Anthelmintic resistance in equine parasites: an epidemiological approach to build a framework for sustainable parasite control

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by:

Hannah Elizabeth Lester

October 2015

Abstract

Faecal egg count (FEC) directed targeted anthelmintic treatment programmes and regular efficacy testing using the faecal egg count reduction test (FECRT) have been advocated to support evidence-based helminth control in horses. One major hurdle to their widespread application is that horse owners/managers and those that prescribe anthelmintics may have insufficient knowledge on which to base evidence-based protocols. The ultimate aim of this study was to create a framework for a decision support system (DSS) to support evidence-based helminth control in horses. To create the framework, the diagnostic performance of FEC and FECRT methodologies were evaluated. In addition, the efficacy of the three licensed anthelmintic classes was tested in several equine populations. The prevalence and distribution of helminths was determined in these populations, and an analysis undertaken to investigate factors associated with different levels of strongyle egg shedding in individuals. The consistency of egg shedding patterns in individuals over time was evaluated and the resource implications of following a FEC directed targeted treatment investigated. The FEC analysis findings support the rationale of FEC directed targeted anthelmintic treatments in horses to reduce treatment frequency in order to mitigate the impact of anthelmintic resistance. Moreover, the results show that such a strategy may be cost effective. The efficacy studies revealed that the macrocyclic lactone anthelmintics were highly effective in reducing strongyle egg output at two weeks after treatment, but further studies are required to analyse the strongyle egg reappearance period after treatment with these anthelmintics. In summary, this study validates the use of FEC directed treatment protocols in the field and the next step will be to use the derived information to design user-friendly online support tools.

Declaration

This thesis contains original work conducted by the author as part of a collaborative research project. Faecal egg count data was generated primarily by Hannah Lester and Claire Stratford, with additional laboratory assistance from several undergraduate students; Tasmin Siu, Sheena Tarrant, Emma Wood and Rachel Cookson. This work has not been submitted for any other degree or to any other University than the University of Liverpool.

Hannah Elizabeth Lester

October 2015

Dedication

To my family, for their unfailing love and support

Acknowledgements

I would like to thank the Elise Pilkington Trust for their generous funding. Jacqui Matthews for encouraging me to undertake a PhD when I first met her at Whipsnade Safari Park back in 2010. My PhD supervisors Jacqui Matthews, Eric Morgan, Jane Hodgkinson and Dave Bartley for their excellent guidance, patience and encouragement. My friend and collaborator Claire Stratford for all her help and enthusiasm. Dan Nussey for the generous provision of his time and guidance. Colleagues at the Moredun Research Institute for their support. Sheena Tarrant, Emma Wood and Rachel Cookson for their help in generating the FEC data. Tim Mair, Karen Stevenson, Gemma Lemon and Becky Swan from the Bell Equine Veterinary Clinic and Jess Spanton from House and Jackson Veterinary Surgeons for help recruiting yards and collecting samples. The many horse owners who allowed their horses to partake in this study. The medical staff at NHS Lothian who fixed me!

List of publications and presentations

Peer-reviewed papers

Lester, H.E., Spanton, J., Stratford, C.H., Bartley, D.J., Morgan E.R., Hodgkinson, J.E., Coumbe, K., Mair, T., Swan, B., Lemon, G., Cookson, R., Matthews, J.B. (2013) Anthelmintic efficacy against cyathostomins in horses in Southern England. *Veterinary Parasitology* 197, 189-196. (Chapter 3)

Lester, H.E., Bartley, D.J., Morgan, E.R., Hodgkinson, J.E., Stratford, C.H., Matthews, J.B. (2013) A cost-comparison of faecal egg count directed anthelmintic delivery versus interval programme treatments in horses. *Veterinary Record* 173, 371. (Chapter 6)

Lester, H.E., Matthews, J.B. (2014) Faecal worm egg count analysis for targeting anthelmintic treatment in horses: points to consider. *Equine Veterinary Journal* 46, 139-145. (Chapters 1 & 2)

Stratford, C.H., Lester, H.E., Pickles, K.J., McGorum, B.C., Matthews, J.B. (2013) An investigation of anthelmintic efficacy against strongyles on equine yards in Scotland. *Equine Veterinary Journal* 46, 17-24. (Chapter 3)

Lester, H.E., Matthews, J.B (2014) Getting the most out of equine faecal egg counts. In Practice. *In Press.* (Chapter 2)

Peer-reviewed abstracts

Lester, H., Stratford, C., Matthews, J.B. 2012. Effect of five-day fenbendazole or moxidectin on strongyle faecal egg counts in a population of welfare horses. *Journal of Veterinary Internal Medicine* 26, 432-433 (Chapter 3)

Lester, H.E., Bartley, D.J., Morgan, E.R., Hodgkinson, J.E., Matthews, J.B. 2012a. The spatial distribution of strongyle eggs in horse faeces. *Journal of Equine Veterinary Science* 32, S33-S34 (Chapter 2)

Lester H.E., Bartley D.J., Morgan E.R., Hodgkinson J.E., Stratford C.H., Matthews J.B. 2012b. A comparison of three methods for calculating anthelmintic efficacy in equids. *Journal of Equine Veterinary Science* 30, S34-35 (Chapter 4)

Contents

List of abbreviations

5d FBZ	Five-day course of fenbendazole
AR	Anthelmintic resistance
BHS	British Horse Society
Bwt	Body weight
BZ	Benzimidazole
CF	Centrifugal-flotation
CL	Confidence limit (95%)
Cm	Centimetre
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CV	Coefficient of variation
Cy-CID	Cyathostomin immunodiagnostic antigen
Cy-GALA	Cyathostomin gut associated larval antigen
DL4	Developing fourth stage larvae
Dl	Detection limit
DNA	Deoxyribose nucleic acid
DSS	Decision support system
EC50	Effective concentration 50
EHT	Egg hatch test
EL	Encysted larvae
EL3	Encysted third stage larvae
ELISA	Enzyme-linked immunosorbent assay
EPG	Eggs per gram
ERP	Egg reappearance period
ES	Excretory/Secretory
FBZ	Fenbendazole
FEC	Faecal egg count
FECR	Faecal egg count reduction
FECRT	Faecal egg count reduction test
g	Gram
G	Unit of gravity
GABA	Gamma amino butyric acid
GLM	Generalised linear model
GLMM	Generalised linear mixed model
HES	High egg shedder
IFN-γ	Interferon-γ
IgG	Immunoglobulin-G
IL-10	Interleukin-10
IL-4	Interleukin-4
ITS2	Internal Transcribed Spacer 2
IVM	Ivermectin
k	Aggregation parameter
kDa	Kilo Dalton
L1	First stage larvae
L2	Second stage larvae
L3	Infective third stage larvae
L4	Fourth stage larvae
L5	Fifth stage larvae
LCL	Lower 95% confidence limit

LDT	Larval development test
LES	Low egg shedder
LL3	Late third stage larvae
Lm	Linear model
LMI50	Larval migration inhibition 50
LMIT	Larval migration inhibition test
LRT	Likelihood ratio test
MAb	Monoclonal Antibody
MAFF	Ministry of Agriculture Food and Fisheries
McM	McMaster
McM15	McMaster with a 15 egg per gram (EPG) detection limit
McM50	McMaster with a 50 egg per gram (EPG) detection limit
MCMC	Markov Chain Monte Carlo
MES	Medium egg shedder
mg/kg	Milligrams per kilogram
Min	Minute
Ml	Millilitre
ML	Macrocyclic lactone
MLE	Maximum likelihood estimation
Mm	Millimetre
MOX	Moxidectin
MRI	Moredun Research Institute
N	Number
NaCl	Sodium Chloride
NBD	Negative binomial distribution
NBGLM	Negative binomial generalised linear model
Od	Optical density
OD	Over-dispersion
OXB	Oxbendazole
PCR	Polymerase chain reaction
PGP	P-glycoprotein
PRZ	Praziquantel
PYR	Pyrantel embonate
R	The R project for statistical computing
R(d)SVS	Royal Dick School of Veterinary Studies
rRNA	Recombinant ribose nucleic acid
SNP	Single nucleotide polymorphism
SQP	Suitably Qualified Person
THP	Tetrohydropyrimidine
TNF-α	Tumour necrosis factor-α
TCBZ	Triclabendazole
UCL	Upper 95% confidence limit
WAAVP	World Association for the Advancement of Veterinary Parasitology
$\sum$	Summation
μ	Mean
χ^2	Chi-square
Γ	Gamma function

List of tables

List of figures

List of equations

CHAPTER 1: Introduction

Introduction

Gastrointestinal helminths present a serious challenge to the health and welfare of equids worldwide. With virtually all grazing equids at risk of infection (Nielsen et al., 2006), there is a need for control strategies that help reduce the threat of clinical disease. As early as the 1600's, products were being administered to horses in order to 'control' worms (Poynter and Hughes, 1958) and since the early 1900's and the advent of scientific testing, a number of anthelmintic compounds, with increasing spectrum, efficacy and safety have been developed and licensed for use in horses (Lyons et al., 1999). To break nematode life cycles and reduce pasture contamination, interval dosing treatment programmes were suggested (Drudge and Lyons, 1966); however, while the frequent administration of anthelmintics has significantly reduced parasite-associated morbidity (Matthews, 2014), it is widely believed to have contributed to the development of anthelmintic resistance, particularly in cyathostomins (Kaplan, 2002, 2004), and as such, these approaches to parasite control are no longer sustainable. As helminth infections and worm egg excretion are highly over-dispersed amongst horses (Relf et al., 2013), targeted treatment programmes are being advocated to reduce anthelmintic use and hence selection pressure for drug resistance. As part of targeted treatment programmes, faecal egg count (FEC) analysis is increasingly being used to direct treatment decisions (Matthews, 2014). The major caveat to the application of targeted treatment programmes is that many horse owners/managers have insufficient knowledge on which to base such protocols (Stratford et al., 2014a; Matthews, 2014). For example, often they have little idea of the relative contribution that individual horses make to pasture contamination or of the true drug sensitivity of the parasite population. Furthermore, advice is seldom sought from veterinary surgeons and often, when it is, the information provided is based on out-dated

concepts (e.g. interval treatment protocols developed in the 1970's) (Matthews, 2014). The aim of this project is to build a framework for sustainable helminth control for horse owners, Suitably Qualified Persons (SQP), veterinary pharmacists and veterinary surgeons to use to help them to develop rational parasite control programmes on an evidence basis. Such a system will be underpinned by tools such as the FEC to promote the targeted treatment of horses in the field and will help sustain the effectiveness of the currently available anthelmintic classes.

1.1 Equine helminths

There are several gastrointestinal helminth species that can affect horses; they can be broadly categorised into the following classes; Nematoda and Cestoda. In addition, there are nematode species that can affect the lungs, and the trematode, *Fasciola hepatica*, that can affect the liver but these infections are seldom seen in horses. For this reason, the focus of this thesis will be on gastrointestinal nematodes and cestodes, in particular the small strongyles because of their prevalence and potential pathogenicity.

1.1.1 Nematodes

Cyathostomin spp. are by far the most prevalent group of nematodes found in equids (Herd, 1990a; Herd, 1990b; Lyons et al., 2000; Nielsen et al., 2006) and have been recognised as the main contributor of worm eggs shed in faeces (Herd et al., 1981; Stratford et al., 2014a). Presently, these are considered the most important parasite species to affect equids (Love et al., 1999; Matthews, 2008); this is due to their high prevalence, potential pathogenicity and ability to develop anthelmintic resistance (Lester and Matthews, 2014). There are more than 50 recognised cyathostomin species (Lichtenfels et al., 2008), with most horses infected with between five and 10 common species (Ogbourne 1976; Reinemeyer, 1986). Most infections are well tolerated;

however the most serious consequence of cyathostomin infection is the mass emergence of encysted cyathostomin larvae from the large intestinal wall, which can result in larval cyathostominosis, a colitis characterized by diarrhoea, rapid weight loss and ventral oedema, which can be fatal in up to 50% of cases (Love and McKeand, 1997; Hillyer and Mair, 1997; Love et al., 1999). Cyathostomins are commonly referred to as small strongyles; adult worms are less than 1.5cm in length. They undergo a direct lifecycle and do not require an intermediate host (Figure 1.1). Infective larvae (L3) are ingested by the horse from pasture and develop and mature in the large intestine to adulthood. Adult females may start to lay eggs within 5 - 6 weeks of infection and oviposit in the caecum and large colon (Reinemeyer, 1986). Eggs are passed via faeces onto pasture, where their hatching and development is influenced by climatic conditions. Under optimum conditions (25 - 33˚C; Ogbourne, 1972; Mfitilodze et al., 1987) eggs may hatch and develop into infective third stage larvae (L3) within 3 - 4 days (Ogbourne, 1972). Once the eggs hatch, the first stage larvae (L1) undergo two parasitic moults to become infective third stage larvae (L3). L1 and L2 stages, survive by ingesting bacteria and organic material from the environment (Rupashinge and Ogbourne, 1978). L2 then moult to become L3, which have a protective sheath that is formed from the cuticle of the L2. This is thought to help to protect them from adverse environmental conditions such as freezing and desiccation. However, as the L3 cannot ingest nutrients they must survive on lipids stored within intestinal cells (Reinemeyer, 1986). The L3 can persist for long periods of time in faeces, and can survive for up to 14 – 21 weeks during summer months as the faeces protect the larvae from desiccation (Mfitilodze et al., 1988; Reinemeyer, 1986). Rainfall is necessary for L3 to migrate from faeces to herbage (English, 1979; Ogbourne, 1973; Ludwig, 1982; Craig et al., 1983; Mfitilodze et al., 1988; Reinemeyer, 1986). During periods of prolonged hot weather, L3 perish on pasture within weeks, as a higher temperature leads to an increase in

metabolism and the larvae use food reserves more quickly (Duncan, 1974a; Ogbourne, 1973). After eight days at 38°C, it has been observed that L3 motility stops and the amount of lipids in the intestinal cells significantly decrease (Medica and Sukhedo, 1997). On the other extreme, L1 and L2 have been shown to be more susceptible to freezing conditions than L3 (Lucker, 1941a), and over-winter, long term freezing damages eggs and significantly reduces the number of larvae present on pasture downstream (Ober-Blobaum, 1932; Enigk, 1934; Lucker, 1941b; Nielsen et al., 2007).

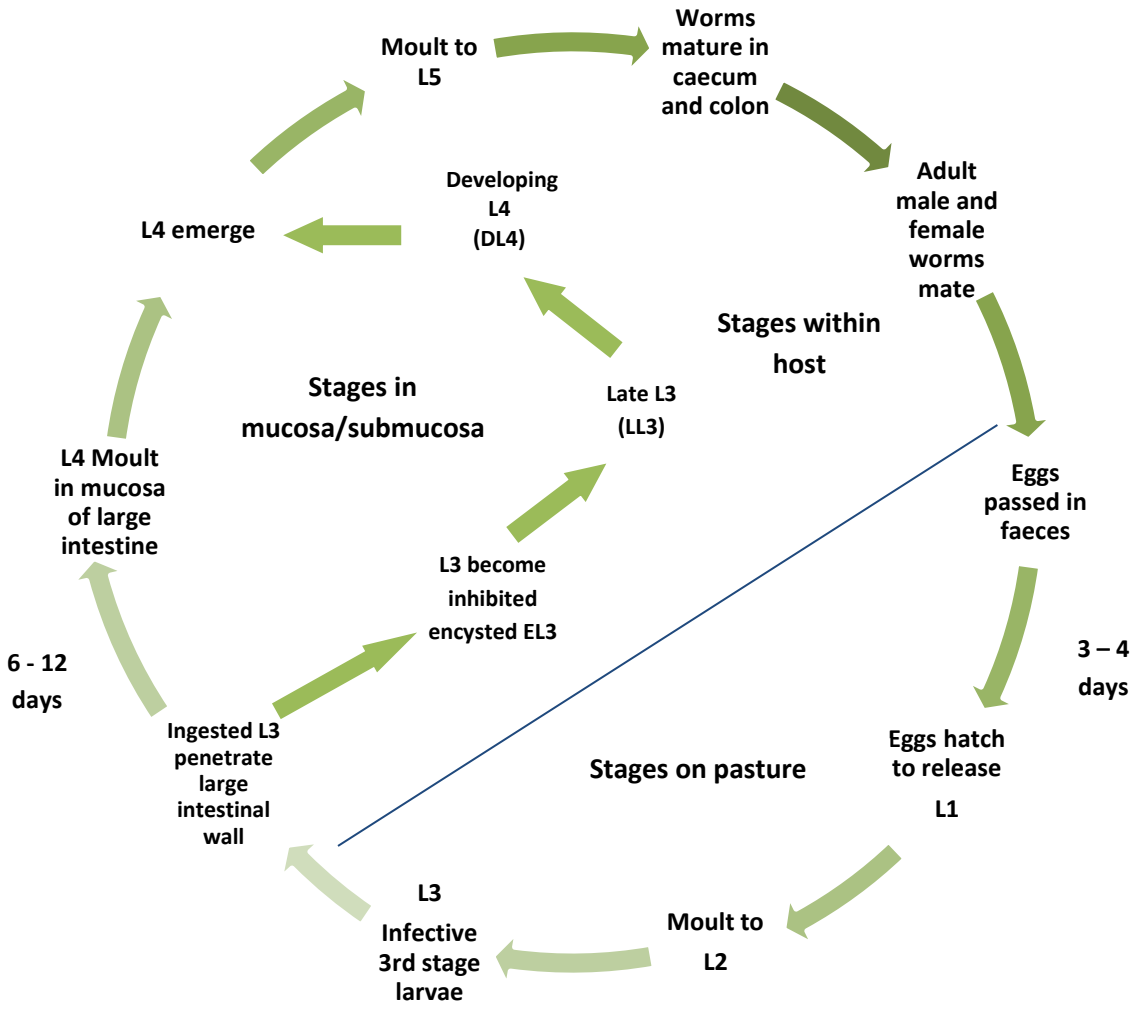


Figure 1.1. The cyathostomin lifecycle

Ingested L3 are thought to exsheath in the small intestine and penetrate mucosal cells at the base of the glands of Lieberkuhn in the caecum and colon, and, depending on the species, may be found in the mucosa or submucosa (Tiunov, 1953; Mathieson, 1964; Reinemeyer, 1986). A cyst is formed around the developing larvae, by fibroblast accumulation (Mathieson, 1964) and L3 can moult to L4 within 6 - 12 days of infection (Tiunov, 1953). The L4 subsequently emerge from the cysts and enter the lumen of the caecum or colon; the time that this takes is thought to vary considerably (Reinemeyer, 1986). The presence of L4 in the gut lumen has been reported to follow a seasonal distribution in the UK, with a peak in the late winter/early spring and late summer/early autumn (Ogbourne, 1976; Dowdall et al., 2002). In many cases, after ingestion, encysted larvae enter a state of prolonged development in the gut wall and this has been measured from four months to periods of up to two years (Reinemeyer et al., 1986). In the UK, larval encystment has been observed to occur predominantly during the winter months when it is presumed that conditions for larval development on pasture are less favourable (Herd and Willardson, 1985). In this country, it has been reported that up to 90% of the total cyathostomin burden may exist as encysted larvae, with several million present in individual horses (Murphy and Love, 1997; Dowdall et al., 2002). The emergence of larvae from the intestinal wall is thought to be influenced by environmental conditions, host immunity and/or worm population density, and may also coincide with the removal of adults from the lumen following anthelmintic dosing (Gibson, 1960; Smith, 1976). The emergence of encysted stages and their subsequent development to adulthood has been proposed as responsible for the spring rise of FEC observed in April and May in temperate climates, coincident with a rise in environmental temperature, which favours larval development on pasture (Reinemeyer, 1986). The exact mechanisms triggering the mass emergence of encysted larvae are still largely unknown, but when larvae emerge in large numbers from the

caecal and colon wall, this can cause an intense inflammatory reaction, leading to a potentially fatal syndrome called 'larval cyathostominosis'. The emergence of larvae can severely damage the gut wall, causing diarrhoea, oedema, anorexia, acute weight loss, pyrexia and protein losing enteropathy (Giles et al., 1985; Love et al., 1999; Lyons et al., 2000). In temperate climates, cases of larval cyathostominosis are usually seen between January and May, and usually in horses under the age of five (Reinemeyer, 1986; Reid et al., 1995). Figure 1.2a shows a three year-old mare that presented with larval cyathostominosis in April 2011 to the Equine Hospital at the Royal (Dick) School of Veterinary Studies (R(d)SVS) (Case Number 1.1). Clinical signs the horse presented with included; chronic diarrhoea, with larvae visible in the faeces, anorexia and pyrexia. Unfortunately, the mare did not respond to treatment with anthelmintics, fluids and corticosteroids and was euthanased. Upon post mortem examination, there was pronounced thickening and oedema of the ventral colon (Figure 1.2b), and many larvae visible on the mucosa, highlighting the real threat of larval cyathostominosis in young horses that have not been treated appropriately with anthelmintics.

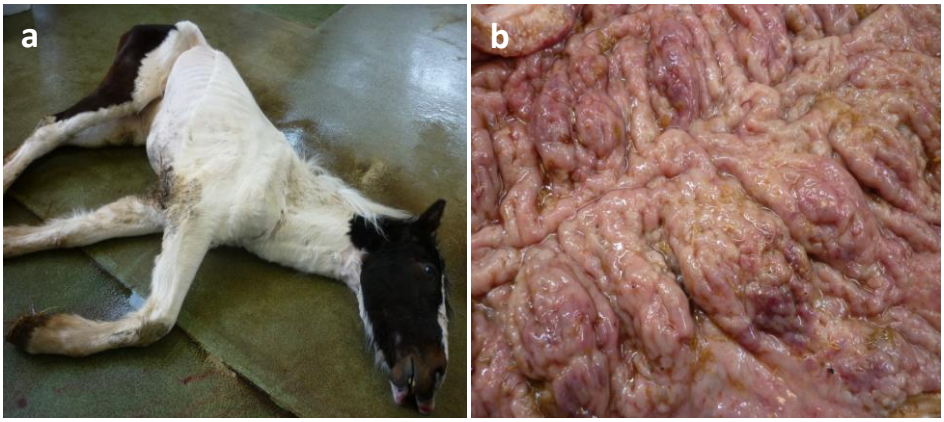


Figure 1.2 The image on the left (a) shows a three year-old mare (Case Number 1.1) that presented with larval cyathostominosis. The image on the right (b) shows pronounced thickening and oedema of the ventral colon at post mortem as a result of the mass emergence of cyathostomin larvae.

The equine intestinal immune response to cyathostomin infection is poorly understood. Collobert-Laugier et al. (2007) suggested that intestinal mast cell responses were involved in the acquisition of immunity (Collobert-Laugier et al., 2007). Further studies identified a linear correlation observed between magnitude of caecal cyathostomin burden and specific mast cell populations, indicating little association with immunity (Pickles et al., 2010). Davidson et al. (2005), measured cytokine responses in cyathostomin infected horses. In these studies, interleukin-4 (IL-4) and interleukin-10 (IL-10) were cited as indicators of a T helper cell (Th) 2 response and tumour necrosis factor- α (TNF-α) and interferon- γ (IFN-γ) as indicators of Th1 type responses. These authors found a significant correlation between IL-4 and IL-10 levels with numbers of cyathostomin EL3 and developing larvae and no correlation between larval numbers and IFN-γ levels. TNF-α was identified at a few sites in the caecum of horses with inflammatory enteropathy associated with emerging/emerged larvae, but this cytokine was not detected at sufficient sites for the observation to be significant. From the results, it was concluded that Th2 responses predominate in mucosal cyathostomin infections prior to larval reactivation (Davidson et al., 2005). However, this was a small study in a limited number of horses, and further work is required to understand the host immune response to cyathostomin infection and to understand the role it plays in larval encystment and subsequent re-emergence. All published studies available indicate that immunity is relatively slow to develop and requires prolonged exposure to cyathostomin challenge and heavy worm burdens may be seen in horses of all ages (Klei and Chapman, 1999). However, strongyle egg shedding tends to be higher in younger horses compared to adult horses (von Samson -Himmelstjerna et al., 2007; Relf et al., 2013). Understanding the equine intestinal immune response to cyathostomins is essential for developing effective control programmes (Klei and Chapman, 1999; Pickles et al., 2010).

