migration began to decrease, but the majority of the L3 were still alive and presumably still capable of transcribing RNA), based on data from the LMIT in preliminary experiments, see Appendix 1.9. All aliquots were incubated for 2 h at 26 ºC as for the LMIT. After incubation, all aliquots were centrifuged for 2 min at 200 *g*, the supernatant removed and the L3 re-suspended in 20 ml of dH_2O. This was repeated three times. Finally, L3 from each aliquot were transferred to an eppendorf and centrifuged for 5 min at 11,000 *g*. The remaining supernatant was removed from each aliquot and they were air dried, re-suspended in 100 % ethanol (Sigma-Aldrich, UK) and stored at -20 ºC. RNA extraction and cDNA synthesis was carried out on each L3 sample as described in Section *6.2.3.1*. In the cDNA synthesis step,

reverse transcriptase-free negative controls were included for each aliquot. The cDNA concentration in each sample was quantified using the Picogreen DNA quantification kit (Promega), as per manufacturer's instructions. The cDNA concentration in each aliquot was standardised to 2 ng/µl using RNase/DNase-free water (Sigma-Aldrich, UK). Real time PCR for 18S and *pgp-9* was then performed alongside standard curves for all samples according to the method outlined in Section *6.2.3.5*, except, in order to keep cDNA concentrations close to the optimum, 2 µl of cDNA were added to each reaction to give a total of 4 ng DNA. Duplicates were included for each sample, as well as reverse transcriptase-free negative controls and non-template controls. For each incubation (i.e. dH_2O, 3 % DMSO or 0.15 µg/ml IVM), there were two technical (within assay) repeats of each sample from the three biological repeats described above.

6.2.3.7. Analysis of real time PCR data

Raw data from the real time PCR was evaluated using Opticon Monitor 3 version 3.1.32.0 software. Standard graph correlations were checked and melting curves examined for each sample; those which did not have a single peak at the expected temperature were excluded from further analysis. Figure 6.2.1. shows the *pgp-9* melting curves for samples used in the analysis. Raw data from the qPCR reactions were entered into a Microsoft Excel spreadsheet. The percentage genomic contamination in each sample was calculated from the reverse transcriptase negative control for the raw 18S data, this was used to correct the raw data for 18S and *pgp-9* so that any genomic DNA was not counted. The average copy number between each technical repeat was taken for each sample for 18S and *pgp-9*, and the relative transcript level of *pgp-9 gene* compared to the 18S gene, calculated for each biological repeat. The data was entered into SPSS 21 to generate a box and whisker plot to represent the data graphically.

This data was analysed with two way analysis of variance (ANOVA), to compare *pgp-9* transcription between treatments and equid populations, using R statistical software (R Core Team, 2014).

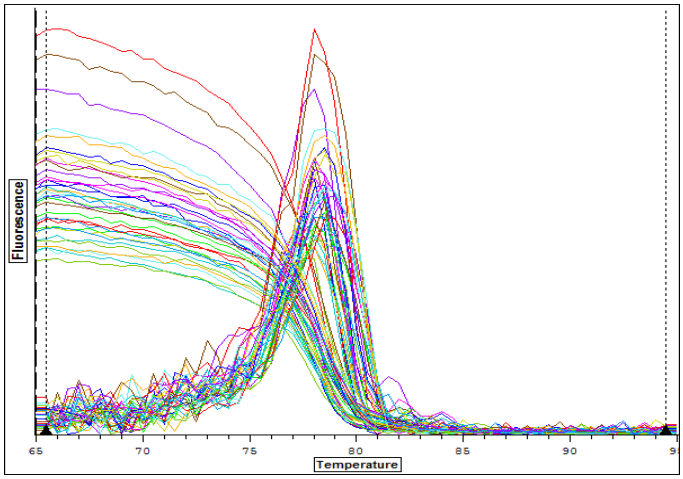

Figure 6.2.1. *Pgp-9* real time PCR melting curves for all third stage larval (L3) cDNA samples from Populations 1 and 2.

6.2.4. Evaluating the effect of P-glycoprotein inhibitors on ivermectin efficacy in the larval development test and larval migration inhibition test

6.2.4.1. Pharmaceutical preparations for use in the larval development test and larval migration inhibition test

IVM (Sigma-Aldrich, UK) was dissolved in DMSO (Sigma-Aldrich, UK) to give stock solutions of 5000 nM for the LDT and 3000 µg/ml for the LMIT. These were serially diluted in DMSO as described in Sections *2.5.1.* and *2.4.1.* respectively to give working concentrations for use in the two tests. The concentration of three P-gp inhibitors was optimised, as described in Sections *2.5.2.* and *2.4.3.* (see Appendix *1.17.*, *1.12.* and *1.13.* for results of optimisation),

for use in both the LDT and LMIT, so that it was used at the optimal concentration for P-gp inhibitory activity, without having an effect on its own against cyathostomins. Ketoconazole (Sigma-Aldrich, UK) was used at 10 µM in all tests with the LDT and LMIT. For the LDT a stock solution of 2000 µM in DMSO was used and added in a 0.5:100 ratio to the test. For the LMIT a 1000 µM in DMSO was used and added in a 1:100 ratio at each stage of the test. Pluronic 85 (P85) (BASF, USA) was used at 22 µM in the LDT and LMIT. For the LDT a stock solution of 4400 µM in dH_2O (Sigma-Aldrich, UK) was added in a 0.5:100 ratio to the test. For the LMIT a stock solution of 2200 µM in dH_2O was used and added in a 1:100 ratio at each stage of the test. IVM-AG (synthesised at Toxalim, INRA, Toulouse, and kindly donated by Dr Anne Lespine) was used at 8 nM in the LDT and, for this, a stock solution of 1600 nM was used and added in a 0.5:100 ratio to the test. In the LMIT IVM-AG was used at a concentration of 60 nM and, for this, a stock solution of 6000 nM was used, which was added in a 1:100 ratio at each stage of the test.

6.2.4.2. The larval development and larval migration inhibition test with pluronic 85, ketoconazole and ivermectin aglycone

The LDT and LMIT with IVM was performed with and without the addition of three P-gp inhibitors, ketoconazole, P85 and IVM-AG, in samples from Pop 1 and Pop 2. The LDT was performed with increasing concentrations of IVM as described in Section *2.5.1*. However, for each parasite sample, the test was repeated with the addition of 10 µM ketoconazole, 22 µM P85 and 8 nM IVM-AG in all wells, including the negative control. The working concentrations of IVM were adjusted to keep the DMSO concentration constant at 0.5 %. The LDT, with the addition of each P-gp inhibitor, was repeated using two parasite samples from each Population. The LMIT was performed as described in Section *2.4.1*. with increasing concentrations of IVM.

For each sample, the test was repeated with the addition of 10 µM ketoconazole, 22 µM P85 or 60 nM IVM-AG in all wells, including the negative control. Again, the working concentrations of IVM were adjusted to keep the DMSO concentration constant at 3 %. The LMIT with the addition of each P-gp inhibitor was repeated using four parasite samples from each population.

6.2.4.3. Analysis of dose response data from the larval development test and larval migration inhibition tests

Dose response data was analysed using general linear mixed models (GLMM). Conventional PROBIT analysis was also performed on the data for comparison, see Appendix *3.1*. It was not possible to analyse some of dose response data with the PROBIT model, and hence GLMM was chosen as the optimal method here. For the GLMM method, the data was grouped according to drug combination and test, including IVM alone in each group for comparison of P-gp inhibitor addition within each model, i.e. model 1 = IVM alone and IVM + P85 in Pops 1 and 2 in the LDT, model 2 = IVM alone and IVM + keto in Pops 1 and 2 in the LDT and so on. The GLMM was constructed with concentration IVM, p-gp inhibitor (present or absent) and Population (Pop 1 or 2) as fixed factors and animal/repeat as random effects. An interaction term was introduced between all pairs of fixed factors. The GLMM was performed using R (R Core Team, 2014) with the Lme4 package (Bates et al., 2015) according to the following code:

```
model <- glmer cbind (ndl3.r,dl3.r) ~ conc*pgpi*pop - conc:pgpi:pop + (1|animal), data = d, family="binomial")
```

Where ndl3.r = undeveloped or non-migrated larvae, dl3r = developed or migrated larvae, conc = concentration IVM, pgpi = presence/absence of P-gp inhibitor, pop = population 1 or 2, animal = repeats between and within animal.

The coefficients for each variable in the model output were used to calculate an odds ratio (OR) for the likelihood of larval death at each concentration, taking into account the Population and whether or not they had been exposed to P-gp inhibitor. The ORs were calculated and plotted in Microsoft Excel, 2013.

6.3. Results

6.3.1 Design of real time PCR for cyathostomins pgp-9, and measurement of its transcription in populations of differing ivermectin sensitivity

6.3.1.1. PCR for beta tubulin and Pgp-9 on cDNA from archived cyathostomins samples

PCR for the small amplicon of beta-tubulin was positive for all cDNA samples tested, with the expected fragment size of 160 bp being observed (Figure 6.3.1a). However, for the larger amplicon, only DSW showed a positive result at the expected size of 1100 bp (Figure 6.3.1b). PCR for *pgp-9* using primers designed from a consensus sequence of *C. elongatus* and *H. contortus* was negative in all samples except DSW (Figure 6.3.1.c). It was assumed that the RNA and cDNA quality in the negative samples was poor and hence DSW was used for all further work.

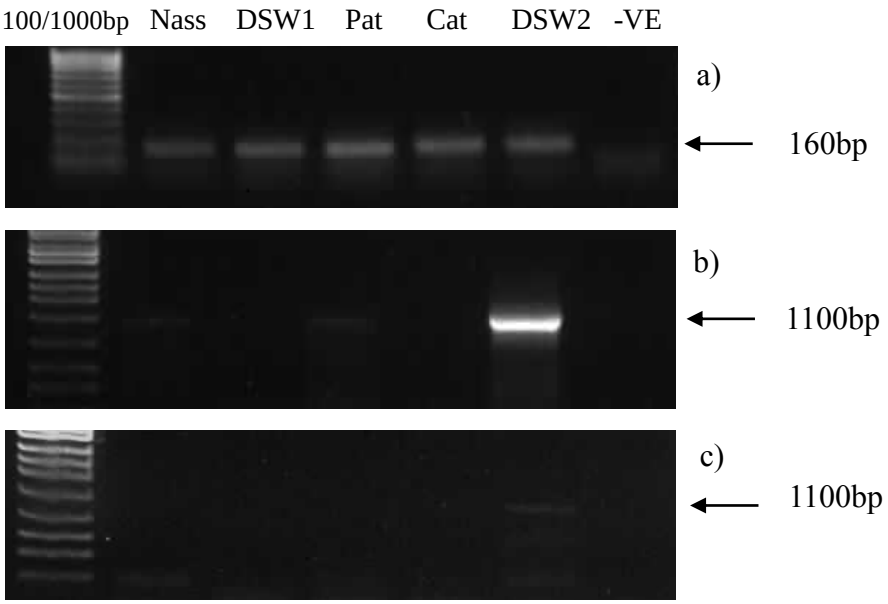

Figure 6.3.1. Composite gel image showing the PCR product for the beta-tubulin 160bp (a), beta-tubulin 1100bp (b) and *pgp-9* (c) amplicons for cyathostomin cDNA from *Cylicocyclus nassatus,* (Nass) *Cyathostomum pateratum* (Pat), *Cyathostomum catinatum* (Cat) and mixed species, DSW1 and 2.

6.3.1.2. Analysis of sequence data from PCR for pgp-9

The sequence was found to have 92 % identity to the *pgp-9* genes previously identified for *C. elongatus* across 100 % of the amplicon. BLAST analysis showed 77 % and 74 % identity to *H. contortus* and *T. circumcincta pgp-9* respectively, and lower levels of identity to other nematode species (Appendix *3.2*). A nucleotide sequence alignment of all 10 sequences (A1-5 and B1-5) from the DSW PCR product showed the range of identity to be between 91.1 % (between *C. elongatus* and DSW sequence B4 and A4) and 99.8 % (between DSW sequence B2 and B3). The maximum likelihood phylogenetic tree for nucleotide sequence (Figure 6.3.2.) bootstrap values indicated that the model predicts with 100 % certainty that the sequences from DSW are closely related to *C. elongatus* in comparison to sequence from other nematodes. For the 10 sequences from DSW there was 80 % certainty that these separated into two clusters. Within the clusters there were few differences between sequences and the likelihood that these represented phylogenetically distinct sequences fell, as reflected by the lower bootstrap values

seen in Figure 6.3.2. The alignment of a deduced consensus amino acid coding sequence for the 10 DSW sequences, and the corresponding gene region of *C. elongatus*, is shown in Figure 6.3.3. The sequence reveals the typical domain arrangement of one half of a P-gp gene with an ABC transporter transmembrane domain containing transmembrane helices followed by the first part of the first nucleotide binding domain containing the highly conserved Walker A/P loop (CDD accession number cd03249), highlighted in Figure 6.3.3. Other highly conserved regions lie just outside of the sequenced region (Figure 6.3.3.). The sequence identity ranged between 91.1 % (between *C. elongatus* and DSW B4 and A4) and 99.8 % (between sequence DSW B2 and B3).

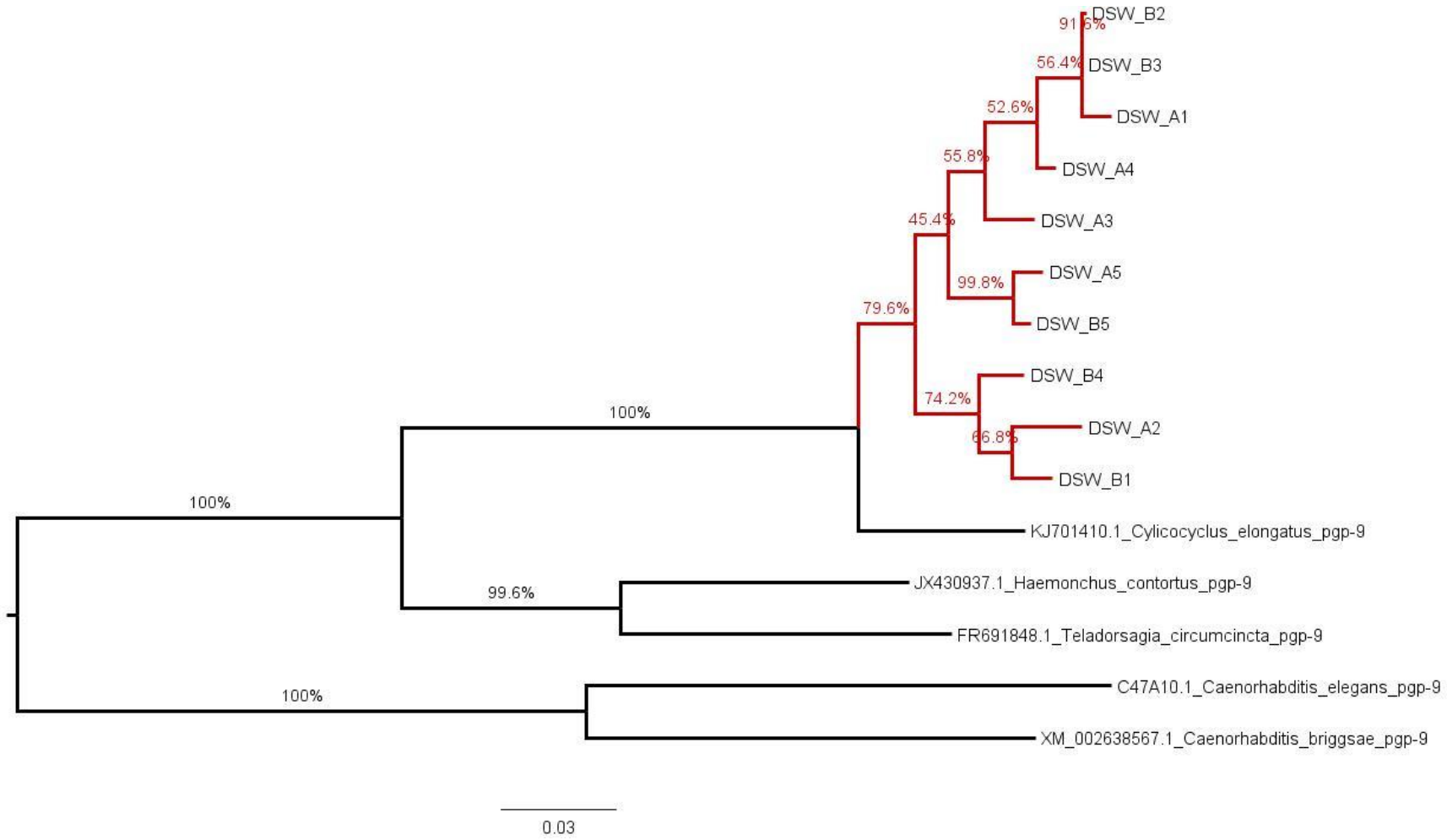

Figure 6.3.2. Maximum likelihood phylogenetic tree demonstrating the relationship between nucleotide sequences for DSW (A1-5 and B1-5) (highlighted red) and published *pgp-9* sequences from *Cylicocylus elongatus pgp-9* (A.N. KJ701410.1), *Haemonchus contortus pgp-9* (A.N. JX430937.1), *Teladorsagia circumcincta pgp-9* (A.N. FR691848.1), *Caenorhabditis elegans pgp-9* (C47A10.1 wormbase i.d.) and *Caenorhabditis briggsae pgp-9* (XM_002638567.1).

Figure 6.3.3: Amino acid alignment of translated sequence from a consensus of all DSW sequences (DSW_C) and *Cylicocyclus elongatus pgp-9* amino acid sequence (A.N. AJM87336.1) (C_elongatus_pgp9). Amino acid changes are indicated underneath the alignment, with * indicating conserved, : indicating strongly similar, . indicating weakly similar and blank indicating different. The characteristic regions of ABC transporters are highlighted with text boxes in the two nucleotide binding domains. Colour coding is a follows: red = Walker A/P loop; black = Q loop; yellow = ABC transporter signature motif; green = Walker B; Purple = H loop (CDD A.N. cd03249).

6.3.1.3. Pgp-9 transcription in L3 from two mixed species populations of cyathostomins of differing ivermectin sensitivity

Absolute and relative copy numbers of *pgp-9* transcript in L3 samples from Pop 1 (IVM-resistant) and Pop 2 (IVM-naive) after incubation with either dH_2O, 3% DMSO or 0.15 µg/ml IVM for 2 h are shown in Table 6.3.1; these are represented in a box and whisker plot showing the transcript levels of *pgp-9* mRNA relative to the housekeeping gene, 18S (Figure 6.3.4). After exposure to dH_2O there was slightly higher transcript expression in Pop 1 than Pop 2, suggesting higher baseline levels of *pgp-9* transcription in Pop 1, although the difference was not significant ($p=0.159$). After exposure to DMSO there was a statistically significant increase in *pgp-9* transcription in Pop 1 compared to Pop 2 ($p=0.03$). Finally, after exposure to IVM there was a highly statistically significant increase in *pgp-9* transcription in Pop 1 compared to Pop2 ($p<0.001$). Within each population there was a significant effect of the different treatments for Pop 1 ($p=0.005$), but not for Pop 2 ($p=0.313$). The fold increase in *pgp-9* transcripiton between the DMSO and IVM treatment in Pop 1 was 2.44

Table 6.3.1. The results, in terms of absolute and relative copy numbers, of real time PCR for the house keeping gene 18S and *pgp-9* in Populations 1 and 2, exposed to distilled water (dH_2O), 3 % dimethyl sulfoxide (DMSO) or ivermectin at 0.15 µg/ml (IVM).

Sample	Treatment	Biological repeat (average of 2 technical repeats)	Copy number of house keeping gene 18s	Copy number of *pgp-9*	Relative copy number for *pgp-9*
Population 1	DH_2O	1	1.83E+08	156.14	8.52E-07
		2	1.33E+09	891.79	6.68 E-07
		3	8.65E+08	1657.53	19.17 E-07
	DMSO	1	1.53E+09	2675.41	17.53 E-07
		2	3.23E+08	301.65	9.34 E-07
		3	2.43E+08	440.50	18.10 E-07
	IVM	1	2.37E+08	737.13	31.13 E-07
		2	1.32E+08	458.68	34.63 E-07
		3	1.21E+08	532.03	44.02 E-07
Population 2	DH_2O	1	9.67E+08	340.79	3.53 E-07
		2	1.64E+09	717.24	4.38 E-07
		3	3.68E+09	2189.31	5.95 E-07
	DMSO	1	5.55E+08	209.81	3.78 E-07
		2	1.4E+09	411.78	2.94 E-07
		3	9.17E+08	674.28	7.35 E-07
	IVM	1	9.71E+08	699.05	7.20 E-07
		2	9.26E+08	544.00	5.88 E-07
		3	8.62E+08	561.11	6.51 E-07

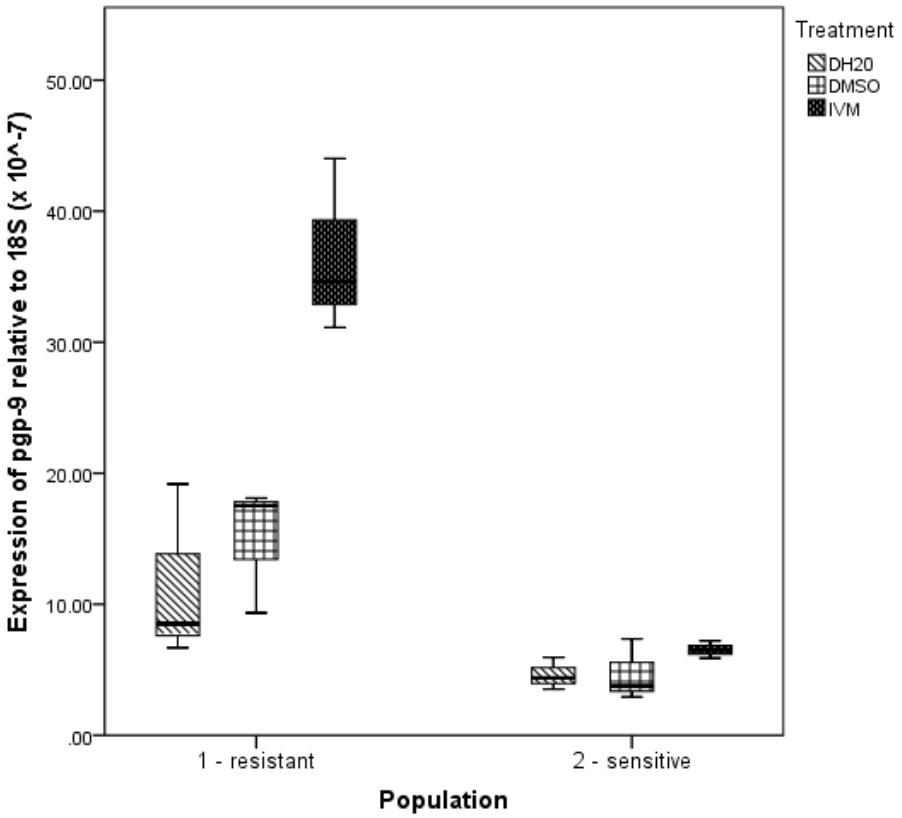

Figure 6.3.4. Box plot showing the transcript levels of *pgp-9* mRNA relative to the housekeeping gene, 18S, in third stage larvae from Populations 1 and 2, which had been incubated with distilled water (DH_2O), dimethyl sulfoxide (DMSO) or ivermectin (IVM).

6.3.2. Evaluating the effect of P-glycoprotein inhibitors on ivermectin efficacy in the larval development test and larval migration inhibition test

The results of the GLMM for each P-gp inhibitor are shown in Table 6.3.2., with coefficients and P-values for each variable. For P85 in the LMIT a piecewise GLMM was performed to model the effect at low and high IVM concentrations. Concentration of IVM and the presence of P-gp inhibitor had a significant positive effect for all tests (p values between 0.002 and <0.001), except with IVM-AG in the LDT (p=0.561). There was also a significant effect of Population in all LMIT tests (p<0.001), except in the piecewise model for P85. There was no effect of Population in the LDT (p=0.270 (with P85), p=0.907 (with ketoconazole), p=0.561 (with IVM-AG)). There were significant interactions between concentration and P-gp inhibitor

addition for all models except IVM-AG in the LDT, which indicated that the effect of P-gp inhibitor altered depending on IVM concentration. There were significant interactions between concentration and Population in all models except P85 in the LMIT at concentrations lower than 0.6 µg/ml, which indicated that the effect of Population changed depending on IVM concentration. Finally there were significant interactions between Population and P-gp inhibitor in all models except P85, which indicated that the effect of the addition of P-gp inhibitor differed between Populations in most cases. The overall outcome of all of these effects and their interactions was represented by the OR for larval death at each concentration of IVM and are represented graphically for each Pgp-inhibitor in the LDT or LMIT in Figures 6.3.5., 6.3.6. and 6.3.7. These graphs demonstrate that IVM-AG and ketoconazole increase the likelihood of larval death in the LMIT (Figure 6.3.5.). The effect is greater in Pop 1 for IVM-AG and ketoconazole. The OR graphs for the piecewise model for P85 are presented in Figure 6.3.6 and show that, concentrations less than 0.6 µg/ml (Figure 6.3.6.a), P85 increases the likelihood of larval death, whereas high concentration greater than 0.6 µg/ml (Figure 6.3.6.b) P85 reduces the likelihood of larval death. Figure 6.3.7. shows the effect of adding P-gp inhibitors in the LDT, on the likelihood of larval death. There was a clear increase in OR for larval death in both Pop1 and 2 with P85 and ketoconazole, there was no differential effect of Population. No effect was observed for IVM-AG.

Table 6.3.2. Results of a general linear mixed model for the dose response data in the LMIT and LDT with the addition of three p-glycoprotein inhibitors (PGPI); pluronic 85 (P85), ketoconazole (keto) and ivermectin aglycone (IVM-AG), in Populations 1 (Pop1) and Population 2 (Pop2). There is a separate model for each PGPI in each assay and within this, the coefficient, standard error and p-value are given for each fixed factor and interaction between factor (indicated by :).

Test	P-gp inhibitor	FIXED Factor	Coefficient	Standard Error	P-value
LDT	P85	Concentration	0.32	0.01	<0.001*
		PGPI addition	-0.47	0.15	0.002*
		Pop 2	-0.70	0.63	0.27
		Concentration: PGPI	1.29	0.06	<0.001*
		Concentration: Pop 2	0.52	0.04	<0.001*
		Pop 2: PGPI	2.98	0.20	<0.001*
	Keto	Concentration	0.36	0.01	<0.001*
		PGPI addition	-0.76	0.16	<0.001*
		Pop 2	-0.09	0.84	0.907
		Concentration: PGPI	1.13	0.05	<0.001*
		Concentration: Pop 2	0.40	0.03	<0.001*
		Pop2: PGPI	2.56	0.19	<0.001*
	IVM-AG	Concentration	0.35	0.01	<0.001*
		PGPI addition	-0.08	0.14	0.561
		Pop 2	-0.09	062	0.885
		Concentration: PGPI	-0.02	0.02	0.321
		Concentration: Pop 2	0.35	0.02	<0.001*
		Pop 2: PGPI	0.72	0.15	<0.001*
LMIT	P85	Concentration < 0.6	2.34	0.36	<0.001*
		Concentration >= 0.6	0.10	0.00	<0.001*
		PGPI addition	0.22	0.03	<0.001*
		Pop 2	0.13	0.18	0.489
		Concentration<0.6: PGPI	2.67	0.40	<0.001*
		Concentration>=0.6: PGPI	-0.08	0.00	<0.001*
		Concentration<0.6: Pop 2	0.17	0.40	0.670
		Concentration>=0.6: Pop 2	-0.03	0.00	<0.001*
		Pop 2: PGPI	0.07	0.04	0.077
	Keto	Concentration	0.13	0.00	<0.001*
		PGPI addition	0.53	0.03	<0.001*
		Pop 2	0.46	0.09	<0.001*
		Concentration: PGPI	0.04	0.00	<0.001*
		Concentration: Pop 2	0.07	0.00	<0.001*
		Pop 2: PGPI	0.66	0.04	<0.001*
	IVM-AG	Concentration	0.06	0.00	<0.001*
		PGPI addition	0.40	0.04	<0.001*
		Pop 2	0.66	0.09	<0.001*
		Concentration: PGPI	0.01	0.00	<0.001*
		Concentration: Pop 2	0.06	0.00	<0.001*
		Pop 2: PGPI	-0.49	0.06	<0.001*

*indicates the significance in the model

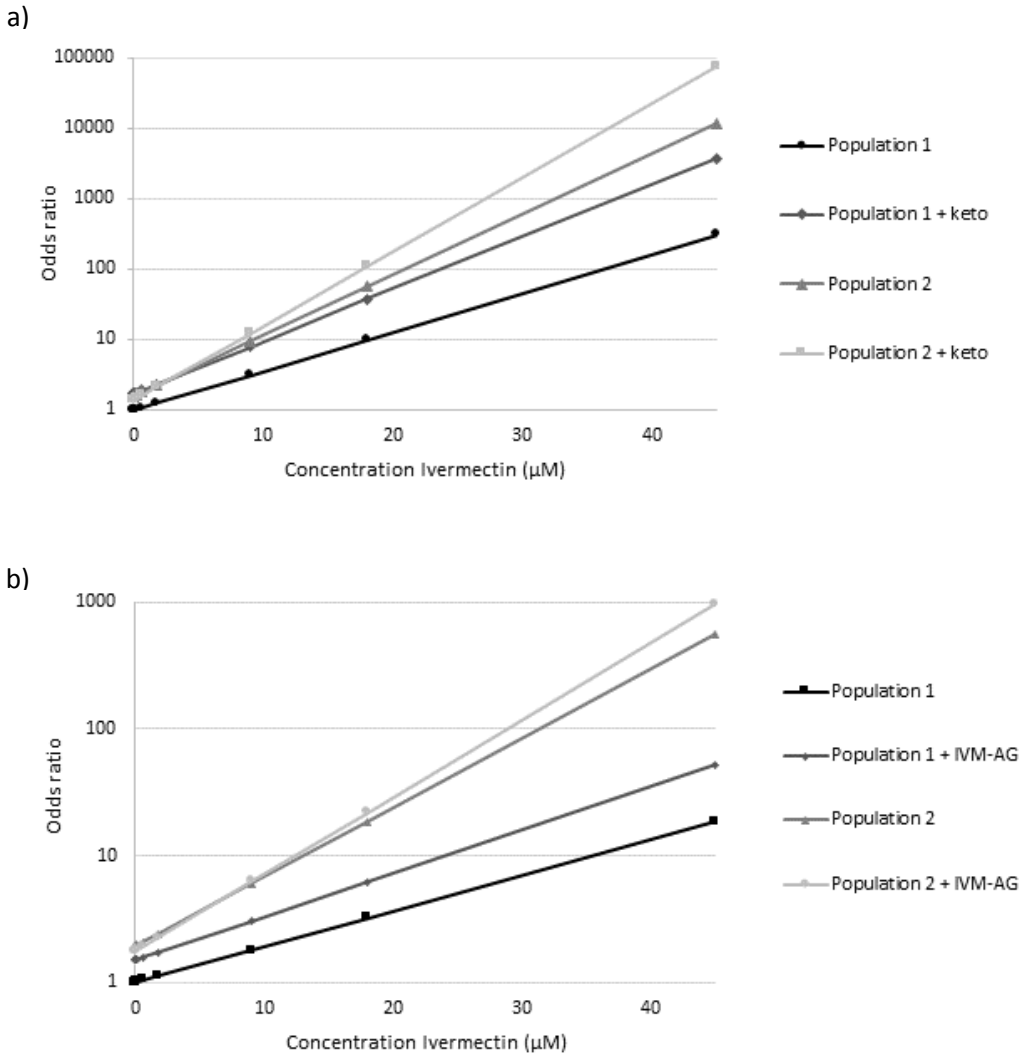

Figure 6.3.5. Graphical representation of the odds ratio for larval death (calculated from the output of the general linear mixed model) plotted against ivermectin concentration, for the larval migration inhibition test, with the addition of ketoconazole (keto) (a) and ivermectin aglycone (IVM-AG) (b). Each graph shows Populations 1 and 2, with and without the addition of the p-glycoprotein inhibitor plotted as separate lines.

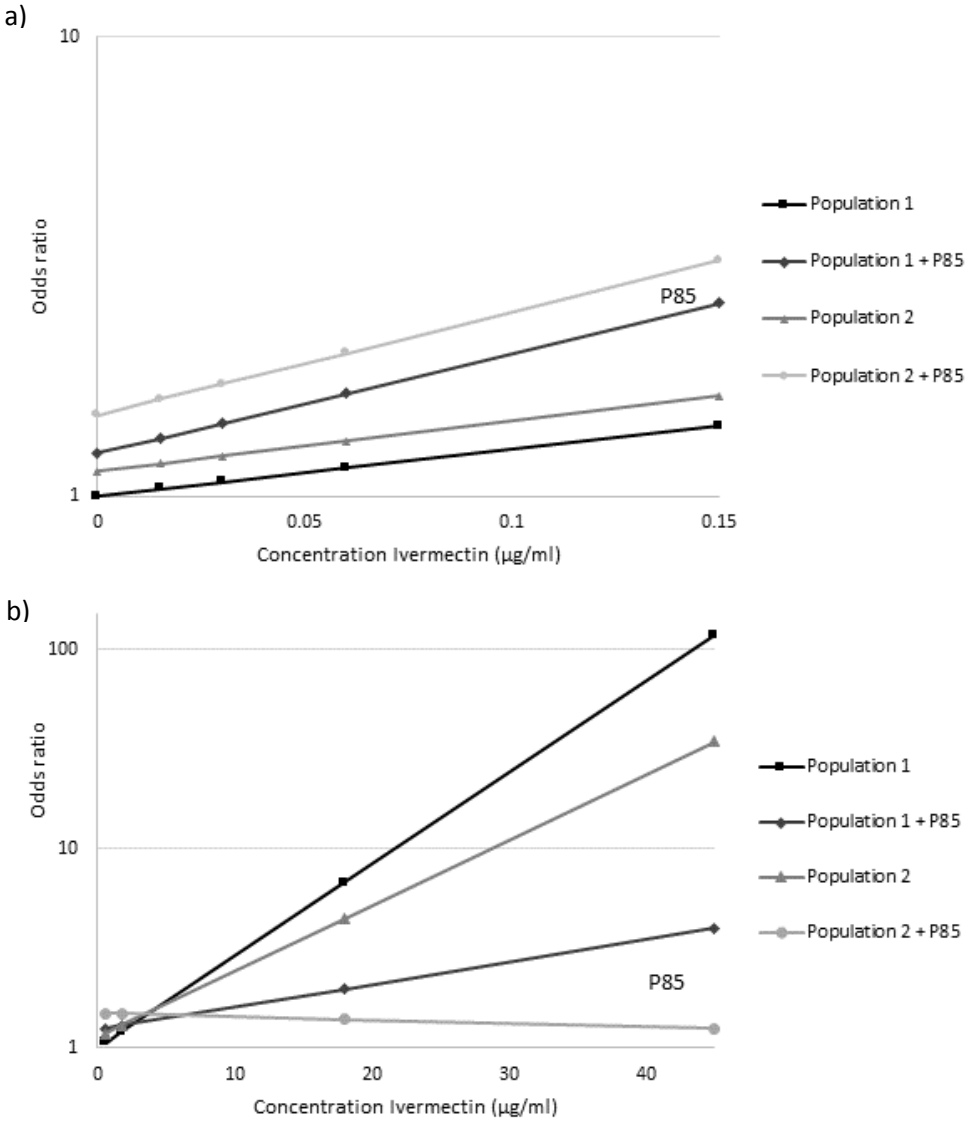

Figure 6.3.6. Graphical representation of the odds ratio for larval death (calculated from the output of a piecewise general linear mixed model) plotted against ivermectin concentration, for the larval migration inhibition test (LMIT), with the addition of pluronic 85 (P85) at a concentration of less than 0.6 µg/ml (a) and greater than 0.6 µg/ml (b). Each graph shows Populations 1 and 2, with and without the addition of the p-glycoprotein inhibitor plotted as separate lines.

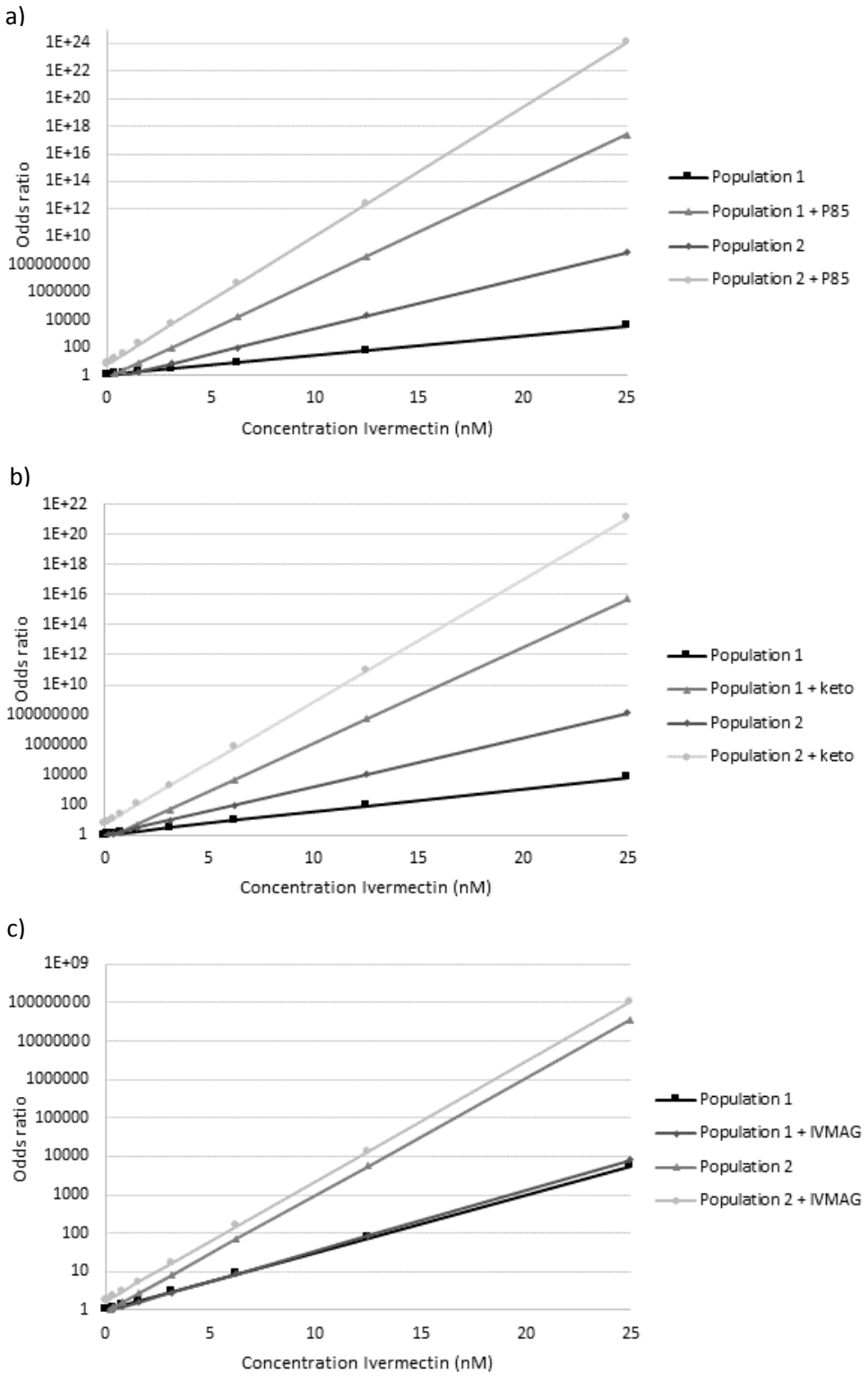

Figure 6.3.7. Graphical representation of the odds ratio for larval death (calculated from the output of the general linear mixed model) plotted against ivermectin concentration, for the larval development test (LDT) with the addition of pluronic 85 (P85) (a) and ketoconazole (keto) (b) and ivermectin aglycone (c). Each graph shows Populations 1 and 2, with and without the addition of the p-glycoprotein inhibitor plotted as separate lines.

6.4. Discussion

This study has demonstrated higher levels of *pgp-9* gene transcript after IVM exposure for cyathostomin L3 with a 'resistant' phenotype (defined as an increased ERP *in vivo*). In addition it has demonstrated that IVM efficacy in the same population can be increased by its combination with certain P-gp inhibitors *in vitro*. Together this evidence suggests a role for P-gps in emerging IVM resistance in cyathostomins, and raises the possibility that the use of P-gp inhibitors in equids *in vivo* may increase the susceptibility of IVM-resistant parasite populations, as has been shown for sheep and cattle (Lifschitz et al., 2010a; Lifschitz et al., 2010b).

6.4.1. Identification of pgp-9 sequence from a cyathostomin population of unknown species

In order to design a real time PCR assay to measure transcription of *pgp-9* in cyathostomins, DNA was amplified from a cDNA sample, produced from cyathostomin populations of unknown species. *Pgp-9* was chosen specifically as increased transcription of this gene has been associated with ML resistance in closely related parasitic nematodes (Dicker et al., 2011b; Williamson et al., 2011). The results of the BLAST and phylogenetic analysis supported that the cyathostomin *pgp-9* gene had been successfully isolated. Preliminary analysis of the sequence data showed that there were high levels of nucleotide identity (91.1-99.8 %) amongst DSW sequences and with the recently published, *C. elongatus pgp-9* sequence (Kaschny et al., 2015). Preliminary analysis of the data, in a maximum likelihood phylogenetic model, predicted with 100 % certainty that there was a true difference between DSW A1-5 and B1-5 nucleotide sequence and *C. elongatus* sequence; this may reflect intra-specific variation but may also be due to inter-specific variation due to the presence of multiple different cyathostomins species in parasites present with the equid GI tract (Ogbourne, 1976). The fact

that analysis of nucleotide sequence showed they clustered into two groups, raises the possibility that *pgp-9* from two species of cyathostomin was amplified here. Previous work on morphological identification of adult cyathostomins isolated from donkeys at the Donkey Sanctuary identified 80 % *Cyathostomum tetracanthum, C. catinatum* and *C. pateratum* with some *Cylicostephanus longibursatus* and *Cylicocyclus nassatus* (Cwiklinski et al., unpublished data). Further work is required to identify levels of intra- and inter-specific variation of *pgp-9* in cyathostomins and which species we have represented here.

6.4.2. Pgp-9 transcription in cyathostomin populations with differing ivermectin sensitivity

Importantly, from the sequence data identified here, it was possible to design consensus primers for, and validate, a real time PCR assay which would measure transcript levels in *pgp-9* sequence from cyathostomin cDNA. Subsequently it was shown that *pgp-9* transcription in cyathostomin L3 exposed to IVM was increased three fold in cDNA from Pop 1 (IVM 'resistant'), whilst no change was measured in that from Pop 2 (IVM 'sensitive'). As noted above, in this study cyathostomin species composition was not known for the two L3 populations. As the primers were designed from consensus sequence for all DSW sequences and *C. elongatus*, it is possible that the PCR would have measured transcription of *pgp-9* from the range of cyathostomin species in each population. The real time PCR was also carried out on cDNA from *C. nassatus, C. pateratum, C. catinatum* as part of the validation process, with positive results and melting curves identical to the *pgp-9* standard (data not shown here). However further work in identifying the exact species mix in the two populations, and validating the PCR on each of these species individually, is required to fully validate the real time PCR. Regardless of species differences, within the context of each population, it is clear that IVM induced higher levels of *pgp-9* transcript in Pop 1 compared to the baseline with

dH_2O, whereas it has no effect in Pop 2, indicating that this gene is upregulated in cyathostomins with a 'resistant' phenotype. This is in agreement with studies in two closely related species, *T. circumcincta,* and *H. contortus,* which also demonstrated increased transcription of this gene in drug resistant strains (Dicker et al., 2011b; Williamson et al., 2011). In Williamson et al (2011), transcription levels were measured in L3 cultured from multi-drug resistant *versus* susceptible parasites that had been passaged through goats, the fold change in *pgp-9* was similar to that seen here, at 2.65. Dicker et al. (2011) measured transcription levels in all life cycle stages of *T. circumcincta* from a multidrug resistant v*ersus* susceptible strain, a much larger fold change of 17.49 was recorded in this study. The reason for the higher levels of transcription observed for *T. circumcinta,* in comparison to the findings here, may be that the data was generated using a homogenous multidrug resistant strain, with FECR values of only 60 % for MLs, whereas in this study a reduced ERP was used as a proxy for ML resistance. In addition the samples used here were mixed samples from multiple cyathostomin species, which may have introduced variation if those species had different transcription levels. Unfortunately in cyathostomins there are currently no established parasite populations with unequivocal ML resistance for performing this type of study.