The large strongyles form a clinically significant group of helminths that can affect horses, and historically, were the focus of parasite control programmes (Drudge and Lyons, 1966). However, their prevalence has diminished significantly over the past 20 years due to intensive anthelmintic worming regimens and the introduction of the broad-spectrum macrocyclic lactone (ML) class of anthelmintics (Love et al., 1999; Nielsen et al., 2012a). The large strongyles, so named because adults of certain species can reach 5 cm in length, are large dark-red bursate nematodes that undergo a direct lifecycle. The main species are *Strongylus vulgaris*, *Strongylus edentatus*, *Strongylus equinus* (Figure 1.3) and *Triodontophorus* spp. *S. vulgaris* (Looss, 1900) is the most pathogenic of these species. Infective L3 are ingested from pasture and enter the intestinal mucosa where they moult to L4 in the submucosa (Duncan, 1974b). From there, they enter small arteries and migrate to the predilection site of the cranial mesenteric artery and its main branches (Duncan and Pirie, 1972), where they dwell for approximately four months (Wetzel, 1940). The larvae return to the intestinal wall via the arterial lumen. The prepatent period is 6 - 7 months (Duncan and Pirie, 1972). Migrating larvae are responsible for major pathology, where thrombus formation can occur around the larvae (Duncan, 1974b). Thrombolic emboli may detach causing damage to smaller arteries and arterioles downstream (Enigk, 1950), leading to localised ischemia and subsequent infarction and necrosis to areas of the large intestine (Enigk, 1950; Duncan and Pirie, 1972), resulting in a painful thromboembolic colic. Clinical signs include; tachycardia, profuse sweating and endotoxic shock (Enigk, 1950; Drudge and Lyons, 1966; Duncan and Pirie, 1972). In acute cases the prognosis is poor (Drudge and Lyons, 1966). *S. vulgaris* was once highly prevalent, with prevalence reported to be 80% - 100% (Bollinger, 1870; Robertson, 1939; Slocombe and McCraw, 1975; Tolliver et al., 1987; Nielsen et al., 2012). In the 1960's, interval dosing protocols were recommended to reduce transmission (Drudge and Lyons, 1966) and as a result,

the prevalence of this parasite has decreased significantly to individual prevalence rates below 5% (Höglund et al., 1997; Craven et al., 1998; Lind et al., 1999; Boxell et al., 2004) and is rarely observed in populations of horses that receive regular anthelmintic treatment. The focus of parasite control then shifted to the small strongyles (Herd, 1990a; Love; Nielsen et al., 2012a). *Strongylus edentatus* larvae have been reported to cause diarrhoea, peritonitis, colic and death (Wetzel, 1952; Wetzel and Kersten, 1956; Phillips and Koltviet, 1958; Slocombe and McGraw, 1975). Infective larvae are ingested from pasture and penetrate veins in the intestinal mucosa where they travel to the liver (McCraw and Slocombe, 1974). Here, the larvae moult to L4. Migration through the liver can cause mononuclear cells and eosinophils to accumulate leading to foci and tortuous tracks which can give a rough and mottled appearance to the liver, with pronounced thickening (Wetzel and Kersten, 1956). Larvae exit the liver to their predilection site of the hepatic ligaments and the flank. The hepatic ligaments may become thickened and fibrosed and the flanks may look oedematous and thickened (McCraw and Slocombe, 1974). The final moult occurs after approximately four months, where the L5 migrate subperitoneally, to the large intestinal wall. Larvae returning to the caecum and ventral colon can form purulent nodules and haemorrhagic foci, which subsequently rupture releasing young adults into the lumen (McCraw and Slocombe, 1974). Less is known about *S. equinus*, but the pathology and clinical signs are similar to those reported for *S. edentatus* (Wetzel, 1940; McCraw and Slocombe, 1974; Slocombe, 1985). Ingested L3 penetrate the wall of the caecum and ventral colon, causing the formation of nodules, where they moult to L4. Larvae then use the peritoneal cavity to migrate to the liver, where they then migrate through the liver parenchyma for six weeks or more (Wetzel, 1940; McCraw and Slocombe, 1974). Following migration through the liver, L4 and L5 may be found in the pancreas. Here, they can become encased in a fibrous capsule, causing the pancreas to become firm and

nodular, resulting in a disruption to lobular architecture, a reduction in parenchyma and atrophy of secretory cells (McCraw and Slocombe, 1974).

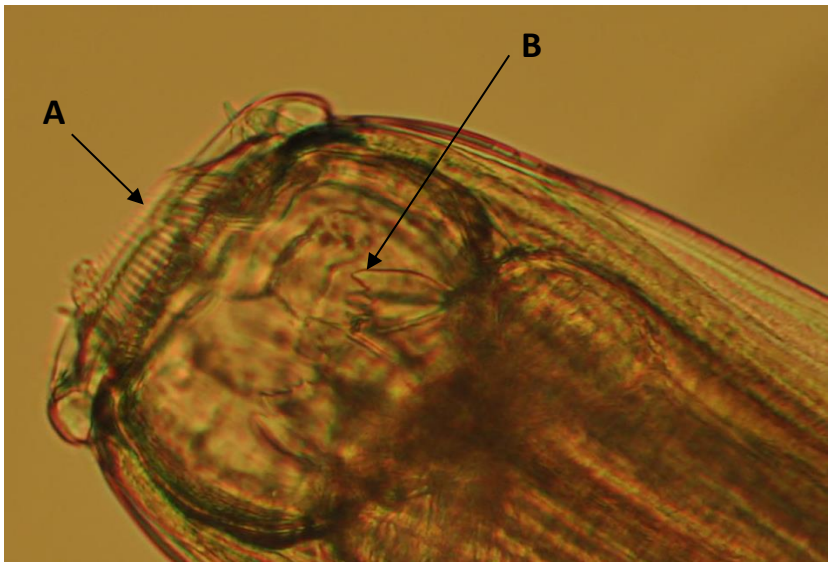


Figure 1.3. A photograph showing the buccal capsule of *Strongylus vulgaris* at x 100 magnification. Arrow A points to the leaf crown and B points to the teeth (H. E. Lester)

Triodontophorus spp. are a group of non-migratory large strongyles. Pathology is caused by damage to the caecum and colon wall from the ingestion of plugs of mucosa by feeding adult parasites; in particular *Triodontophorus tenuicollis*, which feeds in groups and may cause large deep ulcers (Slocombe, 1985).

The ascarid, *Parascaris equorum* is regarded as the most important parasite of young equids (Drudge and Lyons, 1966; Clayton, 1986; Reinemeyer, 2009). It is a ubiquitous nematode, found in the small intestine of young horses worldwide (Reinemeyer, 2009; 2012). Larvae emerge from the ingested egg into the alimentary tract, after which they undergo hepato-tracheal migration before arriving at the predilection site of the small intestine, approximately one month later as L4 (Clayton and Duncan, 1977). The larvae then mature in the small intestine and can achieve patency within 75 -80 days post-infection (Clayton and Duncan, 1977). Adult females are highly fecund and are capable

of producing 200,000 eggs per day, which are passed in faeces (Clayton, 1986). Second stage larvae (L2) then develop in the eggs which have been measured as infective in 10 days at 25 - 35°C (Clayton, 1986). Larvated eggs can survive in the environment for five to 10 years providing a persistent source of environmental contamination (Clayton, 1986). The L2 emerging from ingested eggs migrate through the liver causing focal haemorrhages and eosinophillic tracts to the lungs where they cause mucous formation and eosinophilic alveolitis, bronchiolitis and bronchitis (Nichols et al., 1978; Slocombe, 1985). Heavy burdens may lead to intestinal impaction or perforation of the intestine (Slocombe, 1985). Clinical signs include a transitory cough with purulent nasal discharge, poor growth, emaciation, a pot-bellied appearance, poor coat, dullness, inappetence, lassitude and death (Clayton, 1986; Slocombe, 1985; Reinemeyer, 2009; Reinemeyer, 2012). Protective immunity to *P. equorum* is thought to develop on exposure and after six months of age (Clayton and Duncan, 1979a): infections are seldom seen in horses above the age of two (Reinemeyer, 2012). However, older horses that have not been exposed when younger can harbour patent infections at low levels (Gawor, 1996; Kornas et al., 2006; Hinney et al., 2011a). Studies have reported the prevalence of *P. equorum* within populations of young horses to range from 22.4% to 80% (Gawor, 1996; Hinney et al., 2011; Laugier et al., 2012; Relf et al., 2013).

Trichostrogylus axei (Cobald, 1879) is a nematode found in the stomach of horses and ruminants (Slocombe, 1985). It is not considered to be highly pathogenic unless present in vast numbers, where it can cause profuse diarrhoea, especially in foals (Leland et al., 1961; Slocombe, 1985). *T. Axei* eggs are undistinguishable from strongyle eggs under microscopic examination; therefore determination of prevalence by FEC analysis is not possible.

Strongyloides westeri is a fine hair-like worm that dwells in the small intestine of foals and weanlings (Lyons et al., 1973). This nematode can be parasitic or free-living (Lyons et al., 1973). The parasitic phase is exclusive to female worms. The L3 can become parasitic, infecting the host by skin penetration or ingestion (Greer et al., 1974). They then migrate via the venous system and develop into adult females in the small intestine. In the small intestine, the females produce larvated eggs through parthenogenesis, and the eggs are then passed in faeces (Lyons et al., 1973; Greer et al., 1974). The prepatent period for *S. westeri* is 8 - 14 days (Lyons et al, 1973). Foals are at risk immediately after birth as inhibited larvae can become mobilized from the ventral abdominal wall of the mother and infect the foal by vertical transmission through milk (Greer et al., 1974). Clinical signs include diarrhoea, anorexia, dullness and poor growth rates and are unusual but may be seen in very young animals (Lyons et al., 1973). Mature parasites found in the duodenum and proximal jejunum may cause inflammation with oedema and erosion of epithelium if present in large numbers and cause catarrhal enteritis with impairment of digestion and absorption (Lyons et al, 1973). A recent study investigating helminth egg excretion in populations of Thoroughbred horses in the UK detected *S. westeri* eggs on 45% (9/20 yards) of the yards tested and reported that 8% of the overall horse population tested positive and 1% were measured as excreting ≥200 EPG (Relf et al., 2013).

The equine pinworm, *Oxyuris equi,* is fairly common but of limited pathogenic significance in the intestine (Reinemeyer, 2012). Erosion of the mucosa may occur in heavy infections and an inflammatory response may be seen (Enigk, 1949). Eggs are ingested and larvae are released into the small intestine. They then travel to the large intestine and migrate to mucosal crypts in the caecum and colon, where they develop into L4 (Enigk, 1949). The L4 emerge and feed on the mucosa before reaching maturity.

The adults are found in the lumen of the dorsal and descending colons. After fertilisation, gravid females migrate to the hosts' anus, and, extruding their anterior end lay their eggs around the perianal area. Allergens in the proteinaceous liquid in which the eggs are laid, cause intense anal pruritis and tail-rubbing (Reinemeyer, 2012). The reported prepatent period is five months (Hasslinger, 1990). There have been no recent studies performed in the UK looking into the prevalence of this parasite.

Dictyocaulus arnfieldi is the only lungworm found in equids (Round, 1976). This parasite is relatively common in donkeys (Matthews and Burden, 2013), where large numbers of parasites can accumulate in the lungs without causing clinical disease (Boyle and Houston, 2006). Patent infection in horses is rare; however, infection can lead to a persistent cough, increased respiratory rate and nasal discharge (Veneziano et al., 2011). *D. arnfieldi* undergoes a direct life cycle, with a reported prepatent period of approximately 12 weeks (Round, 1976; Clayton and Duncan, 1981). Eggs containing L1 are laid by adult females residing in the lung bronchi, where they ascend the mucociliary apparatus, up the trachea. They are then swallowed, and eggs and L1 are passed in faeces (MacKay and Urquhart, 1979; Burks, 1998; Boyle and Houston, 2006). Migrating larvae and adult worms in the parenchyma and bronchi may result in an immune-mediated bronchopneuemonia (Beech, 1979). Adult parasites cause chronic catarrhal bronchitis with hyperplasia, thickened epithelium as well as focal oedema and haemorrhage (Beech, 1979; Boyle and Houston, 2006). This can lead to an increased risk of secondary bacterial infections (Burks, 1998; Boyle and Houston, 2006). Infection is more common in horses that are co-grazed with donkeys in cold, wet climates (Beech, 1979). Clinical signs in horses can develop within 12 days of infection (MacKay and Urquhart, 1979) and are usually seen in adult horses rather than foals (Clayton and Duncan, 1981). Typically, coughing, exercise intolerance and

bilateral mucopurulent nasal discharge are seen, the latter is particularly noted if there is a concurrent secondary bacterial infection (Boyle and Houston, 2006). The prevalence of lungworm in UK equid populations is largely unknown due to a lack of recent published literature.

1.1.2 Cestodes

Historically in the UK, equine tapeworm infections were considered to be of little clinical relevance, and were rarely associated with disease (Soulsby, 1968; Proudman and Trees, 1999). A number of reports emerged in the 1980's, citing circumstantial evidence of an association between *Anoplocephala* spp. and interssusception of the ileocaecal region, caecal rupture and spasmodic colic (Barclay et al., 1982; Beroza et al., 1983; Cosgrove et al., 1986; Owen et al., 1989; Proudman and Edwards, 1993; Proudman et al., 1998; Proudman and Trees, 1999). There are three species of equine tapeworm belonging to the family Anoplocephalidae, these are: *Anoplocephala perfoliata* (Goeze, 1782), *Anoplocephala magna* (Abilgarrd, 1978) and *Anoplocephaloides mamillana* (formerly *Paranoplocephala mamillana*). *A. perfoliata* is the most common tapeworm found in equids (Proudman et al., 1998; Reinemeyer, 2012). *A. magna* is the largest and is found in the posterior small intestine. *A. mamillana* the smallest and is found in the anterior small intestine and occasionally in the stomach (Proudman and Trees, 1999). The life cycle is indirect, requiring an intermediate forage mite host to complete its lifecycle. Each species possesses a scolex for attachment to the intestinal mucosa and a flattened strobila comprised of proglottids, through which nutrients are absorbed. The proglottids contain both male and female reproductive organs and the most posterior proglottid becomes gravid, containing embryonated eggs. The gravid proglottid then detatches and is passed in faeces releasing embryonated eggs, which can survive for up to 9 months on pasture

(Dunn, 1978). Embryonated eggs are ingested by the intermediate oribatid forage mite and develop into infective immature cysticercoids, which contain the scolex. This stage of development takes approximately 2 - 4 months (Bashkirova, 1941). Oribatid mites are ingested and are digested, releasing the cysticercoids. The scolex attaches to the intestinal mucosa, where proglottids grow from the base of the scolex and mature in 6 - 10 weeks (Arundel, 1985). At the site of attachment, *A. perfoliata* cause ulceration of the mucosa and submucosa, leading to localised inflammation which may involve the entire thickness of the caecal wall (Bain and Kelly, 1977; Pavone et al., 2011). It is believed that the local inflammation interferes with gut motility, leading to an increased chance of interssusception and spasmodic colic (Lee and Tatchell, 1964; Bain and Kelly, 1977; Burns et al., 1990; Summers et al., 1995; Pavone et al., 2011). A recent post mortem study performed on 31 horses in Italy found a significant relationship between parasite burden and histopathological lesions in the mucosa and submucosa, with hypertrophy of circular muscle (Pavone et al., 2011). In addition, horses with a moderate-to-high infection expressed degenerative regressive changes in neuronal cells in the intestines with a decreased number of myenteric ganglia and neuronal cells; supporting the theory that infection with *A. perfoliata* interferes with gut motility (Pavone et al, 2011). The presence of local fibrous connective tissue can mechanically constrict the ileocaecal orifice, which can also lead to intestinal impaction (Reinemeyer and Nielsen, 2009). Complete protective immunity does not develop against tapeworm species and horses in the UK are at risk of infection throughout their lives (Ihler et al., 1995; Nilsson et al., 1995; Rehbein et al., 2013).

1.1.3 Trematodes

Fasciola hepatica is a common zoonotic trematode found worldwide (Rojo-Vazquez et al., 2012; Gordon et al., 2013), and is responsible for economic losses in food-producing

animals due to reduced growth, fertility and milk yield, death and the condemnation of livers at slaughter (Mezo et al., 2011; Sargison and Scott, 2011; Borji et al., 2012; Gordon et al., 2013). Recently, an increase in the prevalence and incidence of *F. hepatica* has been observed in the UK in ruminants, with parasite distribution increasing from the west of the country, eastwards (Mitchell, 2002; Gordon et al., 2013). This has been attributed to the warmer winters and wetter summers that have occurred, which are favourable climatic conditions for the propagation of the intermediate snail host, *Galba truncatula* (Kenyon et al., 2009; Taylor, 2012; Gordon et al., 2013). *F. hepatica* has a wide host range, which includes equids. A study looking at experimental infection of horses with liver fluke concluded that the horse exhibits resistance to the establishment of *F. hepatica* infection, and found that the majority of parasites were eliminated or immobilized at an early stage of infection (Nansen et al., 1974). In addition, plasma aminotransferase levels were within a normal range throughout infection indicating limited damage to the liver parenchyma by migrating immature fluke and, neither eosinophilia nor precipitating antibodies were detected suggesting that the antigenic stimulus was insignificant and/or transient (Nansen et al., 1975). In contrast, in 38 cases, *F. hepatica* infection has been associated with clinical signs in horses that had been co-grazing with cattle and sheep known to harbour liver fluke (Owen, 1977). The horses showed signs of lowered performance and/or a loss of condition, capricious appetite and acute diarrhoea (Owen, 1977). A recent coprological survey in donkeys in the UK also estimated the prevalence in these hosts at approximately 4% (Matthews and Burden, 2013). Earlier studies reported that 33 - 91% of donkeys examined excreted *F. hepatica* eggs (Pankhurst, 1963; Kearney, 1974). In the same studies, prevalence in horses was recorded at 0.1 - 77% (Pankhurst, 1963; Kearney, 1974). Interestingly, the latter study was performed in the west of Ireland, which has been recognised as an endemic focus for liver fluke, with prevalence

reported to be high in cattle (Ross, 1966; Murphy et al., 2006). However, there is paucity of information regarding the prevalence of this parasite in horses, but there have been several reports of disease and this is usually associated with co-grazing with infected livestock or deer (Rehbein et al., 2002).

Of all the helminth species to affect equids, the cyathostomins are now the focus of control programmes (with the exception of foals). This is due to their high prevalence, potential pathogenicity and ability to develop anthelmintic resistance. However, when considering equine parasite control, it is necessary to consider other helminth species. To do this, it is necessary to have up-to-date epidemiological information and validated tools for their detection.

1.2 Detection of parasites in equids

The detection and enumeration of parasite eggs and larvae in faeces has formed the cornerstone of diagnostic parasitology since the methodology for identifying human hookworm infection was first described (Stoll, 1946). Since then, a number of faecal egg count (FEC) methodologies for detecting and enumerating parasite eggs in faeces have been published. Such methodologies range from a simple direct smear (Beaver, 1950) to more complicated methods involving centrifugation and flotation. The most widely used standard quantitative technique is the McMaster (McM) method (Gordon and Whitlock, 1939; Pereckiene et al., 2007; Rinaldi et al., 2014). This method or related modifications (Henriksen and Aagaard, 1976; MAFF, 1986; Dunn and Keymer, 1986; Thienpont et al., 1986; Gronvold, 1991; Kassai, 1999) are relatively easy to perform and are used widely worldwide. FEC analyses give an approximate estimation of levels of egg excretion and do not provide an accurate reflection of total nematode burden within individuals (Shaw and Dobson, 1995; Nielsen et al., 2010; Lester and

Matthews, 2014). In essence, they can only indicate a patent infection, and are of no use in detecting prepatent infection such as migrating or encysted larval stages, which are often responsible for the major pathology associated with infection (Andersen et al., 2013). Further, traditional FEC methods do not reliably detect tapeworm, pinworm, fluke or lungworm eggs (Lester and Matthews, 2014). There are 64 strongyle species that can infect equids, 50 of which are small strongyle species and these eggs cannot be differentiated morphologically (Andersen et al., 2013) and, as such, coproculture of eggs to the L3 stages is necessary to differentiate between large and small strongyles; however, this does not permit differentiation of small strongyles to species level (Andersen et al., 2013).

The majority of FEC methods are based on flotation, and relies on the use of solutions of specific gravity that act to separate the nematode eggs from debris. The eggs are then quantified under a microscope, usually at x 40 magnification. Each test differs in diagnostic sensitivity, the time required to perform the test, the type of laboratory equipment required and the level of expertise needed to execute the technique with accuracy (Table 1.1). 'Detection limit' is the term used to describe the diagnostic sensitivity of the FEC method. If the egg detection limit is high, the method will not be particularly sensitive to changes in egg abundance (Morrison, 2004) below or around the detection limit and is more likely to give false negative results. A lower detection limit means that the method is more sensitive, as one uses a lower (or no) multiplication factor for the conversion of the number of eggs seen to provide an estimation of EPG. Larger multiplication factors artificially inflate the variance observed in the FEC, leading to more varied results (Torgerson et al., 2012) and so it is considered best practice to use a method with a lower detection limit, particularly when assessing efficacy of anthelmintics (Kaplan and Nielsen, 2010; Vidyashankar et al., 2012).

Table 1.1. The detection limit, equipment, approximate cost of equipment, time taken/sample, ease of method and number of steps taken for each faecal egg count method method.

FWEC method	Detection limit of test*	Special equipment needed	Cost of equipment	Time taken: from weighing out subsample to FWEC result	Relative ease of method (based on number of steps, expertise required)	Number of steps in protocol from weighing subsample to result
Centrifugal-flotation[a]	1-9 EPG	Miller eye piece graticule, centrifuge, cuvettes, polymer tubes	Approximately £500 (not including centrifuge or microscope)	~10-15 min	Complex	7
FECPAK[b]	20 EPG	FECPAK system	£760	~5-10 min	Moderate	4
miniFLOTAC[c]	5 EPG	FLOTAC device	NA*	12-15 min	Easy	5
McMaster[d]	15-100 EPG	McMaster slides	£15 to 150/slide	~5-10 min	Easy	4
Ovatec[e]	Only recorded as + or -	Ovatec devices	£95 for 50 tests	~10 min	Easy	6

[a] Bartley and Elsheikha, 2011
[b] Innovis Ltd
[c] Contact Professor Giuseppe Cringoli for information
[d] MAFF, 1986
[e] Zoetis UK Ltd

* *a-d* FWEC methods are quantitative and give and EPG estimate, method *e* is qualitative and gives a positive or negative result

The McMaster method and its modifications (Gordon and Whitlock, 1939; MAFF, 1986) are the most widely used standard quantitative tests for estimating the number of strongyle eggs in equine faecal samples. These are relatively quick and straightforward to perform and are the method currently recommended by the World Association for the Advancement of Veterinary Parasitology (WAAVP) for determining anthelmintic efficacy in domestic species (Coles et al., 1992). The modified McMaster method (MAFF, 1986) is based on the flotation-dilution principle and assumes that eggs are randomly distributed in solution, if the sample has been well-mixed prior to dispensing the filtrate into the 'McMaster' counting slide (Torgerson et al., 2012). If the faecal suspension is allowed to sit in saturated salt solution for a time prior to loading of the slide, eggs will float to the surface and will no longer be randomly distributed. By not mixing the faecal suspension and removing only the surface layer, relatively more eggs will be placed into the slide, overestimating EPG and leading to an inaccurate result. In the authors' experience, this is a relatively common error in practice. A number of modifications of the McMaster method exist; two recent studies have attempted to determine which of these is the most accurate and reliable (Table 1.2). Both studies found that McMaster adaptations that use a larger amount of faeces and a lower dilution ratio (i.e. g of faeces/ml of water) and subsequently, a lower multiplication factor when converting the number of eggs seen into an estimation of EPG, gave more reliable results with increased diagnostic sensitivity (Table 1.2).

Table 1.2. Results from two studies that investigated different modifications of the McMaster method. Study 1 (Vadlejch et al., 2011) compared seven published modifications and Study 2 (Pereckiene et al., 2007) evaluated which of three McMaster modifications was the most reliable.

Study	Details	Methods investigated	Vol. of faeces (g)	Vol. of water (ml)	Centrifugation (min:rpm)	Flotation solution (specific gravity)	Flotation time (min)	Multiplication factor	Detection Limit (EPG)	Major findings
1	Comparison of modifications of the McM methods for counting *Ascaris suum* eggs in pig faeces	1. Henriksen & Aagaard (1976)	4	56	7:1200	NaCl + sugar (1.27)	2-3	20	20	When counting all chambers, methods 1, 2, 5 and 6 showed 100% sensitivity. Method 7 showed the least sensitivity (83.3%). Method 7 was the easiest and quickest to perform but the least sensitive, Method 1 was the most complex and most sensitive. Counting eggs in the chambers increased sensitivity of all methods
		2.Kassai (1999)	3	42	3 :1500	NaCl (1.2)	3	50	50	
		3.Urquhart et al. (1996)	3	42	2 :2000	NaCl (1.2)	2-3	50	50	
		4.Urquhart et al. (1996)	3	42	None	NaCl (1.2)	2-3	50	50	
		5.Gronvold (1991)	4	56	None	NaCl (1.2)	2-3	50	50	
		6.Gronvold (1991)	4	56	None	NaCl + sugar (1.27)	2-3	50	50	
		7.Thienpont et al. (1986)	2	60	None	NaCl (1.2)	2-3	100	100	
2	Investigation of sensitivity and reliability of three different McM techniques for counting *Teladorsagia circumcincta* eggs	8.Wetzel (1951)	2	60	None	NaCl (1.2)	2-3	67	67	Method 10 demonstrated the greatest sensitivity and reliability compared to the other methods.
		9.Zajicek (1978)	1	15	2:2000	$MgSO_4$ + $Na_2S_2O_3$ (1.28) (Breza, 1959)	5	33	33	
		10.Roepstorff and Nansen (1998)	4	56	5:1200	NaCl + glucose (1.3)	3-5	20	20	

The FECPAK™ (Innovis UK Ltd) method was developed in New Zealand initially as an on-farm tool for assessing sheep FEC (Presland et al., 2005). Here, 10g faeces are examined and there is no centrifugation step (Table 1.1). The number of eggs seen is multiplied by a factor of 20 to provide an EPG estimate. When the FECPAK method was compared with the modified McMaster method for counting nematode eggs in equine faecal samples, the former was found to display higher sensitivity and shown to be less likely to provide an underestimate of mean EPG for data with similar characteristics of mean EPG and sample variation in their study (Presland et al., 2005).

Centrifugal-flotation methods (Christie and Jackson, 1982; Egwang and Slocombe, 1982; Bartley and Elsheika, 2011) have detection limits down to 1 EPG. These methods are technically more complex than the others outlined in Table 1.1 and require more specialist equipment.