Different P-gps have been implicated in ML resistance in other parasitic nematodes; for example, *pgp-11* in *Cooperia oncophora* and *Parascaris equorum* (De Graef et al., 2013; Janssen et al., 2013). De Graef et al (2013) demonstrated increased transcript levels of *pgp-11* in adult worms isolated from calves that had been treated with IVM or MOX. In addition they were able to induce increased transcript levels in L3 by exposing them to IVM *in vitro* in resistant, but not susceptible, isolates, which is identical to our findings here for *pgp-9*. Janssen et al (2013) demonstrated increased transcription of *pgp-11* in pre-adult stages of ML resistant *P. equorum,* in addition to three SNPs causing missense mutations. Gene knockout studies in

C. elegans have also shown that different combinations of ABC transporter are associated with IVM and MOX sensitivity, indicating that the drug type affects which transporters are likely to be associated with resistance (Ardelli and Prichard, 2008). In summary, the variations in P-gp subtype associated with ML resistance probably reflect a difference in the functionality of corresponding genes, such that the specific P-gp involved for their efflux may differ among nematode species and ML subclass.

6.4.3. The effect of P-glycoprotein inhibition on the effect of ivermectin against cyathostomins in vitro

In the second part of this study *in vitro* bioassays were used to investigate the effect of a range of P-gp inhibitors on IVM efficacy in cyathostomins. Specifically the LDT and LMIT were chosen as they are established methods for evaluating IVM efficacy in parasitic nematodes (Demeler et al., 2010a; Demeler et al., 2010b). The LDT measures the ability of L1 to develop to L3 in the presence of increasing IVM concentrations, and the LMIT measures the motility of L3, through their ability to migrate across a mesh in the presence of increasing IVM concentrations. An interesting observation here was that overall there was no statistically significant difference between Pop 1 and 2 in the LDT, whereas the LMIT consistently differentiated between the two with respect to IVM efficacy. This is in agreement with previous studies in cyathostomins where the LDT has shown poor correlation with the *in vivo* phenotype (Lind et al., 2005; Tandon and Kaplan, 2004). In other parasitic nematodes such as *H. contortus*, the LDT is an established technique for detecting differences in drug sensitivity (Dolinska et al., 2013). This may indicate that cyathostomins differ in the specific mode of action of IVM and potential mechanisms of resistance. A recent study has validated the use of the LMIT to detect differences in *in vivo* sensitivity to IVM (McArthur et al., 2015). The data

herein confirm that, in cyathostomins, the LMIT is a better *in vitro* tool for identifying reduced IVM efficacy, and consequently for investigating resistance mechanisms in cyathostomins.

This study demonstrated that the addition of P-gp inhibitors impacts the effect of IVM on the larval stages of cyathostomins, as measured in the LMIT and LDT. This finding is consistent with studies in other parasitic nematodes (AlGusbi et al., 2014; Ardelli and Prichard, 2013; Bartley et al., 2009; Demeler et al., 2013; Raza et al., 2015). Overall the effect was dependent on the parasite isolate, the test (and hence the life cycle stage of the parasite) and the P-gp inhibitor used. P-gp inhibitors have been shown to have different modes of action and levels of affinity for different ABC transporters. Ketoconazole, an antifungal imidazole used in veterinary medicine, is a potent inhibitor of cytochrome p450 in humans (Maurice et al., 1992) and it has been shown to alter the pharmacokinetics of several P-gp substrate drugs by competitive inhibition at P-gp channels (Bartley et al., 2009; Bartley et al., 2012; Hugnet et al., 2007; Kageyama et al., 2005; Ward et al., 2004). Specifically it has been shown to compete with IVM at the cyathostomin *pgp-9* channel (Kaschny et al., 2015). IVM-AG is an avermectin derivative that is a substrate for P-gp, and therefore also acts by competitive inhibition. It has been shown to be as potent as verapamil, one of the best P-gp inhibitors, and importantly it has greater affinity for nematode *versus* mammalian P-gp (Lespine et al., 2013). P85 has a multimodal action, it belongs to a group of compounds called polaxomers, which are used as nanocarriers in drug delivery systems. They incorporate into cell membranes changing their microviscosity, this has the effect of inhibiting ATPase activity, thus reducing the activity of ABC transporters (Batrakova and Kabanov, 2008). They have been shown to be potent inhibitors of P-gps and also other ABC transporters such as MRP1 and MRP2 (Shaik et al., 2008).

In this study, in the LMIT IVM-AG and ketoconazole increased the effect of IVM to a greater extent in Pop 1 than Pop 2. In other words, substrate inhibitors of P-gp increased the effect of IVM on L3 with a 'resistant' phenotype, but had less effect on L3 with a 'sensitive' phenotype. This suggests that P-gp activity is upregulated in the 'resistant' phenotype, which supports the earlier finding of increased *pgp-9* transcription in L3 from the same population. A study by Demeler et al (2013), tested different P-gp inhibitors against *C. oncophora* in the LDT and LMIT, and reported similar results to those found here; i.e. in the LMIT only there was an increased effect on the resistant population *versus* a susceptible one. In this study, the LMIT P85 had a bi-modal effect, whereby it increased the effect of IVM in both populations at low IVM concentrations, and at IVM concentrations above 0.6µg/ml the effect was reversed. One study in *C. elegans* has also shown that the effect of different P-gp inhibitors can be dependent on IVM concentration, although there was not a clear reversal of the effect as seen here (Ardelli and Prichard, 2013). Another study using P85 as a P-gp inhibitor with IVM in a larval feeding assay in *H. contortus* and *T. circumcinta*, did not note this bimodal effect (Bartley et al., 2009), and in addition it was not seen here in the LDT for P85. One point to note is that much higher concentrations are used in the LMIT than in assays on earlier larval stages, and hence the effect seen may be an interaction specific to these high concentrations. Relating the mode of action of P85 to the effects seen here is clearly not straightforward, and further work is needed to elucidate them.

In the LDT, ketoconazole and P85 increased the effect of IVM in both populations to a similar extent. As mentioned above, the LDT did not differentiate between the 'resistant' and 'sensitive' population, and therefore it follows logically that it also did not differentiate

between the effects of P-gp inhibitors on IVM efficacy in these populations. Demeler et al. (2013), found a similar effect in *C. onchophora* and Bartley et al. reported little differential effect of P-gp inhibitors between resistant and sensitive in a larval feeding assay, which is also carried out on the early larval stages. What can be concluded from the LDT here, is that P-gp inhibitors markedly increase the effect of IVM on early larval stages of cyathostomins, which suggests that they do express P-gps and that they are involved in the metabolism of IVM. In contrast to ketoconazole and P85, it was found that IVM-AG did not have an effect in the LDT. This was surprising given its effect in the LMIT, however it was used at a much lower concentration in the LDT than LMIT, as during optimisation it was found that it had an independent effect at higher concentrations. IVM-AG is known to exert some paralytic effect on early larval stages of GI nematodes, although it is significantly less potent than IVM, and has no effect on adults (Sheriff et al., 2002). It is probable that it was not at a high enough concentration to cause significant competitive P-gp inhibition here. Further work here might include increasing concentrations in the LDT to investigate any possible synergistic effect.

There have been a number of other studies reporting the effects of different P-gp inhibitors on the *in vitro* effect of IVM in parasitic nematodes (AlGusbi et al., 2014; Ardelli and Prichard, 2013; Bartley et al., 2009; Demeler et al., 2013; Raza et al., 2015). As with this study there are reports of differing effects dependent on parasite population, test and P-gp inhibitor used. For example Raza et al. (2015) tested some P-gp inhibitors, tariquidar, zosuquidar, elacridar, verapamil and valspodar, against *H. contortus* larvae of differing drug sensitivity, in the LDT and LMIT. They found that several of these increased sensitivity of drug resistant and susceptible isolates, for example, tariquidar with IVM in the LMIT and zosuquidar with IVM in the LDT, whereas others had a significant effect on the resistant isolate only, for example, zosuquidar with IVM in the LMIT and verapamil with IVM in the LDT. There is also *in vitro*

evidence that there are differences between nematode species, for example a recent study investigating the effect of verapamil on IVM in the LMIT showed there to be a positive effect in both resistant and sensitive isolates from *Ostertagia ostertagi* but only in the resistant isolate of *C. oncophora* (AlGusbi et al., 2014). In summary these studies, combined with the data collected here, suggest that some inhibitors interact with P-gps representing intrinsic pathways present across nematode populations with different drug sensitivities, and others interact with P-gps of significance only to resistant nematodes, and represent an acquired resistant mechanism. These interactions also appear to alter depending on test (and therefore life cycle stage) and nematode species.

6.4.4. The potential use of P-glycoprotein inhibitors in combination with ivermectin in vivo to increase ivermectin efficacy

Due to the evidence from *in vitro* studies, there is increasing interest in the potential use of P-gp inhibitors to provide more effective control of GI nematodes *in vivo*. The proposed mechanisms of such combinations *in vivo* are: (i) reducing the efflux of drug from nematodes, and hence increasing the amount of drug reaching its target site within the nematode, and (ii) reducing the excretion of drugs by host animals, and hence increasing their bioavailability. Based on the results here, IVM-AG and ketoconazole are possible options for taking forward to *in vivo* trials in equids as they have both increased the effect of IVM in the 'resistant' population. Even though the effects of P85 were not specific to the 'resistant' population, it is also worth considering, as its effects were potent in the LDT, and at lower concentrations of IVM in the LMIT. The concentrations that the worm is exposed to *in vivo* are likely to be significantly lower than 0.6 µg/ml, where a reversal of the effect of P85 was seen in the LMIT (Lloberas et al., 2012; Perez et al., 1999; Perez et al., 2002). To date, the results of *in vivo* trials

in other species have been equivocal. For example, a study in sheep showed that, although the plasma concentration of IVM was significantly increased by its co-administration with ketoconazole, there was no effect on efficacy against *H. contortus* as measured by the FECRT (Bartley et al., 2012). The same study did show a moderate effect of P85 on IVM efficacy, even though the plasma concentration was similar to that seen with ketoconazole, which might indicate that P85 had a direct effect on parasite P-gp function. P85 may have an advantage over other drugs such as ketoconazole, in that it is an excellent drug delivery agent, and therefore is more likely to be delivered to the parasite with IVM (Batrakova and Kabanov, 2008).

The study which has shown the most convincing effect of an IVM/P-gp combination has used loperamide in combination with IVM in sheep against resistant GI nematodes, and shown a markedly improved efficacy (Lifschitz et al., 2010a). Loperamide has been shown to increase plasma concentrations of MLs (Lifschitz et al., 2002); it also reduces faecal transit time and therefore may increase exposure time of parasites to MLs in the gut. Despite the lack of clarity of the evidence, given that no new anthelmintics are likely to be licenced in equids in the short to medium term, there is certainly cause to investigate the use of P-gp inhibitors in combination with MLs for treatment of cyathostomin infections in future. Some of the P-gp inhibitors tested here have already been used systemically in equids, which may facilitate future *in vivo* trials, for example ketoconazole for the treatment of fungal infections (Nappert et al., 1996), and loperamide for diarrhoea (Sanchez, 2014). It is also important to be aware of the potential side effects of drug interactions, for example increased toxicity of IVM due to increased bioavailability and plasma levels, however, reassuringly toxicity has not been noted in the *in vivo* trials discussed here.

6.4.5. The use of a general linear mixed model to analyse dose response data

In this study two different approaches were initially taken to analyse dose response data from *in vitro* tests. The first is the conventional PROBIT analysis, which gives an EC-50 value for comparison between tests, see Appendix 3.1. The potential issue with using this method is that, especially in tests subject to variability, the results do not always fit a PROBIT model, a good example in this study is the case of P85 in the LMIT. If the results were analysed using PROBIT then the EC-50 values would not truly represent the bimodal relationship between IVM concentration and larval migration in the presence of P85. Other methods are also described in the literature, such as the four parameter model, which offer greater flexibility in model fitting (AlGusbi et al., 2014; Demeler et al., 2013). Here, the use of a GLMM was explored to allow statistical comparison of the effect of each PGPI on the two populations in each test. A piecewise break down of this model allowed successful modelling of the data from the LMIT with P85. The results correlated with what was seen from the EC-50 ratios (Appendix 3.1., Table A.3.1) thus validating the methodology. The GLMM can therefore be considered in future to be a flexible and statistically powerful method for analysing this type of data.

6.5. Conclusion

The results strongly implicate a role for P-gps in reduced sensitivity to IVM in cyathostomins. Specifically, increased *pgp-9* transcription in response to IVM in mixed species L3 populations was identified. The *in vitro* data confirm that IVM interacts with P-gps in cyathostomins, as the effect of IVM was increased by three different P-gp inhibitors in the LMIT and LDT. Specifically ketoconazole and IVM-AG increased the effect of IVM in the 'resistant' cyathostomin isolate to a greater extent than in the 'sensitive' population, implicating interference with an acquired resistance mechanism. These data support the possibility that P-

gp inhibitors could be used in combination with IVM *in vivo*, as a novel approach to improving control of IVM resistant cyathostomins. The expression of various P-gps at different lifecycle stages in multiple cyathostomin species represents a highly complex system and it is clear that further work is needed to identify specific resistance mechanisms that can be targeted in future attempts to improve ML efficacy against these important parasitic nematodes.

7. FINAL DISCUSSION

7.1. Overview

Multidrug resistance is emerging in cyathostomins, and a lack of novel therapeutics on the horizon is putting the control of these important gastrointestinal (GI) nematodes under threat. This thesis examined the potential of novel control options for cyathostomins, with a focus on bioactive plants and combinations of ivermectin (IVM) and P-glycoprotein (P-gp) inhibitors. It was found that a number of crude plant extracts from plants sourced both in the UK, and Ethiopia, showed good anthelmintic activity *in vitro* (Chapters 4 and 5). It was also found that, not only was *pgp-9* transcription increased in cyathostomins with a 'resistant' phenotype, but P-gp inhibitors significantly increased IVM activity against cyathostomins *in vitro* (Chapter 6). This thesis provides vital preliminary *in vitro* data in order to take some of these novel therapeutic options forward into *in vivo* trials.

7.2. Do bioactive plant extracts represent a realistic option for the control of cyathostomins?

Here via a participatory rural appraisal (PRA) for Ethiopia and a literature review for the UK, several plant species were chosen for *in vitro* evaluation of any anthelmintic effects against cyathostomins. These are established techniques for gathering information on the potential anthelmintic activity of plants (Hussain et al., 2008; Lans et al., 2000; Nabukenya et al., 2014a; Tolossa et al., 2013). During this work several issues arose which highlighted potential problems with the uptake of plant-based treatments for cyathostomins in equids. For Ethiopia the PRA data suggested that people do not treat donkeys and horses as they do other livestock, see Section 3.3.1., and hence there may be a fundamental lack of awareness of the need to administer treatment, even if a plant was to be recommended. As discussed in Section 3.4.4., it may be of use to identify proxies to indicate that treatment is needed in equids such as has

been done for parasitic disease in livestock (Nabukenya et al., 2014a; Vilela et al., 2012), or alternatively recommend regular treatment at certain times of year when transmission intensity is known to be at its highest (Getachew et al., 2008). It may be necessary to implement education programs imparting this information, before recommending any plant based treatments. In addition, in a thematic analysis of opinions from the PRA, it was found that there was a move away from traditional plant treatments in favour of chemical anthelmintic preparations available at government clinics, due to concerns regarding efficacy and side effects (Scantlebury et al., 2013). This has also been noted in other recent ethnobotanical studies (d'Avigdor et al., 2014). It seems that peoples' opinions regarding ethnoveterinary medicines are changing, and this may make it difficult to implement plant-based treatment recommendations in future. The PRA also called into question the ethics of recommending plant based treatments to poor rural communities in developing countries. There was wide variability in the preparation and dosing of plants and the side effects reported, in addition some of the methods of dosing were potentially dangerous, see Table 3.3.4.a and b. Any future *in vivo* trial would need to address these factors, and ensure that the proposed treatment was not dangerous in any form.

In developed countries, such as the UK, such issues are largely obsolete as people regularly treat equids with anthelmintics and there is access to better diagnostics. However there are similar safety considerations surrounding dosing and administration of bioactive plants. There are also issues surrounding the development of any plant based product for use in the UK as a novel pharmaceutical product. For example, to develop new an anthelmintic preparation with efficacy greater than 95 %, it would be necessary to identify the active component/s in each extract, perform toxicity tests and *in vivo* efficacy tests using a refined preparation (Shi et al., 2009; Wang et al., 2011). Research arising from this study could include such techniques,

however large scale production and testing in equids would be costly and better suited to a pharmaceutical company. It has already been highlighted in the introduction to this thesis, that the novel synthetic anthelmintics, emodepside and monepantel, do not appear to be being developed by pharmaceutical companies for use in equids (Section *1.3.4*) (Epe and Kaminsky, 2013), hence it seems unlikely that there would be uptake from this industry for developing plant extracts.

An alternative approach would be to evaluate the merit of supplementing equine feed with crude dried extract, or allow equids to graze forage with anthelmintic component, such as has been done for bioactive forages in ruminants (Heckendorn et al., 2007; Hoste et al., 2015). This approach would also eliminate some of the safety considerations surrounding methods of administration which apply in both the UK and Ethiopia, making it potentially the most feasible option for improving control of cyathostomins in equids using bioactive plants.

7.3. Which of the bioactive plants tested here should be considered for in vivo trials in equids?

As discussed above, the most feasible option in the short term, for developing a plant based anthelmintic treatment for use in equids in both the UK and Ethiopia, would be supplementation of the diet with a forage or crude extract with some anthelmintic activity. The aim would be similar to that of many of the novel approaches that were discussed in the introduction to this thesis, i.e. to reduce reliance on the currently available synthetic anthelmintics, and thus reduce selection pressure for further development of drug resistance

(Matthews, 2014). The choice of plant for future *in vivo* trials, will depend on which species is best suited to this therapeutic goal, whilst taking into account safety considerations.

The *in vitro* findings here showed that one of the most efficacious extracts from Ethiopia was *Acacia nilotica* fruit pods, whose anthelmintic effect was found to be due to the action of condensed tannins. In support of the use of *A. nilotica* for *in vivo* trials in equids, there is also evidence that it is efficacious against GI nematodes *in vivo* in sheep (Bachaya et al., 2009). In addition, in tropical and sub-tropical regions other tannin rich legume forages, such as *Desmodium intortum* (Mill.) Urb. and *Sesbania sesban* (L.) have been found to have anthelmintic activity *in vivo* (Debela et al., 2012). *Acacia nilotica* pods are seasonal, but in theory could be harvested for use in equids throughout the year and provided as forage, as described for other livestock species in Djibuti (Audru et al., 1993). Major advantages of forages with anthelmintic activity, are that they also have some nutritional value, and can be fed as part of a balanced diet (Hoste et al., 2015) and, in addition, they are usually consumed by choice, thus alleviating issues over methods of administration. This approach could be an extremely useful addition to cyathostomin control in equids in Ethiopia. In temperate regions such as the UK, forages containing high levels of tannins have also been found to be effective in reducing parasite burdens in small ruminants (Heckendorn et al., 2007; Lange et al., 2006). Examples of these are *Onobrychis viciifolia* Scop., *Lotus corniculatis L.* and *Lespedeza cuneate* G. Don., some of which were highlighted in the literature review for the UK in Chapter 3, see Appendix 2.2.. The bioactive forages were originally rejected for testing on donkey cyathostomins in the UK, due to concerns over their high nutritional value and the tendency of donkeys to obesity (Burden, 2012). However considering the evidence of the effect of tannins on cyathostomins, they deserve further consideration in future studies with horses and donkeys.

Other plants which were identified here as having an anthelmintic effect *in vitro*, included *R. abyssinicus* and *C. prophetarum* from Ethiopia, and *Allium sativum, Zingiber officinale* and *Chenopodium album* from the UK. These could also be considered for future *in vivo* trials given as a crude dried extract, as equids are unlikely to eat them in significant quantities voluntarily. Toxicity studies and detailed consideration of dosing and method of administration would be needed before embarking upon this work. The advantage of the *A. sativum* and *Z. officinale* for use in developing countries, is that they are already manufactured as dietary supplements in equids (Williams and Lamprecht, 2008), which would facilitate *in vivo* trials. Although these extracts are commonly given to equids, no study has reported their effects on GI nematode burdens in equids. This is an obvious area for further investigation that has been highlighted by this study.

If the aim were to develop a novel therapeutic with high efficacy for use in developed countries, the best candidate for taking forward to *in vivo* trials based on the results of this thesis, would be papaya latex supernatant (PLS), which contains high levels of cysteine proteinases (CPs). PLS was found to be effective against multiple stages of cyathostomins at low concentrations, thus, of all of the plant extracts tested, it showed the most potent effect against cyathostomins (Chapter 5). In addition there is a great deal of supporting evidence for its anthelmintic efficacy from *in vivo* studies in other veterinary species, and significant adverse side effects are not reported (Buttle et al., 2011; Levecke et al., 2014; Mansur et al., 2014a; Satrija et al., 1994; Stepek et al., 2006, 2007b, c). In the event of multidrug resistance becoming widespread in cyathostomins, PLS represents an excellent candidate for consideration as a novel therapeutic for cyathostomins in equids. Unfortunately a major factor preventing the development of extracts containing CPs as novel anthelmintics in other veterinary species, is that they may not be patentable, due to the fact they have been widely used for decades in traditional medicine.

This may make it unlikely that a pharmaceutical company would develop PLS as a product for treatment of cyathostomins in equids, even if there were a dire need.

7.4. The challenge of defining anthelmintic efficacy

This study has highlighted some general issues surrounding testing plants for anthelmintic activity. It was noted in Chapter 4, that the effect of extracts differed depending on the *in vitro* test and solvent used. Similarly there are reports of such variations in the literature (Marie-Magdeleine et al., 2009; Marie-Magdeleine et al., 2010a; Marie-Magdeleine et al., 2010b). There are also many other variables when testing plant extracts *in vitro.* The efficacy of extracts has been shown to depend on the part of the plant that is used: for example, an *in vitro* study assessing the anthelmintic activity of *Peltophorum africanum* found the root to be five times more efficacious than the leaf (Bizimenyera et al., 2006). The preparation of the plant material and method of extraction of plant compounds also varies between studies. For example there is a large variation in the time in which the plant compounds are extracted for: from as little as 15 minutes to 120 hours (Githiori et al., 2003a; Hordegen et al., 2003). In addition a wide variety of solvents are used for extraction including water, ethanol, methanol, acetone, ethyl acetate, hexane, DMSO, dichloromethane and acetic acid. Commonly aqueous and hydro-alcoholic solvents such as methanol and ethanol have been compared, with the latter usually proving more efficacious (Iqbal et al., 2006c; Marie-Magdeleine et al., 2010b). These differences probably account for the variation in results of studies in the literature for both *in vitro* and *in vivo* data. For example Hordegan et al. (2003) performed an *in vivo* study looking at the potential effects of 5 plant extracts, which had shown good efficacy in previous studies and only one plant, *F. parviflora,* showed significant efficacy. In addition the efficacy of *Albizia anthelmintica in vivo* has been assessed in different studies and found to be 34 % and

90 % depending on the study design (Gathuma et al., 2004; Githiori et al., 2003a). It is therefore important that initial plant screening should include testing on multiple life-cycle stages of parasites, with extracts from different extraction techniques, *in vitro* and in *vivo*. Future work here could include repeating extractions with different solvents and in more *in vitro* tests, but ultimately randomised controlled trials *in vivo* are needed to confirm anthelmintic efficacy.

Another area for future consideration is the use of high throughput screening techniques to look at the effect of plant extracts. Here the number of plants that could be screened was limited by the optimisation and repeated screening of extracts in tests that take up to three days to complete. Recently, specialist laboratories have validated high throughput automated systems for some nematode species (Marcellino et al., 2012; Smout et al., 2010). These techniques were designed to aid the screening of large libraries of synthetic compounds for anthelmintic activity, but would also be applicable to screening plant extracts. Microfluidic techniques are also under development for analysing the effect of drugs on nematodes, and could be of use in future for immature stages cyathostomins (San-Miguel and Lu, 2013). At present none of these techniques have been evaluated for use in cyathostomins and this is an important area of future research for the development of novel therapeutics.

7.5. Should P-glycoprotein inhibitors in combination with ivermectin be taken forward to *in vivo* trials in equids?

As described in Chapter 6, *in vitro* tests were used to test a different hypothesis; that P-gps are involved in IVM metabolism in cyathostomins, and the use of inhibitors of these protein channels can potentially be used to improve the effect of IVM against cyathostomins as a novel

control option. Initially the transcription of *pgp-9* was measured in 'resistant' *versus* 'sensitive' cyathostomin third stage larvae (L3), and found to be significantly increased in the 'resistant' population after exposure to IVM. This is in agreement with studies from other closely related GI nematodes (Dicker et al., 2011b; Williamson et al., 2011) and indicates that *pgp-9* expression is associated with ivermectin resistance in cyathostomins. In support of this data, *in vitro* tests described in Chapter 6 showed that the P-gp inhibitors pluronic 85 (P85), ketoconazole and ivermectin aglycone (IVM-AG) increased the effect of IVM in the larval migration inhibition test (LMIT) and/or larval development test (LDT), and that this effect was specific to 'resistant' cyathostomins in the LMIT with ketoconazole and IVM-AG. Based on this evidence, *in vivo* trials in equids should be considered as a direct progression of this work.

The important question with respect to novel options for controlling cyathostomins, is whether modulating P-gp activity *in vivo* will improve IVM efficacy. The evidence from *in vivo* studies in other host species is not fully supportive, as discussed in Chapter 6, Section *6.4.4.* (Bartley et al., 2012). There are several possible reasons for this. Firstly the ML resistance trait is thought to be multigenic and may differ between field isolates of resistant nematodes, and therefore P-gps, although genuinely upregulated in some examples of resistance, may not represent the major mechanism of resistance in others. For example a study evaluating the transcriptome of ML resistant *Teladorsagia circumcincta* reported that there was no increase in transcription of P-gps after IVM exposure *in vitro* (Dicker et al., 2011a), whereas another study found a marked increase in transcription of *pgp-9* in the same species (Dicker et al., 2011b). Similarly one study in *Haemonchus contortus* showed no changes in transcription of P-gp genes in an ML resistant isolate (Williamson and Wolstenholme, 2012), whereas another study showed that both *pgp-2* and *pgp-9* expression were increased (Williamson et al., 2011). There is also the possibility that the P-gp inhibitors that have not worked *in vivo,* have not been

given at the right dose, or by the best route of administration. For example, there is evidence from drug combinations with anthelmintics that certain routes of administration result in an improved synergistic effect (Lanusse et al., 2015). In addition the choice of P-gp inhibitor used appears to affect efficacy *in vivo*; studies to date indicate that loperamide has a better effect than ketoconazole and P85 (Bartley et al., 2012; Lifschitz et al., 2010a; Lifschitz et al., 2010b). Based on these observations future *in vivo* trials in equids should aim to evaluate a range of P-gp inhibitors at different doses, administered by different routes.

Should it be proven that P-gp inhibitors improve efficacy of IVM *in vivo* in equids, careful consideration must also be given to the consequences of such treatments. One theory is that, by effectively increasing the anthelmintic dose that parasites are exposed to *in vivo,* selection pressure for multidrug resistance might be increased (Lespine et al., 2012). This hypothesis could be tested *in vitro* but this would be difficult in cyathostomins as currently, there are no *in vitro* models of resistance. This may mean that drug combinations with MLs and P-gps would not be suitable for use as a commercial preparation for widespread use, however they could still offer a vital treatment alternative in cases of clinical disease due to multi resistant cyathostomins.

7.6. Final conclusions

This thesis has highlighted several avenues for further investigation that hold promise for improving control of cyathostomins infections in equids. After consideration of the data here in combination with supporting evidence it can be recommended that PLS, P-gp/IVM combinations and tannin rich forages, should be seriously considered for *in vivo* trials in equids

in developed countries, where anthelmintic resistance is a major threat to control. Plants such as *A. nilotica* and bioactive legume forages, which contain high levels of condensed tannins are good options for taking forward to *in vivo* trials for use in developing countries, where access to anthelmintics is often limited.

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9. APPENDICES

Appendix 1

1.1. Optimisation of egg storage method

Three different methods of nematode egg storage have been described; anaerobic storage at room temperature, or 4 ºC and aerobic storage at 4 ºC (Coles et al., 1992; Ihler and Bjorn, 1996). The optimal method for cyathostomins has not been established, and hence was tested here. Figure A.1.1. shows the mean percentage egg hatch in cyathostomins egg samples after seven days under these different conditions. One way analysis of variance (ANOVA), performed in SPSS version 21 (SPSS Inc., Chicago IL), on the data demonstrated that there was no significant difference in percentage egg hatch between different storage methods after 7 days of storage, $p = 0.067$, all samples had >80 % egg hatch. It was noted at microscopy that the majority of eggs that were stored aerobically and anaerobically at 4 °C were showing signs of development, and thus for all future experiments eggs were stored anaerobically at room temperature.

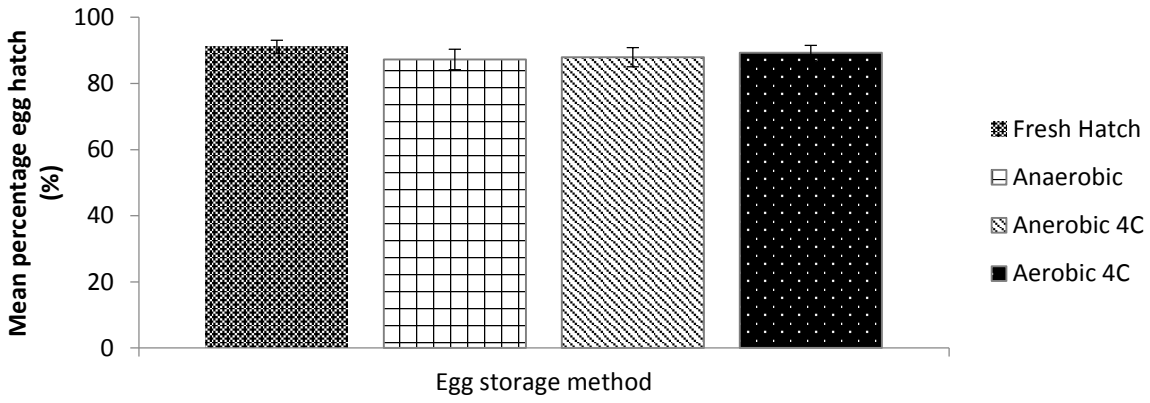

Figure A.1.1. The effect of different storage methods (fresh hatch, anaerobic, anaerobic 4 ºC, aerobic 4 ºC) on egg samples at seven days, on mean percentage egg hatch (taken from an average of a sample from horse and donkey faeces).

1.2. Comparison of percentage egg hatch in tap water and distilled water

The chemical composition of the tap water (H_2O) can have an inhibitory effect on egg hatch. It was therefore necessary to assess whether the tap H_2O available in the laboratory at the University of Liverpool altered percentage egg hatch. A student T test, performed in SPSS version 21, demonstrated that a significantly greater percentage of eggs hatched when using distilled H_2O (dH_2O) rather than tap H_2O, $p = 0.016$. The data are represented in a bar chart in Figure A.1.2. Thus dH_2O was considered optimal for use in all further tests.

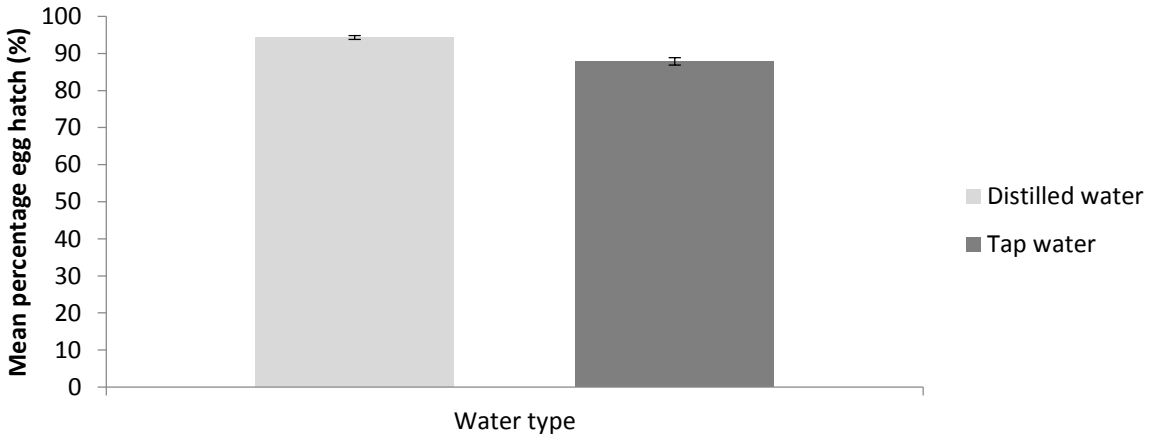

Figure A.1.2. Percentage egg hatch at 48 h after incubation in distilled water (dH_2O) and tap water (H_2O)

1.3. Evaluation of the effect of dimethyl sulfoxide in the egg hatch test

Dimethyl sulfoxide (DMSO) is the most commonly used solvent to dissolve anthelmintic compounds in pharmacological studies using the egg hatch test (EHT), hence as part of the validation process the effect of DMSO on egg hatch was evaluated. The ability of eggs to hatch in increasing concentrations of DMSO (between 0-10 % v/v) was evaluated, the results are shown in Figure A.1.3. This was repeated throughout the thesis in each new parasite population used, in case of any differential effect amongst populations. There was a significant reduction in egg hatch at DMSO concentrations of > 5% ($p<0.001$), measured by repeated measures analysis of variance (ANOVA), performed in SPSS version 21.

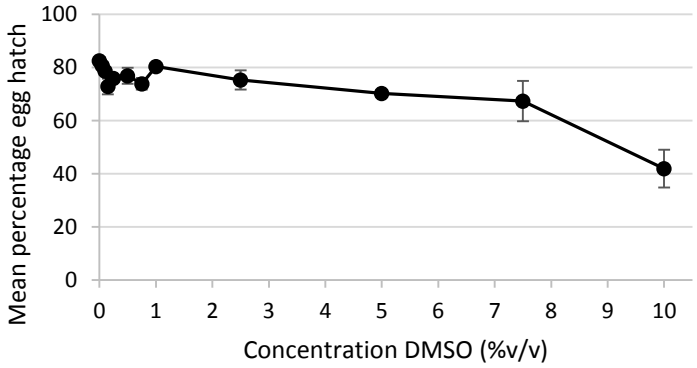

Figure A.1.3. Graphical representation of the effect of increasing dimethyl sulfoxide (DMSO) concentration on percentage egg hatch in cyathostomins

1.4. Preliminary examples of the egg hatch test with thiabendazole

Dose response curves for the EHT with thiabendazole (TBZ) for cyathostomins eggs from horse and donkey samples are shown in Figures A.1.4.a and b respectively. The results clearly demonstrate that percentage egg hatch in cyathostomins can be reduced in a dose dependent fashion by TBZ, and thus the EHT can be used with confidence to assess whether other compounds, such as plant extracts, have any effect on cyathostomin egg hatch. EC-50 values were calculated for each test using PROBIT analysis (SPSS 21), and were 0.052 µg/ml (confidence interval (CI): 0.049-0.055) and 0.043 µg/ml (CI: 0.033-0.050) for the horse and donkey samples respectively.

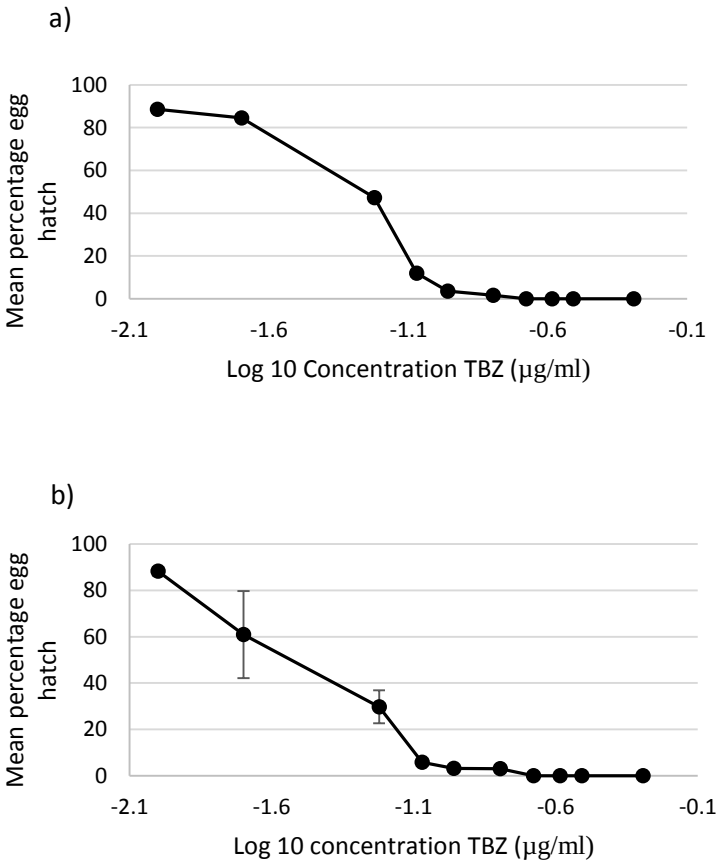

Figure A.1.4. Logarithmic dose response curves showing the effect of an increasing concentration of thiabendazole (TBZ) on percentage egg hatch in a horse (a) and donkey (b).

1.5. Preliminary examples of the egg hatch test with papaya latex supernatant

Figure A.1.5. shows dose response curves for preliminary EHTs with papaya latex supernatant (PLS). EC-50 values (calculated in GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com)) were 0.09 (CI:0.08-0.08) and 0.09 (CI:0.08-0.10) µM, for the two repeats. There was no significant difference between repeats for PLS, according to the sum of squares F test (Graphpad Prism 6).

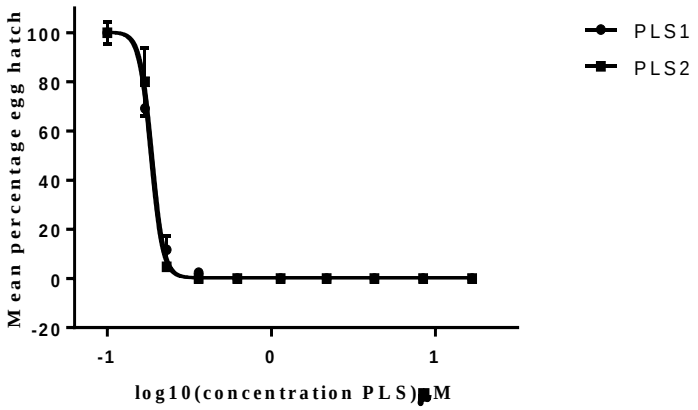

Figure A.1.5. Graphical representation of the dose response relationship between mean percentage cyathostomin egg hatch and concentration of papaya latex supernatant (PLS) in two preliminary repeats.

1.6. Assessment of suitability of mesh pore size by heat treatment of sheathed and ex-sheathed third stage larvae

To ensure that the mesh size used in the larval migration inhibition test (LMIT) protocol was appropriate for the cyathostomin populations used in this thesis, a heat treatment assay was performed. Sheathed and ex-sheathed third stage larvae (L3) were killed by exposure to 70 ºC heat for 20 min. Their ability to migrate across a 25 µm nylon mesh (HPC gears), was then compared to live L3. Figure A.1.6., demonstrates that for both sheathed and ex-sheathed L3 the pore size of 25 µm prevents dead or immotile L3 from crossing the nylon mesh, whilst allowing the majority of live L3 to migrate.

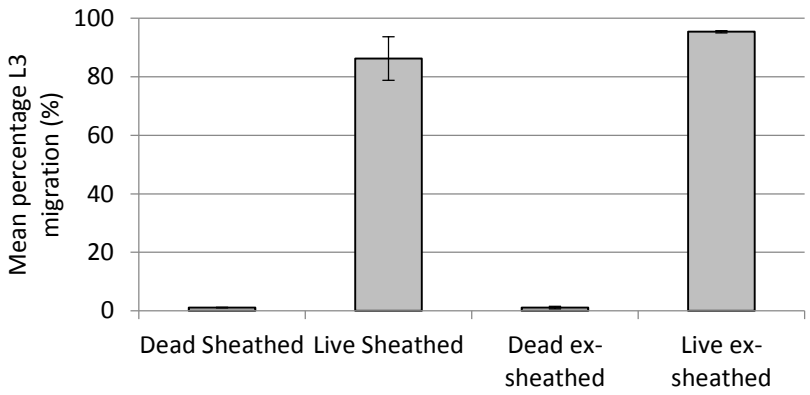

Figure A.1.6. The mean percentage larval migration in sheathed and unsheathed third stage larvae (L3) across a 25 µm nylon mesh before (live) and after (dead) heat treatment

1.7. Comparison of sheathed versus ex-sheathed third stage larvae in the larval migration inhibition test

The LMIT was repeated with and without ex-sheathment of L3. There was no significant difference in percentage migration in controls (p=0.344), as identified by the students T test (SPSS 21) of sheathed and ex-sheathed L3 through the nylon mesh. However there was a significant difference in the total number of migrators and non-migrators that were counted at the end of the tests, p<0.001. A mean of 82 ± 10 sheathed and 207 ± 14 ex-sheathed L3 were counted. This indicated that there had been a loss of sheathed L3 during the experiment due to the sheathed L3 becoming trapped in the nylon mesh. A comparison of sheathed and ex-sheathed L3 in the LMIT with IVM is shown in Figure A.1.7.

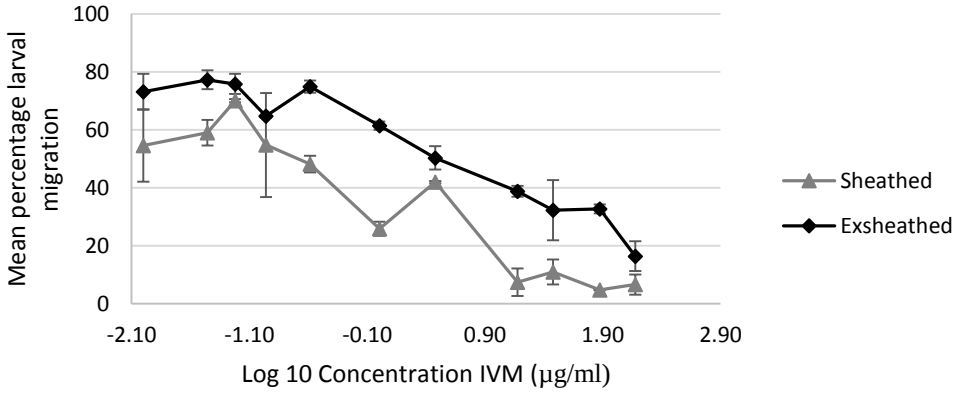

Figure A.1.7. The effect of ivermectin (IVM) concentration on sheathed and ex-sheathed cyathostomin third stage larvae (L3).