FLOTAC™ (Cringoli, 2010) apparatus, including mini-FLOTAC and fill-FLOTAC, offer sensitive FEC methods. The standard FLOTAC method requires investment in specialist equipment, and requires two-weeks' training at the FLOTAC™ laboratory based at University of Naples (for further information contact Professor G. Cringoli: cringoli@unina.it). Several studies have been performed to evaluate FLOTAC for estimating nematode eggs and larvae in the faeces of different host species (Cringoli, 2010; Cringoli et al., 2010; Rinaldi et al., 2011; Levecke et al., 2012b). In each, a greater level of diagnostic sensitivity was achieved in comparison to the other methods investigated. For example, the diagnostic accuracy of a simple flotation, McMaster and

FLOTAC FEC methods for counting helminth eggs in sheep faeces was investigated. The study reported a lower coefficient of variation in FLOTAC compared to the other methods, emphasizing the potential higher accuracy of this method (Rinaldi et al., 2011). The major limitations of the FLOTAC method are the number of steps required

to process one sample and the requirement of a centrifuge, which means each sample takes a relatively long time to analyse, and this is not always practical if high volumes of samples have to be processed. The mini-FLOTAC is an alternative; less specialist equipment is required and there is no centrifugation step, making it less complex to perform. For equine samples, it has been proposed that 45 ml of saturated salt solution be added to 5 g of well-mixed faeces, before loading into a mini-FLOTAC chamber. After 10 min, the sample can be read. The detection limit can be adapted by counting different areas of the slide and a detection limit of 5 EPG has been reported [G. Coles, *pers. comm*.]. The mini FLOTAC system will be available commercially in the near future for use in practice, but further validation is required to investigate reproducibility for its use with equine faecal samples.

The Ovatec™ (Zoetis UK Ltd) system provides a relatively quick method to examine whether or not nematode eggs are present. The output of this method is a 'positive' or 'negative' result rather than an EPG estimate. If no eggs are observed, this system reports a 95% certainty that the FEC of the sample is less than 150 EPG and 100% certainty that it is less than 250 EPG (Ronsyn et al., 2012). A relatively small amount of faeces is examined (less than 3 g), which is likely to render this method inaccurate. In this context, it has been shown that the larger the volume of equine faeces examined in the FEC test, the lower the variability in the true mean count of a sample (Denwood et al., 2012). Thus, whilst Ovatec may be useful for identifying medium to high FEC, due to its current inherent lack of sensitivity, is not recommended for assessing anthelmintic efficacy.

Several studies have compared relative values of the different FEC methodologies. Most recently, a study investigating bias, accuracy and precision of the modified McMaster (10 EPG detection limit), Cornell-Wisconsin (CW, 1 EPG detection limit) and

FLOTAC™ (1 EPG detection limit) methods with regard to the outcome of faecal egg count reduction test data in cattle found that FLOTAC™ gave the most precise results, whilst the McMaster and Cornell-Wisconsin (CW) methods gave similar, but less precise results (Levecke et al., 2012b). FEC methods with the same detection limit (for example, FLOTAC and CW, detection limit = 1EPG) do not necessarily have an equal level of precision, with differences due to the egg recovery method. For example, in the CW method, eggs are recovered by placing a cover slip onto the meniscus of the flotation solution, which may result in a loss of eggs through spillage. In addition, the area for eggs to float onto for counting is smaller with CW and if egg density is high, eggs may be stacked in multiple layers, reducing the number counted, therefore producing an inaccurate estimate of EPG (Levecke et al., 2012b).

There are a number of factors that affect the variability of FEC data, which in turn impact the precision of the result. These can broadly be split into biological factors and technical factors. Biological factors include, faecal consistency, fluctuation in egg shedding over time, worm fecundity and host immunity. Technical factors include the underlying Poisson distribution of egg count data, aggregation of eggs in faeces, storage and handling of the faecal samples and the faecal egg count method used. Studies have shown that faecal consistency affects the variability of FEC, whereby diarrhoea increases faecal moisture and may dilute the number of eggs observed (Le Jambre et al., 2007), leading to a lower observed FEC. In a number of host–parasite systems, it has been reported that parasite eggs are not laid at a constant rate and diurnal fluctuations in egg excretion over time have been observed (Engels et al., 1996; Yu et al., 1998; Giver et al., 2000; Oju and Mpoame, 2006). Typically, parasite eggs are shed at a higher rate during the day compared to the night (Villanouna et al., 2006; Dolnik et al., 2011; Coelho et al., 2013), and this impacts variability of FEC as the concentration of eggs differs over time leading to differences in observed FEC depending on the time of day

the sample is collected, therefore, samples taken for FEC should be taken at the same time of day to reduce potential variation.

Density dependent fecundity is commonly observed in helminth species (Anderson and May, 1985). Typically, egg output decreases as parasite burden increases and a greater degree of variation in fecundity is observed at low worm burdens (Kotze and Kopp, 2008). However, density dependent effects are not common to all helminth species, for example *Haemonchus contortus* in sheep (Coyne et al., 1991) and *Trichostrongylus tenuis* in red grouse (Shaw and Moss, 1989) do not exhibit density dependent fecundity. It has been suggested that density dependent fecundity results from competition between parasites, host immunity and direct parasite-parasite interactions (Anderson and May, 1985; Keymer, 1982). A recent study investigated the fecundity of 10 common cyathostomin species by extracting eggs from the uterus of mature female worms and found that *Cylicocyclus insigne* and *C. nassatus* were the most fecund species (average of 445 and 212 eggs, respectively), while *C. leptostomum* and *C. longibursatus* were the least fecund (average 63 and 49 eggs, respectively) and a positive correlation between worm size and fecundity was observed (Kuzmina et al., 2012).

Host immunity can influence FEC via the preventing the establishment of incoming larvae, inhibiting or delaying maturation of larvae to the adult stage, and the subsequent reduction in the size of the adult females (Smith et al. 1983a, b, 1984; Stear et al. 1995; Garnier et al., 2015).

The Poisson distribution describes the underlying distribution of count data, where the mean and variance are equal (Torgerson et al., 2012). In terms of FEC, fixed weights of faeces are mixed with a fixed volume of flotation solution and a known volume of the diluted faecal suspension is placed into a counting slide. The number of eggs observed

in the counting slide are then multiplied by the appropriate egg detection limit to estimate the number of egg per gram (EPG). Eggs within the slide should follow a Poisson distribution if the sample has been well mixed. The egg detection limit is dependent on the dilution factor of faeces and the volume of faecal suspension counted. However, when raw egg counts are transformed to EPG estimates, inflated variance between repeated counts of the same sample is observed because the multiplication factor/egg detection limit artificially inflates the variance (Torgerson et al., 2012). For example, five raw egg counts from drawn from a Poisson distribution with a mean of 4 eggs would be 0, 4, 6, 2, and 3. If each if the counts are multiplied by an egg detection limit of 50 EPG the observed EPG values would be 0, 200, 300, 100 and 150 EPG, demonstrating the variability in FEC. This variability is due to the Poisson process, which is inflated by the multiplication factor (Torgerson et al., 2012). As such, FEC methods that use a lower multiplication factor/egg detection limit produce less variable FEC.

Nielsen et al. (2010) investigated the effect of storage factors on strongyle FEC in horses and found that optimum counts could be achieved if faeces were <12 hours old and if the sample was refrigerated after collection and processed within five days (Nielsen et al., 2010).FEC method.

A major caveat to the application of FEC is the evidence that there is no significant positive relationship between cyathostomin worm burden (including the larval stages) and faecal egg count (FEC) (Dowdall et al., 2004; Nielsen et al., 2010; Kuzmina et al., 2012), and that horses measured as shedding 100 EPG may harbour more than 100,000 worms (Nielsen et al., 2010). That said, these studies have been focussed on horses less than two years of age, which are more likely to have higher burdens of encysted larvae (Lyons et al., 2011), which will have impacted the results as the

immature stages do not lay eggs. To date, there have been no recent studies looking at the relationship between worm burden and FEC in adult horses. In adult horses however, the relationship between adult worm burden and FEC could be influenced by immune responses. For example, in sheep, immunity to the nematode *Teladorsagia circumcincta* results in a reduction in the size of adult female worms (Smith et al., 1983; Stear et al., 1995), which results in a reduction in fecundity (Stear and Bishop, 1999), therefore, adult female worms may be present but may not be detected if they are laying fewer eggs. This phenomenon however, has not been demonstrated in cyathostomins.

In support of FEC, strongyle egg shedding has been shown to be consistent in adult horses over time (Dopfer et al.,2004; Nielsen et al., 2006), thus supporting the rationale for targeting treatments in adult horses. Furthermore, Nielsen et al. (2010) reported that horses with a FEC of up to 500 EPG had significantly higher strongyle worm counts, supporting the usage of treating horses with FEC in this range and demonstrates that deworming horses with high FEC can potentially reduce the level of environmental contamination.

Currently, there are no non-invasive methods for the detection of cyathostomin encysted larvae (EL) (Matthews, 2014). A routine diagnostic test would help to identify the horses with high EL burdens, which may have low or negative FEC, and would aid the identification of horses requiring larvicidal anthelmintic treatment. Recently, serum IgG(T) responses to two larval native antigen complexes (of 20 and 25 kDa in size) were observed to be significantly higher in clinical cases than in cyathostomin-naïve or negative animals, and in experimentally infected animals, anti-25 kDa complex IgG(T) levels correlated positively with field exposure to cyathostomin species, burdens of EL3 and total mucosal parasites (Dowdall et al., 2002; Dowdall et al., 2003;

Dowdall et al., 2004). In addition, antigen-specific IgG(T) responses were significantly higher in infected compared to uninfected horses (p=0.0001 and 0.002 for anti-25 and anti-20 kDa responses, respectively) in naturally infected horses, whose parasite burdens were quantified at post mortem. In infected horses, anti-25 kDa IgG(T) levels correlated positively with mucosal and luminal burdens (p<0.05) (Dowdall et al., 2004). The antigens were shown to be specific to cyathostomins and cross reactivity between other helminth species was limited (Dowdall et al., 2003). Recently, genes encoding two antigenic proteins, that are produced by EL cyathostomins; gut associated larval antigen (Cy-GALA) (McWilliam et al., 2010) and cyathostomin immunodiagnostic antigen (Cy-CID, Matthews, *pers. comm.*) have been isolated and the proteins expressed in *Escherichia coli.* These proteins exhibit no cross reactivity to serum from horses specifically infected with other non-cyathostomin helminth species, and antiserum raised to one Cy-GALA, does not bind to extracts from other helminth species (McWilliam et al., 2010). It is anticipated that a diagnostic ELISA based on detection of antibodies to a cocktail of these recombinant proteins will be developed for commercial use in the next 3 years (Matthews, 2014), with further development focussing on increasing cyathostomin species coverage for the proteins Cy-GALA and Cy-CID, to ensure that the assay is sensitive to the presence of less common cyathostomin species.

There is no validated FEC method for the detection of *Oxyuris equi* as adult female worms lay their eggs around perineum, so eggs are not consistently detected in faeces. The recommended method for the identification of *O. equi* eggs is the tape test, whereby sticky tape (i.e. Sellotape®) is pressed around the perineum and then removed carefully and examined under a microscope for the presence of the distinctive eggs (Reinemeyer, 2012).

Tapeworm eggs are not reliably detected by the traditional FEC methods outlined above, (Nilsson et al., 1995). FEC counts performed by the McMaster method give low sensitivity (8 – 61%) for tapeworm (Proudman and Edwards, 1992; Abbott and Barrett, 2010). Modifications by using a larger amount of faeces (30 - 40 g) have been suggested (Proudman and Edwards, 1992; Ihler et al., 1995; Nilsson et al., 1995; Meana et al., 1998; Kjaer et al., 2007), and by adopting a more sensitive method (i.e. centrifugal-flotation) (Proudman and Edwards, 1992). Sensitivity using these modifications has been reported to range between 37 to 61% and a specificity of 98% (Proudman and Edwards, 1992; Ihler et al., 1995; Nilsson et al., 1995; Meana et al., 1998; Proudman and Trees, 1999). However, other reports suggest that there is no clear relationship between adult tapeworm burden and FEC (Meana et al., 1998). An ELISA for the detection of *A. perfoliata* specific antibody responses is commercially available (Diagnosteq, Liverpool, UK). This serological assay detects IgG(T) antibodies against 12 and 13 kDa excretory/secretory (ES) antigens (Proudman and Trees, 1996). Proudman and Trees (1996) demonstrated a correlation between ELISA optical density (Od) and *A. perfoliata* infection intensity, and reported that horses with high infection intensity (in this case, Od > 0.6) were at greater risk of tapeworm-associated colic. The sensitivity and specificity of this ELISA was reported as ~70% and ~95%, respectively (Proudman and Trees, 1996; Traversa et al., 2008). Od in the range of 0.2 and 0.6 are difficult to interpret as one study found that 66% of tapeworm-negative horses had an ELISA Od of 0.2 or more (Kjaer et al., 2007), demonstrating that the circulating antibodies from a previous infection results in a lack of sensitivity. The tapeworm ELISA has been available for over ten years (http://www.liv.ac.uk/diagnosteq/tapeworm_test.htm) and has proved useful as a monitoring tool, but is not widely used due to the associated cost. A lack of diagnostic sensitivity has been observed due to the long half-life of antigen-specific

immunoglobulin in equine serum in response to infection and this must be taken into consideration when interpreting the test (Abbott and Barrett, 2010). If the deworming history of a horse is unknown and tapeworm infection is suspected, rising Od levels in serial blood samples over a 2-month period is likely to indicate infection (Abbott and Barrett, 2010). Recently, a diagnostic ELISA test that measures tapeworm antibodies in saliva has been developed and is available to buy commercially in the UK (Equisal: http://equisal.co.uk/The-Test). This test costs £17.95 (price at time of writing this thesis), does not require a veterinary surgeon, and therefore could be a more favourable option to the horse owner. The information associated with the test currently claims that it is able to identify a negative/low burden or a moderate/high burden with 83% sensitivity and 85% specificity. This was determined by comparing the results of the test with the number of adult tapeworm recovered at post mortem in 104 horses. (http://equisal.co.uk/WebRoot/Store5/Shops/d7497350-0c56-4e11-a207-b31ca0b55b54/MediaGallery/Equisal_Vets_lft_A5_single_page.pdf). At the time of writing, there were no peer-reviewed scientific publications available to support these data. A coproantigen ELISA, able to detect ES antigens in horse faeces as been developed (Kania and Reinemeyer, 2005), and has demonstrated a positive correlation between antigen concentration and the number of adult tapeworm (Skotarek et al., 2010) giving a reported sensitivity and specificity of 74% and 92%, respectively. This test is not available commercially as yet. A PCR-based assay has been proposed for the detection of tapeworm infections based on the amplification of Internal Transcribed Spacer 2 (ITS2) of parasite rDNA, which has been identified as a genetic marker for the detection of *A. perfoliata* in horse faeces (Drogemuller et al., 2004; Traversa et al., 2008). Initial reports are promising, but more work is required to validate this method (Traversa et al., 2008; Andersen et al., 2013).

The detection of *F. hepatica* eggs is commonly based on a standard sedimentation FEC technique (McCaughey and Hatch, 1964; Gordon et al., 2013). In addition, standard FEC techniques such as the McM and centrifugation-flotation techniques can be used with saturated zinc sulphate as the flotation medium as this offers a lower specific gravity (1.18), which enables the flotation of larger eggs (MAFF, 1986). The test is confounded by the long prepatent period (9 to 15 weeks) of this helminth species and sporadic egg shedding observed due to the fact that adult fluke lay their eggs in the gall bladder (Chowaniec and Darski, 1970; Mezo et al., 2004; Valero et al., 2009; Gordon et al., 2013). Recently, a coproantigen ELISA based on a monoclonal antibody (MM3 MAb) that detects cathepsin L protease, secreted from the luminal surface of the fluke's gut (Mezo et al., 2004; Muino et al., 2011) has been developed and is available commercially for use in ruminants (BIO-X Diagnostics, Belgium). In sheep, claims have been made that this test is able to detect a single, live fluke (Mezo et al., 2004). Moreover, following experimental infection, liver fluke were detected five weeks post-infection (Mezo et al., 2004; Flanagan et al., 2011; Gordon et al., 2012), indicating value in detecting immature infections before they reach patency, which will help to reduce damage caused by migrating immature fluke. There is a lack of information regarding the detection of fluke infections in equids, as such investigations into FEC methods and the usefulness of the coproantigen ELISA are warranted, particularly because of the rise of prevalence of *F. hepatica* in ruminant species (Mitchell, 2002; Gordon et al., 2013) and the increased number of reports of suspected failure of the anthelmintic triclabendazole (TCBZ), which has been confirmed in sheep (Gordon et al., 2012; Winkelhagen et al., 2012) and in cattle (Olaechea et al., 2011). TCBZ is the only flukicide with activity against all stages of liver fluke, most importantly the immature stages (Fairweather et al., 2012). Little is known about the clinical significance of *F.*

hepatica in horses and donkeys and a rise in prevalence and infection intensity of this helminth could potential have a bearing on their health and welfare.

The Baermann technique is routinely used for the detection of *D. arnfieldi* (Boyle and Houston, 2006). The eggs when laid, are embryonated, and can hatch rapidly liberating L1, so FEC methods are less useful. Detection via faecal examination in the horse can be inaccurate as patent infections are seldom seen, but the inflammatory process associated with immature larvae can cause disease without a patent infection (Boyle and Houston, 2006).

Many of the recommended FEC techniques for the identification of equine helminth infection have not been fully validated or optimised. If such techniques are to underpin evidence-based parasite control programmes there is a need to ensure that recommended methods are fit for purpose.

1.3 Control of parasites in equids: anthelmintics

Certain species of gastrointestinal helminths are ubiquitous in grazing equids (Andersen et al., 2013) and infection can potentially lead to clinical disease (Nielsen et al., 2014). Consequently, there is a need for their control. As early as the 1600's a variety of different organic and non-organic substances such as hen's eggs, the intestines of young hen's and pigeons, human faeces, aniseed, aloes, liquorice, linseed and vitriolated mercury were administered to horses for the control of intestinal parasites (Poynter, 1958). In 1915, the oil of chenopodium was recommended, and excellent results *versus* strongyles were reported when used in combination with linseed oil after the horse had been fasted for 36 hours prior to treatment (Lyons et al., 1999). Later, Colonel Floyd Sager, wrote in his book, in which he recorded anecdotes from his 60 years as an equine practitioner, that following treatment with oil of

chenopodium, horses would lose weight and not be able to eat or drink for 3 - 4 days post-treatment (Lyons et al., 1999), indicating the questionable safety of such compounds. Since the early 1900's and the advent of the scientific testing of drugs to assess efficacy and safety, 25 anthelmintic compounds have been developed and licensed for the control of gastrointestinal helminths (Table 1.3).

Table 1.3. The history of anthelmintic compounds licensed for use in horses and their claimed activity against different gastrointestinal parasite species

Year	Class	Compound/s	Activity	Reference/s
1917	Carbon disulphide	Carbon disulphide*	Ascarids Bots	Hall, 1917
1940	Phenothiazine	Phenothiazine*	Strongyles	Habermann et al., 1941
1950	Piperazines	Piperazines*		
		Used with carbon disulphide	Ascarids, bots, pinworm, strongyles Increased activity *versus* large strongyles and phenothiazine-resistant small strongyles	
		Used with phenothiazine		Drudge et al., 1963
1960's	Organophosphates	Trichlorfon*	Ascarids, bots, mature pinworm	
		Dichlorvos*	Ascarids, bots, large and small strongyles, pinworm	
1960's	Benzimidazoles	Thiabendazole* Cambendazole* Fenbendazole Mebendazole* Oxfendazole* Oxibendazole*	Ascarids, pinworm, strongyles	Drudge et al., 1963 Colglazier et al., 1977
1970's	Tetrohydropyrimidines	Pyrantel tartrate	Ascarids, *Strongylus vulgaris*, small strongyles, pinworm	Cornwell and Jones, 1968
		Pyrantel pamoate	Ascarids, small strongyles, pinworm, *Strongylus vulgaris* and *Strongylus equinus* *Anoplocephala perfoliata*	Lyons et al., 1975
		At double the nematocidal dose		Slocombe, 1979
1980's	Macrocyclic lactone (avermectin)	Ivermectin	Ascarids, bots, small and large strongyles, pinworm	Klei et al., 1993; Xiao et al., 1994; DiPietro et al., 1989
1997	Macrocyclic lactone (milbemycin)	Moxidectin	Ascarids, bots, adult and larval stages of large strongyles, adult, EL3 and developing stages of small strongyles, pinworm	Monahan et al., 1995, 1996; DiPietro et al., 1997; Duncan et al., 1998; Bairden et al., 2001; Bairden et al., 2006

* compound no longer licensed for use in horse

1.3.1 Anthelmintics

Carbon disulphide was the first compound to be tested and developed for use against ascarids and bots (Hall, 1917) and over the years there have been a number of anthelmintic compounds discovered, tested and licensed for use against equine parasites. Currently there are three classes of broad-spectrum anthelmintic licensed for use in horses in the UK; these are the benzimadazoles (BZ), tetrahydropyrimidines (THP) and the macrocyclic lactones (ML). The ML group is split into two ML derivatives known as the avermectins and the milbemycins, to which ivermectin (IVM) and moxidectin (MOX) belong, respectively. Praziquantel (PRZ) is a narrow-spectrum anthelmintic specifically licensed for use against cestodes.

Anthelmintics may be effective against one or more parasitic stages. For example they may possess adulticidal activity, i.e. they are effective against the adult worm population, larvicidal activity, i.e. they are effective against the larval stages and ovicidal, i.e. they are effective against the eggs. The effect of anthelmintics on different parasite stages has implications for control.

1.3.3.1 Benzimidazoles

BZ inhibit the synthesis of microtubules in nematodes (Lacey, 1990). By binding to the nematode β-tubulin subunit, BZ prevent dimerization and subsequent polymerisation during microtubule assembly (Lacey, 1990). The progressive loss of microtubule function disrupts cell division, depletes energy stores and causes starvation (Prichard, 1990). Fenbendazole (FBZ) is the only BZ licensed for use in horses in the UK. Initial controlled efficacy studies found that FBZ administered orally as a single dose (5mg/kg body weight (bwt)) was 100% effective against adult cyathostomins (Colglazier et al., 1977) (Table 1.4), while a dose of 7.5 mg/kg bwt administered for five consecutive

days reported >95% efficacy against total musosal larvae and >91% efficacy against EL3 (Bairden et al., 1998). In addition, FBZ was reported to be completely effective against adult *O. equi* and, 50% against L4 (Colglazier et al., 1977) and highly effective against *P. equorum* (Malan et al., 1981).

1.3.3.2 Tetrahydropyrimidines

The THP class of anthelmintics includes the pyrantel (PYR) salts pyrantel embonate and pyrantel tartrate, with the former being licensed for use in the UK. PYR is a depolarizing neuromuscular blocker that exerts a cholinergic action causing paralysis by massive contraction of the parasite musculature (Brady and Nichols, 2009). Through mimicking acetylcholine action, it changes the permeability of the post-synaptic membranes leading to sustained muscle contraction (spastic paralysis) (Elsheikha et al, 2011). PYR was shown to eliminate 89 - 96% of adult cyathostomins, when administered orally at a dose-rate of 19 mg/kg bwt (Lyons et al., 1974) but was not effective against mucosal stages. At the same dose rate, PYR was reported to be >90% effective against adult *O. equi* and *P. equorum* (Lyons et al., 1974). In addition, PYR given at 38 mg/kg is highly effective against *A. perfoliata* (Slocombe, 1979) (Table 1.4).

1.3.3.3 Macrocyclic lactones

The introduction of the ML class marked a key step in the control of parasites as these compounds possess broad-spectrum anti-nematode and -arthropod activity but they are not effective against tapeworm or fluke species (Schumacher and Taintor, 2008), because these helminths lack the high affinity binding site for ML (Neal, 2002). ML are highly lipohilic and are excreted in bile and eliminated in faeces (Zulalian et al., 1994). The ML were originally thought to potentiate the action of the inhibitory

neurotransmitter gamma-aminobutyric acid (GABA) by blocking the hyperpolarisation of nematode somatic muscle by opening GABA-gated chloride channels (Holden-Dye and Walker, 1990), although this hypothesis has yet to be supported (Martin et al., 2002). It is thought that the ML probably act by binding to and permanently opening glutamate-gated chloride channels found only in the neurones and monocytes of invertebrates (Cully et al., 1994). This causes the influx of chloride ions leading to neuromuscular paralysis (flaccid paralysis) and death (Cully et al., 1994). IVM, an avermectin, was the first ML to be licensed for use in horses in 1981, and, at a dose rate of 0.2 mg/kg, was demonstrated to have high efficacy (>99%) against adult cyathostomins and luminal larvae (98%), but showed little activity against EL3, even at an elevated dose rate of 1 mg/kg (Klei et al., 1993; Xiao et al., 1994). IVM was also effective (>90% efficacy) at killing adult and immature stages of *O. equi*, adult large strongyle species (including arterial and tissue larval stages), adult, L3 and L4 stages of *P. equorum* (DiPietro et al., 1989), adult *S. westeri*, adult and immature *D. arnfieldi*, adult *T. axei* and oral and gastric stages of *Gasterophilus* spp (Coles et al., 2003). MOX, a milbemycin, was first introduced to the market in 1997 (Schumacher and Taintor, 2008), and is indicated for use for the control of adult and larval stages of large and small strongyles, including EL3, *P. equorum* (Monahan et al., 1995), *T. axei*, and *G. intestinalis* (Coles et al., 1993). MOX has demonstrated good efficacy (>95%) against adult and larval stages of *S. vulgaris*, *S. equinus* and *S. edentatus* (Monahan et al., 1995). Early studies investigating efficacy against cyathostomin mucosal larvae found MOX to be ineffective (Xiao et al., 1994; Monahan et al., 1995, 1996). These data were in contrast to later studies that reported 90.8% efficacy against cyathostomin EL3 (Bairden et al., 2001, 2006; Reinemeyer et al., 2003) and 99.9% against other developing stages of these parasites (Bariden et al., 2001). The discrepancy in reported efficacy against cyathostomin mucosal larval stages is possibly explained by differences

in the interval between dosing and necropsy, with the earlier studies performing necropsy two weeks post-treatment, which is believed to be too short a time to allow elimination of mucosal larvae (Monahan and Klei, 2002; Matthews, 2008), therefore the results obtained in the study by Bairden et al. (2001) would appear to be more accurate. MOX has persistent activity, with prolonged plasma bioavailability (Perez et al., 1999), and may provide protection from cyathostomin reinfection for 2 - 3 weeks post-administration (Vercruysse et al., 1998). There have been concerns over the safety of MOX, for use in foals and debilitated horses, and since it became licensed, there have been several adverse drug reactions reported (Hampshire et al., 2004). MOX is 100 times more lipophilic than IVM (Hayes, 1994), and may become highly concentrated in the serum of equids with little body fat. In this situation, MOX may cross the blood-brain barrier, leading to toxicity (Johnson et al., 1999; Muller et al., 2005). Reported clinical signs of MOX toxicity include dyspnoea, depression, ataxia, weakness, seizures and coma (Johnson et al., 1999; Hampshire et al., 2004; Muller et al., 2005). Given that MOX is the only anthelmintic effective with larvicidal activity and given that the pathogenic risk of the larval stages, MOX should be considered for use for the control of cyathostomins despite the apparent safety risks. To mitigate against this, if MOX is to be used in foals and equids with little body fat, it is essential that the correct dose is administered based on an accurate body weight.