1.8. Evaluation of the effect of dimethyl sulfoxide on larval migration in the larval migration inhibition test

The LMIT was repeated with DMSO (1-10 %) in order to establish the effect of the solvent on migration, and hence determine limits of DMSO concentration for further experiments. This was repeated throughout the thesis in each new parasite population used, in case of any differential effect between populations. The results of this titration are shown in Figure A.1.8. There was a significant effect on larval migration at DMSO concentrations higher than 5 % ($p=0.02$), measured by repeated measures ANOVA (SPSS version 21) thus 5 % was the maximum used in all further tests.

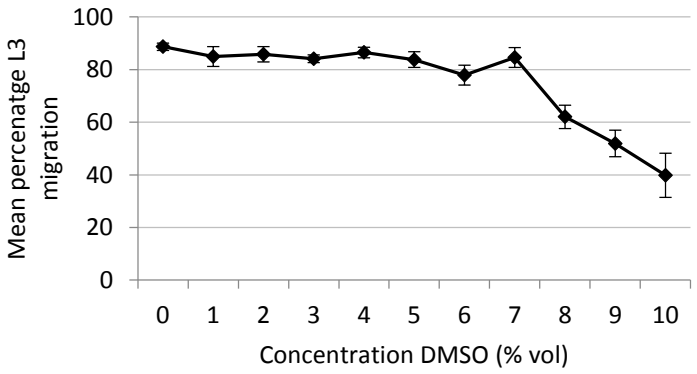

Figure A.1.8. The effect of increasing concentrations of dimethyl sulfoxide (DMSO) on larval migration in the larval migration inhibition test.

1.9. Preliminary examples of the larval migration inhibition test with ivermectin

Figure A.1.9. shows dose response curves for preliminary repeats of the LMIT with increasing concentrations of ivermectin (IVM) in horses and donkeys. The EC-50 values calculated by PROBIT analysis (SPSS 21 version 21) were 1.928 µg/ml (CI: 0.365-4.595), 8.391 µg/ml (CI: 1.374-19.834), 0.472 µg/ml (CI: 0-8.859) and 1.684 (CI: 0.150-7.923) for donkey 1, donkey 2, horse 1 and horse 2 respectively.

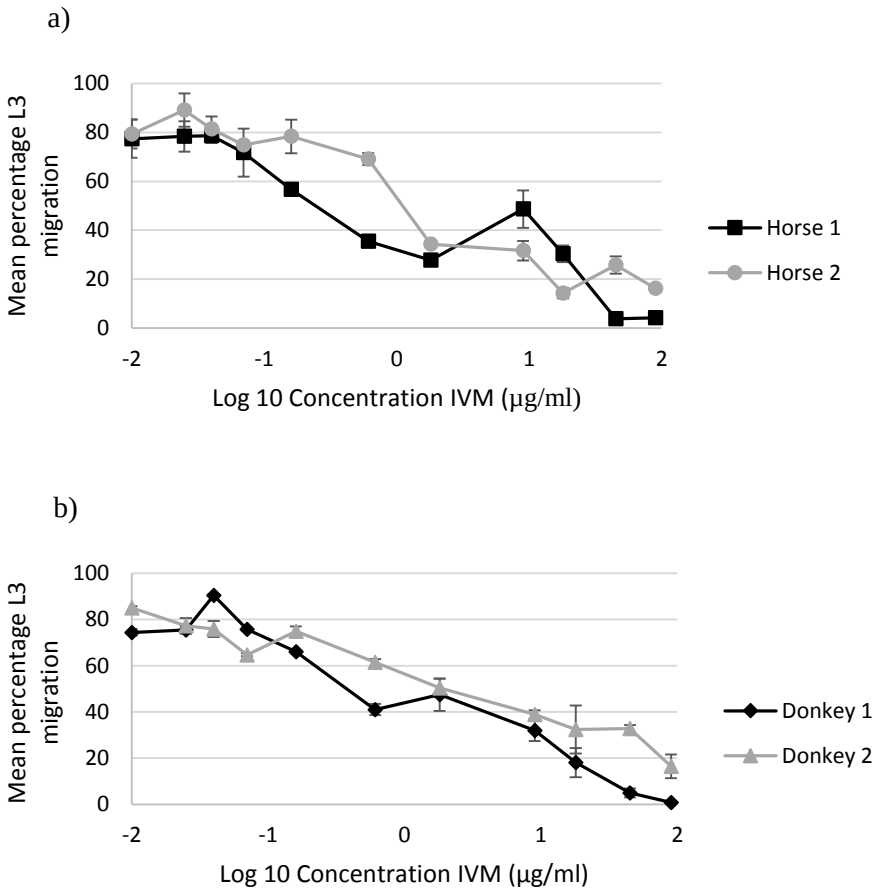

Figure A.1.9. The effect of an increasing concentration of ivermectin (IVM) on the mean percentage migration of third stage larvae (L3) from 2 horses (a) and 2 donkeys (b).

1.10. Optimisation of incubation time with papaya latex supernatant in the larval migration inhibition test

The LMIT was repeated for PLS with four different incubation times (2, 6, 16 and 24 h), to establish the optimal time for demonstration of a good effect. The results are shown in Figure A.1.10. An increasing effect was seen at increasing incubations.

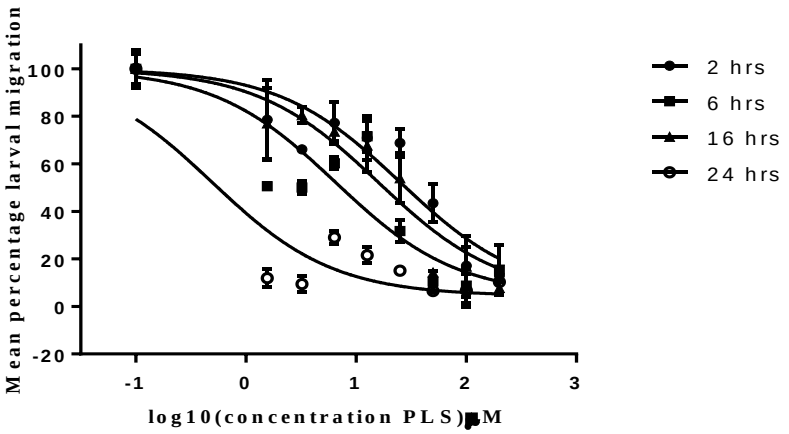

Figure A.1.10. The effect of increasing incubation times with papaya latex supernatant (PLS) in the larval migration inhibition test.

1.11. Preliminary examples of the larval migration inhibition test with increasing concentrations of papaya latex supernatant and stem bromelain

Preliminary repeats of the LMIT were performed for PLS, with incubation time 16 h. The dose response curves are shown in Figure A.1.11. EC-50 values (calculated using Graphpad Prism 6) were 18.69 (CI:12.58-27.76) and 15.42 (CI:10.35-22.91) µM for PLS repeats 1 and 2. There was no significant difference between repeats for PLS, calculated using the sum of squares F test (Graphpad Prism 6).

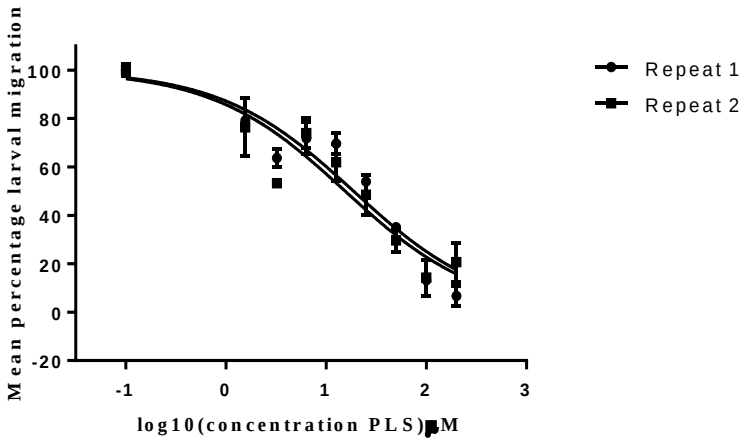

Figure A.1.11. Graphical representation of the dose response relationship between mean percentage larval migration and papaya latex supernatant (PLS) concentration in two repeats.

1.12. Dose titration of pluronic 85 and ketoconazole in the larval migration inhibition test

For optimisation of concentration in the LMIT, pluronic 85 (P85) and ketoconazole were diluted in dH_2O or DMSO respectively and used in the LMIT in a 1:100 ratio to give final concentrations of 500.0, 250.0, 125.0, 62.5, 31.3, 15.6, 7.8, 3.9 and 2.0 µM in the test. The DMSO concentration was kept constant at 3 %, at all drug concentrations. Figure A.1.12. shows the results of these dose titrations. No significant effect was seen at any concentration tested for either P85 or ketoconazole except 500 µM.

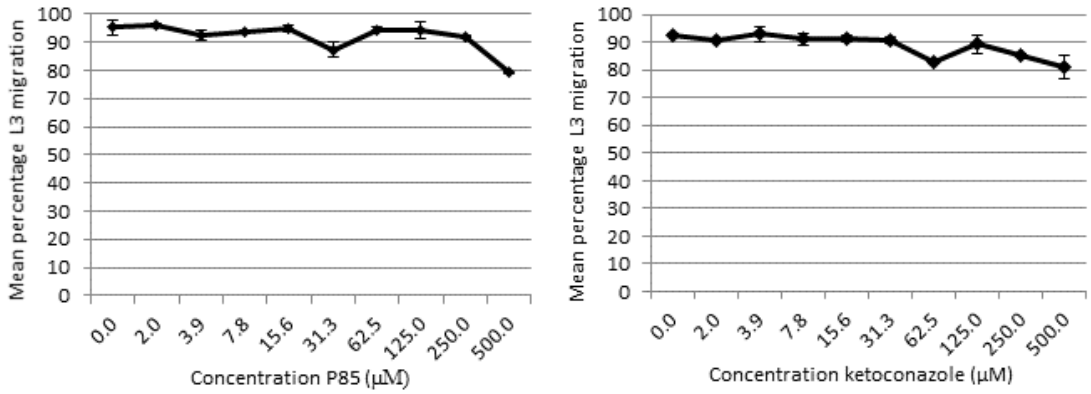

Figure A.1.12. Dose titrations of pluronic 85 (P85) and ketoconazole in the larval migration inhibition test

1.13. Dose titration and establishing optimal concentration for ivermectin aglycone in the larval migration inhibition test

For ivermectin aglycone (IVM-AG) a dilution was made in DMSO to give working concentrations which were added to the LMIT in a ratio of 0.5:100 to give the final concentrations, 200, 180, 160, 140, 120, 100, 80, 60, 40 and 20 nM. The concentration of DMSO was kept constant at 3 %. As the optimal concentration has not been previously reported, an additional test was run where increasing concentrations of IVM-AG were compared with increasing concentrations of IVM-AG plus 1 µM IVM. This was done to determine the concentration at which the addition of IVM-AG optimally improved inhibition of larval migration compared to IVM alone. The effect of co-incubation with IVM-AG and IVM, peaked at 60 nM and 120 nM IVM-AG. The lower of these concentrations, 60 nM, was chosen for further tests.

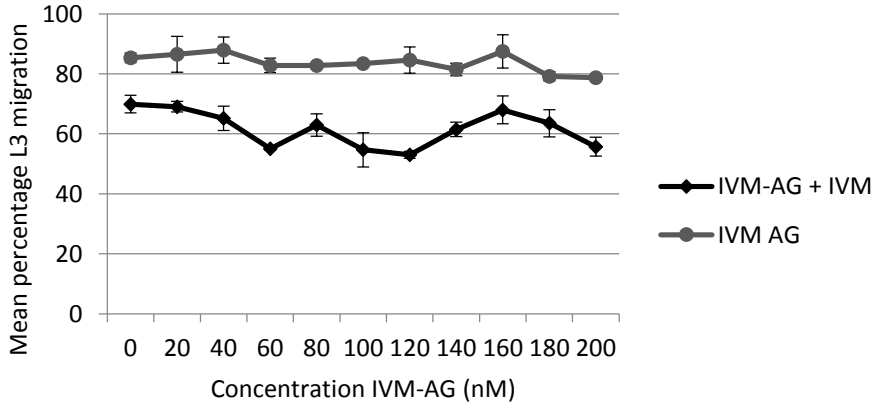

Figure A.1.13. Graph showing the effect of increasing concentrations of ivermectin aglycone (IVM-AG) on mean percentage third stage larvae (L3) migration, with and without, the addition of 1 µM ivermectin (IVM).

1.14. Optimisation of growth medium and amphotericin B concentration in the larval development test for cyathostomins

Two growth mediums were used in preliminary experiments. The first (growth medium A (GMA)) consisted of 10 x Earles salt solution (Sigma-Aldrich, UK) and yeast solution (1g yeast (Sigma-Aldrich, UK) autoclaved in 90 ml 0.9 % sodium chloride (NaCl) (Sigma-Aldrich, UK)) in a 1:9 ratio. This was combined with 1.5 mg/ml autoclaved lyophilised *Escherichia coli* K12 (Sigma-Adlrich, UK) in a 2:1 ratio. The second (growth medium B (GMB)) consisted of 10 x Earles salt solution and yeast solution (1g yeast autoclaved in 90 ml 0.9 % NaCl) in a 1:9 ratio. This solution was diluted 2.5 times with dH_2O and combined with autoclaved 1.5 mg/ml lyophilised *Escheria coli* K12 in a 2:1 ratio. A larval development test (LDT) was set up to compare growth mediums and dose titration of amphotericin B (Sigma-Aldrich, UK) (final concentrations 0-58 µg/ml). Figure A.1.14. shows the effect of increasing concentrations of amphotericin B on percentage larval development in GMA and GMB. Amphotericin B had no effect up to 33 µg/ml. Percentage larval development was consistently higher with GMA compared to GMB. It was also observed that the motility of larvae was greater in GMA and the medium was less cloudy, suggesting less bacterial growth. Hence GMA was used in all LDTs.

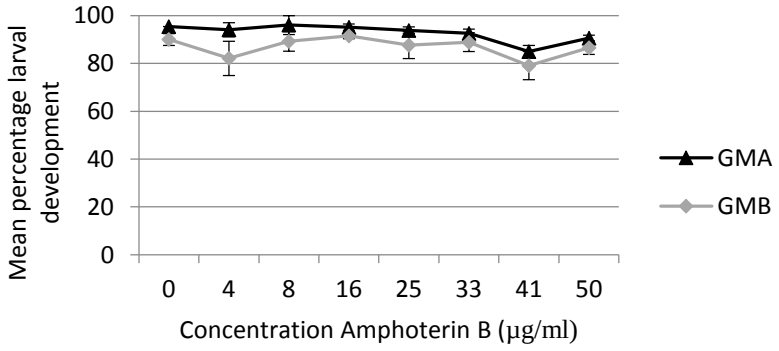

Figure A.1.4. Graph showing mean percentage larval development in growth medium A (GMA) and growth medium B (GMB) at increasing concentrations of amphotericin B.

1.15. Evaluation the effect of dimethyl sulfoxide in the larval development test

The LDT was performed with a dose titration of drug solvent DMSO (between 0-1% v/v) to establish the threshold for the concentration of DMSO in each well. The results are shown in Figure A.1.5. There was a significant effect on larval migration at DMSO concentrations higher than 0.5 % (p=0.001), measured by repeated measures ANOVA (SPSS version 21). Therefore a concentration of 0.5 %, in accordance with that described in Demeler et al (2010), was used in all further tests.

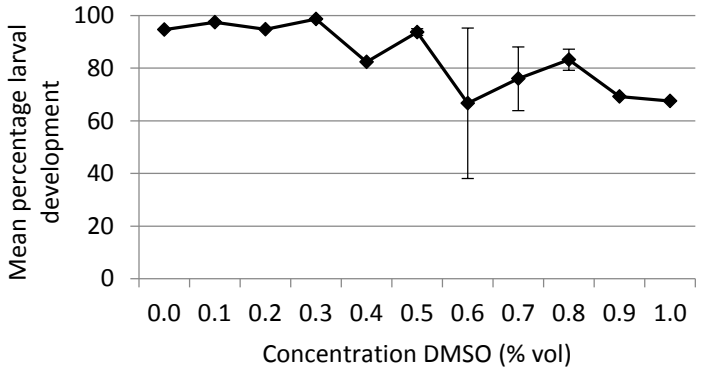

Figure A.1.15. Graph showing the effect of increasing concentrations of dimethyl sulfoxide (DMSO) on percentage larval development in the larval development test.

1.16. Preliminary examples of the larval development test with ivermectin

Examples of the results of preliminary LDTs in cyathostomins samples from horses and donkeys are shown in Figure A.1.16. EC-50 values calculated with PROBIT analysis (SPSS version 21) for the IVM LDT in four separate samples were: donkey 1 = 6.823 nM (CI: 5.407-8.310), donkey 2 = 11.396 nM (CI: 10.106-12.842), horse 1 = 3.727 nM (CI: 2.842-4.875), horse 2 = 4.240 nM (CI: 3.808-4.706).

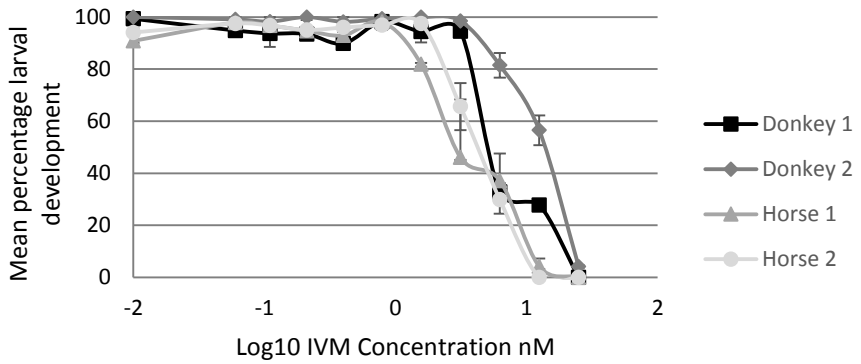

Figure A.1.16. The effect of increasing concentrations of ivermectin (IVM) on larval development in the larval development test on samples from two donkeys and two horses.

1.17. Dose titration of pluronic 85, ketoconazole and ivermectin aglycone in the larval development test

For optimisation of concentration in the LDT, P85 and ketoconazole were diluted in dH_2O or DMSO respectively to give working solutions which were added in a 0.5:100 ratio in the LDT to give final concentrations of 250.0, 125.0, 62.5, 31.3, 15.6, 7.8, 3.9, 2.0, and 1.0 µM. For optimisation of concentration in the LDT IVM-AG was dissolved in DMSO to give working solutions which were added to the LDT in a 0.5:100 ratio to give the final concentrations 100.00, 50.0, 25.00, 12.50, 6.25, 3.13, 1.56, 0.78, 0.39 and 0.20 nM. Figure A.1.17. shows the results of these dose titrations. For ketoconazole there was a marked effect of larval development at concentrations above 15.6 µM, and for IVM-AG there was an effect on larval development at concentrations greater than 12.5 nM.

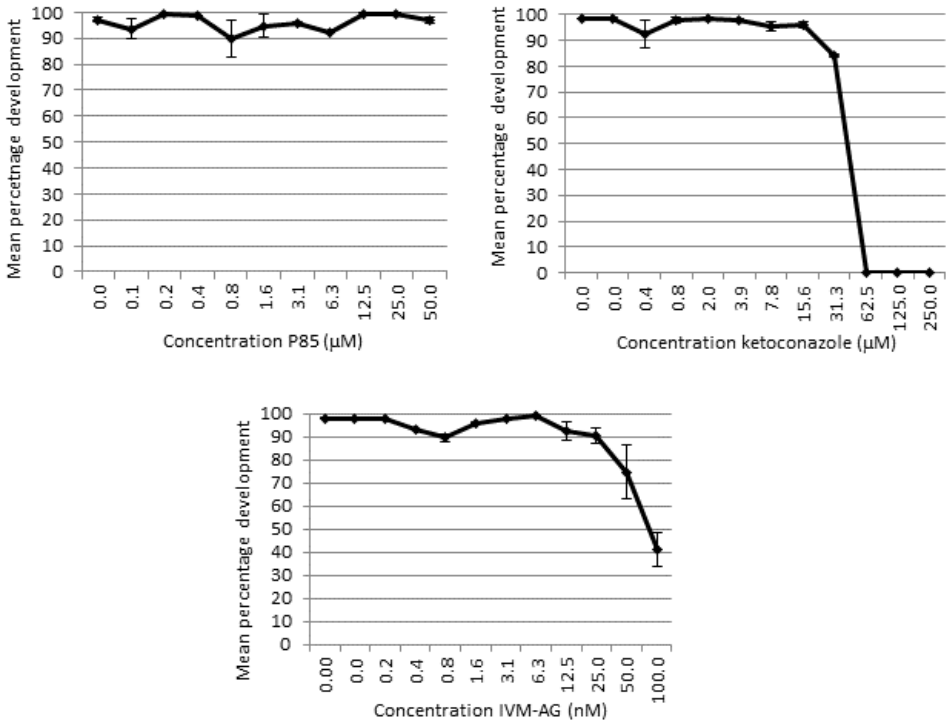

Figure A.1.17. Dose titrations of pluronic 85 (P85), ketoconazole and ivermectin aglycone (IVM-AG) in the larval development test

Appendix 2

2.1. Key informant interview outline

Date:		Audio:	Village:	Key Informant Code:	

Plant name (local / Latin)	

Where it grows?

Time of year / availability?

Part of plant used?

Any features of plant used for potency?

Effectiveness?

Preparation	Time of year

Part / amount of plant

Preparation details: How harvested, how dried, ground, extraction (in what liquid, how long, what temperature, crushed?), how long kept for, storage?

Administration	Dosage? (e.g. for size of animal)

How administered?

How often?

What time of year given?

What effects are seen after giving this plant? Any negative effects?

Which plants do you think are effective?
Which do you use?
What are the benefits of using plant based medicine?
If you could test one of these in the lab, which would you choose and why?
Where does your knowledge of plant based treatments come from?

2.2. Summary of the results of the PubMed literature review for identification of UK plant candidates

Table A.2.1. Summary of the results of the PubMed literature review for identification of UK plant candidates for in vitro testing, including 138 references reporting anthelmintic effects of plants against nematodes

Efficacy	Moderate/low Efficacy	No Efficacy	Animal	Nematode spp	Extract with	Reference
- Annona squamosa ***- Eclipta prostrate*** ***- Solanum torvum*** ***- Terminalia chebula*** ***- Catharanthus roseus*** ***In vitro***				*Haemonchus contortus*	Ethyl acetate, acetone, methanol	(Kamaraj and Rahuman, 2011)
- Andrographis paniculata ***- Anisomeles malabarica*** ***- Datura metel*** ***- Solanum torvum*** ***- Annona squamosa*** ***In vitro***				*Haemonchus contortus*	Ethyl acetate, acetone, methanol	(Kamaraj et al., 2011)
		Artemisia spp. *In vivo*	Gerbil	*Haemonchus contortus*	Ethanol, Aqueous	(Squires et al., 2011)
- Anogeissus leiocarpus ***- Khaya senegalensis*** ***- Euphorba hirta*** ***- Annona senegalensis nigrescens*** ***In vitro***				*Caenorhabditis elegans*	Ethanol	(Ndjonka et al., 2011)
- Trichosanthes dioica Roxb. ***In vitro***				*Ascaridia galli*	Dichoromethane' Methanol, Aqueous	(Bhattacharya et al., 2010)
- Manihot esculenta				*Haemonchus contortus*	Dichoromethane, Methanol, Aqueous	(Marie-Magdeleine et al., 2010b)
- Eucalyptus staigeriana ***In vivo***			Goats (toxicity in rats)	*Haemonchus contortus*	Essential oil	(Macedo et al., 2010)

		- *Lysiloma latisiliquum* *In vivo*	Sheep	*Haemonchus contortus*	Fodder	(Martinez-Ortiz-de-Montellano et al., 2010)
	- *Zanthoxyylum rhoifolium* *In vivo*		Sheep	*Haemonchus contortus*	Leaf extract	(Peneluc et al., 2009)
Orange oil emulsion ***In vivo***	`		Sheep Gerbil	*Haemonchus contortus*		(Squires et al., 2010)
- Lotus pedunculatus ***- Lotus corniculatus*** ***- Dorycnium pentaphyllum*** ***- Dorycnium rectum*** ***- Rumex obtusifolius*** ***In vitro***				*Teledorsagia circumcincta*	Extracts	(Molan and Faraj, 2010)
- Anacardium humile ***In vitro***				Gastrointestinal nematodes	Aqueous, Ethanolic	(Nery et al., 2010)
- Balanites aegyptiaca ***In vivo***			Rats	*Trichinella spiralis*	Methanol	(Shalaby et al., 2010)
	- *Rubus niveus* *In vitro*			*Meloidogne incognita* (root knot nematode)	Alcohol Petroleum	(Sultana et al., 2010)
- Melia azedarach ***In vitro***				*Haemonchus contortus*	Aqueous, Hydroalcholic, Leaves and seed	(Kamaraj et al., 2010)
- Lysimachia ramosa ***In vitro***				*Fasciolopsis buski* *Ascaris suum* *Raillietina echinobothrida*	Alcoholic extract	(Challam et al., 2010)
- Tabernaemontana citrifolia ***In vitro***				*Haemonchus contortus*	Dichloromethane. aqueous, methanol	(Marie-Magdeleine et al., 2010a)
- Combretum molle ***In vitro***				*Haemonchus contortus*	Ethanol, Acetone, Butanol, Hexane, Chloroform, Methanol	(Ademola and Eloff, 2010)
	- *Morinda citrifolia* *In vivo*		Chickens	*Ascarida galli*	Aqueous, Ethanol	(Brito et al., 2009)

- Zingiber officinale **extracts** ***In vitro***			*Angiostrongylus cantonensis*	Extracted compounds	(Lin et al., 2010)
	- Azadirachta indica *In vitro*		*Haemonchus contortus* *Trichostrongylus spp.*	Crude Aqueous Methanol	(Iqbal et al., 2006a)
- Acacia polyacantha ***- Anogeissus leiocarpus*** ***- Bridelia micratha*** ***- Cassia seiberiana*** ***- Combretum nigricans*** ***- Grewia bicolour*** ***- Strychnos spinosa*** ***- Ziziphus mucronata*** ***In vitro***			*Caenorhabditis elegans*	Aqueous Organic	(Waterman et al., 2010)
- Quercus coccifera ***- Ceratonia siliqua*** ***In vitro/in vivo***	*-Pistacia lentalis* *-Castanea sativa* *- Onobrychis viciifolia* *- Olea europaea* *- Ceratonia siliqua* *In vitro/in vivo*	Sheep	*Haemonchus contortus*		(Manolaraki et al., 2010)
- Canthium manni ***In vitro/in vivo***		Mice	*Heligosomoides polygyrus*		(Wabo Pone et al., 2010; Wabo Pone et al., 2009)
	- Carica papaya (latex) *In vivo*	Chickens	Mixed gastrointestinal nematodes		(Chota et al., 2010)
- Khaya senegalensis ***In vitro***			*Haemonchus contortus*	Ethanol (Separation of fractions)	(Ademola et al., 2009)
	- Prosopis laevigata *In vivo*	Gerbils	*Haemonchus contortus*	Hexane	(De Jesus-Gabino et al., 2010)
- Cocos nucifera ***In vivo***		Mice	Gastrointestinal nematodes	Butanol	(Costa et al., 2010)
- Sericea lespedeza ***In vivo***		Goats	Gastrointestinal nematodes	Fed hay	(Terrill et al., 2009)

- Acacia nilotica ***- Ziziphus mummularia*** ***In vitro/in vivo***			Sheep	Gastrointestinal nematodes *in vivo* *Haemonchus contortus* *in vitro*	Methanol	(Bachaya et al., 2009)
- Maesa lanceolata ***In vitro***	*- Plectranthus punctataus* *In vitro*			*Haemonchus contortus*	Aqueous, Hydroalcoholic	(Tadesse et al., 2009)
	-Cleistopholis patens			*Rhabditis pseudoelongata*		(Akendengue et al., 2009)
	- Coreopsis lanceolata			*Caenorhabditis elegans*	Spectroscopic methods	(Kimura et al., 2008)
- Cucurbita moschata ***In vitro***				*Haemonchus contortus*	Dichloromethane Methanol Aqueous	(Marie-Magdeleine et al., 2009)
- Artemisia absinthium ***In vivo***			Sheep	Gastrointestinal nematodes	Aqueous Ethanol	(Tariq et al., 2009)
		- Phormium tenax *In vivo*	Cattle	Gastrointestinal nematodes	Chopped leaves	(Litherland et al., 2008)
- Cocos nucifera ***In vitro***		*Cocos nucifera* *In vivo*	Sheep	Gastrointestinal nematodes	Ethyl acetate	(Oliveira et al., 2009)
		- Allium sativum *- Carica papaya* *In vivo*	Goats	Gastrointestinal nematodes	Garlic bulbs Papaya seed in water	(Burke et al., 2009)
	- Lantana camara			*Meloidogyne incognita*	Extracted compounds	(Begum et al., 2008)
- Albizia anthelmintica			Sheep	Gastrointestinal nematodes	Traditional extraction	(Grade et al., 2008)
- Allium sativum			Mice	*Aspiculuris tetraptera*	Crushed garlic	(Ayaz et al., 2008)
	- Coriander orientalis *- Valeriana wallichi* *In vitro*			*Bursaphelenchus xylophilus* (pine wood nematode)	Essential oils	(Kim et al., 2008)
- Annona squamosa ***In vitro***				*Haemonchus contortus*	Ethyl acetate	(Souza et al., 2008)
	- Sericea lespedeza		Goats	Gastrointestinal nematodes	Hay	(Moore et al., 2008)

	- *Lippia sidoides* *In vivo*		Sheep	*Haemonchus contortus* *Trichostrongylus colubriformis*	Essential oil	(Camurca-Vasconcelos et al., 2008)
- *Achillea millifolium* ***In vivo***			Sheep	Gastrointestinal nematodes *Haemonchus contortus in vitro*	Aqueous Ethanol	(Tariq et al., 2008a)
	- *Camellia sinensis* *In vitro*			*Caenorhabditis elegans*	Extraction of polyphenols	(Mukai et al., 2008)
- *Acacia pennatula* **- *Lysiloma latisiliquum*** **- *Leucaena leucocephala*** ***In vitro***				*Haemonchus contortus* *Trichostrongylus colubriformis*		(Alonso-Diaz et al., 2008)
- *Iris hookeriana* ***In vivo***				*Trichurus ovis* Gastrointestinal nematodes	Aqueous Ethanol	(Tariq et al., 2008b)
	- *Prosopis laevigata* *In vitro*			*Haemonchus contortus*	Hexane Acetone Ethanol Methanol	(Lopez-Aroche et al., 2008)
		- *Azadirachta indica* *In vivo*	Sheep	Gastrointestinal nematodes	Dried leaves	(Chagas et al., 2008)
- *Sacoglottis gabonensis* ***In vivo***			Mice(toxicity IP) Rats	*Heligmosomoides polygyrus*	Aquous bark extract	(Nwosu et al., 2008)
- *Caesalpina crista* **- *Chenpodium album*** ***In vitro/in vivo***			Sheep	*Trichostrongylus colubriformis*	Aqueous Methanol (hydroalcoholic)	(Jabbar et al., 2007)
- Cysteine protease extracts ***In vitro***				*Meloidogyne Globodera* (plant nematode)		(Stepek et al., 2007a)
	- *Lippia sidoides* - *Croton zehntneri* *In vitro/vivo*		Mice	*Haemonchus contortus in vitro* Mice Gastrointestinal nematodes *in vivo*		(Camurca-Vasconcelos et al., 2007)
		- *Manihot esculenta* *In vivo*	Goats	Gastrointestinal nematodes	Fed grass	(Seng et al., 2007)

- Pectis oligocephala ***- Pectis apodocephala*** ***In vitro***				*Meloidogyne incognita* *Aedes aegypti*	Essential oils	(Albuquerque et al., 2007)
- Carica papaya* latex** ***In vivo			Rodents	*Heligmosomoides polygyrus*		(Stepek et al., 2007c)
	- Hedera helix *In vivo*		Sheep	Gastrointestinal nematodes	Aqueous, Hydroalcoholic	(Eguale et al., 2007a)
	-Onobrychis viciifolia *In vitro/in vivo*			*Haemonchus contortus* *Trichostrongylus colubriformis*		(Brunet et al., 2008)
- Chicorium intybus ***In vivo***	- Lotus corniculatus - Onobrychis viciifolia *In vivo*		Sheep	*Haemonchus contortus* *Cooperia spp.*	Fed plant	(Heckendorn et al., 2007)
- Myristica fatua ***In vitro***				*Caenorhabditis elegans*	Almond extract	(Desrivot et al., 2007)
	- Cissampelos capensis *In vitro/in vivo*		Mice	Gastrointestinal nematodes	Extracted compounds of	(Ayers et al., 2007)
	- Spigelia anthelmia *In vivo*		Sheep	Gastrointestinal nematodes	Ethanol	(Ademola et al., 2007)
- Coriandrum sativum ***In vitro***		*- Coriandrum sativum* *In vivo*	Sheep	*Haemonchus contortus*	Aqueous hydroalcoholic	(Eguale et al., 2007b)
	- Acacia negra *In vivo*		Sheep	Gastrointestinal nematodes		(Cenci et al., 2007)
	Azadirachta indica *In vivo*		Sheep	Gastrointestinal nematodes	Fresh leaves	(Chandrawathani et al., 2006)
	- Chicorium intybus *In vivo*		Sheep	Gastrointestinal nematodes	Pasture	(Athanasiadou et al., 2007)
	-Melia azadarach *In vitro*					(Szewczuk et al., 2006)
	- Spigelia anthelma *In vivo*		rats	*Nippostrongylus brazilienses*	Aqueous	(Jegede et al., 2006)
- Sericea lespedeza ***In vivo***			Sheep	*Haemonchus contortus*	Hay	(Lange et al., 2006)

- Swertia chirata ***In vivo***			Sheep	Gastrointestinal nematodes *Haemonchus contortus*	Aqueous Methanol	(Iqbal et al., 2006e)
- Jasminum fruticans ***-Mentha longifolia*** ***- Pinus nigra*** ***- Zea mays*** ***- Citrillus lanatus*** ***- Juniperus drupacea*** ***- Juniperus oxycedrus*** ***- Plantago lanceeolata*** ***In vitro***			Mice	Pinworm	Ethanol Aqueous	(Kozan et al., 2006)
-Ananas comosus ***- Azdarachta indica*** ***- Caesalpinia crista*** ***- Vernonia anthelmintica*** ***- Fumaria parviflora*** ***- Embelia ribes*** ***In vitro***				*Haemonchus contortus*		(Hördegen et al., 2006)
- Momordica charantia				*Caenorhabditis elegans*	Crude extract	(Das et al., 2006)
- Melia azedarach				*Haemonchus contortus*	Hexane Ethanol	(Maciel et al., 2006)
	- Leucaena leucocephala (Ademola and Idowu, 2006) *In vitro*			*Haemonchus contortus*		(Ademola and Idowu, 2006)
- Sericea lespedeza ***In vivo***			Goats	Gastrointestinal nematodes	Hay	(Shaik et al., 2006)
		- Azadirachta indica *In vivo*	Sheep	Gastrointestinal nematodes	Dried leaves	(Costa et al., 2006)
- Carica papaya **latex** ***In vivo***			Mice	*Trichuris muris*		(Stepek et al., 2006)
- Zingiber officinale ***In vivo***			Sheep	Gastrointestinal nematodes	Aqueous	(Iqbal et al., 2006a)

- Nicotiana tabacum ***In vivo***		Sheep	Gastrointestinal nematodes	Aqueous Methanol	(Iqbal et al., 2006c)
- Butea monosperma ***In vivo***		Sheep	Gastrointestinal nematodes	Crude powder	(Iqbal et al., 2006d)
- Peltophorum africanum ***In vitro***			*Trichostrongylus colubriformis*	Aqueous	(Bizimenyera et al., 2006)
- Onobrychis viciifolia ***In vitro***			*Haemonchus contortus*	Fractionation of extracts	(Barrau et al., 2005)
- Cardiospermum halicacabum ***In vitro***			*Strongyloides stercoralis*	Aqeous Alcohol	(Boonmars et al., 2005)
- Calotropis procera ***In vivo***		Sheep	Gastrointestinal nematodes	Aqueous Methanol	(Iqbal et al., 2005)
	- Nigella sativa *- Allium cepa* *In vivo*	Rats	*Trichinella spiralis*	oil	(Abu El Ezz, 2005)
	- Lantana camara *In vitro*		*Meloidogyne incognita* (root knot nematode)	Bioassay fractionation	(Qamar et al., 2005)
- Zanthoxylum xanthoxyloides ***- Newbouldia leavis*** ***- Morinda lucida*** ***- Carica papaya*** ***In vitro***			*Haemonchus contortus*		(Hounzangbe-Adote et al., 2005)
- Spondias mombin		Sheep	Gastrointestinal nematodes	Ethanol Aqueous	(Ademola et al., 2005)
	- Zanthoxylum fagara *In vivo*	Sheep		Fresh	(Hounzangbe et al., 2005)
Cysteine proteinases ***In vitro***		Mouse	*Heligmosomoides polygyrus*	Extracts from papaya, fig, etc	(Stepek et al., 2005)
	- Onobrychis viciifolia *In vivo*	Goats	Gastrointestinal nematodes	Hay	(Paolini et al., 2005)

		- *Lotus pedunculatus* - *Hedysarium coronarium* - *Onobrychis viciifolia* - *Cichorium intybus* *In vivo*	Sheep	*Trichostrongylus colubriformis*	Grazed on forage rich pasture	(Athanasiadou et al., 2005)
		- *Hagenia abyssinica* - *Olea europaea* - *Annona squamosa* - *Ananas comosus* - *Dodonea angustifolia* - *Hildebrandtia sepalosa* - *Azadirachta indica* *In vivo*	Lambs	*Haemonchus contortus*	Aqueous Fresh	(Githiori et al., 2004)
- Artemesia brevifolia ***In vivo***			Sheep	*Haemonchus contortus*	Crude Aqueous Methanol	(Iqbal et al., 2004)
- Khaya senegalensis ***In vivo***			Sheep	Gastrointestinal nematodes	Aqueous Ethanol	(Ademola et al., 2009)
- Myrsine africana ***- Albizia anthelmintica*** ***- Hildebrandtia sepalosa*** ***In vivo***			Sheep	Gastrointestinal nematodes	Traditional methods	(Gathuma et al., 2004)
	- *Myrsine africana* - *Azdirachta indica* - *Rapanea melanophloeos* *In vivo*		Mice	*Heligmosomoides polygyrus*	Aqueous Ttraditional	(Githiori et al., 2003b)
	- *Mangifera indica* *In vivo*		Mice	*Trichinella spiralis*	Aqueous	(Garcia et al., 2003)
- Fumaria parviflora ***In vivo***		- *Vermonia anthelmintica* - *Embelia ribes* - *Caesalpinia crista* *In vivo*	Lambs	*Trichostrongylus colubriformis* *Haemonchus contortus*	Ethanol	(Hordegen et al., 2003)
- Spigelia anthelmia ***In vitro***				*Haemonchus contortus*	Hexane Chlorophorm Ethyl acetate	(Assis et al., 2003)

					Methanol	
	- *Albizia anthelmintica* *In vivo*		Sheep Mice	*Haemonchus contortus* *Heligmosomoides polygyrus*	Aqueous	(Githiori et al., 2003a)
- Quebracho extracts ***In vivo***			Goats	*Haemonchus contortus*	Fed 5% dry matter	(Paolini et al., 2003)
- Annona senegalensis ***In vitro***		- *Vernonia amygdalina* *In vitro*		*Haemonchus contortus*	Whole ground plant in faecal culture	(Alawa et al., 2003)
	- *Lotus corniculatus* - *Cichorium intybus* *In vivo*			Gastrointestinal nematodes	Grazing	(Marley et al., 2003)
- Artemesia santonica ***In vitro***	- *Albizzia lebbek* - *Inula helenium* *In vitro*			*Ascaris lumbricoides*	Aqueous	(El Garhy and Mahmoud, 2002)
	- *Ocimum gratissimum* *In vitro*			*Haemonchus contortus*	Oil	(Pessoa et al., 2002)
		- *Myrsine Africana* - *Rapanea melanophloeos* *In vivo*	Sheep	*Haemonchus contortus*	Aqueous	(Githiori et al., 2002)
	- *Occimum sanctum* *In vitro*			*Caenorhabditis elegans*	Essential oil	(Asha et al., 2001)
- Quebracho ***In vivo***			Sheep	Gastrointestinal nematodes	Aqueous	(Athanasiadou et al., 2001)
	- *Carica papaya* *In vitro*			*Caenorhabditis elegans*	Aqueous	(Kermanshai et al., 2001)
- Berlina grandiflora ***In vitro***				*Caenorhabditis elegans*	Ethyl acetate MethanolHexane	(Enwerem et al., 2001)
		- *Lotus corniculatis* *In vivo*	Lambs	Gastrointestinal nematodes	Grazing	(Bernes et al., 2000)
	- *Calotropis procera* *In vivo*		Sheep	*Haemonchus contortus*		(Al-Qarawi et al., 2001)
- Nauclea latifolia ***In vivo***			Sheep	Gastrointestinal nematodes	Aqueous	(Onyeyili et al., 2001)

	- *Lotus corniculatus* - *Lotus pendunculatus* - *Hedysarum coronarium* - *Onobrychis viciifolia* *In vivo*		Deer	*Dictyocaulus viviparous* Gastrointestinal nematodes	Acetone Aqueous Ascorbic acid	(Molan et al., 2000a)
	- *Hedysarum coronarium* - *Lotus corniculatus* *In vivo*		Deer	Gastrointestinal nematodes Lungworm	Fresh	(Hoskin et al., 2000)
	- *Lantana camara* *In vitro*			*Meloidognyne incognita* (root knot nematode)	Extracts-lantanoside, lantanone	(Begum et al., 2000)
CT containing forages ***In vitro***						(Molan et al., 2000b)
Lit rev Italy: ***-Ruta graveolens*** ***-Cucurbita maxima*** ***- Artemesia absinthium*** ***- Allium sativum***						(Guarrera, 1999)
		- *Ficus carica* - *Ficus insipid* *In vivo*	Mice			(de Amorin et al., 1999)
	- *Zanthoxylum liebmannianum* *In vivo* Toxic mice		Sheep	*Ascaris suum*		(Navarrete and Hong, 1996)
	- *Carica papaya* latex *In vivo*		Mice	*Heligmosomoides polygyrus*	Crude latex	(Satrija et al., 1995)
	-*Tavierniera abyssinica* *In vitro*			*Caenorhabditis elegans*		(Stadler et al., 1994)
	- *Curcuma comosa* *In vitro*			*Caenorhabditis elegans*		(Jurgens et al., 1994)
- Caesalpina crista ***In vivo***			Chickens	*Ascaridia galli*		(Javed et al., 1994)

- Annona muricata ***- Annona cherimolia*** ***In vitro***				*Molinema dessetae*		(Bories et al., 1991)
- Saussurea lappa ***In vivo***			Human	Gastrointestinal nematodes	Methanolic extract	(Akhtar and Riffat, 1991)
- Ficus glabrata ***In vivo***			Humans	Ascaris Ancyclostoma Trichuris	Fresh	(Hansson et al., 1986)
		- *Embelia ribes* - *Hagenia abyssinica* (toxic) *In vivo*	Chicks			(Low et al., 1985)
		- *Chenopodium ambrosoides* *In vivo*	Humans	Ascarids	Powdered dry plant	(Kliks, 1985)
	- *Stemona japonica* - *Sophora flavescens* *In vitro*			*Angiostrongylus* *Diplydium caninum* *Fascioloa hepatica* *Schistosoma japonica*	Extracted tuberostemonine	(Terada et al., 1982)
	- *Artemisia herba-alba* *In vivo*		Goats	*Haemonchus contortus*	Powdered roots	(Idris et al., 1982)
- Carica papaya ***- Butea frondosa*** ***- Momordica charantia*** ***In vivo***			Chickens	*Ascardia galli*		(Lal et al., 1976)
- Curcubita maxima						(Bose et al., 1961; Srivastava and Singh, 1967)
- Carica papaya						Bose et al. 1961

Appendix 3

3.1. PROBIT analysis method and results for comparison with general linear model outcome in Section 6.2.4.3 and 6.3.2.

Data were grouped according to drug combination and test, i.e. ivermectin (IVM) alone in the larval development test (LDT), IVM + pluronic 85 (P85) in the LDT etc. Data from each group were analysed using PROBIT regression analysis with correction for natural mortality based on the data, to enable calculation of an effective median concentration (EC-50) of IVM. The independent covariate, concentration, was transformed by the natural logarithm to enable best fit of the model. This analysis was performed using SPSS version 21 (SPSS Inc., Chicago IL). EC-50 ratios were calculated for comparison of IVM alone with IVM plus P-gp inhibitor, defined as the synergy ratios (SR). EC-50 ratios were also calculated for comparison between parasite populations in each assay, defined as resistance ratios (RRs). Table A.3.1. shows the EC-50 values and the ratio of EC-50 values IVM alone: IVM + PGPI, defined as the synergy ratio (SR). From these results it can be seen that, in the LDT, P85 and ketoconazole increased the effect of IVM in both Pops 1 and 2, but to a lesser extent in Pop 1 than in Pop 2. In the LDT, IVM-AG has no positive effect on IVM efficacy. In the LMIT, IVM-AG and ketoconazole both increased the effect of IVM in Pop 1, but had little effect in Pop 2. In the LMIT, P85 increased the effect of IVM at lower anthelmintic concentrations but decreased the effect at high concentrations; hence it was not possible to model the data meaningfully using PROBIT regression. The resistance ratio (RR), i.e. the ratio of Pop 1: Pop 2, was 1.1 in the LDT and between 3.0-18.3 in the LMIT. There was a range of RRs for the LMIT because each P-gp inhibitor was tested on L3 sampled at a different time of year.