Table 1.4. Summary of the mode of action, dose rate and delivery route and efficacy claims for each of the broad-spectrum anthelmintics licensed for use in horses in the UK

Anthelmintic	Mode of action	Recommended dose rate and delivery route	Efficacy claim	Reference/s
Fenbendazole	Binds to the nematode β-tubulin subunit, disrupting microtubule assembly leading to starvation	5 mg/kg *per os*	100% efficacy vs. cyathostomins 100% vs. adult pinworm 95-100% vs. L4 pinworm	Colglazier et al., 1977
			100% vs. *Parascaris equorum*	Malan et al., 1981
		7.5 mg/kg *per os* for 5 consecutive days	90.7% vs. luminal cyathostomins 95.3% vs. mucosal larvae 99.4% vs. LL3 and D4 91.5% vs. EL3	Duncan et al., 1998
			80% and 100% vs. migrating *S. vulgaris* and *S. edentatus* 95% vs. mucosal small strongyles 100% vs. adult small and large strongyles	Duncan et al., 1980
Pyrantel	Mimics acetylcholine action, changing the permeability of the post-synaptic membranes leading to sustained muscle contraction (spastic paralysis)	19 mg/kg *per os*	89-96% vs. adult cyathostomins >90% vs. *O. equi* and *P. Equorum*	Lyons et al., 1974
		38 mg/kg *per os*	Highly effective vs. *A. Perfoliata*	Slocombe, 1979
Ivermectin	MLs bind to glutamate-gated chloride channels causing the influx of chloride ions leading flaccid	0.2 mg/kg *per os*	>99% vs. adult cyathostomins; 98% vs. luminal cyathostomin larvae >90% vs. adult and immature stages of *O. equi* >90% vs. adult, arterial and tissue stages of large strongyles >90% vs. adult L3 and L4 *P. equorum*, adult *S. westeri*, *T.*	Klei et al., 1993; Xiao et al., 1994 DiPietro et al., 1989

	paralysis and death		*axei* and adult and immature *D. Arnfieldi*	
Moxidectin	Flaccid paralysis – see above	0.4 mg/kg *per os*	90-100% vs. adult and larval stages of large strongyles, adult ascarids, cyathostomins, *T. axei* and *S. westeri* 90.8% vs. cyathostomin EL3 99.9% vs. other developing stages of cyathostomin	Monahan et al.,1995, 1996; Coles et al., 1998; Bairden et al., 2001, 2006; Reinemeyer et al., 2003

1.3.3.4 Narrow-spectrum

PRZ exerts its effect by primarily by invoking tetanic contractions of parasite musculature and intense irreversible focal vacuolization and disintegration of the tegument (Elsheikha et al, 2011). The damage is probably caused by interaction with phospholipids and proteins that create an imbalance in the ion transport of cations through the tegument. This affects metabolism and leads to strong contraction of the tegument muscles leading to paralysis (Elsheikha et al, 2011). PRZ has been licensed for concomitant use with IVM and MOX.

1.3.3.5 Herbal preparations

In the UK, there is an herbal preparation (Verm-X) that claims to offer 'natural control and daily protection'. However, apart from claiming 'veterinary approval' there have been no formal registration type efficacy studies performed to substantiate efficacy of this product in equids. An efficacy study in nine donkeys using Verm-X reported no reduction in strongyle egg count after treatment and concluded that this product should not be used for the control of strongyles (Bernard and van Doorn, 2011). Further, a comparative study that examined the efficacy of the Verm-X preparation for chickens and flubendazole against four species of chicken nematode, found that Verm-X efficacy against adult worms ranged from <0 - 11% and was not significantly different to the untreated control group (Squires et al., 2012).

1.4 Anthelmintic resistance

Anthelmintic resistance (AR) in nematodes is well documented (Kaplan and Nielsen, 2010). AR is defined as 'when a greater frequency of individuals in a parasite population, usually affected by a dose or concentration of a compound, is no longer affected, or a greater concentration of drug is required to reach a certain level of efficacy' (Pritchard et al., 1980). The first case of AR reported in cyathostomins was to phenothiazine in the late 1950's (Drudge and Elam, 1961), and since then, AR to all four modern, broad-spectrum anthelmintic compounds used in equids has been identified (Stratford et al., 2011). AR arises through genetic polymorphisms, which are selected for over time and are passed on to subsequent generations (Pritchard et al., 1980). The most serious consequence of resistance is complete treatment failure leading to persistent infection and, when high burdens are present, clinical disease.

A reduction in anthelmintic efficacy may be identified *in vivo* by the faecal egg count reduction test (FECRT), which is considered the gold standard for determination of anthelmintic efficacy in domestic species (Coles et al., 1992). FECRT datasets derived from horses are often characterised by low precision and poor reproducibility (Craven et al., 1999). Currently, there are no clearly defined guidelines regarding appropriate cut-off limits for determining efficacy for the anthelmintic classes used in horses (Vidyashankar et al., 2012). The WAAVP initially defined the threshold for resistance as a FECR of less than 95% reduction in FEC taken 10 days apart using arithmetic group means (Coles et al., 1992). However, this singular cut-off value did not take into consideration differing original efficacies of the various anthelmintic classes in anthelmintic-sensitive nematode populations at the time of licensing, leading to the potential for misclassification of sensitivity. Recently, FECR cut-offs have been recommended for the different anthelmintic compounds used in horses; for example,

90% for FBZ (and other BZ anthelmintics) and PYR and 95% for ML anthelmintics (Kaplan and Nielsen, 2010). A mean population FECRT above these cut-offs indicates acceptable efficacy, whilst a mean FECRT below these thresholds indicates AR (Kaplan and Nielsen, 2010). In cases of suspected AR, where the overall group mean FECR is close to the threshold for resistance and this is due to effect of FECR observed in one or two individuals, the test should be repeated in these individuals to rule out possible under-dosing or administration error in the initial study (Stratford et al., 2013). The use of 95% confidence limits (CL) has been suggested to provide a better indication of the range of the FECRT data (Vidyashankar et al., 2007). Some recent studies have used both the FECR and 95% lower CL (LCL) to determine the presence or absence of AR (Craven et al., 1998; Ihler et al., 1995; Osterman Lind et al., 2007).

The standard method of FECR analysis (Coles, 1992) currently used in efficacy studies worldwide involves calculation of the group arithmetic mean before and after treatment to estimate arithmetic mean percentage FECR, from which, 95% CL are then derived. However, this method makes the assumption that FEC data are normally distributed, which may affect statistical validity, and lead to inaccurate inferences regarding the presence, or absence, of resistance. To overcome the problem of over dispersion in faecal worm egg excretion amongst horses, log and arcsine transformations have been suggested (Fulford, 1994; Pook et al., 2002). Arcsine transformation of individual FECR has been reported to reduce variation in efficacy calculations as demonstrated when applied to field data, this method generated LCL closer to the mean in comparison with the WAAVP method (Pook et al., 2002). Non-parametric bootstrapping has been suggested as an alternative method as it does not require underlying knowledge of the distribution of the data, however, it assumes the data obtained are representative of the whole population (Vidyashankar et al., 2007). Bayesian methods such as the profile-likelihood method (Torgerson et al., 2005) and

Markov chain Monte Carlo (MCMC) (Denwood et al., 2010) have also been proposed to account for the variability in FECRT data. The MCMC has been reported to outperform non-parametric bootstrapping when sample size is small and the pre-treatment FEC is low (Denwood et al., 2010). These methods are associated with increased power and a reduced chance of incorrectly rejecting the null hypothesis. However, they are computationally intensive, requiring a high level of statistical knowledge and the ability to use the statistical modelling package R.

These issues highlight the potential for inappropriate data analysis that may lead to misclassification of resistance in equine worm populations and clearly a validated, standardised and easily computable method of FECR data analysis is required.

Another method for identifying a reduction in anthelmintic efficacy is to measure the strongyle egg reappearance period (ERP). The ERP is the time post-anthelmintic treatment that helminth egg shedding remains negligible, or below a certain threshold (Duncan, 1985), and varies with each anthelmintic class. The ERP in anthelmintic-sensitive populations can vary but have been previously generally specified as 6 - 8 weeks for BZ, 6 weeks for PYR (Borgsteede et al., 1993; Herd and Gabel, 1990b; Mercier et al., 2001). For the MLs, an ERP of between 8 and 12 weeks was observed for IVM (Borgsteede et al., 1993; Boersema et al., 1996) and between 12 and 25 weeks for MOX (diPietro et al., 1997; Demeulenaere et al., 1997). The initial ERP were determined when each of the drugs were first introduced and based on the assumption that helminth populations were sensitive to that specific compound. It is widely accepted that a decreased ERP is the first indicator of resistance (Sangster, 2001), but the definition and interpretation of ERP differs. Some studies define ERP as the week of the first positive FEC post-treatment (Little et al., 2003; Lyons et al., 2008). Others define ERP as a fixed threshold of the mean egg count such as 100 or 200 EPG

(Boersema et al., 1996; Mercier et al., 2001) or use FECRT to calculate weekly efficacy and use a designated threshold to define the ERP (Tarigo-Martinie et al., 2001; von Samson-Himmelstjerna et al., 2007). Ideally, a FECRT should be followed by measuring the ERP, that way if egg shedding resumes earlier than the expected ERP this could act as an early indicator of a lack of efficacy and decisions about the future use of that particular anthelmintic can be made.

BZ were first introduced in the 1960s. Since then, reduced efficacy against cyathostomins has been reported in more than 21 countries (Lyons et al., 1999; Kaplan et al., 2004). Studies have reported the prevalence of FBZ resistance to be >70% of premises tested (Fisher et al., 1992; Craven et al., 1998; Lind et al., 2007) and, in some areas, the prevalence measured approaches 100% (Kaplan, 2004). Many researchers believe that FBZ resistance is ubiquitous in cyathostomin populations (Kaplan et al., 2004; Lind et al., 2007; Traversa et al., 2009). Compared to the situation with FBZ, identification of cyathostomin resistance to PYR has been less frequently reported, although the phenomenon is common in certain countries such as the USA (Kaplan et al., 2004). However, the true prevalence of PYR resistance may be underestimated due to a lack of studies in the field (Stratford et al., 2011). The ML class dominate the equine anthelmintics market worldwide (Nielsen et al., 2006; Comer et al., 2006; Lind et al., 2007; Allison et al., 2011). Reduced efficacy of IVM has been measured in cyathostomins in the UK (Traversa et al., 2007), the US (Lyons et al., 2008) and Germany (von Samson-Himmelstjerna et al., 2007). A recent multinational study performed in UK, Germany and Italy reported IVM resistance (FECR<95%) on 3% of farms, with on one farm, resistance demonstrated to all three classes in small strongyles (Traversa et al., 2009). There have been several reports of a reduction in the strongyle ERP following IVM administration in Europe, Brazil and the USA (von

Samson-Himmelstjerna et al., 2007; Molento et al., 2008; Lyons et al., 2008). For example, Lyons et al. (2008) reported a strongyle ERP of four weeks following IVM treatment in 27 yearlings and 19 foals on 6 yards in Kentucky. The same authors performed a follow-up study looking into the probable reasons for the observed ERP by performing critical tests, which involve counting and identifying worms that survived treatment. In this study, the researchers necropsied four yearlings that had previously been enrolled as foals in the study above (Lyons et al., 2008) and had been born and raised under the same conditions and grazed on the same pasture (Lyons et al., 2009). In three horses that were necropsied six days post-treatment, efficacy (as measured by a reduction of >90%) *versus* cyathostomin fourth stage larvae (L4) was 36 – 80%, whilst efficacy against adult cyathostomins was 99 – 100%. The authors concluded that the probable reason for the observed reduction in IVM ERP in the previous study was due reduced efficacy of IVM *versus* L4 and L5 allowing these stages to survive treatment and resume maturation and egg laying much sooner than when the anthelmintic was first licensed (Lyons et al., 2009). The first report of potential cyathostomin resistance to MOX was reported in donkey populations on a welfare sanctuary in the UK (Trawford et al., 2005; 2012). Here, there had been a history of use of a MOX formulation licensed for use in cattle, which had been administered orally to the donkeys. In two trials, the first of which monitored strongyle ERP over the grazing season following MOX treatment, a mean strongyle ERP of eight weeks was measured. In a second trial, two groups were studied; one in which FEC were measured 14 days after administration and the other 25 days post-treatment. Mean reductions in strongyle FEC in these two trials were 77 and 87%, respectively. The authors concluded that the effect was likely to be attributed to using a MOX formulation not licensed in donkeys as this could have affected pharmacokinetics of the compound leading to sub-optimal concentrations of the anthelmintic at the site of action

(Trawford et al., 2005). A recent study in Kentucky reported a shortened strongyle MOX ERP of five weeks in 15 yearling horses which had naturally acquired infections (Rossano et al., 2010), while a another in which MOX and IVM were evaluated found the strongyle ERP to be as low as 4 and 6 weeks, respectively (van Doorn et al., 2014). Similarly a mean strongyle ERP following MOX administration has been reported as four weeks in Brazil (Molento et al., 2008). Generally, there is little strongyle ERP data available and as such, there is a real need to generate additional data to further assess the effectiveness of the MLs across regions.

Ivermectin resistance in *P. equorum* was first recorded in 2002 in Europe (Boersema et al., 2002) and Canada (Hearne and Peregrine, 2003; Slocombe et al., 2007). The FECRT study carried out by Boersema et al. (2002) was in response to a report of failure of ML in controlling *P. equorum* on a stud farm. Here, seven foals that tested positive for *P. equorum* eggs by FEC were treated with MOX; 19 days later FEC were performed to reveal that the MOX did not reduced pre-treatment FEC by >90% in any foal. Two foals with the highest FEC were treated with IVM and the FEC were not reduced >90 % at 19 days post-treatment indicating resistance (Boersema et al., 2002). In 2003, a further report of IVM resistance was made (Hearne and Peregrine, 2003). Here, 16 Thoroughbred foals with low to medium *P. equorum* FEC were treated with IVM. Post-treatment FEC at 13 days post-treatment revealed that FEC had increased in seven foals and remained the same in one. A further 21 foals were treated with IVM and 12 had a positive FEC 13 days post-treatment (Hearne and Peregrine, 2003). Subsequently, a critical test was performed using the isolate from the Canadian FECRT study (Kaplan et al., 2006). Here, 11 foals that had been raised helminth free were inoculated orally with larvated eggs at six weeks to three months of age. They were treated with IVM and necropsied 13 days post-treatment. The results revealed that IVM

did not significantly reduce the pre-treatment FEC and on average only reduced worm number by 26%, (Kaplan et al., 2006). Subsequently, there have been several reports of ML resistance in *P. equorum* in young horses (Craig et al., 2007; Slocombe et al., 2007; von Samson-Himmelstjerna et al., 2007; Veronesi et al., 2009), which is of concern given the potential pathogenicity of this helminth. It has been suggested that the development of IVM resistance in ascarids may be attributable to the heavy use of this compound in young foals and weanlings on horse farms (Kaplan, 2004; Reinemeyer, 2012). There have been anecdotal reports of ML treatment failures against *O. equi* (Durham and Coles, 2010; Rock et al., 2013) and recently, Wolf et al. (2014) reported continuous *O. equi* egg shedding in two groups of horses following MOX treatment. In one horse, continuous egg shedding was recorded 13 weeks post-treatment. Due to the long prepatent period of this parasite (quoted as 4.5 months), post-treatment egg shedding was not attributed to new infections. To date there have been no reports of AR in the large strongyle or tapeworm species.

Taken together, the evidence summarised above suggests that AR is an increasing problem in the helminth species that affect equids, based on the current guidelines for classifying resistance. This is of particular concern as there are no new anthelmintic classes likely to become available in the short to medium term for horses, and it is now essential to preserve efficacy of the currently effective ones, particularly MOX, which is the only anthelmintic effective against larval stages of cyathostomins. The primary aim of control regimens should focus on the preservation of anthelmintic-sensitive nematode populations and this needs to be balanced with minimising the risk of parasite-associated disease.

1.4.1 Factors affecting anthelmintic efficacy in equids

The current opinion of many parasitologists is that one of the most important factors associated with the development of AR is the proportion of parasites in refugium (van Wyk, 2001; Kaplan, 2004). Parasites in refugia include stages in the host that are potentially not exposed to anthelmintics (i.e. encysted cyathostomin larvae), free-living stages on pasture and parasites in untreated hosts (Nielsen et al., 2014a; Nielsen et al., 2014b). Maximising levels of refugia includes reducing the frequency of treatments, especially when levels are presumed to be low on pasture (i.e. during cold winters or hot summers or clean, non-grazed pasture). Using FEC to identify horses that are shedding moderate-to-high numbers of eggs in their faeces, and targeting treatments to these individuals while leaving low egg shedders untreated provides refugia (Kaplan and Nielsen, 2010). It has been proposed that nematodes that exist in refugia are not exposed to selection pressure for AR and thereby provide a source of susceptible alleles that in the next generation would act to 'dilute' resistant alleles in the population that are theoretically selected for in worms that survive treatment (Sangster, 2001; van Wyk, 2001, Nielsen et al., 2007). The concept of maintaining a population of helminths in refugia, thus providing a pool of anthelmintic susceptible genotypes was originally suggested in the 1980's (Martin et al., 1981), and is central to current recommendations for the management of anthelmintic resistance worldwide (van Wyk, 2001; Pomroy, 2006; Waghorn et al., 2008). However, evidence demonstrating the effectiveness of this strategy is scant in horses, with experiments to-date conducted in small ruminants. In sheep, Martin et al. (1981) demonstrated that development of thiabendazole resistance could be slowed by exposing smaller proportions of each generation of *Haemonchus contortus* to anthelmintic treatment. In further studies (Leathwick et al., 2006a; Leathwick et al., 2006b; Waghorn et al., 2011),

it was reported that leaving up to 20% of lambs untreated with anthelmintic could delay resistance development. The main caveat in leaving a proportion of lambs untreated was the increase in pasture contamination leading to a reduction in productivity as FEC analysis was not performed to identify high egg shedding animals. In horses, targeted approaches that have been proposed are based on the measurement of individual FEC to identify moderate-to-high egg shedders, so that anthelmintics can be targeted appropriately. This is not as practical in ruminant species, as often, there are hundreds of animals per flock/herd, which are rarely handled individually, whereas horses are usually kept in small groups and are handled daily making collecting faeces from individuals more achievable.

Anthelmintic exposure selects for the survival of individuals possessing alleles that reduce susceptibility to a particular anthelmintic class. Resistance can only occur when the relevant allele(s) are present in the population (Sangster, 2001). Parasitic nematodes have high levels of genetic diversity (Gilleard and Beech, 2007) and large population sizes, which means that within populations, resistance-conferring alleles may be present in sensitive populations even if they have not been previously exposed to a particular anthelmintic (Matthews, 2008). A simple mutation can lead to a switch from a susceptible genotype to a resistant one when an alteration in nucleotide sequence at a single site (known as a single nucleotide polymorphism (SNP)) occurs (Matthews, 2008), as is the case with resistance to the BZ class of anthelmintics. Once such a SNP is present in a population, each time a horse is treated with the same anthelmintic, the majority of worms that survive are resistant and subsequent treatments will eventually lead to a higher proportion of the population with the resistant genotype. It is the current opinion that resistance is a permanent trait and resistant genotypes do not revert to susceptibility even when a population has not

been exposed to the associated anthelmintic class for a number of years (Jackson and Coop, 2000). However, evolutionary pressure could result in a loss of resistance if resistance was not a favourable trait for survival.

There is evidence to suggest that anthelmintics are not as effective when used in younger horses. Early studies conducted on a Thoroughbred breeding farm between 1982 and 1988 found that oxbendazole (OXB), PYR and IVM were significantly less effective when administered to yearlings compared to adult mares (Herd and Gabel, 1990b). Here, the authors measured how effective each anthelmintic was at suppressing strongyle FEC output at for four-week intervals for OXB and PYR and eight-week intervals for IVM. Acceptable efficacy was classified as the mean FEC being measured as less than 100 EPG at the defined interval. OXB was found to be significantly (p<0.05) less effective at suppressing FEC in yearlings compared to mares. PYR was administered on 10 occasions, and on each occasion, the mean FEC exceeded 100 EPG in the yearlings but not the mares. IVM was administered on four occasions and a significant (p=0.001) group mean reduction in IVM efficacy was observed in mares compared to the yearlings (Herd and Gabel, 1990b). A recent study conducted on UK Thoroughbred studs, reported that PYR was effective in two groups of mares (group mean FECR 98% - 99%), compared to five groups of yearlings in which efficacy ranged from 0 - 73% (Relf et al., 2014). A reason for the observed reduced efficacy in younger horses is the fact that younger horses lack acquired immunity, which allows a greater accumulation of encysted cyathostomins (Lyons et al., 2009). When anthelmintics such as BZ, PYR and IVM are administered, which do not possess high efficacy against these stages, once luminal adult worms have been eliminated, encysted stages may emerge and resume development and eggs are seen in faeces sooner compared to adult horses, which harbour fewer encysted stages (Herd, 1986; Herd and Gabel, 1990b). A study looking at PYR efficacy on 64 Danish horse farms also found that

PYR ‘efficacy’ increased with age and that, as pre-treatment egg counts increased, pyrantel efficacy appeared to decrease (Nielsen et al., 2013). It is well documented that strongyle FEC are higher in younger horses compared to adult horses (Relf et al., 2013). However, Nielsen et al. (2013) proposed that the explanation for the observed lack of efficacy in younger horses with a higher FEC is technical not biological, as using a FEC method with an egg detection limit of 20 EPG, a horse with a 200 EPG count treated with a drug with a 90% efficacy will be likely to exhibit 0 EPG post-treatment, because the low level egg count is likely to go undetected due to a lack of FEC method sensitivity. However, if a horse has a pre-treatment FEC of 1000 EPG as measured using the same FEC method and is treated the same drug, eggs are likely to be detected post-treatment leading to a lower observed efficacy.

The rotation of anthelmintic classes over time was originally advocated to reduce selection pressure to any one class (Drudge and Lyons, 1966). The rationale being, by using an anthelmintic with a different mode of action, any parasites surviving the previous treatment will be killed. Two rotation plans have been advocated; slow rotation, where a single anthelmintic class is used for a year and fast rotation, whereby the class is rotated after each deworming occasion. The majority of studies looking into anthelmintic rotation and the effect it has on resistance levels have been performed in sheep and goats (Martin et al., 1981; Lawrence et al., 2006; Hughes et al., 2007). These studies found that fast rotation of classes may mask the emergence of AR. In horses, slow rotation could be challenging, especially in light of the high levels of resistance to FBZ in cyathostomins, and increasing reports of resistance to PYR in these nematodes, making rotation difficult (Kaplan and Nielsen, 2010).

The dose and move strategy, whereby animals are treated with anthelmintic and then moved immediately to ‘clean’ pasture with low levels of parasite infestation has been

recognised for many decades and widely advocated as a control practice in many species, including horses (Brunsdon, 1980). The rationale being that grazing animals will have a reduced exposure to challenge with larvae, resulting in a slower rate of reinfection after anthelmintic treatment (Brunsdon, 1980). Clean pastures can be maintained by management practices such as re-sowing grass, grazing with alternative stock classes, or resting pastures (Brunsdon, 1980; Barger, 1999). However, it has been recognised that 'dose and move' strategies can place strong selection for resistant parasites, because worms surviving treatment become the major source of subsequent contamination of clean pasture (Le Jambre, 1978; Cawthome and Whitehead, 1983; Michel, 1985; Taylor and Hunt, 1988; Taylor and Hunt, 1989; Martin, 1989) and levels of refugia are diminished. Despite this, the practice has been widely implemented, particularly in small ruminants (Martin, 1989; Anderson, 1990; van Wyk, 2001; Besier and Love, 2003). Evidence of AR in sheep was found to be associated with treatment prior to movement to 'clean' pastures (Vlassoff and Kettle, 1980; Cawthome and Whitehead, 1983; Martin et al., 1985; Taylor and Hunt, 1988). Modelling studies have also indicated the selective potential of treatment prior to movement onto low-contamination pastures (Leathwick et al., 1995; Barnes et al., 1995), but the most compelling evidence suggesting that dose and move strategies increase resistance have come from field studies in sheep. In a study by Martin (1989), lambs were treated with an anthelmintic in late spring followed by a move to 'clean' pasture, and subsequently received two further treatments with the same anthelmintic in summer. Changes in the levels of AR in parasite populations in those lambs, as measured by the Egg Hatch Test (EHT), were compared with lambs that were moved to clean pasture but not treated with anthelmintic. The authors found that levels of resistance increased dramatically after even a single treatment associated with a move to clean pasture (Martin, 1989). Two recent studies in New Zealand have provided further evidence. In the first, the

authors found that when 100 % of lambs were treated prior to a move onto clean pasture, levels of resistance in the resulting larval populations on pasture increased compared to when 10 – 20% of lambs were left untreated (Waghorn et al., 2008). In the second study, lambs were treated and moved to pastures prepared with differing levels of contamination with infective stage nematode larvae. The authors found that following treatment, moving lambs onto pastures with the lowest levels of contamination resulted in significantly higher levels of resistance compared with moving onto pastures with larger levels of contamination (Waghorn et al., 2009). These studies provide evidence that dose and move strategies increase the development of resistance and support the notion of maintaining levels of parasite refugia. There are no data to demonstrate the impact of dose and move strategies on the development of resistance in equine parasites, but given that the biology of equine strongyles and ovine trichostrongyles is similar it could be postulated that reducing levels of refugia after treatment of horses could lead to the same observations.