Table A.3.1. EC-50 values calculated for each Population in the larval development test (LDT) and larval migration inhibition test (LMIT), with ivermectin (IVM) and IVM plus Pluronic 85 (P85) (22 µM), ketoconazole (keto) (10 µM) and ivermectin aglycone (IVM-AG) (8 and 60 nM for the LDT and LMIT, respectively). For each p-glycoprotein (P-gp) inhibitor a ratio of the EC-50 with IVM alone and the EC-50 with P-gp inhibitor is shown, defined as the synergy ratio (SR)

	Assay (LDT or LMIT)	**P-gp inhibitor**	**EC-50 IVM alone, (confidence interval)**	**EC-50 IVM plus p-gp inhibitor (confidence interval)**	**Synergy ratio**
Population 1	LDT	P85	3.645 (2.464-5.093) nM	0.765 (0.412-1.55) nM	4.8
		keto	3.645 (2.464-5.093) nM	1.619 (0.751-2.278) nM	2.3
		IVM-AG	3.645 (2.464-5.093) nM	6.665 (4.994-8.301) nM	0.5
	LMIT	P85*	-	-	-
		keto	3.014 (2.63-3.737) µg/ml	1.849 (1.450-2.287) µg/ml	1.7
		IVM-AG	11.141 (7.633-15.616) µg/ml	2.363 (1.642-3.245) µg/ml	4.7
Population 2	LDT	P85	3.977 (3.455-4.518) nM	0.406 (0.346-0.470) nM	9.8
		Keto	3.977 (3.455-4.518) nM	0.665 (0.487-0.871) nM	6.0
		IVM-AG	3.977 (3.455-4.518) nM	3.726 (3.103-4.414) nM	1.1
	LMIT	P85*	-	-	-
		Keto	0.610 (0.450-0.798) µg/ml	0.538 (0.387-0.717) µg/ml	1.1
		IVM-AG	0.989 (0.737-1.291) µg/ml	0.802 (0.598-1.046) µg/ml	1.2

*For P85 in the LMIT, an atypical dose response relationship was observed and the data did not fit a PROBIT model and hence an EC-50 value is not shown.

3.2. Results of BLAST analysis of sequence data for cyathostomin pgp-9

Table A.3.2. The top 11 results of a NCBI nucleotide BLAST search using sequence data from the PCR product for *pgp-9* using DSW cDNA

Nucleotide sequence hit	Accession	Max Score	Total Score	Query cover	E-value	Identity
Cyclocylcus elongatus P-glyocprotein (pgp9) mRNA, complete cds	KJ701410.1	1418	1418	100%	0.0	92%
Haemonchus contortus isolate Weybridge P-glycoprotein 9a mRNA partial cds	JX430937.1|	778	778	99%	0.0	77%
Teladorsagia circumcincta partial mRNA for p-glycoprotein 9, nucleotide binding domain 1 (pgp-9 gene)	FR691848.1	677	677	99%	0.0	74%
Necator americanus ABC transporter transmembrane region mRNA	XM_013451424.1	333	586	79%	3e-88	80%
Caenorhabditis briggsae C CBR-PGP-9 protein (Cbr-pgp-9) mRNA complete cds	XM_002638567.1	284	284	89%	1e-73	67%
Caenorhabditis remanei hypothetical protein (CRE_22140) mRNA, complete cds	XM_003091578.1	269	269	92%	3e-69	66%
Caenorhabditis remanei hypothetical protein (CRE_27936) mRNA, complete cds	XM_003090002.1	266	266	96%	3e-68	66%
Cyclostephanus goldi genome assembly C_goldi_Cheshire, scaffold CGOC_contig0005008	LL376643.1	262	392	41%	4e-67	74%
Cyclostephanus goldi genome assembly C_goldi_Cheshire, scaffold CGOC_contig0000757	LL362081.1	255	1051	88%	6e-65	75%
Cyclostephanus goldi genome assembly C_goldi_Cheshire, scaffold CGOC_contig0002917	LL371363.1	230	580	58%	2e-57	90%

10. PUBLICATIONS ARISING FROM THIS THESIS

Scantlebury et al. BMC Veterinary Research 2013, 9:179
http://www.biomedcentral.com/1746-6148/9/179

BMC Veterinary Research

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Participatory study of medicinal plants used in the control of gastrointestinal parasites in donkeys in Eastern Shewa and Arsi zones of Oromia region, Ethiopia

Claire E Scantlebury[1], Laura Peachey[1], Jane Hodgkinson[1], Jacqui B Matthews[2], Andrew Trawford[3], Getachew Mulugeta[3], Gebre Tefera[4] and Gina L Pinchbeck[1*]

Abstract

Background: Gastrointestinal nematode infections constitute a threat to the health and welfare of donkeys worldwide. Their primary means of control is via anthelmintic treatments; however, use of these drugs has constraints in developing countries, including cost, limited availability, access to cheaper generic forms of variable quality and potential anthelmintic resistance. As an alternative, bioactive plants have been proposed as an option to treat and control gastrointestinal helminths in donkeys. This study aimed to use participatory methodology to explore donkey owner knowledge, attitudes and beliefs relating to the use of plant-based treatments for gastrointestinal parasites of donkeys in Ethiopia.

Results: In focus groups, 22/29 groups stated they knew of plants used for the treatment of gastrointestinal parasites in donkeys. All groups volunteered plants that were used in cattle and/or small ruminants. In total, 21 plants were named by participants. 'Koso' (*Hagenia abyssinica*) 'Grawa' (*Vernonia amygdalina*) and a mixed roots and leaves preparation were the most frequently named plant preparations. 'Enkoko' (*Embelia shimperi*) and 'a mixture of roots and leaves' were ranked highly for effectiveness in donkeys. However, 'Grawa' and 'Koso' were the highest ranked when taking into account both the rank position and the number of groups ranking the plant.
Thematic analysis of participants' current attitudes and beliefs surrounding traditional plant-based remedies for gastrointestinal parasites revealed that anthelmintics obtained from clinics were generally favoured due to their ease of administration and perceived higher effectiveness. There was doubt surrounding the effectiveness of some plant-based treatments, but there were also perceived advantages including their low cost, ease of cultivation and availability. However, plant-based treatments were considered a *"past trend"* and people favoured *"modern"* medicine, particularly among the younger generation.

Conclusions: There was extensive knowledge of plant-based treatments for gastrointestinal parasites in livestock in Ethiopia. In donkeys, Koso (*Hagenia abyssinica*), Grawa (*Vernonia amygdalina*), Enkoko (*Embelia shimperi*) and 'mixed roots and leaves' were the most frequently named and/or highest ranked plants with reported efficacy against gastrointestinal parasites. Further *in vitro* and *in vivo* investigation of these plants is now required to determine viable alternatives for the treatment and control of gastrointestinal parasites in Ethiopia.

Keywords: Ethnoveterinary, Anthelmintic, Nematode, Participatory, Donkey, Equid, Ethiopia, Thematic analysis

* Correspondence: ginap@liv.ac.uk
[1]Institute of Infection and Global Health and School of Veterinary Science, University of Liverpool, Leahurst Campus, Cheshire, UK
Full list of author information is available at the end of the article

Background

Gastrointestinal nematode infections constitute a major threat to the health and welfare of donkeys worldwide. The strongyle nematode species, in particular the cyathostomins, are the most numerous and pathogenic parasites of equids both in the UK and in developing countries [1,2]. These parasites can be responsible for considerable morbidity and mortality in horses [2,3] and may have negative effects on performance and productivity in donkeys [4,5]. Helminthiasis has been documented as a significant problem in working equids, many having polyparasitism [1,6-8]. There is a high prevalence and, often, high infection intensities in donkeys [1,9], making this a significant health concern in Ethiopia. As most animals do not acquire 100% immunity to intestinal nematodes [10], there is a need for life-long control strategies to reduce the burden of infection, particularly in those individuals who remain susceptible to high levels of infection throughout their lives [3].

The currently available anthelmintics (benzimidazoles, tetrahydropyrimidines and macrocyclic lactones) have been widely used against equine gastrointestinal helminths prophylactically and chemotherapeutically for many years. However, anthelmintic resistance is thought to be present in many populations and threatens sustainable control in future [3,11]. Further, in some developing countries, where donkeys are relied upon for transportation, there may be other constraints to the use of manufactured anthelmintics, such as limited availability and excessive cost. Anthelmintics may be diluted before being sold or may be used at incorrect dose rates, which may further accelerate the development of resistance in these populations [12]. Hence, there is a need to explore alternative methods of control of gastrointestinal helminths in donkeys in these parts of the world.

According to circumstances and depending on their relative efficacy, bioactive plants with anthelmintic properties offer an alternative that may overcome some of these problems [13]. Ethnoveterinary medicines (including bioactive plants) have been used for centuries for the treatment of a variety of health problems in humans and animals and it is estimated that up to 80% of Africa's population use traditional medicine for their health needs [14]. A number of studies from Ethiopia and elsewhere have reported plants that are believed to have efficacy against internal parasites in ruminants, chickens or people [15-23]; however, none have identified plant-based anthelmintics specifically for use in donkeys or other equids.

In Ethiopia, horses and donkeys play a crucial role in both urban and rural communities, where they are used to transport a variety of goods including crops, firewood, household consumables and water [24]. The UN Food and Agriculture Organisation estimate that there are over 7 million donkeys, mules and horses in Ethiopia [25], and it has the second largest donkey population in the world. Added to this, there is a rich diversity of plant species among Ethiopia's varied topography [26], generating a long tradition of the use of plants for medicinal purposes. This study aimed to use participatory approaches [27] to explore donkey owner knowledge, attitudes and beliefs relating to the use of plant-based treatments for gastrointestinal parasites in these animals in Ethiopia. In addition, owner recognition of gastrointestinal parasitic disease was explored.

Results

In total, 182 donkey owners participated in 29 focus group discussions. The majority of these (94%) were male participants ranging in age from >16 to <80 years.

Participant reported signs of gastrointestinal parasites

In cattle, small ruminants and donkeys, the most frequently named sign recognised as indicative of the presence of gastrointestinal parasites was observing worms in faeces (Table 1), followed by loss of body condition. In all groups, a combination of signs were volunteered, possibly indicating that a number of signs are recognised and assessed together before an animal is regarded as having gastrointestinal parasites. The local term used for internal parasites in animals was 'Maga'. In donkeys, two types of worms were described as being present in the faeces, which were long white worms and 'alive' red worms.

Table 1 Signs of gastrointestinal parasites in donkeys and ruminants reported by 29 groups of donkey owners

Signs attributed to gastrointestinal parasites	Donkey n groups (%)	Cattle n groups (%)	Sheep and goats n groups (%)
Worms in faeces	28 (96.6)	25 (86.2)	20 (69.0)
Loss of body condition	25 (86.2)	22 (75.9)	10 (34.5)
Colic	3 (10.3)	5 (17.2)	3 (10.3)
Bloat	5 (17.2)	4 (13.8)	2 (6.9)
Rough hair coat / loss of hair	5 (17.2)	3 (10.3)	1 (3.4)
Cough	11 (37.9)	4 (13.8)	8 (27.6)
Loss of appetite	9 (31.0)	8 (27.6)	1 (3.4)
Diarrhoea	-	14 (48.3)	5 (17.2)
Gut sounds	4 (13.8)	-	2 (6.9)
Eggs on mane	2 (6.9)	-	-
Lice / external parasites	-	2 (6.9)	3 (10.3)
Bottle jaw	-	5 (17.2)	7 (24.1)
Cysts (internal organs)	-	-	1 (3.4)
Pungent smell faeces	-	2 (6.9)	-
Bloody urine	-	2 (6.9)	-
Miscellaneous	8 (27.6)	4 (13.8)	-

Plant-based treatments for gastrointestinal parasites

All groups volunteered plants that were used as anthelmintics in cattle and/or small ruminants, but only 22/29 groups stated they knew of plants for use in donkeys. Table 2 summarises all the plants named in the focus groups believed to be efficacious against gastrointestinal parasites for use in donkeys, cattle and small ruminants. 'Koso' (*Hagenia abyssinica*) 'Grawa' (*Vernonia amygdalina*) and 'mixed roots and leaves' were the most frequently named plant preparations. The unnamed 'mixtures of roots / leaves' varied considerably and were often recipes handed down through the generations that were prepared and sold for use by local villagers. The plant ranking data for donkeys (Table 3) shows that although 'Enkoko' *(Embelia schimperi)*, 'mixture of roots and leaves' and 'Abdul salim' (unknown) had a high average rank score, 'Grawa' and 'Koso' were the highest ranked when taking into account both the rank position and the number of groups ranking the plant (reported as combined rank score). The informant consensus (calculated at group level in this study) was 0.621. There was considerable variation in the described modes of preparation; these included techniques such as crushing, drying, mixing with water or salt, fermentation and smoking (inhalation). Aside from the group of remedies presented as 'mixed roots / leaves', 10 plants were prepared by crushing and infusion in water, 5 were infused with water with or without salt or other plants, 2 were prepared as concoctions, 1 crushed, 1 seeds of the plant were mixed with salt, 1 was in a powder form dissolved in water and 1 was smoked. Plant preparations were often administered by drenching the donkey using a glass bottle (for example 'Coca-Cola™ bottle). Additionally, dosage information was difficult to obtain, with estimations of amounts or volumes being illustrated in measures such as 'handfuls' for leaves, 'finger lengths' for roots and drinks bottles for liquids. A range of side effects were reported and these were sometimes attributed to variation in preparation methods and dose (Table 2).

Attitudes towards 'pharmaceutical' and 'traditional' medicine

Table 4 details the super-ordinate (or key) themes and examples of sub-themes within each category. It was apparent that, although plant-based 'traditional' medicines were familiar to the current generation, pharmaceutical anthelmintic preparations obtained from the animal health clinics were generally favoured. Numerous reasons were cited; for example, pharmaceutical preparations derived from clinics were widely available at all study sites and were perceived to be more *"modern"* and even *"civilised"* making them a more attractive choice. This was because *"professional people"*, whose advice was held in high regard, prescribed them and there was also the reassurance that the products had been tested experimentally. Additionally, it was reported that these professionals (referring to local development agents/doctors/veterinarians/pharmacists/animal health workers) advised against the use of 'traditional' medicines due to the risk of side effects, uncertainty surrounding correct dosing and risks associated with drenching donkeys.

> *..."Whenever there is any problem with their animal they will take (the animal) to the clinic and the first question by the professionals is 'have they given any traditional medicine to this animal', such a question is not good for us they said so they have already stopped giving any traditional medicine to these animals because the animal health professionals do not advise them to use"...*

There was additional confidence in pharmaceutical anthelmintic preparations as they were prescribed after specific diagnosis by a clinician, whereas 'traditional' medicines were less specific, often with the same plant preparation used for a range of different problems. Pharmaceutical preparations were reportedly easy to administer and effective after one treatment whereas plant-based 'traditional' medicines often required multiple or prolonged dosing before any efficacy was observed along with a risk of side effects. Practical problems with administering plant-based treatments also made them less favourable, as often it required drenching the animal with large volumes of fluid.

Reported disadvantages of pharmaceutical preparations were the, albeit, small number of observed side effects. Nevertheless, there were some positive reports in favour of plant-based 'traditional' medicines including, their low cost (even no cost in some cases), ease of cultivating at home and availability where no clinics were accessible. One traditional healer described villagers coming to him in the night for his plant remedies. However, in some areas, the reduction in demand for specific plant-based preparations meant that they were no longer available at the market. One participant voiced concern about deforestation in some areas resulting in difficulties sourcing some plant species.

In some instances plant-based 'traditional' medicines were believed to be more effective than pharmaceutical preparations; for example, some people considered 'Koso' to be a superior treatment, particularly in people. However, the reported side effects were a major consideration and ranged from diarrhoea to death if overdosed.

> *..."'koso' works better than a tablet but the only problem is the side effect, that is why they prefer to use tablet even though Koso is more effective"...*

Table 2 Plants used as anthelmintics in donkeys and ruminants volunteered by 29 focus groups (made up of 182 individuals)

Scientific name	Traditional name	Frequency of groups (%) volunteering plants for use in donkeys	Frequency of groups (%) volunteering plants for use in cattle and/or small ruminants	Reported potential side effects
Hagenia abyssinica (Bruce) J.F. Gmel	Koso (Am)	9 (31)	9 (31)	Diarrhoea can kill if overdose
Unknown	Mixed roots / leaves / traditional remedy	8(27.6)	12 (41.4)	
Vernonia amygdalina Delile	Grawa (Am)	7 (24.1)	13 (44.8)	Diarrhoea can kill if overdose
Embelia schimperi Vatke	Enkoko (Am) / Hanko (Or)	4 (13.8)	0	Abdominal pain and diarrhoea, bitter taste
Cucumis prophetarum C.A. Mey ex. Cogn	Holoto (Or)	3 (10.3)	2 (6.9)	Severe diarrhoea and can kill if overdose
Verbascum sinaiticum Benth.	Gura Harre (Or) / Yeahiya joro (Am) Donkey ear	3 (10.3)	1 (3.4)	None reported
Withania somnifera (L.) Dunal	Wahale (Or) / Gizawa (Am)	3 (10.3)	1 (3.4)	None reported
Tapinanthus globiferus Tiegh.	Harmuu (Or)(parasitic plant)	1 (3.4)	0	None if correct dose
Phytolacca dodecandra L'Hér	Endod (Am) / Handode (Or)	1 (3.4)	0	Acidic effect in stomach, can create burning sensation
Unknown	Yeare Geleba (Am) / Geleba atara (Or) Bean Straw	1 (3.4)	0	
Trigonella foenum-graecum L.	Abish (Am)	1 (3.4)	2 (6.9)	None reported
Unknown 'root'	Buri (Or)	1 (3.4)	0	Severe diarrhoea and can kill if overdose
Unknown	Abdul Salim	1 (3.4)	1 (3.4)	Bitter taste if overdose can kill
Dodonaea angustifolia L.F.	Kitkita (Am)	1 (3.4)	0	Severe diarrhoea and can kill
Unknown	Sara-aja (Or)	1 (3.4)	2 (6.9)	None reported
Croton macrostachyus Hochst. ex. Delile	Bisana (Am)	1 (3.4)	0	Burning sensation and severe diarrhoea
Unknown	Chobi (Or)	0	1 (3.4)	None if use root, juice from plant is irritant to skin
Unknown	Feto (Or/Am)	0	1 (3.4)	None reported
Nicotiana tabacum L.	Tobacco	0	10 (34.5%)	Diarrhoea if overdose unconsciousness and can kill
Melia azedarach L.	Milia	0	1 (3.4)	None reported
Capparis cartilaginea Decne.	Delensisa (Or)	0	2 (6.9)	Diarrhoea overdose can kill
Unknown	Keskesae (Am)	0	1 (3.4)	None reported

NB: International plant name index (IPNI) names including latin and author names for each plant species are given where available.
Where a plant is labelled 'unknown', we were unable to obtain a voucher specimen that was positively identified by a respondent either due to season or locality.
Where known, the language for each traditional name given is noted as either Or (Oromic) or Am (Amharic).

In some cases plant-based treatments for donkeys had developed from treatments that were previously used in cattle or sheep and goats.

..."When they do not have any option, they use it as an option for donkeys, they assume it will probably treat donkeys but they are not sure"...