1.5 Control of equine parasites

Due to the prevalence of helminths in equids and the pathogenic potential of certain species, control is necessary to protect their health. To break transmission of helminths, control methods broadly fall into two categories; anthelmintic administration and environmental control. Multiple anthelmintic dosing regimens have been recommended in the past namely, interval dosing, strategic- and targeted dosing reviewed by (Proudman and Matthews, 2000). Moreover, various management practices have been suggested to reduce the build up of parasite eggs and infective larvae in the environment (Drudge, 1966; Herd , 1993): for example, the regular removal of dung from pastures (Herd, 1993).

Interval dosing regimens were first proposed in the 1960's and were based on the application of broad-spectrum anthelmintics to all horses in a population at regular intervals (Drudge and Lyons, 1966). These programmes were designed to reduce the morbidity and mortality associated with infection with *S. vulgaris*, which was present at high prevalence at the time. The treatment intervals were designed around the ERP identified at the time each anthelmintic was first licensed with the aim of eliminating nematode egg excretion and pasture contamination. This approach successfully reduced large strongyle and other worm related disease in horses (Kaplan, 2002); however, it is believed that this approach has significantly contributed to, and accelerated, the rate of development of AR. Strategic dosing was first suggested in the 1980's (Reinemeyer, 1986) and promotes the administration of anthelmintics at specific times of year based on knowledge of helminth epidemiology and life cycles, with the overall aim of disrupting seasonal transmission (Proudman and Matthews, 2000). While this approach might be considered to offer a more rational approach than interval dosing, it does not consider the distribution of parasites amongst host populations, or different levels of nematode egg excretion between individuals, nor does it address changes in weather, which can cause early or late peak pasture infectivity (Proudman and Matthews, 2000). Targeted dosing relies on administering anthelmintics at the most appropriate time of year, whilst considering the life cycle and epidemiology of parasites and taking into account variation in nematode egg excretion amongst individuals. This approach includes the measurement of individual FEC of horses within populations at specific times of the year to facilitate targeting of anthelmintics to only those excreting moderate-to-high levels of nematode eggs in their faeces (Duncan and Love, 1991; Gomez and Georgi, 1991). Helminth infections tend to be highly over-dispersed amongst hosts (Crofton, 1971; Anderson and May, 1978; Shaw and Dobson, 1995; Shaw et al., 1998), and equine nematode infections are no

exception (Kaplan and Nielsen, 2010; Relf et al., 2013). This has been demonstrated in a recent study looking at helminth egg excretion on UK Thoroughbred stud farms. Here, Relf et al. (2013) reported that 11% of 1,221 horses tested by FEC analysis were responsible for excreting 80% of eggs detected in samples collected over the sampling frame. Such levels of over-dispersion provide an opportunity for sustainable control via targeted anthelmintic therapy (Sangster, 1999). The EPG threshold value used to prompt treatment is usually between 200 - 500 EPG (Uhlinger, 1993; Larsen et al., 2011). Some studies have provided evidence that horses tend to sustain strongyle egg shedding status; i.e. those that shed high numbers of eggs in their faeces when first assessed often have higher FEC than co-grazing horses over time, despite similar levels of parasite exposure (Nielsen et al., 2006; Becher et al, 2010). For targeted treatment protocols to be successful, it is necessary to understand the factors that affect strongyle egg shedding. Such knowledge will facilitate decision making and create optimum protocols to determine frequency of FEC testing and to identify risk factors associated with increased egg shedding so that only horses that require treatment are targeted.

Major caveats to the application of a FEC directed targeted treatment programme include the fact that FEC do not detect encysted cyathostomin larvae and do not reliably detect tapeworm eggs. Because of the potential pathogenicity of cyathostomin EL, the current recommendation is that all horses should receive an anthelmintic with larvicidal activity (i.e. moxidectin) and PRZ for tapeworm in late autumn or early winter in the UK (Matthews, 2008). Further, reducing anthelmintic treatment frequency and in low egg shedding horses, totally withholding anthelmintic treatment, creates a risk for the re-emergence of *S. vulgaris* and other large strongyle species (Nielsen et al., 2012). A recent study conducted on 42 horse farms in Denmark looked at the prevalence of *S. vulgaris* at individual horse level and farm level by culturing the faeces of 662 horses. They found that at individual level and farm level, the prevalence

of S*. vulgaris* was significantly higher on farms that practiced FEC directed strategic treatments compared to farms that treated at regular intervals (Nielsen et al., 2012). This highlights the need for routine surveillance of *S. vulgaris* when following FEC directed targeted treatment programmes especially if anthelmintics are totally withheld from 'low shedding' individuals.

1.5.1 Parasite management practices

A number of studies have shown that many horses are dewormed too frequently and that veterinarians have become dissociated from equine parasite control practices (Matthews, 2008; Stratford et al, 2011). In racing yards in the UK, Earle et al. (2002) found that only 29% of racing yard trainers involved a veterinary surgeon in their deworming programmes (Earle et al., 2002) and in a later survey, 84% of horses from Thoroughbred training yards were found to be administered with an anthelmintic even when a FEC performed prior to treatment was shown to be less than 50 EPG (Comer et al., 2006). A questionnaire study conducted in Ireland reported that of 55 yard owners questioned, none of the respondents left any animals untreated, 72% administered anthelmintics based on a treatment interval of <8 weeks, indicating that if they were administering MLs, they would be treating horses within the standard ERP, thus potentially exerting a strong selection pressure for resistance (O'Meara and Mulachy, 2002). Further, only 40% of recipients owned an accurate weighing device, which could lead to inaccurate dosing and could potentially lead to parasites being exposed to a sub-lethal dose of anthelmintic, thus increasing selection pressure for resistance (Stratford et al., 2014a). In a more recent study carried out on 61 UK Thoroughbred studs, the authors found that despite many respondents indicating a high level of concern about AR, many were not aware of the risk factors for development of resistance (Relf et al., 2012). In the preceding 12 months, 98% of respondents stated

that they had administered an ML, and 100% of respondents administered anthelmintics on an interval basis. None of the yards performed any regular FEC analysis (Relf et al., 2012). Considering that some respondents were administering up to nine ML treatments per year, there will clearly be selection pressure for AR. An online study performed in the UK targeting the general horse owning population (Allison et al., 2011) found that <60% of respondents (*n* = 574) believed that their current programme was not as effective as it could be. In this study, 49% of the horses were kept on livery yards and the owners indicated that they followed a deworming regime imposed by the yard manager. In total, 40% of owners indicated that were 'not happy' with the imposed programme. Further, 84% of owners stated that they were aware of AR, 89% stated that they would be interested in finding out if their horses are affected by AR, and 83% said they would be willing to pay two pounds or more per month to regularly check for resistance, which is encouraging. However, while 94% of respondents believed their horses were reasonably or well protected from worms, only 25% wanted to reduce the amount of anthelmintic they used and only 31% followed a FEC directed regimen (Allison et al., 2011). A recent questionnaire study conducted in Scotland (Stratford et al., 2014a) reported that ML or related combination products were most commonly administered and that treatments licensed for use against cyathostomin EL and tapeworms were administered to horses by 80% and 90% of respondents (*n* = 193), respectively (Stratford et al., 2014a). In this study, it appeared from the responses that veterinarians had the greatest influence on control practices (40% of 193 respondents), which contrasts to the findings of the earlier study by Allison et al. (2011). While 40% respondents believed they practiced 'targeted dosing', this was not associated with delaying treatment beyond the standard ERP of the anthelmintic used (Stratford et al., 2014a).

The evidence from these questionnaire studies indicates that horse owners are becoming increasingly aware of the issue of AR and the need to adopt more sustainable approaches to control. Nevertheless, it was clear from the results that the majority are confused by these concepts and few are actually following robust FEC directed targeted treatment programmes. The challenge now is to educate and encourage those that prescribe anthelmintics to support the application of FEC directed targeted programme, and to convince horse owners to deviate from traditional approaches to control. A greater emphasis needs to be placed on diagnostic tests such as the FEC and FECRT to identify which horses need treatment and to improve surveillance of AR.

1.5.1.1 Dung removal

The regular removal of dung from pasture has been advocated to help reduce pasture contamination (Herd, 1993; Herd and Coles, 1995). The rationale being to remove strongyle larvae before they develop into L3, which migrate from the faeces onto the pasture (Mathee et al., 2004). This approach, in conjunction with reducing the frequency of anthelmintic treatments, is now widely recommended to reduce selection pressure for AR (Corbett et al., 2014). A recent study, conducted at the Donkey Sanctuary in the UK, investigated the effectiveness of dung removal methods to control small strongyle burdens in donkeys (Corbett et al., 2014). Here, faecal samples and pasture samples were collected on a monthly basis for seven months from donkeys managed under three different pasture management strategies. The results revealed that twice weekly removal of faeces from pastures, either by manual or automated means, significantly reduced the number of strongyle eggs shed in faeces of co-grazed donkeys (Corbett et al., 2014). It has been suggested that regular dung removal may reduce refugia, which may lead to an increase in the likelihood of AR (Nielsen et al., 2007) however; further research is needed to investigate this. The study by Corbett et

al. (2014) was conducted in donkeys, which only received anthelmintic treatment if their egg count exceeded 2000 EPG (i.e. higher that the arbitrary 200 EPG threshold currently recommended for horses) therefore, studies need to be performed in horses to evaluate how effective dung removal is and to determine suitable EPG thresholds for anthelmintic treatment for animals that graze pastures which are regularly cleaned.

1.5.1.2 Co-grazing with ruminants

Allowing ruminants to co-graze with horses has been recommended as a way to reduce pasture contamination (Herd, 1986). Few nematode species are shared between horses and ruminants, with the exception of *Trichostrongylus axei*, which is of little pathogenic significance to equids. A caveat to this approach is the increasing prevalence of *F. hepatica* in ruminants (Mitchell, 2002; Gordon et al., 2013). *F. hepatica* has a wide host range, including humans and equids. Currently, routine surveillance of *F. hepatica* in equids is not performed and further, routine FEC analysis will not reliably detect fluke eggs. There is a lack of information on the prevalence of *F. hepatica* and the clinical significance of fluke in equids in the UK and, in light of the increased prevalence of this trematode, there is a need for further research. In addition, there are no licensed flukicides available for use in equids in the UK, with treatment only allowed by a veterinary surgeon under the 'cascade' option: a legal flexibility that allows veterinary surgeons to prescribe an unlicensed product in the absence of a suitable licensed product. In areas where fluke prevalence is high and in areas where the intermediate snail host is found, ruminants should be tested and treated with an appropriate anthelmintic before being turned out with horses or donkeys or used in rotational grazing plans with horses (Matthews and Burden, 2013).

1.5.1.3 Quarantine

Newly introduced horses have the potential to disseminate anthelmintic resistant parasites and should be treated with an effective broad-spectrum anthelmintic with activity against encysted cyathostomin and large strongyle larvae (Kaplan, 2002; Matthews, 2008, 2011; Lester and Matthews, 2014). It has been suggested that after quarantine treatment, horses should not be turned out onto pasture for 72 h, to ensure all eggs have been excreted in faeces (Nielsen et al., 2010). This is viewed as best practice and should be recommended. To date, there have been no studies conducted to investigate the impact of not quarantining horses in terms of spreading resistant nematodes, but data from surveys in New Zealand indicated that sheep farms that had introduced large numbers of purchased stock were significantly more likely to test positive for IVM resistance than those that did not (Lawrence et al., 2006; Hughes et al., 2007). In a further study, on sheep farms in Western Australia, the prevalence of IVM resistance was linked with failure of farmers to quarantine-treat stock after purchase (Suter et al., 2004). These data support the view that failure to adequately quarantine-treat animals is responsible for the dissemination of anthelmintic resistant nematodes (Coles and Roush, 1992).

1.6 Project aims

The evidence presented in this Chapter, indicates that of all the helminth species to affect horses, the cyathostomins present the greatest challenge while, in foals, *P. equorum* is the greatest threat. The frequent, prophylactic administration of anthelmintics has contributed to the development of widespread resistance in cyathostomins and *P. equorum* and recent evidence suggests that resistance to MLs in *O. equi* is a potential issue and chemical options for control are becoming limited and

unsustainable. With no new anthelmintic classes likely to be licensed for use, in the near future, control programmes must be designed to preserve efficacy of the currently effective ones. The primary aim of control programs must now be the preservation of anthelmintic-sensitive nematode populations, coupled with minimising the risk of parasite-associated disease. FEC directed targeted treatment programmes and regular efficacy testing have been advocated; however, if FEC are to become the cornerstone of control, there is a need to optimise the methodologies to ensure that they are fit for purpose. Results from questionnaire studies have demonstrated a large gap in knowledge regarding evidence-based helminth control. In light of the aforementioned issues, there is a real need to build a supportive framework for sustainable helminth control for horses.

The aim of this study was to do this by investigating the following areas:

1. Assessing sources of variation in equine FEC, which underpin anthelmintic-targeted treatment programmes and anthelmintic efficacy evaluation, with the aim of producing guidelines to reduce variation and improve accuracy of these tests.
2. Studying in detail, efficacy of commonly used anthelmintics in populations of horses to determine the current prevalence of AR in the UK.
3. Investigating the true value the FECRT by comparing different methodologies to determine which is the most accurate and reliable to ensure that resistance is not misclassified.
4. Identifying risk factors associated with nematode egg excretion. This was achieved by comparing FEC test results with questionnaire study answers on relevant management practices.
5. Undertaking studies into the practical utility of these protocols in the field.

The information derived from the above areas was then used to build a framework for a decision support system (DSS) to facilitate sustainable helminth control in future.

CHAPTER 2: Performance of faecal egg count methods for the detection of equine parasites

2.1 Introduction

There are a number of underlying factors that can lead to variability in faecal egg count (FEC) data. Biological factors include; fluctuation in egg excretion over time (Oju & Mpoame, 2006; Rinaldi et al., 2009), faecal consistency (Uhlinger, 1993; Le Jambre et al., 2007; Turner et al., 2010) and differences in the biotic potential of different parasite species (Mfitilodze and Hutchinson, 1987). Technical factors include; aggregation of eggs within faeces (Yu et al., 1998; Denwood et al., 2012), variation in the collection, storage and handling of samples (Nielsen et al., 2010b), the Poisson process as well as the type of FEC method used (Mes, 2003; Cringoli et al., 2004; Pereckiene et al., 2007; Vadlejch et al., 2011; Levecke et al., 2012a; Torgerson et al., 2012). Each of these factors forms the potential for aggregation of helminth eggs at each level, potentially leading to variability in faecal egg count (FEC) data and this may confound interpretation of the data. Downstream consequences might include under- or over-estimating the requirement for treatment, leading to unnecessary treatments or not treating horses that require treatment and/or the misclassification of anthelmintic efficacy i.e. classifying a drug as efficacious when it's not or misclassifying a drug as resistant . If FEC analysis is to underpin evidence-based helminth control in horses, there is a need to investigate sources of variation to ensure that recommended methods are fit for purpose and minimise potential misclassification.

2.2 Aims and objectives

Here, technical factors associated with FEC variability were investigated, namely, the distribution of strongyle eggs in equine faeces at different levels where egg aggregation could occur. The levels investigated were the entire faecal motion, between faecal boluses, within boluses at individual bolus level and the distribution of eggs in

suspension. The biotic potential was not investigated here as there are over 50 species of cyathostomin (Lictenfels et al., 2008), each with varying biotic potential (Kuzmina et al., 2012) and there is no way of differentiating between cyathostomin species using FEC analysis. Two FEC methods were compared, the McMaster (McM) technique (Gordon & Whitlock, 1939), which is the most widely used technique in practice and a centrifugal-flotation (CF) technique (Christie and Jackson, 1982), which offers increased diagnostic sensitivity to assess the impact of variability on decisions to administer anthelmintics and on estimates of efficacy. Practical solutions are proposed to reduce FEC variability in practice in support of targeted anthelmintic use and efficacy monitoring.

2.3 Materials and methods

2.3.1 Faecal sample collection and storage

All faecal samples were obtained from freshly voided material. A minimum of three faecal boli from each sample were collected and placed into a zip-lock bag, expelling as much air as possible before sealing. All samples were stored at approximately 4°C until being processed and analysed, which occurred within four days of collection to minimise the degradation of nematode eggs in the samples (Nielsen et al., 2010b).

2.3.2. Faecal egg counting techniques

2.3.2.1 McMaster method

A McM technique (Gordon and Whitlock, 1939), described in the Ministry of Agriculture, Fisheries and Food technical manual (MAFF, 1986), was employed. In brief, the method involved adding 3 g faeces to 42 ml of saturated sodium chloride

solution (NaCl; specific gravity 1.204; MAFF, 1986) homogenising and then pouring the faecal suspension over a sieve (1mm aperture) into a beaker. Two different volumes of faecal suspension were examined using a standard McM slide (Fig. 2.1), either 1.0 ml (two entire chambers) resulting in an egg detection limit (dl) of 15 eggs per gram (EPG) (McM15) or 0.3 ml (two grids), resulting in an egg dl of 50 EPG (McM50). The total number of eggs counted using each variation of the method was recorded separately.

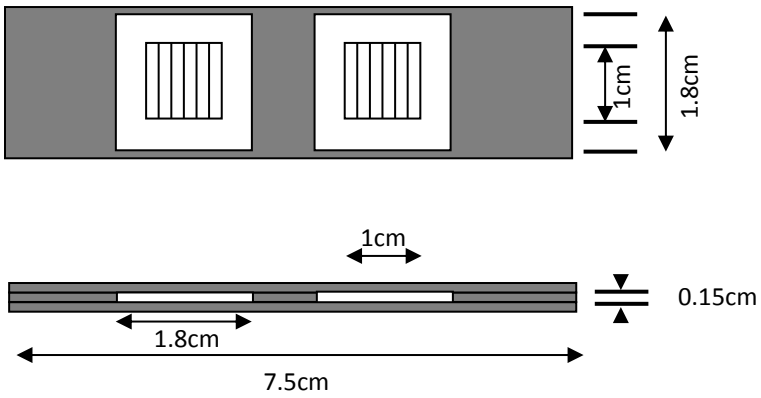


Figure 2.1. The dimensions of a standard McMaster slide: C = chamber (1 chamber = 0.5 ml) G = grid (1 grid = 0.15 ml) (modified from MAFF, 1986)

2.3.2.2. Centrifugal-flotation method

A modification of a CF method described by Christie and Jackson (1982), with an egg dl as low as 1 EPG was used for investigations into egg distribution. In brief, each gram of faeces was thoroughly mixed with 10 ml tap water. From this, a 10 ml aliquot was dispensed over a 1 mm sieve and washed through with a further 5 ml tap water. The resulting suspension was poured into plastic polymer tubes (Beckman Coulter Ltd. UK) and then centrifuged (203 x *g* for 2 min). After centrifugation and removal of the supernatant, the faecal pellet was re-suspended in 10 ml saturated NaCl solution (specific gravity 1.204; MAFF. 1986) and centrifuged at 203 x *g* for 2 min. Artery

forceps were used to clamp the tube just below the meniscus, isolating the eggs from the sample, and the contents above the forceps poured into a cuvette. The cuvette (2.5 ml volume, Sigma-Aldrich® Co. UK) was inverted to ensure even distribution of the eggs and topped up with NaCl solution and then a lid applied. The cuvette was positioned horizontally under a compound microscope (x 40 total magnification) and, using a Miller square eyepiece graticule (Graticules Ltd. UK) (Fig. 2.2), eggs were enumerated depending on egg density. Where eggs were present at low density (i.e. ~< 25 eggs in the cuvette), all eggs in the cuvette were counted, and no multiplication factor was used equating to an egg dl of 1 EPG. If eggs were at a moderate density (i.e. ~25 - 50 eggs in the cuvette), two traverses of the cuvette were counted using the large square of the Miller eyepiece graticule, equating to a third of the total cuvette volume (Fig. 2.3A). The number of eggs in each traverse were added together and multiplied by a factor of three to give an EPG estimate, equating to an egg dl of 3 EPG. When eggs were at high density (i.e. ~> 50 eggs in the cuvette), two whole traverses of the cuvette were counted using the smaller square of the eyepiece, equating to one ninth of the total cuvette volume (Fig. 2.3B). The total number of eggs in two traverses were then added together and multiplied by a factor of nine to give EPG estimate equating to an egg dl of 9 EPG. Parasite eggs were differentiated into the following types: strongyles, *Parascaris equorum*, *Oxyuris equi*, *Anoplocephala* spp. and *Strongyloides westeri* (Thienpont, 1986).

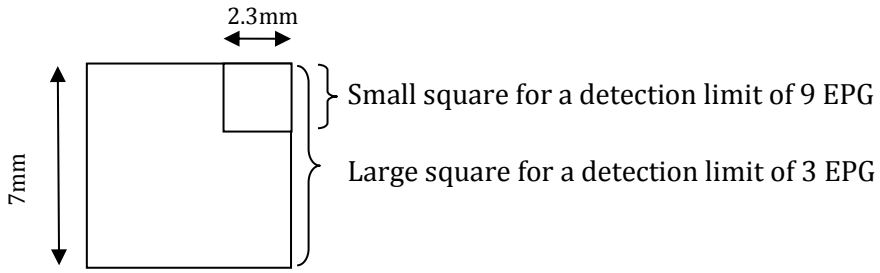


Figure 2.2. Diagrammatic representation of Miller square eyepiece graticule (7 x 7 mm), the small square is exactly $^{1}/_{3}$ of the width of the larger square. The large square was used when the egg density was moderate, resulting in a detection limit of 3 eggs per gram (EPG). The small square was used when the egg density was high, giving a detection limit of 9 EPG

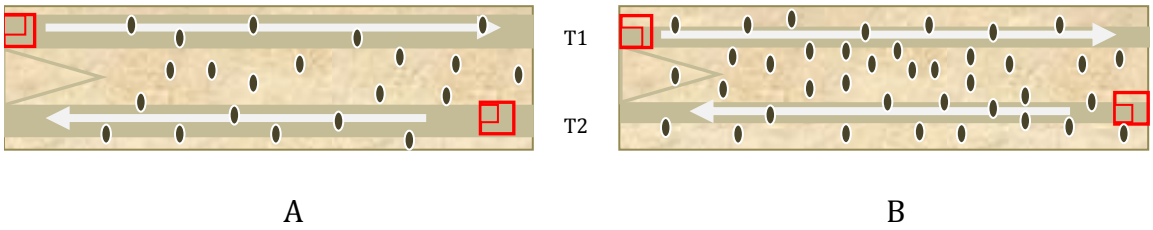


Figure 2.3. Diagrammatic representation of the different areas examined depending on the egg density in the cuvette. A = medium egg density, here the number of eggs seen in two traverses (traverse 1 (T1) and 2 (T2)) using the large square of the Miller eye piece were recorded and multiplied by 3. B = high egg density, here the number of eggs seen in two traverses using the small square of the Miller eye piece were recorded and multiplied by 9 (adapted from Bartley and Elsheikha, 2011)

2.3.3 Populations of equids used for the provision of faeces for analysis

The populations of horses used in the different analyses were selected opportunistically and were not consistent between investigations. Faecal samples used for investigations into the distribution of strongyle eggs in faeces (Section 2.3.4) were collected from three horses (H1, H2 and H3) resident at the equine hospital at the Royal (Dick) School of Veterinary Studies (R(d)SVS), University of Edinburgh. These

samples were collected in October 2011. The horses were selected on the basis of having positive FEC during initial screening. The horses were aged between 5 and 15 years. The samples used for investigations into the distribution of strongyle eggs at cuvette level (Section 2.3.5) were collected from 31 donkeys resident at the Donkey Sanctuary, Sidmouth, Devon, UK. The samples were collected in August 2013 and posted to Moredun Research Institute. All samples were analysed within 4 days of collection. All donkeys were over the age of 5 years (5 – 32 years), and were managed under the same conditions. The faecal samples used for studies into the effect of mixing (Section 2.3.6) and subsample size (Section 2.3.7) on the variance in FEC were collected from a population of ponies from Dartmoor and transported to Moredun Scientific for use in a clinical trial. Entire faecal motions were collected on a single occasion from seven ponies (designated H4, H5, H6, H7, H8, H9 and H10) in June 2012. The ponies were approximately 6 months old and had not received anthelmintic treatment prior to faecal collection. The ponies were maintained together and were housed at the time of sampling. The samples used for investigating the effect of FEC method on decisions for targeted treatment (Section 2.3.8) and the effect of FEC method on the estimated anthelmintic efficacy using the faecal egg count reduction test (FECRT) (Section 2.3.9) were collected from 13 horse yards (designated Yard 1 to 13). All yards were based in the southeast of England and were approached to participate in this study through the Bell Equine Veterinary Clinic, Kent, and the House and Jackson Veterinary Clinic, Essex, between March and December 2012. Further information about these horses can be found in Chapter 3, Section 3.3.1.

2.3.4 Distribution of strongyle eggs in faeces

The spatial distribution of strongyle eggs in faeces was investigated, as aggregation or clumping could be an important source of variation (Denwood et al., 2012;

Vidyashankar et al., 2012; Torgerson et al., 2012). The errors arising through the FEC process follow a hierarchical structure (Figure 2.4). Each different level was investigated as a potential source of variation. Single, entire motions were collected from three horses (designated H1, H2 and H3, respectively) residing at the equine hospital at the R(d)SVS, once a day at approximately the same time for three consecutive days, with the exception of one horse (H1), which was discharged from the hospital before the third day. Each motion was divided into individual boli and two, 1 g subsamples were taken from each; one from the surface and one from the centre, and analysed separately using the CF method. The remainder of each motion was thoroughly mixed by hand until all the boluses had been broken down and a 10 g subsample was taken from each and analysed twice by CF to give an estimation of the average FEC from well mixed faeces for each horse on each occasion.

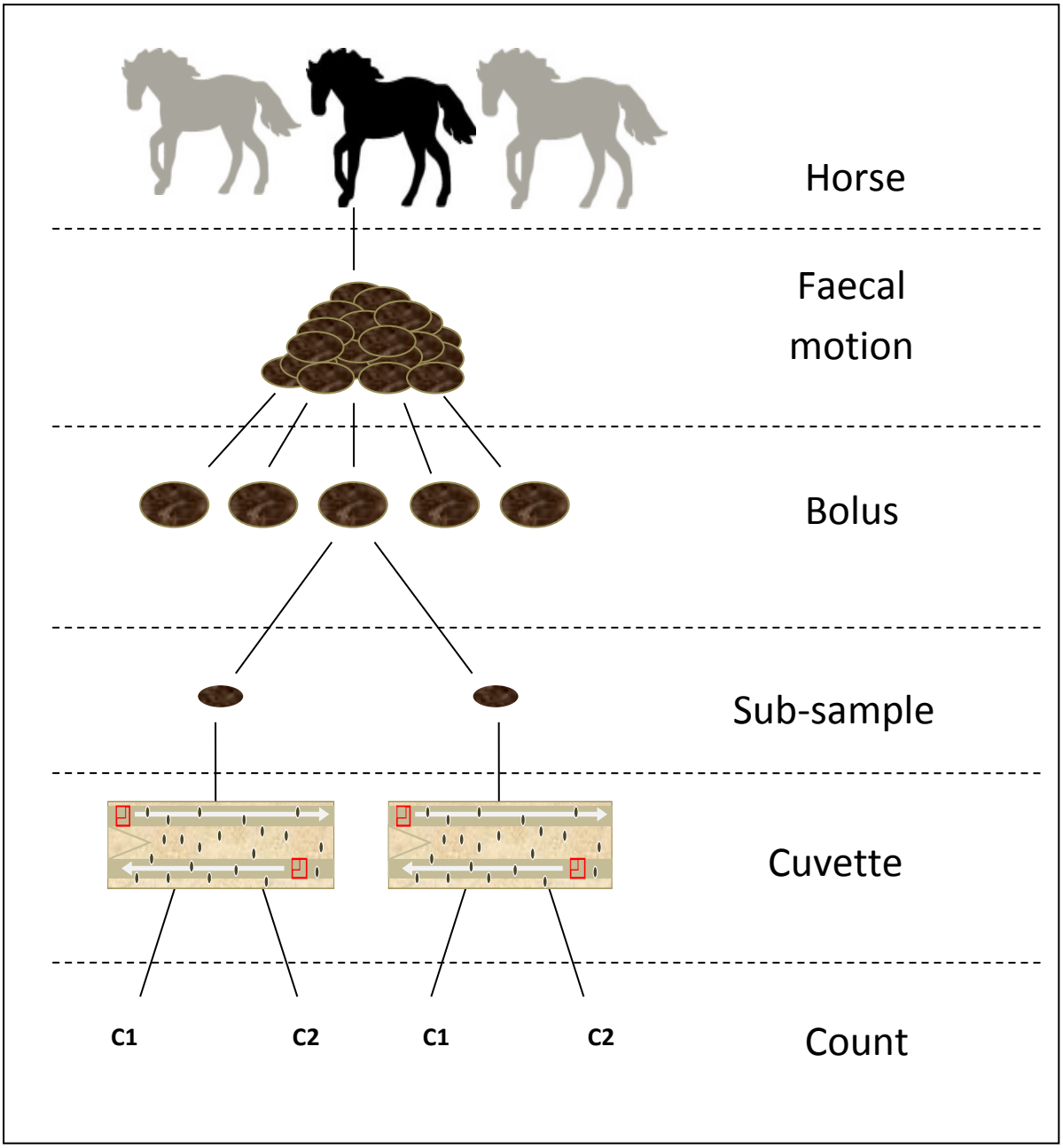


Figure 2.4. The proposed hierarchical levels that could potentially lead to variation during faecal egg count analysis. Count (C1 and C2) refer to the two traverses made when egg density is >25 eggs per cuvette

2.3.5 The distribution of strongyle eggs at cuvette level

The accuracy of the density dependent variations in dilution factor inherent to the CF method depends on the assumption that eggs are randomly distributed across the counting surface of the cuvette. To check this assumption, freshly voided faecal samples were collected from 31 donkeys resident at the Donkey Sanctuary, Sidmouth, Devon. The samples were well mixed before a 10 g subsample was taken for analysis by CF. For each sample, using the large square of the Miller eye piece, the number of eggs in five traverses of the cuvette, were recorded separately. Each cuvette was counted a second time and all eggs present in the cuvette were counted (1 EPG egg dl), then the number of eggs detected in a third of the cuvette were counted (3 EPG egg dl) and finally the number of eggs in a ninth of the cuvette (9 EPG egg dl).

2.3.6 The effect of mixing samples on the variance of strongyle FEC

Entire faecal motions were collected on a single occasion from four ponies (designated H4, H5, H6, H7) harbouring naturally acquired infections that were resident at the Moredun Research Institute. These ponies were from Dartmoor; they were approximately 6 months old and had not received anthelmintic treatment prior to faecal collection. The ponies were maintained together and were housed at the time of sampling. All motions were observed being voided and collected when fresh. From each entire motion, a total of 10 subsamples were randomly taken (i.e. by eye) for CF analysis (10 g per subsample) and a further 10 subsamples were randomly selected for McM analysis (3 g per subsample). The remainder of each unmixed motion was then thoroughly mixed manually and subsamples taken again as described above. For CF, the actual number of eggs observed in each cuvette was recorded. For samples analysed by McM, the actual number of eggs seen in the two grids (McM50) and the

two chambers (McM15) of a McM slide were recorded separately and no multiplication factor was applied.

2.3.7 Effect of sample size on mean and variance of strongyle FEC

Initial faecal sample size taken from an entire motion prior to taking a subsample for FEC analysis could have an effect on variance and repeatability of FEC results, the hypothesis being that a larger sample would be more representative of the entire faecal motion. Freshly voided entire motions were collected on one occasion from the three ponies (designated H8, H9 and H10, respectively) resident at the Moredun Research Institute. From each entire motion, three sample sizes were taken (3 x 7 g, 3 x 10 g and 3 x 15 g). The remainder of each motion was mixed and further samples taken (3 x 7 g, 3 x 10 g and 3 x 15 g). From each sample size, a 3 g subsample was taken and one McM slide per subsample was analysed. The actual number of eggs observed in each grid and in each chamber of the slide was recorded.

2.3.8 The effect of faecal egg count method on decisions for targeted treatment

Personnel at 13 equine yards (Yard 1-13) in the southeast of England were asked to submit faecal samples from all horses resident on each yard between March and December 2012. Large (~200 g), freshly voided, unmixed samples were collected from each horse and placed into sealed bags, carefully ensuring the removal of as much air as possible. Samples were sent to the laboratory by post within 24 h of collection. Once received, each sample was thoroughly mixed by hand and one 10 g and one 3 g subsample taken and analysed by CF and McM50, respectively.

2.3.9 The effect of faecal egg count method on the outcome of anthelmintic efficacy using the faecal egg count reduction test

Faecal samples were obtained from all equids at each yard (Yard 1 to 13) once the minimum strongyle egg reappearance period (ERP) of the previously administered anthelmintic had passed. The standard minimum ERP used were as follows: 6 weeks for fenbendazole (FBZ) and pyrantel embonate (PYR), 8 weeks for ivermectin (IVM) and 13 weeks for moxidectin (MOX) (Herd and Gabel, 1990b, Borgsteede et al., 1993, Mercier et al., 2001, Stratford et al., 2011). Horses for which strongyle FEC of ≥50 EPG (counted by the CF method) were included and administered *per os* with anthelmintic on Day 0 at the following dose rates; FBZ (Panacur® equine paste, MSD Animal Health; 7.5mg/kg body weight (BW)), PYR (Strongid-P™, Elanco animal Health; 19mg/kg BW), IVM (Eqvalan® oral paste for horses, Merial Animal Health; 0.2mg/kg BW) or MOX (Equest®, Zoetis Animal Health; 0.4mg/kg BW). Each horse received a dose appropriate for 110% of individual body weight, as estimated by weigh tape, to minimise the risk of under-dosing (Stratford et al., 2014b). Each individual was treated by their owner and monitored immediately afterwards to ensure that the paste/gel was ingested. All horses on the same yard received the same class and batch of anthelmintic. Faecal samples were collected on Day 0 immediately prior to anthelmintic administration and then at 14 days post-treatment (Stratford et al., 2014b). Each faecal sample was analysed by CF, McM15 and McM50. Testing of anthelmintic classes was performed in succession on several populations, starting with FBZ, followed by PYR, then IVM and finishing with MOX.

2.4 Statistical analyses

2.4.1 Distribution of eggs in faeces

If helminth eggs are randomly distributed within faeces then the number of eggs observed in serial randomly selected subsamples should follow a Poisson distribution (Denwood et al., 2012; Torgerson et al., 2012). The Poisson distribution (Equation 2.1) can be expressed as a discrete probability distribution, in which the probability of finding *s* eggs in a fixed volume of faeces is a function of the mean number of eggs per unit volume, *m*. A feature of the Poisson distribution is that the variance equals the mean. If variance exceeds the mean, this indicates over-dispersion (OD) or clumping of eggs (Hilborn and Mangel, 1997). The Poisson distribution is described by:

$$\boldsymbol{Pr}(\mathbf{Z} = \boldsymbol{s}) = \frac{\boldsymbol{e}^{-\boldsymbol{m}}\boldsymbol{m}^{\boldsymbol{s}}}{\boldsymbol{s}!} \qquad \text{(Equation 2.1)}$$

The mean, variance, variance to mean ratio, and coefficient of variation (CV; i.e. standard deviation divided by mean) were calculated for each entire motion, between boli, between subsamples and between counts at cuvette level (Figure 2.4) for each horse on each day. The distribution of eggs at each level was tested for OD by multiplying the variance to mean ratio by the number of degrees of freedom, and comparing the result with the chi-square distribution (Bliss and Fisher, 1953; Morgan et al., 2005). Where OD was confirmed (p<0.05), the negative binomial distribution (NBD) was fitted to the data by maximum likelihood estimation (MLE) (Williams and Dye, 1994; Shaw et al., 1998; Morgan et al., 2005) to give estimates of the mean, *m,* and the aggregation factor, *k*. The NBD is a discrete probability distribution, which is used to describe the amount of aggregation or OD in data, where decreasing values of *k* correspond to increasing levels of OD (Lloyd-Smith, 2007). The variance of the NBD is equal to ($\mu + \mu^2/k$), so as *k* increases, (effectively $k \geq$c.10) the variance approaches the

mean and the distribution approaches the Poisson (Bolker, 2008). Here, the NBD is used to identify sources of supra-Poisson variability in FEC; thus, the likelihood of individual FEC (Z) from the multiple subsamples throughout the entire motion, given a combination of m and k was estimated using the formula in Equation 2.2 where Γ represents the gamma function. The point likelihoods for each FEC were multiplied and the log of this value was multiplied by -1. This value was minimised by changing m and k iteratively in turn using the Solver function in Excel (Torgerson et al., 2005; Morgan et al., 2005). Ninety-five percent confidence bounds for estimates of m and k were calculated by identifying the corresponding values for the negative log-likelihood plus 1.92 (Hilborn and Mangel, 1997).

$$\Pr(Z = s) = \frac{\Gamma(k+s)}{\Gamma(k)s!}\left(\frac{m}{k+m}\right)\left(1+\frac{m}{k}\right)^{-k}\left(\frac{k}{k+m}\right)^{s} \quad \text{(Equation 2.2)}$$

The chi-square goodness of fit test was used to assess whether the data adequately fitted the NBD (Shaw et al., 1998). The starting point for estimation of k was the corrected moment estimate (Smith and Guerrero, 1993) using Equation 2.3, where m is the mean, v is the variance, and n is the sample size:

$$k = \frac{m^2 - \left(\frac{v}{n}\right)}{v - m} \quad \text{(Equation 2.3)}$$

The actual number of eggs seen, as opposed to the EPG estimations following a multiplication factor, was used for both the MLE and the initial calculation for OD to reduce the inflation of variance introduced by a multiplication factor (Torgerson et al., 2012).

The relationship between the mean number of eggs and *k* at each level was investigated by Poisson regression with a zero intercept using the "glm" function in RStudio version 2.15.1. All models were then plotted using 'ggplot2' (Wickham, 2012).

2.4.2. The distribution of strongyle eggs at cuvette level

The distribution of eggs within the cuvette was investigated because with the CF method, when egg density is medium-high, not all eggs in the cuvette are counted, and if eggs are clumped in suspension within the cuvette this could lead to erroneous FEC results. Initially, the distribution of eggs in the cuvette was investigated by multiplying the mean to variance ratio of the number of eggs detected in each of the five traverses for each sample by the number of degrees of freedom, and comparing the result with the chi-square distribution (Bliss and Fisher, 1953; Morgan et al., 2005). Significant OD was present when $p < 0.05$. To investigate whether counting either a third (3 EPG detection limit) or a ninth (9 EPG detection limit) of the cuvette area was as accurate as counting all eggs in the cuvette (1 EPG detection limit), the total number of eggs counted in the cuvette (1 EPG) was divided by 3 and 9 and rounded to the nearest integer to give the expected (*E*) number of eggs seen in a third and a ninth of the cuvette, respectively. This was compared with the observed (*O*) number of eggs recorded when a third (3 EPG) and a ninth (9 EPG) of the cuvette was counted, using the chi-squared test (χ^2) (Equation 2.4). The relationship between the 1 EPG count and the 3 and 9 EPG counts was analysed by Poisson regression.

$$\chi^2 = \sum \frac{(\boldsymbol{O}-\boldsymbol{E})^2}{\boldsymbol{E}}$$

(Equation 2.4)

2.4.3 The effect of mixing and of sample size on the variance of strongyle faecal egg count

To examine the effect of mixing samples and the effect of sample size on the variance and OD of strongyle FEC for each set of counts from each horse, the mean and variance and CV were calculated and used to test for OD as described in Section 2.3.1. The relationship between mean and k in the unmixed and mixed samples was investigated by Poisson regression (See Section 2.4.1)

2.4.4 The effect of faecal egg count methodology on decisions for targeted treatment

The effect of FEC methodology on decisions for targeted treatment was examined for each yard and each FEC method. Thus, the yard mean EPG and variance were calculated and the NBD fitted to each dataset to gain an estimate of k (as described in Section 2.4.1). In addition, the percentage of horses exceeding the set threshold for treatment (set at 200 EPG) was calculated and a binomial test using the prop.test function in RStudio performed to investigate whether there was a significant ($p<0.05$) difference between the percentage of horses exceeding the 200 EPG threshold depending on which FEC method was used.

2.4.5 The effect of faecal egg count methodology on the outcome of anthelmintic efficacy using the faecal egg count reduction test

FECR datasets derived from the English yards (Yards 1- 11) and used in Chapter 3 were also used for this analysis. On these particular yards, screening samples on Day 0, and follow-up samples on Day 14, were collected and analysed by CF, McM15 and McM50 following the methods outlined in Section 2.3.5. For each yard, the Day 0 and Day 14 FEC derived from each FEC method were used to estimate the percentage reduction in

FEC using the World Association for the Advancement of Veterinary Parasitology (WAAVP) recommended formula (Equation 2.5).

$$\left(\frac{(Day\ 0\ FEC - Day\ 14\ FEC)}{Day\ 0\ FEC}\right) \times 100$$

(Equation 2.5)

The relationship between Mean Day 0 and Mean Day 14 FEC by treatment for McM50, McM15 and CF was investigated by Poisson regression (See Section 2.4.1)

2.5 Results

2.5.1 The distribution of strongyle eggs in faeces

Entire motions were collected from H1, H2 and H3 at approximately the same time each day for three consecutive days, with the exception of H1, which was discharged from the equine hospital on Day 2. The mixed average EPG estimated from 10 g of faeces are shown for each individual in Table 2.1. The number of boli examined from each entire motion differed on each occasion, and ranged from 19 to 30. The mean number of eggs observed from the two subsamples taken from each boli (edge and centre) ranged from 17 to 40 (Table 2.1).

Significant OD of strongyle eggs was observed between counts performed on each entire motion, confirming that eggs are clumped within faeces. Values for *k* ranged from 5.4 to 12.4 and values for CV ranged between 0.3 and 0.5 (Table 2.1). Significant OD was observed in strongyle egg counts for each bolus within an entire motion when the mixed average EPG was less than 50 EPG, suggesting that clumping occurs at bolus level when egg density is low. The values for *k* ranged between 10.6 and 97.0 and the CV ranged from 0.2 to 0.4. Significant OD was observed between the two 1 g

subsamples taken from each bolus for each horse on each day. Values for *k* ranged between 10.6 and 28.5, and the CV ranged from 0.2 to 0.4. When egg density in the faeces collected was moderate-to-high (H1 on Days 1 and 2 and in H2 and H3 on Day 3), either a 3rd or a 9th of the total cuvette volume was counted. Here, the number of eggs seen in two traverses, using the small square of the Miller eye piece to count a 3rd of the cuvette or the number of eggs seen in two traverses using the small square to count a 9th of the cuvette, were recorded as count one and count two separately. There was significant OD of strongyle eggs between the two counts at cuvette level. Values for *k* ranged between 12.7 and 25.4 and the CV ranged between 0.4 and 0.5.

The results show that in the entire motion and on days where two counts at cuvette level were made, the *p*-values and *k* were low, and the CV was greater compared to between boli and between samples. Each measure of OD was in agreement, suggesting that OD of strongyle eggs occurs throughout the entire motion and at cuvette level. The CV was greater throughout the entire motion in all horses on all days compared to between boli and between samples. On days where egg density was high and two counts of the cuvette were made, the CV was the same as in the entire motion (Figure 2.5). A Poisson regression model with *k* as the response variable and the mean number of eggs as the explanatory variable (Figure. 2.6; Table 2.2) showed that the values for *k* were significantly ($p<0.01$) lower in the entire motion compared to between boli and between samples, indicating that the greatest degree of aggregation occurred at motion level, followed by sample level, with the least aggregation observed at bolus level.

Table 2.1. Tests for over-dispersion of strongyle eggs within faeces at each level for each horse on each day where faecal egg counts were measured by the centrifugal-flotation technique

					Entire motion			Between boli			Between samples			Between counts		
Horse	Day	Mixed Av. EPG (10g)*	No. of boli examined on each occasion	Mean no. of eggs **	*p****	*K*	cv	*p****	*k*	cv	*p****	*k*	cv	*p****	*k*	cv
H1	D1	248[a]	29	40	<0.001	8.7	0.4	0.2	60.5	0.3	<0.001	25.0	0.3	<0.001	12.7	0.4
	D2	356[a]	22	39	<0.001	10.9	0.4	0.2	75.0	0.3	0.0007	28.5	0.3	<0.001	14.5	0.4
	D1	35[b]	21	27	<0.001	10.1	0.4	<0.001	11.2	0.3	0.031	11.2	0.3	****	****	****
H2	D2	18[b]	19	22	<0.001	6.3	0.5	<0.001	10.6	0.4	<0.001	10.6	0.4	****	****	****
	D3	56[c]	26	17	<0.001	7.6	0.5	0.2	97.0	0.4	0.0004	17.4	0.4	0.004	12.8	0.5
	D1	45[b]	23	29	<0.001	5.4	0.5	<0.001	14.7	0.4	<0.001	14.7	0.4	****	****	****
H3	D2	39[b]	24	23	<0.001	12.4	0.3	<0.001	47.0	0.2	0.0292	28.0	0.2	****	****	****
	D3	71[c]	30	22	<0.001	7.3	0.4	0.2	84.0	0.3	0.0003	17.8	0.3	0.0085	25.4	0.4

[*] Average eggs per gram (EPG) determined by taking a 10g well mixed subsample from the remainder of each motion, after sub-sampling as described in the methods section.
[a] multiplication factor of 9 used to calculate EPG, [b] no multiplication factor used all eggs counted, [c] multiplication factor of 3 used to calculate EPG
k – aggregation factor cv - coefficient of variance, [**]Actual number of eggs seen in the cuvette either by counting all eggs (low egg density), all eggs seen in 1/3rd of the cuvette (moderate egg density) or all eggs seen in 1/9th (high egg density) *** Variance to mean ratio x degrees of freedom and compared result with chi-square distribution to test for significance. If <0.05 there was over-dispersion, **** Egg density low so all eggs within cuvette counted so OD between counts not tested

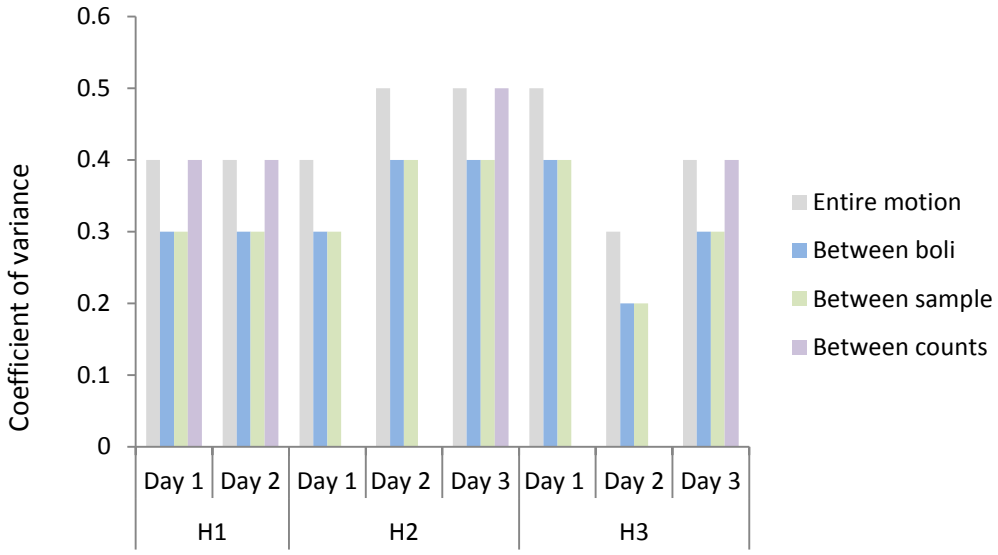


Figure 2.5. The coefficient of variation (CV) of repeated strongyle egg counts taken from the entire motion, between boli, between samples (two samples per bolus) and between counts for each horse (H1, H2 and H3) on each day as measured by centrifugal-flotation

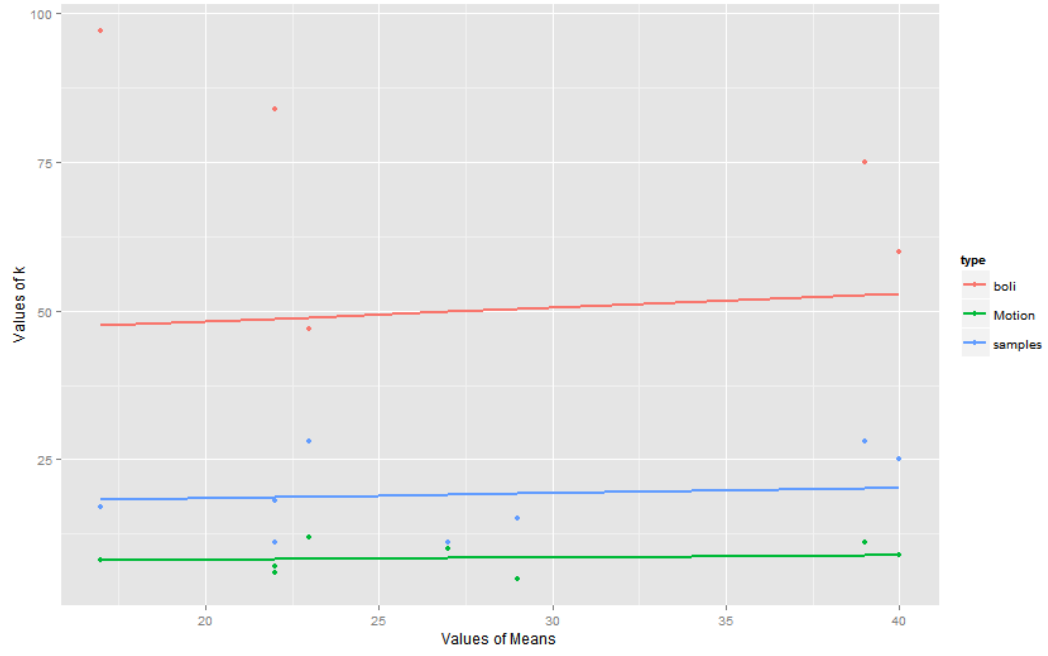


Figure 2.6. Poisson regression model with zero intercept for the mean number of eggs versus k for the entire motion, between boli and between samples

Table 2.2. Poisson regression model of the mean number of eggs versus k for the entire motion, between boli and between samples

Factor	Coefficient	Standard error	Z value	*p*	
Mean	0.004610	0.005122	0.90	0.368	Null deviance : 3342.86 on 24 df* Residual deviance: 212.39 on 20 df
Motion	3.785172	0.150206	25.20	<0.001	
Boli	2.013215	0.186461	10.80	<0.001	
Sample	2.824145	0.163089	17.32	<0.001	

*degrees of freedom

2.5.2. The performance of the centrifugal-flotation method

The number of eggs seen in each traverse (T1 to T5) in the analysis of 31 cuvettes was recorded (Table 2.3). The distribution of eggs between the five traverses for each sample was calculated to test for OD. No significant OD was observed between traverses for any of the samples. There was no significant difference between the expected number of eggs and the observed number of eggs detected for each detection limit (Table 2.4). The EPG estimates for 1, 3 and 9 EPG detection limit for each sample are shown in Figure 2.7. Poisson regression models with 3 EPG as the response variable and 1 EPG as the explanatory variable (Fig. 2.8a) and 9 EPG as the response variable (Fig 2.8b) showed that the model coefficient was smaller for 3EPG compared to 9EPG (0.005 and 0.05, respectively) suggesting that there is a larger unit increase between 1EPG and 9EPG than 3EPG.