Table 3 Plant rankings for perceived effectiveness in donkeys (results from 29 focus groups)

	Vernonia amygdalina (Delile) (Grawa)	*Hagenia abyssinica* (Bruce) J.F. Gmel (Koso)	*Cucumis prophetarum* (C.A. Mey ex. Cogn) (Holoto)	*Embelia schimperi* (Vatke) (Enkoko)	*Withania somnifera* (L. Dunal) (Wahale)	Roots and leaves mixture	Abdul Salim	Roots of plant mix	*Tapinanthus globiferus* (Harmuu) (parasitic plant)	*Verbascum sinaiticum* (Benth) (Gura Harre)	*Trigonella foenum-graecum* (Abish)	*Phytolacca dodecandra* (Sesse & Moc.) (Endod)
Average rank score	2.2	2	3	1	2.5	1	1	2	2	2	3	3
Combined rank score	27	23	14	8	5	4	4	3	3	3	2	2
Number of groups volunteered this plant	6	6	4	2	2	1	1	1	1	1	1	1

Table 4 Summaries of superordinate themes from thematic analysis of discussion surrounding anthelmintic strategies in donkeys and other species

Superordinate (key) themes	Example sub-themes
Attitudes to medicines from the clinic	Preferences for either clinic or traditional plant based medicine.
	Clinic medicines are perceived as more modern, professional or scientific.
Attitudes and beliefs surrounding traditional plant based medicine	Preferences for either traditional or clinic medicines.
	Significance of societal influences upon these preferences.
	Traditional plant based medicines considered a 'past trend'.
	Interpretation of the response to plant based medicines within the animal as indicative of strength / efficacy and reported side effects.
	Spiritual connections and plant based medicine.
Origins of traditional plant based medicine	Evolved from cattle preparations.
	Inherited knowledge from fathers, passed down through the generations.
	Religious texts.
Beliefs of when to worm donkeys	Interpretation of clinical signs as gastrointestinal parasites.
	Selection of individuals or group to de-worm.
	Frequency of worming.
	Sources of advice.
Other non-plant based preparations for de-worming	Including: fermented butter, lake water, rotten egg, oil seed, alcohol.

One of the key questions asked whether people would be likely to return to plant-based treatments for wormers. Responses were orientated towards a preference for scientific justification, including approval by professionals and scientific testing. Additionally if the plant could be grown at home, this would make its use more likely as costs would be reduced. Other participants reported that they would use plant treatments only if they had no other option.

Origins and transmission of knowledge of plant-based medicine

Most knowledge regarding plant-based treatments was passed on via word of mouth, generally through the male family line. However, some female participants reported having been shown by their fathers how to prepare specific remedies.

> *..."he learnt from his father and he will teach his next generation to his children just his family. It is a business and he gets payment from people who use these remedies. His father told him not to charge too much as otherwise it may not work"...*

Generally, people learnt through active participation accompanying their father to collect the plants and assisting with the preparation process. One participant described being able to tell that the preparation of 'Grawa' was correct due to the 'taste' of the mixture. Plant preparations named by traditional healers were kept within their family only and their recipes guarded and not reported here. There were elements of tradition, religion and superstition surrounding the preparation and efficacy of plant preparations and, occasionally, these were sold for a small fee contributing to the household income. In some cases, participants reported they would not pass on some or all of the information in future as clinics were readily available in the area, or that they believed that some of the plant preparations did not work.

> *..."there is no transmission of the knowledge through their children...as this current generation don't want to use those traditional medicines that's why it is not transmitted to their children so everyone converts to modern medicine"...*

A common theme raised by participants was the concept of plant-based medicines as a *"past trend"* and something that people were moving away from in favour of *"modern"* medicine. This was particularly apparent in the younger generation who often reported little interest in learning about plant-based therapies from their elders.

> *..."because they relate it to old-style religion so they don't want to know and also modern medicine is available"...*

Discussion

General knowledge of traditional medicines (TM) used as anthelmintics was high in this study, with all groups naming plants used in livestock species generally and 22/29 groups specifically naming plants for use in donkeys. In total, 21 different plant preparations were named for use in livestock. This is in line with previous

reports of the wide use of TM's in developing countries, often attributable to their accessibility and affordability. In Africa, up to 80% of the population use TM to help meet their health care needs [14] and natural plant-derived products have been known for many decades to possess anthelmintic properties [28]. However, many of the previous studies report the use of plant-based anthelmintics for humans [15] or for ruminants, pigs and poultry livestock [16,18,20,22,23]. Further, the majority of evidence for plant-based anthelmintics is in the form of observations rather than controlled studies [28]. To the authors' knowledge there are no published studies of the use of these materials in equids, despite their immense value to communities in developing countries [29].

Of all the plants named by participants, *V. amygdalina*, was the highest ranked plant for efficacy against gastrointestinal parasites. It is a perennial shrub that is abundant in tropical Africa, including the regions of interest in Ethiopia. It has been used for centuries by humans for the treatment of multiple ailments, and recent research has identified that it may have a number of health benefits such as antimalarial, antimicrobial, antifungal, antitumor, and anti-diabetic effects [30]. There have also been several studies demonstrating its potential as an anthelmintic. For example; a study in puppies in Nigeria demonstrated a significant anthelmintic effect of the aqueous extract of *V. amygdalina* leaves against *Toxocara canis* and *Ancyclostoma caninum* [31] and the aqueous extract of *V. amygdalina* leaves has been shown to reduce faecal egg counts in calves infected with mixed gastrointestinal nematodes by 59.5% [32]. One study investigated the bio-activity of a related species, *V. anthelmintica* and demonstrated a faecal egg count reduction of 73.9% when sheep were administered 3 g/kg of crude aqueous extract of the seeds [33]. *Hagenia abyssinica*, also ranked highly, has well known anti-cestodal properties [15,34] and was reportedly frequently used to treat human infection with tapeworm. Although *H. abyssinica* and *V. amygdalina* were the most frequently named plants here, there were important issues raised about the potential side effects of these two remedies, which ranged in severity and reportedly could include death of the animal if not used correctly. Negative side effects in humans have also been reported with the use of *H. abyssinica*. The most common of these are diarrhoea and abdominal pain. Blindness, changes to the central nervous system, abortion and death, have also been associated with ingestion of a high dose of *H. abyssinica* [15,35].

Amongst the other highly ranked plants, there are reports in the literature of anthelmintic activities, although the evidence is not as compelling as for *V. amygdalina*. *Withania somnifera* has been identified in previous surveys of ethnoveterinary plants [36] and an *in vitro* study assessing the effect of aqueous extracts of this plant against *Pheretima posthuma* (earthworm) showed a significant effect [37]. *Cucumis prophetarum* has also been identified in previous surveys [38,39]. There are no studies assessing the specific anthelmintic activity of this plant species, however plants in the same family, *Cucurbitaceae*, have been used for centuries as taenicidals and a recent study showed a related species *Cucurbita moschata* to be effective against nematodes *in vitro* [40]. The use of *E. schimperi* and evidence for efficacy *in vivo* and *in vitro* is restricted to taenicidal activity [41]. The informant consensus of plants named in this study was relatively high (close to 1) and indicates good homogeneity of cultural knowledge on the use of plants in the treatment of gastrointestinal parasitic disease suggesting that knowledge is shared between communities. This score indicates that relatively few different taxa of plants were reported by the different groups which may suggest that some of these plants could be efficacious. It was not possible to identify the species of plant that was referred to as 'Abdul salim' as the plant itself did not grow in the areas where the study was conducted and, therefore samples could not be collected for specific identification.

The most frequently reported signs associated with gastrointestinal parasites in donkeys were observation of worms in the faeces and loss of body condition. This may indicate a relatively high burden of parasitism prior to any treatment being given. Indeed, previous studies have reported a high prevalence of parasitism within the donkey population in Ethiopia [1,9]. 'Loss of body condition' in a donkey is not necessarily pathognomonic for helminthiasis and it was acknowledged by participants that the same plant based preparations were often used for multiple clinical presentations. This may result in some mis-classification bias within this study however ongoing work is investigating a selected number of highly ranked plants for their bio-activity against *Cyathostome spp. in-vitro*.

The preparation methods described were relatively straight forward and often used leaves or whole plants crushed and mixed with water to make an infusion which was then administered. This is akin to preparation methods reported in other ethnoveterinary medicine studies [42,43]. However between groups there was a variety of methods of preparation and measures of ingredients used resulting in limited useful information relating to how these plants are prepared for use in donkeys. It may be that each family group has slightly different preparation methods or may be indicative of a certain amount of 'trial and error' involved when extending the use of plant-based medicines traditionally established for use in cattle/small ruminants to donkeys as was reported within this study.

There was no report of prophylactic dosing with anthelmintics among donkeys, and animals were treated based on the recognition of clinical signs. This may, in

part, be due to socio-economic pressures influencing the frequency with which medical interventions are sought for donkeys. Further research is required in order to describe how socio-economic and other contextual determinants drive owner decision making regarding preventive health care, particularly given the large population of donkeys within these communities.

Although traditional medicines continue to play a significant role within the community health care system in Ethiopia [44] there appears to be a general shift away from traditional remedies for anthelmintic treatment in this study area due to the availability of clinical services. The perception was that clinics provide a more accurate diagnosis and dosage of medicines and that these represent a modernisation and improvement in practice and had fewer side effects. This finding was similar to that reported by Bussman *et al.* in 2011 [45]. It appears that the younger generation in particular are not as interested in learning about and retaining the knowledge relating to plant-based medicines. Others have shown that ethnoveterinary knowledge is greater in older informants and those with lower education levels [46]. As knowledge of plant-based remedies is passed on through word of mouth and generally stays within family lines, it may be prudent to collect further information for documentation of additional plant-based remedies for use in veterinary species before this information disappears.

There appears to be a widespread practice of drenching donkeys with plant-based remedies for treating many conditions and this poses a significant risk of aspiration pneumonia (cases are regularly reported to the Donkey Sanctuary clinic, *pers comm.*). Additionally, chemical anthelmintics were often reported to be in tablet form which were mixed with water and drenched. Practical alternatives for these problems need to be developed and communicated in order to reduce this risk.

In many cases, people were unable to name the chemical anthelmintic product they used but those that did spoke of "Albendazole". It is unknown whether the helminth population is susceptible to the treatments available in these regions and whether the method, and dose, given to donkeys is sufficient for control. In other parts of the world a range of anthelmintics (benzimidazoles, tetrahydropyrimidines and macrocyclic lactones) have been widely used against equine gastrointestinal nematodes for many years; however, anthelmintic resistance is present in many populations in developed countries and threatens sustainable control in future [11]. Although the degree of anthelmintic resistance has not yet been established in nematodes of donkeys or horses in Ethiopia, continued use of a limited range of chemical anthelmintics, combined with the effects of under or inappropriate dosing, or inferior quality generic products, are all risk factors for promoting anthelmintic resistance. Benzimidazole resistance in small ruminant nematodes has already been demonstrated in Ethiopia [47,48].

Some potential biases may have been introduced due to the roles of the researchers as veterinarians and animal health assistants. This may have influenced the participants' discussions to favour clinical medicine; however, it was considered that a good range of views relating to the benefits and disadvantages of both clinic-based medicine and traditional medicine were obtained so we consider this bias to be minimal. The timing of the study was at the beginning of the wet season and consequently, in some regions there was little vegetation evident. This may result in an amount of recall bias among participants leading to some 'out of season' plants being omitted from the discussion however, we asked the question about plant use in general and given that people recalled annual trends in plant use it is anticipated that this source of bias would be minimal. There may have been some selection bias with more knowledgeable participants selected by the DA's who were influential in selecting the participants; however, they were briefed on which participants to recruit and it is believed that we communicated with a broad range of donkey owners.

Further research is required to determine helminth sensitivity to anthelmintic preparations commonly used in these regions. Additionally, further work is warranted to investigate the potential use of plant-based preparations as anthelmintics in donkeys and other species.

Aside from investigating options to overcome the threat of resistance problems, there are several other advantages of using plant-based anthelmintics in developing countries including cost, availability and environmental aspects [28].

Conclusions

There was extensive knowledge of plant-based treatments for gastrointestinal parasites in donkeys and other livestock in this region in Ethiopia. In donkeys, Koso (*H. abyssinica*) Grawa (*V. amygdalina*), Enkoko (*E. shimperi*) and mixed roots and leaves were the most frequently named and/or highest ranked plants with reported efficacy against gastrointestinal parasites in this species. However, as there appears to be a general shift away from the use of traditional plant-based remedies in the treatment of gastrointestinal parasites in animals it may be prudent to collect further information for documentation of additional plant-based remedies before this knowledge wanes. Results from this study have been triangulated with published research to guide selection of plants for *in vitro* testing against cyathostomins. This may, in future, lead to the identification of an efficacious plant-based remedy that is easily available and readily grown in Ethiopia.

Methods

Study area and participants

The study area consisted of 15 kebeles in the Eastern Shewa and Arsi zones of the Oromia region of Ethiopia (Figure 1). Villages were purposively selected for their varied topography and logistical accessibility and included highland, mid-highland and lowland sites. Six villages had been previously exposed to an equine non-governmental organization (NGO) (which included equine education programmes and clinical services) whilst 9 were unexposed to these services. Data collection was conducted over a 6-week period during June and July 2011, representing the beginning of the wet season in this region of Ethiopia. Participants were eligible for inclusion if they owned a donkey. Participation was entirely voluntary and owners were free to leave at any point. Participants were recruited using local development agents (DA) assigned from the relevant Bureau of Agriculture. Each DA was briefed about the study and asked to select donkey owners of a range of ages (>16 years of age). Two groups per village (with the exception of one where only one group was conducted), with 6–8 people per group, were recruited. The study was conducted in the local language (Afan Oromo/Amharic) and was facilitated by the author (CS) and an Ethiopian animal health worker (AHW) (GT) who acted as co-facilitator and translator. Both had received previous training in participatory approaches and were experienced in facilitating focus group discussions. Participatory methods used in this study were based upon previously published participatory and ethnobotanical study designs [27,49,50].

Focus group discussions

At the beginning of each focus group, GT gave an overview of the study and affiliated research groups and verbal informed consent was gained. The group interviews were semi-structured, including some key questions with opportunities for open discussion and utilised a number of different participatory methodologies (Table 5). Each group participated in constructing 2 sets of matrices, which were drawn on laminated white card and photographed. The first key question was, "which animals / species do you consider are affected by worms / gastrointestinal parasites?" This generated the first row of the matrix labelling animal species that were named by the group. A matrix of signs in each animal species was constructed by asking, "how do you know that an animal has worms-what signs do you see?" Open discussions relating to 'signs of worms', was investigated further with probing questions to explore the nature of worms seen.

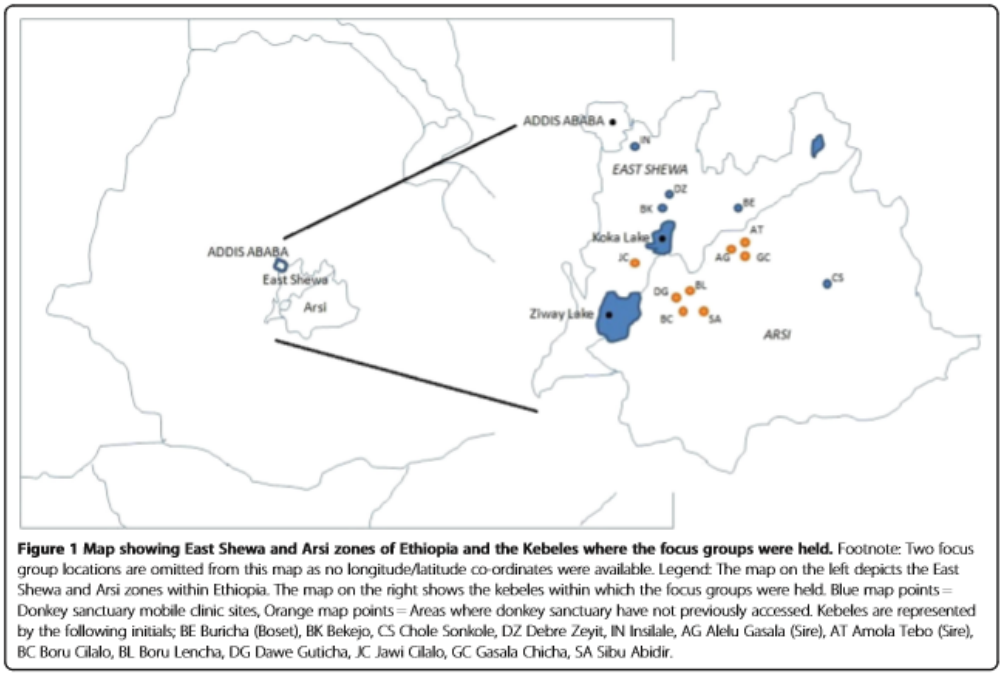

Figure 1 Map showing East Shewa and Arsi zones of Ethiopia and the Kebeles where the focus groups were held. Footnote: Two focus group locations are omitted from this map as no longitude/latitude co-ordinates were available. Legend: The map on the left depicts the East Shewa and Arsi zones within Ethiopia. The map on the right shows the kebeles within which the focus groups were held. Blue map points = Donkey sanctuary mobile clinic sites, Orange map points = Areas where donkey sanctuary have not previously accessed. Kebeles are represented by the following initials; BE Buricha (Boset), BK Bekejo, CS Chole Sonkole, DZ Debre Zeyit, IN Insilale, AG Alelu Gasala (Sire), AT Amola Tebo (Sire), BC Boru Cilalo, BL Boru Lencha, DG Dawe Guticha, JC Jawi Cilalo, GC Gasala Chicha, SA Sibu Abidir.

Table 5 Focus group format: key questions and group tasks initiated within each focus group discussion

Key question	Participatory activity
Which animals / species do you consider are affected by worms / gastrointestinal parasites?	List responses
How do you know that an animal has worms? What signs do you see?	List responses
	Construct matrix of species and signs of worms
What do you use to treat worms in your donkey / cattle and small ruminant? (List and then rank by preference / efficacy within each species)	List treatments
	Rank by preference / efficacy within each species
Why do you use these plants?	Matrix and group discussion
A matrix was constructed with each plant species named and matrix headings were cost, ease of use, availability, side effects, when particular worm seen, animal species treated and benefits working ability.	
The matrix was filled in with + / - or neutral for each column.	
Additional questions were asked during discussions:	
Where do you get the plant from?	
How do you know that it is effective?	
If plant based remedies not volunteered for worms–why not?	
Do you or anyone in your village use plant based treatments for anything else?	
Are there any circumstances where you would return to the use of plant based treatments?	
Would you spend money on worming your donkeys?	Group discussion
If you thought a plant was effective against worms would you grow it specifically to use in your donkeys?	Group discussion
Are there people in the village that know about or supply plant based treatments for wormers?	Group discussion

The next key question was, "what do you do to treat worms in these animals?" Responses were filled in alongside the listed signs and under the appropriate species.

The second matrix focused upon plant-based medicines named for use in donkeys within the first matrix. The names of the volunteered plants were listed along one side of the matrix and for each plant, brief details of where the plant/root or seed was obtained, how owners knew that this was an effective treatment and any side effects were recorded. If more than one plant was named for use in donkeys, the group were asked to arrive at a consensus of how these were ranked in terms of effectiveness. Photographs were taken of the matrices from each group.

During the course of the discussion, further questions were asked to explore general opinions relating to plant-based medicines and the importance of deworming donkeys. Care was taken not to influence the content of the discussion with leading questions. Open-ended questions were used to encourage discussion and exploration of the topic. To optimise data quality, all responses from participants were volunteered and participants were encouraged to contribute freely to the discussion by the facilitators.

Data management

The discussions were translated into English and recorded on a digital Dictaphone and this was then transcribed. Excel software was used to tabulate the photographed matrices. A list of plants named for donkeys was generated including frequency tables of how many groups named each plant type and ranking data to show the perceived effectiveness of the plants for use in donkeys. An average ranking for each plant was produced (by summing the rank positions and dividing by the total number of groups identifying that plant) along with a combined rank score. This was calculated by re-assigning the ranks with a score. For example, if a group named 4 plants for use in donkeys then the plant they ranked 1st and most effective was given a score of 4, the 2nd a score of 3 etc. Subsequently, the total scores for each plant from each group were added together to give a combined rank score. An estimation of the variability and homogeneity of knowledge of plants used to treat gastrointestinal parasites was determined using the Informant consensus factor [51]. This was calculated based on [52] as follows:

$$F_{ic} = (n_{ur} - n_t / n_{ur} - 1)$$

Where n_{ur} was the number of usage reports (or in this case groups naming the plant for use in donkeys) and n_t was the number of taxa used (or named by the groups, excluding mixed preparations). Recent studies have used informant consensus as a means to examine cultural knowledge and diversity of use of plant species for different clinical conditions [38,53].

A thematic analysis [54,55] of the content of the translated discussion relevant to participants' current attitudes and beliefs surrounding traditional plant-based remedies for gastrointestinal parasites was conducted with the aid of NVivo 8 data handling software. This involved reading all transcripts to become familiar with the data, sorting quotes discussing similar aspects relating to plant-based medicine into themes, reviewing the grouped themes and summarising the concepts. This facilitated the analysis and summarisation of the variety of responses relating to the key questions.

Plant specimens and identification

Cutting samples were collected for each plant named in the focus groups including (where possible) the leaves,

stems, flowers or seeds [56]. Where the plant was unknown to the research team, the participant was asked to show an example of the plant if it was locally available. Photographs were also taken of each plant to include close up pictures of the leaf / branch structure and any flowers or seed heads. A note was made of the date and site of collection. All plants were dried and pressed in preparation for formal identification at the National Herbarium, Addis Ababa University. Subsequent to identification of plant species, the results were triangulated with available literature to investigate evidence of bio-activity against gastrointestinal parasites in any species.

This study was reviewed and received ethical approval from the University of Liverpool research ethics committee and the University of Addis Ababa College of Veterinary Medicine and Agriculture.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
GP, JH and JM conceived the study and acquired the funding. All authors contributed to the study design. CS, LP and GT coordinated the study and collected all the data. CS performed all data analysis. GP drafted the manuscript with major input by CS. All authors were involved in revising the manuscript and approved the final manuscript.

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Author details
[1]Institute of Infection and Global Health and School of Veterinary Science, University of Liverpool, Leahurst Campus, Cheshire, UK. [2]Moredun Research Institute, Edinburgh EH26 0PZ, UK. [3]The Donkey Sanctuary, Sidmouth, Devon, UK. [4]College of Veterinary Medicine and Agriculture, Addis Ababa University, Debre Zeyit, P.O. Box 34, Ethiopia.

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