Table 2.3. Number of eggs counted in each traverse of the cuvette (T1 – T5), for 31 independent samples. The distribution of eggs between each traverse was tested for over-dispersion (OD) by multiplying the variance to mean ratio by the number of

degrees of freedom, and comparing the result with the chi-square distribution. OD was confirmed when $p<0.05$

Sample	T1	T2	T3	T4	T5	Mean	Variance	*p*-value
1	0	0	0	0	0	0.0	0	1.00
3	0	1	2	0	0	0.6	0.8	0.25
3	1	0	0	1	1	0.6	0.3	0.096
7	3	2	2	2	3	2.4	0.3	0.97
7	7	12	15	11	6	10.2	13.7	0.25
8	15	12	19	22	13	16.2	17.7	0.36
9	14	10	14	9	9	11.2	6.7	0.70
10	12	7	9	6	15	9.8	13.7	0.23
11	14	16	17	12	20	15.8	9.2	0.68
13	28	28	26	18	19	23.8	24.2	0.40
14	18	19	25	20	27	21.8	15.7	0.58
15	28	35	26	31	29	29.8	11.7	0.80
17	37	29	36	28	18	29.6	58.3	0.069
18	32	24	30	34	34	30.8	17.2	0.69
20	30	27	28	31	30	29.2	2.7	0.98
23	46	46	48	43	47	46.0	3.5	0.99
24	58	55	49	44	42	49.6	47.3	0.43
25	59	62	63	53	54	58.2	20.7	0.84
28	71	53	58	63	65	62.0	47.0	0.60
29	63	62	65	61	79	66.0	55.0	0.50
29	78	69	88	76	83	78.8	51.7	0.62
30	74	67	85	77	70	74.6	48.3	0.62
31	74	81	101	72	87	83.0	136.5	0.16

Table 2.4. The actual number of eggs counted using 1 egg per gram (EPG) detection limit for samples 1 to 31. The expected (*E*) number of eggs at 3 EPG and 9 EPG was calculated by dividing the number of eggs seen at 1EPG detection limit by 3 by 9, respectively. For 3 EPG detection limit and 9 EPG detection limit the chi-squared test

(χ^2) was used to determine whether observed number of eggs differed significantly from that predicted (p <0.05)

Sample	Actual eggs at 1EPG	Expected (E) no. of eggs at 3EPG	Observed (O) no. of eggs at 3EPG	$\frac{(O-E)^2}{E}$	Expected (E) no. of eggs at 9EPG	Observed (O) no. of eggs at 9EPG	$\frac{(O-E)^2}{E}$
1	0	0	0	0	0	0	0
2	2	1	1	1	0	0	0
3	3	1	1	0	0	0	0
4	3	1	1	0	0	1	4
5	12	4	4	0	1	2	1
6	27	9	11	0	3	4	1
7	55	18	14	0	6	5	0
8	53	18	19	0	6	7	0
9	61	20	20	0	7	6	0
10	55	18	23	1	6	6	0
11	88	29	36	0	10	12	0
12	118	39	45	0	13	11	0
13	126	42	41	0	14	12	0
14	111	37	46	0	12	14	0
15	154	51	50	0	17	16	0
16	142	47	53	0	16	19	0
17	167	56	67	0	19	19	0
18	166	55	60	0	18	21	0
19	169	56	75	1	19	19	0
20	176	59	58	0	20	31	1
21	242	81	78	0	27	22	0
22	206	69	81	0	23	32	1
23	246	82	88	0	27	28	0
24	263	88	98	0	29	34	0
25	317	106	106	0	35	31	0
26	283	94	120	1	31	50	1
27	354	118	123	0	39	42	0
28	344	115	142	0	38	47	0
29	429	143	111	0	48	48	0
30	405	135	146	0	45	49	0
31	415	138	125	0	46	58	1
			χ^2	5		χ^2	10
			p	1		p	0.99

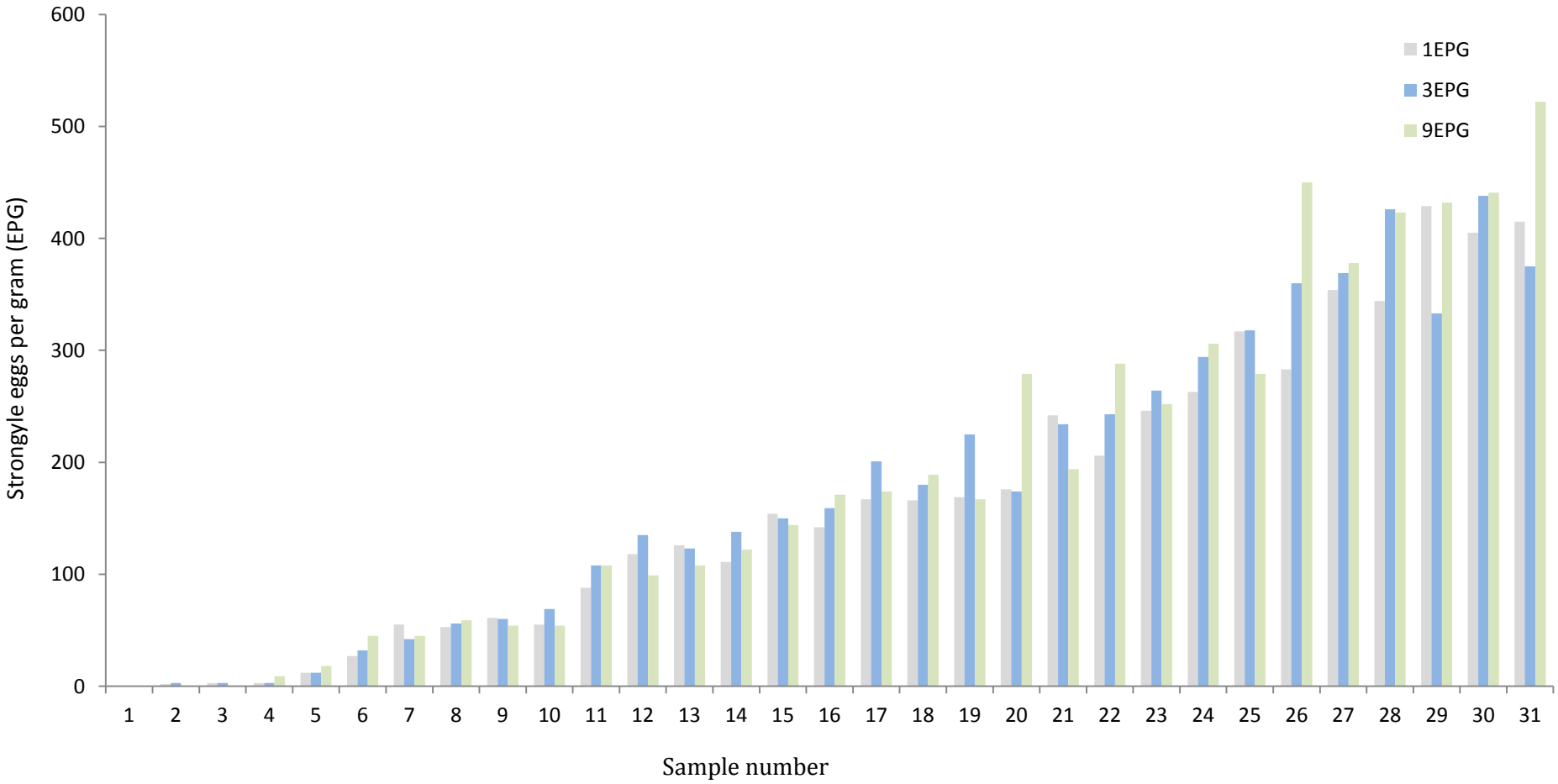


Figure 2.7. The estimated strongyle eggs per gram (EPG) when performing egg counts by the centrifugal-flotation method and applying three different egg detection limits (1 EPG, 3 EPG and 9 EPG) to each sample (1 – 31)

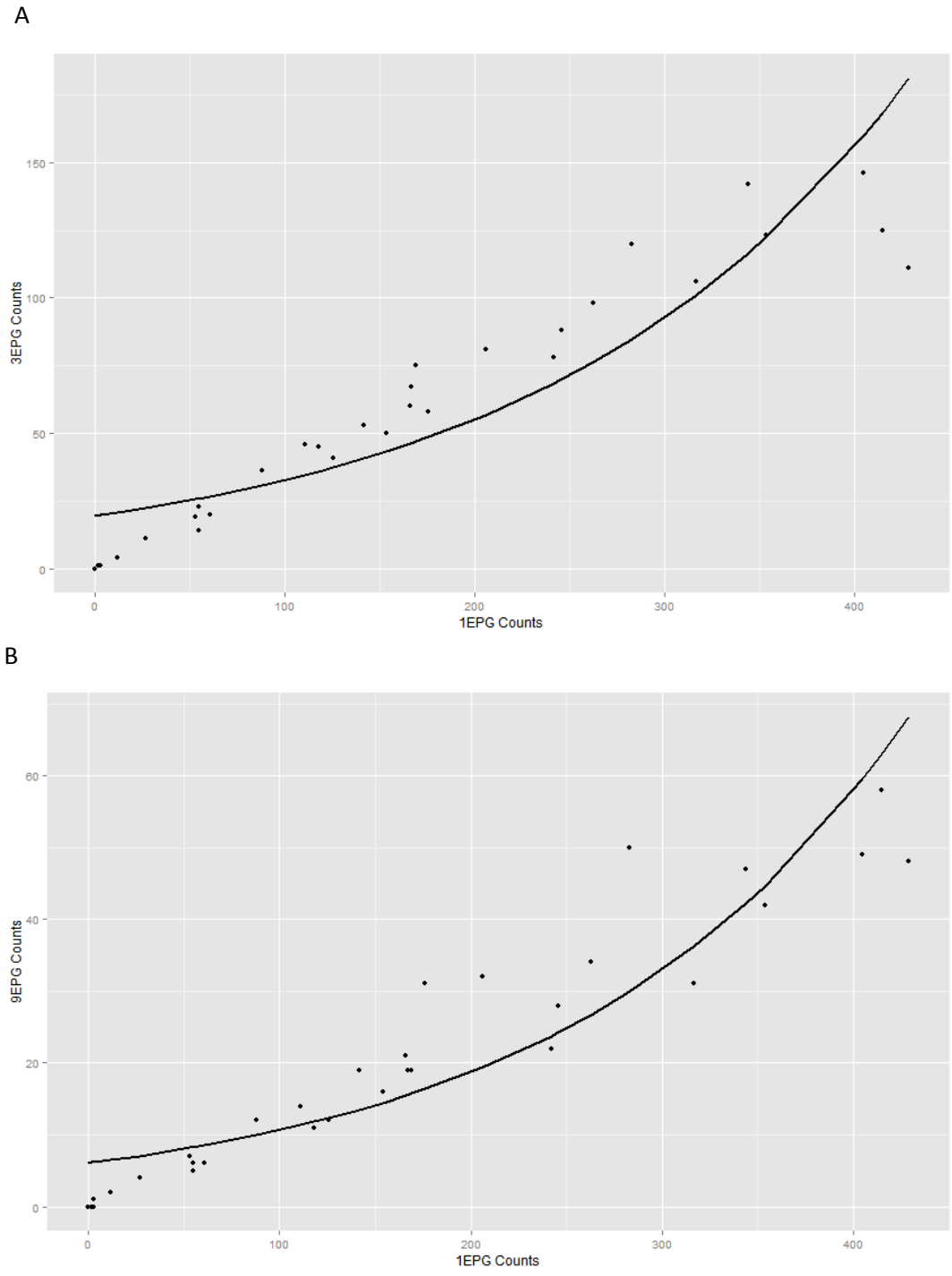


Figure 2.8. Poisson regression with zero intercept of the egg counts derived from 31 horses as measured by 1 egg per gram (EPG) detection limit *vs.* 3 EPG (A: 1EPG = 2.9684493+0.0052001 p<0.001) and 1 EPG *vs.* 9 EPG (B: 1EPG = 1.8042202 + 0.056348 *p*<0.001) using the centrifugal flotation method

2.5.3. The effect of mixing samples on variance and over-dispersion of strongyle faecal egg counts

The effect of mixing faeces before taking a subsample for FEC analysis on variance and OD of eggs in 10 subsamples taken from H4 to H7 was investigated. For the samples counted by CF, in H4 and H6, 1/3 of the total cuvette volume was counted and in H5 and H7, 1/9 of the cuvette was counted. In H4 and H6, the mean total number of eggs counted in 10 samples by CF was less than in H5 and H7 and as such, the variance was greater in H5 and H7 (Table 2.5). For all horses, in the unmixed samples, the variance and CV were greater and *k* lower compared to the mixed samples. The CV ranged between 0.24 and 0.35 in the unmixed samples, and between 0.09 and 0.20 in the mixed samples, and *k* ranged from 10.1 to 37.5 in the unmixed and 80 to 124 in the mixed samples. All measures of OD were in agreement showing that mixing reduced OD in strongyle FEC performed by CF.

Using McM, the total numbers of eggs seen either in two grids or two chambers of a McM slide were recorded (Table 2.5). The CV was greater in the unmixed samples compared to the mixed, where CV ranged from 0.34 to 0.69 and 0.16 and 0.53, respectively. Values for *k* were lower in the unmixed samples compared to the mixed samples, where *k* ranged between 3.88 to 100 and 12 to 344, respectively. In the chamber, variance was greater in the unmixed samples (range, 4 to 819) compared to the mixed samples (range, 1 to 94); the CV was greater in the unmixed compared to the mixed (0.31 to 0.40 and 0.11 to 0.39, respectively). Again, values for *k* were lower in the unmixed samples when compared to the mixed samples and ranged from 7.96 to 348 and 38 to 961, respectively.

These results demonstrate that mixing reduces the OD of strongyle eggs using each counting method. The lowest CV values were observed in the counts performed by CF

compared to McM, and within McM, the lowest CV values were recorded for counts made when the whole chamber areas were counted (Figure 2.9).

Table 2.5. The effect of mixing prior to taking subsamples from entire motions collected from four horses on the mean, variance, coefficient of variation (CV) and over-dispersion of strongyle eggs when faecal egg counts were performed by either centrifugal-flotation (CF) or McMaster (McM)

			Unmixed					Mixed				
		Horse	Mean	Variance	CV	*p*	*k*	Mean	Variance	CV	*P*	*k*
CF	Total eggs observed in cuvette	H4[a]	31	127	0.35	<0.001	12.2	42	69	0.20	<0.001	80
		H5[b]	52	284	0.32	<0.001	10.1	63	80	0.14	<0.001	316
		H6[a]	28	52	0.26	<0.001	37.5	25	16	0.15	<0.001	926
		H7[b]	61	207	0.24	<0.001	21.8	69	36	0.09	<0.001	1245
McM	No of eggs in grid	H4	4	5	0.64	0.16	9.70	6	9	0.53	0.11	12
		H5	24	184	0.56	<0.001	3.88	32	27	0.16	0.53	320
		H6	2	2	0.68	0.59	100	2	1	0.40	0.91	332
		H7	20	47	0.34	0.01	17.8	20	10	0.16	0.83	344
	No of eggs in chamber	H4	13	19	0.32	0.18	55.5	9	12	0.39	0.21	38
		H5	71	819	0.40	<0.001	7.96	55	94	0.18	0.07	93
		H6	5	4	0.38	0.66	348	5	1	0.15	0.1	360
		H7	66	431	0.31	<0.001	13.4	42	20	0.11	0.91	961

[a] 1/3rd of the cuvette was counted
[b] 1/9th of the cuvette was counted

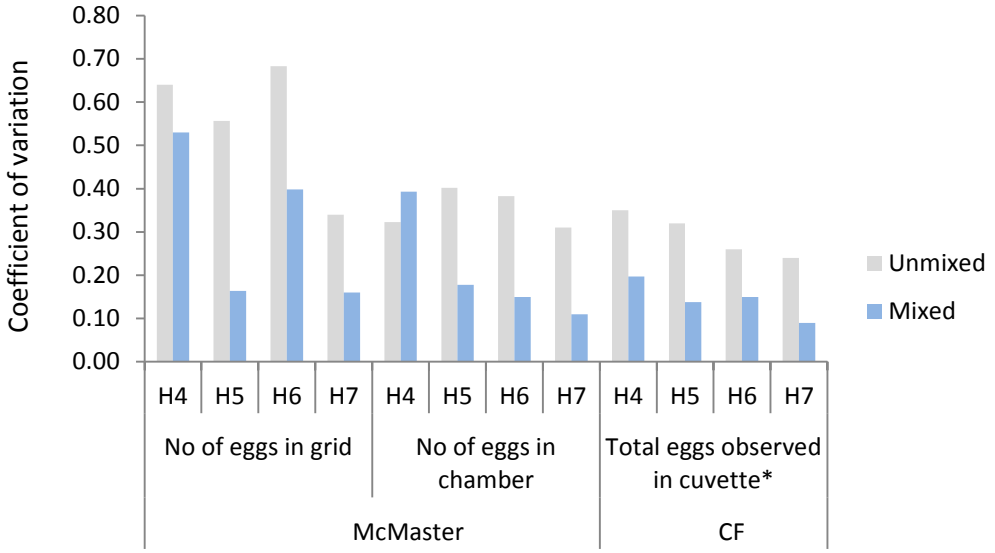


Figure 2.9. The effect of mixing faeces on the coefficient of variation (CV) between 10 subsamples performed by McMaster and centrifugal-flotation (CF) methods on faecal samples collected from horses H4 to H7

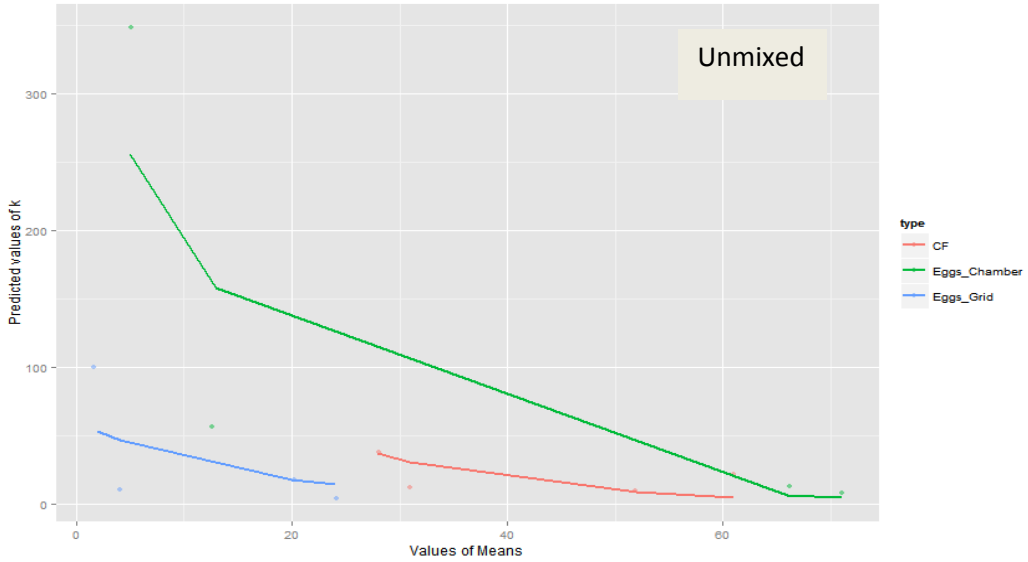
The Poisson regression models for unmixed and mixed samples (Figure 2.10, Table 2.6) revealed that mixing reduced OD. In the unmixed samples, as mean increased, *k* decreased indicating increased OD. In contrast, in the mixed samples, as mean increased, *k* increased indicating reduced OD. In the unmixed samples, the greatest OD was observed with the CF method compared to the mixed samples, where the greatest OD was observed in the grid.

Table 2.6. Poisson regression model for unmixed and mixed samples

		Coefficients	Standard Error	Z value	*p*	
Unmixed	Intercept	5.28600	0.17323	30.515	<0.001	Null deviance: 1144.27 on 11 df* Residual deviance: 256.56 on 8 df
	CF	-0.05978	0.00395	-15.134	<0.001	
	Eggs in Chamber	0.55494	0.15363	3.612	<0.05	
	Eggs in Grid	-1.19864	0.17472	-6.861	<0.001	
Mixed	Intercept	0.0093989	0.0008092	11.61	<0.001	Null deviance: 54887.6 on 12 df Residual deviance: 3328.6 on 8 df
	CF	5.9834045	0.0468663	127.67	<0.001	
	Eggs in Chamber	5.6135021	0.0368771	152.22	<0.001	
	Eggs in Grid	5.3821551	0.0341615	157.55	<0.001	

*df = degrees of freedom

A.



B.

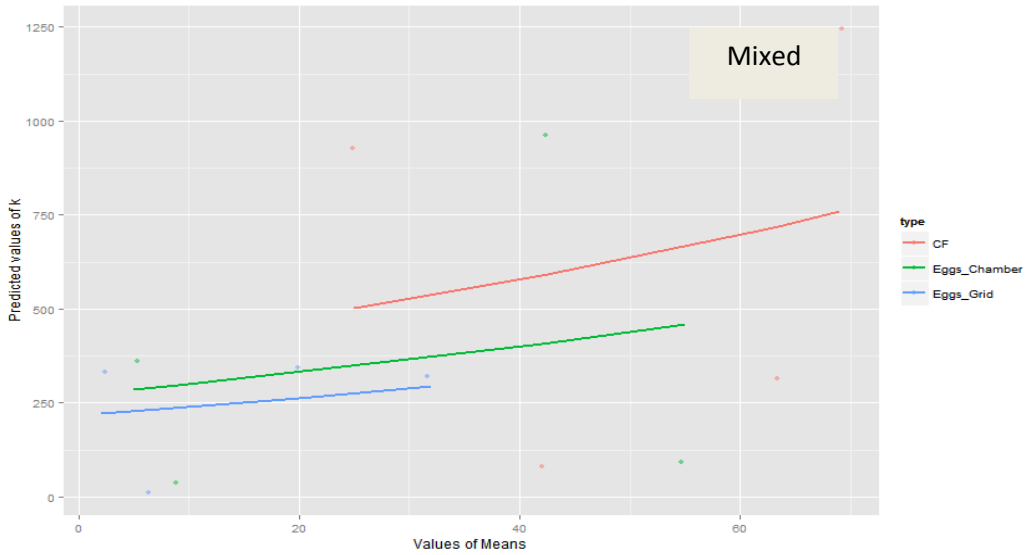


Figure 2.10. Poisson regression with zero intercept for the mean and predicted values of k for Centrifugal Flotation (CF) and McMaster faecal egg counting techniques (Number of eggs in the chamber and the grid) when samples were unmixed (A) and mixed (B)

2.5.4. Effect of subsample size on variance and over-dispersion of strongyle eggs

The effect of three different subsample sizes (7 g, 10 g and 15 g) on the mean, variance, CV and OD of eggs seen was calculated from FEC performed using McM. The variance and CV decreased and *p*-values and *k* were higher in the mixed samples compared to the unmixed subsamples from each horse and for each subsample size in both the grid and the chamber (Table 2.7). The CV was lower when the whole chamber was counted compared to the grid only (Fig 2.11). This was observed over all subsample sizes. No significant OD was observed in either the mixed or unmixed 7 g subsamples in the grid or the chamber. In the sample from H10, OD was detected in 10 g unmixed subsamples in the chamber, and in H9 and H10, OD was observed in 15 g unmixed subsamples in the chamber. No significant OD was detected between any of the mixed subsamples. Increasing subsample size reduced CV in the unmixed subsamples from H8 in the grid, and for all unmixed subsamples in the chamber. With the mixed subsamples, CV decreased with sample size in the samples collected from H9 in the grid and for H8 and H9 in the chamber. In total, CV was reduced by increasing sample size in 7 out of 12 subsamples.

The Poisson regression models of the mean number of eggs versus k for 7, 10 and 15 g subsamples taken from unmixed and mixed faeces (Table 2.8, Figure 2.12) showed that in both unmixed and mixed samples, *k* increased with the mean. In the unmixed samples, values for k were lower in 10 g subsamples compared to 7 and 15 g subsamples, indicating that increasing sample size in unmixed samples does not reduce OD. In the mixed samples, the mean and k were lower for the 7 g subsamples compared to 10 and 15 g subsamples.

Table 2.7. Effect of sample size on variance, coefficient of variation (CV) and over-dispersion of strongyle eggs between faecal samples collected from horses H8 to H10 when performing faecal egg counts by the McMaster technique by counting the number of eggs in both grids of the McMaster slide or both chambers of the McMaster slide

			Unmixed					Mixed				
			Mean	Variance	CV	*p*	*k*	Mean	Variance	CV	*p*	*k*
7 g	No of eggs in grids	H8	21	37	0.29	0.17	121	23	16	0.18	0.49	343
		H9	5	14	0.81	0.05	4.69	8	14	0.45	0.18	41.8
		H10	21	28	0.25	0.26	186	18	1	0.06	0.95	355
	No of eggs in chambers	H8	64	149	0.19	0.1	101	68	56	0.11	0.44	360
		H9	25	70	0.34	0.06	22.4	23	34	0.26	0.23	120
		H10	63	36	0.1	0.64	1110	62	1	0.02	0.98	1305
10 g	No of eggs in grids	H8	25	54	0.29	0.12	51.5	20	2	0.08	0.89	357
		H9	7	8	0.43	0.29	161	8	2	0.2	0.74	343
		H10	16	30	0.35	0.14	49.7	17	4	0.12	0.79	352
	No of eggs in chambers	H8	72	193	0.19	0.07	80.6	68	16	0.06	0.79	1325
		H9	25	9	0.12	0.07	356	21	4	0.1	0.83	356
		H10	54	400	0.37	<0.001	12.9	59	2	0.03	0.96	1246
15 g	No of eggs in grids	H8	24	39	0.26	0.2	101	20	32	0.25	0.19	157
		H9	5	13	0.72	0.07	6.65	10	4	0.2	0.67	344
		H10	27	148	0.45	0.004	7.76	16	3	0.11	0.83	352
	No of eggs in chambers	H8	75	181	0.18	0.09	107	70	17	0.06	0.78	1336
		H9	19	0	0	1	357	27	0	0.02	0.99	365
		H10	80	52	0.09	0.52	1282	55	10	0.06	0.83	1077

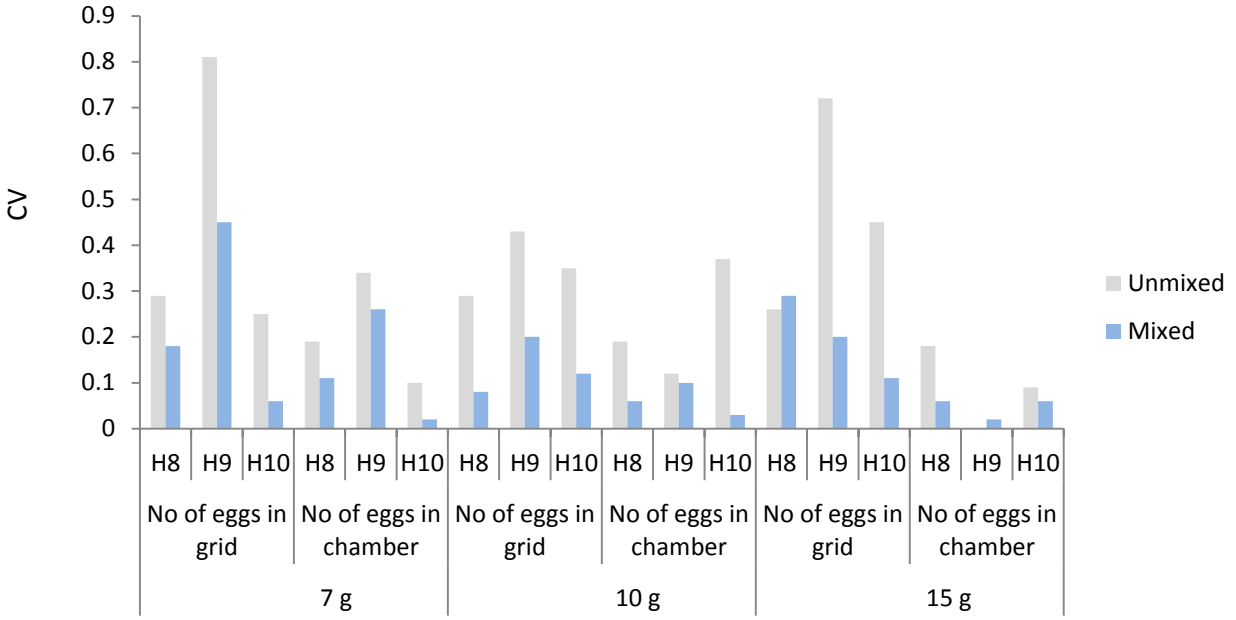


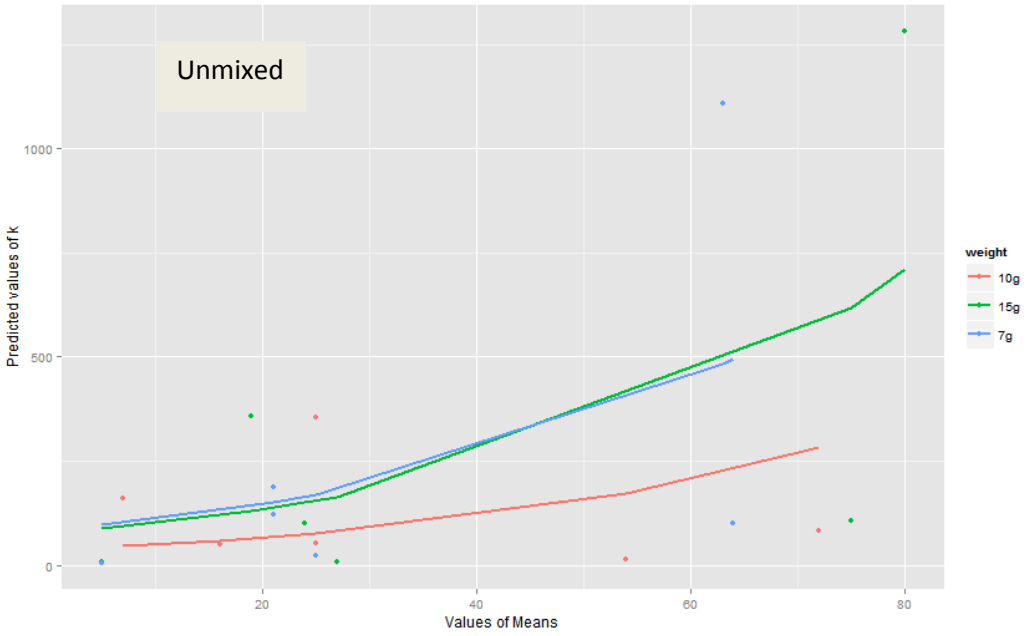
Figure 2.11. Effect of sample size and mixing on the coefficient of variation (CV) between faecal egg counts (FEC) performed by the McMaster method when the number of eggs in two grids or the number of eggs in both chambers of the McMaster slide were enumerated for faecal samples collected from horses H8 to H10

Table 2.8 Poisson regression model with zero intercept for the mean number of eggs and predicted values of k for 7, 10 or 15g subsamples using the McMaster faecal egg counting technique when samples were unmixed and mixed

	Intercept	Coefficients	Standard Error	Z value	*p*	
Unmixed	Mean	3.6437501	0.0489210	74.78	<0.001	Null deviance: 4389.6 on 18 df* Residual deviance: 4290.4 on 14 df
	7g	0.0277752	0.0006468	42.94	<0.001	
	10g	0.7007891	0.0447970	15.64	<0.001	
	15g	0.7864260	0.0452819	17.37	<0.001	
Mixed	Mean	0.0277752	0.0006468	42.94	<0.001	Null deviance: 6923.7 on 17 df Residual deviance: 4290.4 on 14 df
	7g	3.6437501	0.0489210	74.48	<0.001	
	10g	4.3445392	0.0459458	94.56	<0.001	
	15g	4.4301761	0.0398336	111.22	<0.001	

*degrees of freedom

A.



B.

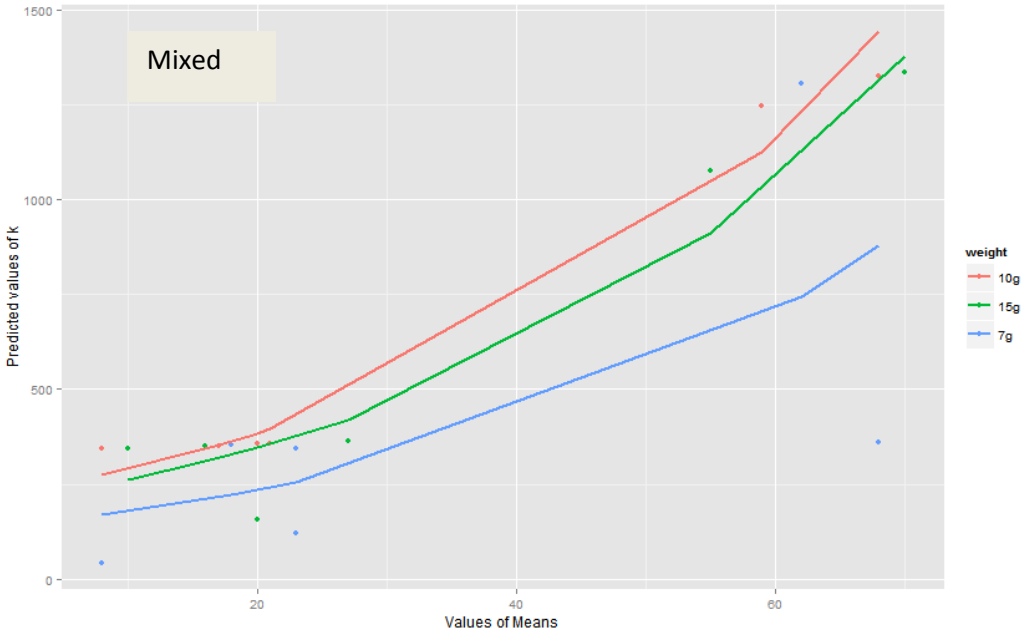


Figure 2.12. Poisson regression with zero intercept for the mean number of eggs and predicted values of k for 7, 10 or 15g subsamples using the McMaster faecal egg counting technique when samples were unmixed (A) and mixed (B)

2.5.5. The effect of FEC methodology on decisions for targeted treatment

The impact of FEC method on the mean, variance, *k*, number of horses and percentage of horses with the FEC of ≥200 EPG was recorded for Yards 1 to 13 (Table 2.9). The number of horses tested on each yard ranged from 12 to 43 individuals. The mean EPG across the yards was 166 EPG (range = 32 - 777 EPG) when FEC analysis was performed by McM, compared to 94 (range = 19 - 482 EPG) by CF. The variance between horses within yards was greater in FEC performed by McM when compared to CF. Distribution (*k*) of FEC between horses within yards, differed between method, where values for *k* were lower on average for FEC performed by McM compared to CF, (0.07; range = 0.03 – 0.17 and 0.19; range 0.06 – 0.38, respectively). The Poisson regression model of group mean EPG versus *k* showed that *k* was significantly (p=0.026) lower when McM was used (Figure 2.13).

These factors had a bearing on the number and percentage of horses meeting a ≥200 EPG threshold set for anthelmintic treatment. On average, 58 (22%) horses would have required anthelmintic treatment when the McM method was used to estimate FEC compared to 31 (11%) horses when FEC were performed by the CF method. On 7/12 yards, significantly more horses met the threshold for treatment when McM was used compared to CF (Table 2.9). Overall, significantly more (p<0.001) horses required treatment when FEC were performed by McM compared to CF.

Table 2.9. The effect of faecal egg count (FEC) method (McMaster or Centrifugal-flotation) on the mean, variance and distribution of FEC between horses on each yard (*k)* on the number of horses with a FEC of more than 200 eggs per gram (EPG) and the percentage of horses with a FEC of more than 200 EPG from faecal samples collected from 13 different yards. A binomial test to compare two proportions was applied to test for significance (p<0.05) between the percentage of horses meeting the 200 EPG threshold for treatment depending on which FEC method was used

	McMaster						Centrifugal-flotation					
Yard	*n*	Mean EPG	Variance	*k*	no. > 200 EPG	% > 200 EPG	Mean EPG	Variance	*k*	no. > 200 EPG	% > 200 EPG	*p*
1	18	175	105956	0.09	6	33	79	24228	0.26	2	11	0.002
2	22	32	8701	0.03	2	9	32	7555	0.1	2	9	1.000
3	17	269	394453	0.05	6	28	132	95183	0.2	3	17	0.015
4	22	120	200157	0.03	2	9	61	36733	0.06	1	5	0.124
5	43	256	115977	0.17	17	55	114	22307	0.38	10	23	0.0004
6	25	128	104808	0.05	4	16	84	42662	0.14	3	12	0.285
7	15	777	1037810	0.14	8	53	482	472264	0.23	7	47	0.744
8	31	29	4962	0.03	3	10	20	1804	0.1	0	0	<0.001
10	21	88	25726	0.10	4	19	57	11606	0.12	3	14	0.344
11	31	28	4828	0.03	3	10	20	1755	0.15	0	0	<0.001
12	12	42	6288	0.05	2	16	19	1124	0.14	0	0	0.008
13	16	53	6156	0.08	1	6	23	888	0.34	0	0	0.053
Totals	273	*166	*167985	*0.07	58	*22	*94	*59842	*0.185	31	*11	<0.001

* Average

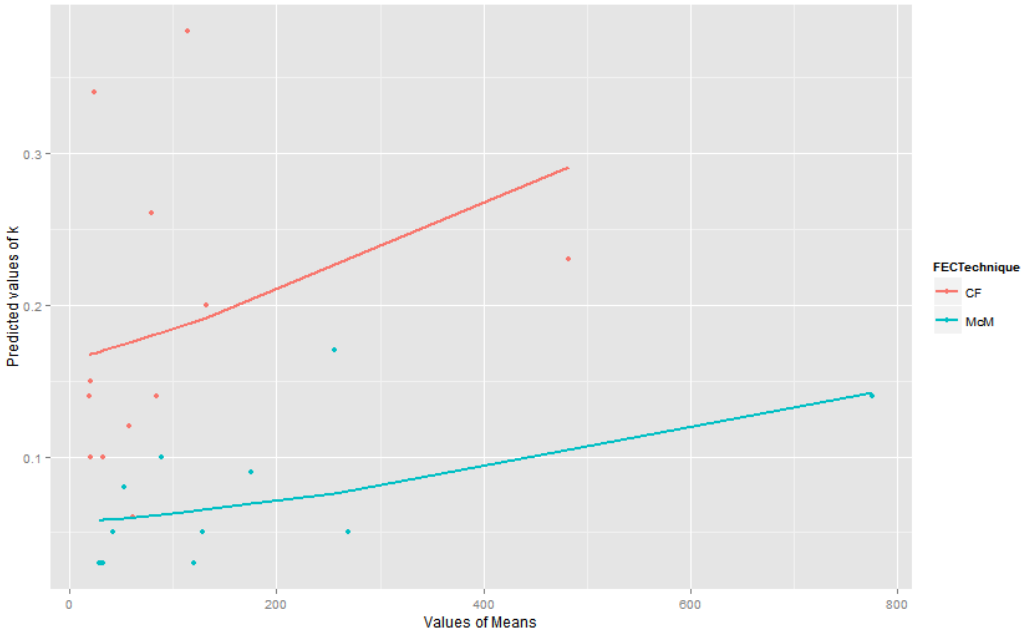


Figure 2.13. Poisson regression with a zero intercept of Mean eggs per gram (EPG) and k for faecal egg counts performed on 13 groups of horses by the centrifugal flotation method (CF: Model, k = 0.001190 + -1.811762) and the McMaster method (McM: Model, k = 0.00190 + -2.881568)

2.5.6. The effect of faecal egg count methodology on the outcome of tests of anthelmintic efficacy against strongyles

A total of 33 FECRT were conducted on 205 individual horses between March and December 2012. MOX FECRT were conducted on 6 yards (*n* = 43 horses); IVM on 8 yards (*n* = 56 horses); PYR on 10 yards (*n* = 43 horses) and FBZ on 9 yards (*n* = 63 horses) (Table 2.10). Faecal samples were collected from all horses on Day 0 prior to anthelmintic administration and on Day 14. Each sample was analysed by the McM method using two different egg detection limits (50 and 15 EPG) and by the CF method, and the percentage reduction in FEC calculated from the EPG estimates derived from each FEC method (see methods Section). All of the MOX FECRT gave a yard mean average of 100% strongyle egg reduction, regardless of FEC method, and

the strongyle population on each yard was classified as sensitive. With the IVM FECRT, a mean faecal egg count reduction (FECR) of >90% was obtained at all sites; however, the average yard FECR differed depending on FEC method. For FEC analysed by McM50, the estimated FECR ranged from 95.9% to 100%; for McM15 this ranged from 95.7% to 100% and for CF FECR, this value was between 99.9% and 100%. The PYR FECRT yielded different results regarding the classification of resistance status between FEC methods on two yards. On Yard 3, the outcome of the PYR FECRT, where FEC were analysed by McM50 was classified as resistant (FECR = 89%), McM15 was classified as susceptible (FECR = 92%) and CF was classified as resistant (FECR = 87%). On Yard 5, the outcome of the PYR FECRT was classified as resistant when McM50 was used to determine FEC (FECR = 86%), and susceptible when McM15 and CF were used (FECR = 98% and 99%, respectively). For yards where FBZ was tested, all FEC methods classified the resident strongyle populations as FBZ-resistant (Fig. 2.14).

Generally, the mean pre- and post-treatment EPG was higher for FEC performed my McM50 compared to McM15, and both McM detection limits gave higher mean EPG compared to the CF method. For faeces collected at Day 14 after PYR treatment, when eggs were detected, the mean EPG was higher using McM50 methodology compared to McM15 methodology and, again, both McM methods calculated a greater mean Day 14 EPG estimate compared to the CF method. On three occasions, the McM50 method failed to detect eggs in samples in which eggs were detected using the CF method and on two occasions the McM15 method failed to detect eggs in samples in which eggs were detected using the CF method. This had a bearing on the outcome of the FECRT: where the mean Day 14 FEC was 0 for both McM methods, the calculated FECR was 100%, while the corresponding FECR calculated using the CF method were below 100%.

The Poisson regression models for Mean Day 0 as the explanatory variable and Mean Day 14 as the response variable by anthelmintic for each FEC method show that there is no relationship between Day 0 mean and Day 14 mean when IVM or MOX are used regardless of the FEC method used (Figure 2.15 a-c). However, there was a significant positive relationship between mean Day 0 FEC and mean Day 14 when PYR ($p<0.001$) and FBZ ($p<0.001$) were used for all three FEC methods (Figure 2.15 a-c) and as the Mean Day 0 FEC increased, the mean Day 14 increased, leading to lower estimates of efficacy.

Table 2.10. The effect of faecal egg count (FEC) method (McMaster (McM) with an egg detection limit of 50 eggs per gram (EPG) (McM50), and 15 EPG (McM15) and by centrifugal-flotation (CF) on the outcome of estimates of faecal egg count reduction (FECR; % efficacy) derived from yard mean Day 0 EPG and yard mean Day 14 EPG from samples collected from horses on Yards 1 to 11. Anthelmintics moxidectin (MOX), ivermectin (IVM), pyrantel (PYR) and fenbendazole (FBZ) were administered on each yard on separate occasions. On each occasion, horses on the same yard received the same anthelmintic. For MOX and IVM, the threshold set for anthelmintic susceptibility was a FECR of >95%. For PYR and FBZ, the threshold was >90%. Results that were classified as resistant are highlighted

		McM50			McM15			CF		
	Yard	Mean Day 0 EPG	Mean Day 14 EPG	% Efficacy	Mean Day 0 EPG	Mean Day 14 EPG	% Efficacy	Mean Day 0 EPG	Mean Day 14	% Efficacy
MOX	1	386	0	100	335	0	100	344	0	100
	2	620	0	100	477	0	100	449	0	100
	4	1064	0	100	744	0	100	318	0	100
	7	1389	0	100	962	0	100	369	0	100
	8	358	0	100	240	0	100	224	0	100
	10	330	0	100	216	0	100	260	0	100
IVM	1	616	10	98.0	541	6.0	98.9	445	0.0	100
	3	462	4	99.2	251	4.6	98.2	345	0.2	99.9
	4	1188	0	100	1039	0.0	100	357	0.0	100
	5	306	13	95.9	264	11.3	95.7	177	0.0	100
	6	314	0	100	230	0.0	100	93	0.0	100
	7	2469	0	100	2040	0.0	100	651	0.0	100
	8	1117	0	100	725	0.0	100	296	0.0	100
	10	1140	0	100	927	0.0	100	344	0.0	100
PYR	1	986	0	100	836	0	100	227	8	97
	2	350	0	100	435	0	100	183	3	98.4
	3	1183	125	89	917	73	92	238	31	87
	4	1050	100	90.5	1025	90	91.2	310	31	90.0
	5	1250	0	100	880	5	99.4	419	8	98.2
	7	1900	88	95.4	1579	64	96.0	484	19	96.1
	8	920	50	97.8	753	15	98.0	242	5	98.0
	9	553	13	86	523	9	98	319	5	99
	10	625	50	92	626	26	95.8	229	11	95.4
	11	793	57	92	663	30	94	374	17	95
FBZ	1	1263	863	31.7	1091	726	30.8	324	209	57.2
	2	450	410	8.9	396	312	21.2	251	106	57.9
	4	742	442	40.4	653	413	36.8	365	261	28.5
	5	713	925	-29.8	439	791	-80.3	461	218	52.7
	7	533	508	4.7	435	355	18.4	263	228	13.1
	8	1080	950	12.0	930	795	6.7	255	264	-3.3
	9	867	497	42.6	743	400	46.2	512	263	48.6
	10	913	688	25	724	521	28	216	106	51
	11	957	593	38	748	441	41	426	304	29

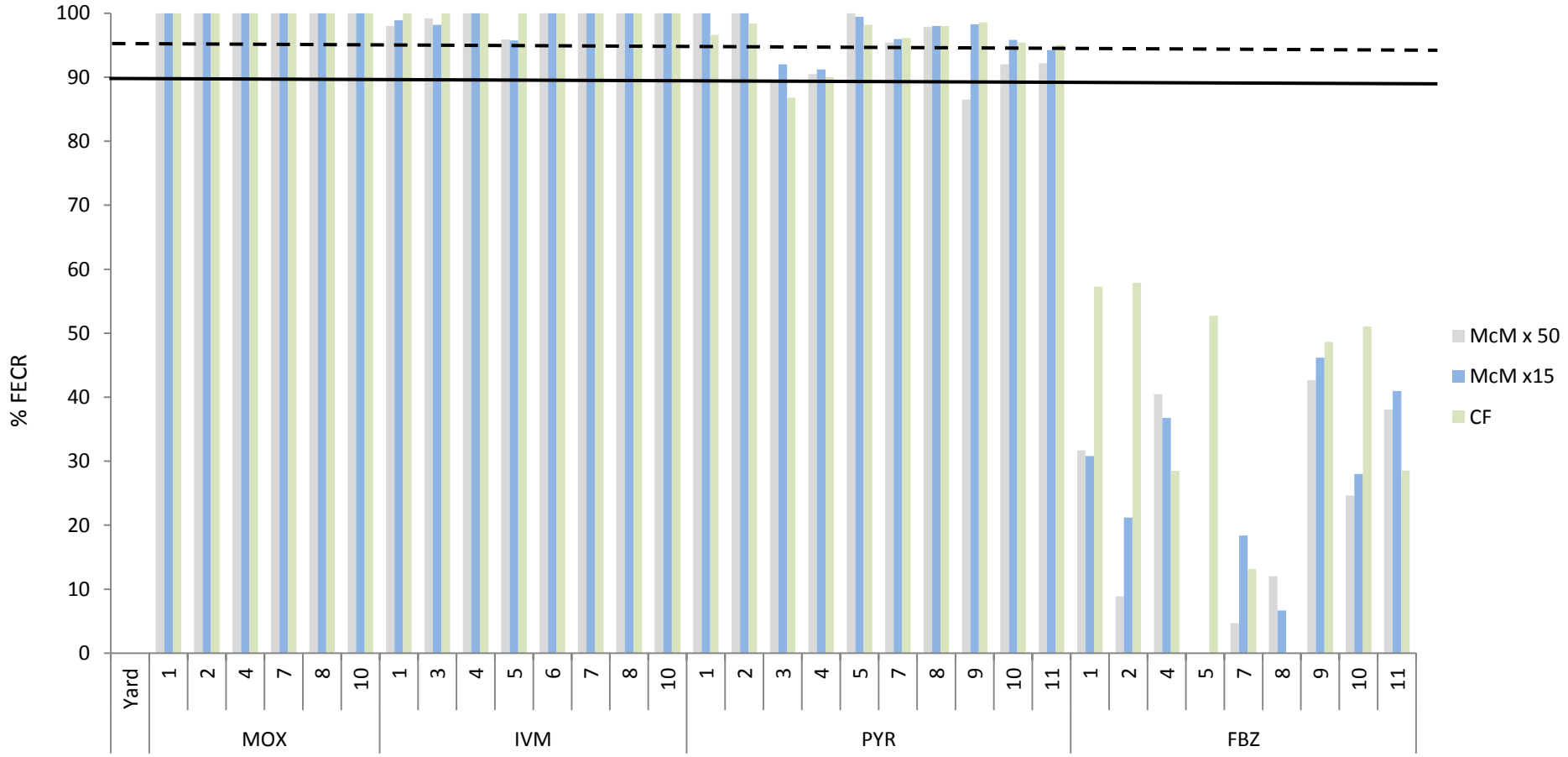


Figure 2.14. The effect of faecal egg count method (McMaster (McM) egg detection limit 50 eggs per gram (EPG) (McM50), McM egg detection limit 15 EPG) or centrifugal-flotation)) on the interpretation of faecal egg count reduction tests (FECRT) (% faecal egg count reduction (FECR)) conducted using moxidectin (MOX), ivermectin (IVM), pyrantel embonate (PYR) and fenbendazole (FBZ) on Yards 1 to 11. The threshold for anthelmintic susceptibility for MOX and IVM was >95% as shown by the dashed line and for PYR and FBZ; the threshold for anthelmintic susceptibility was > 90% as represented by the solid black line

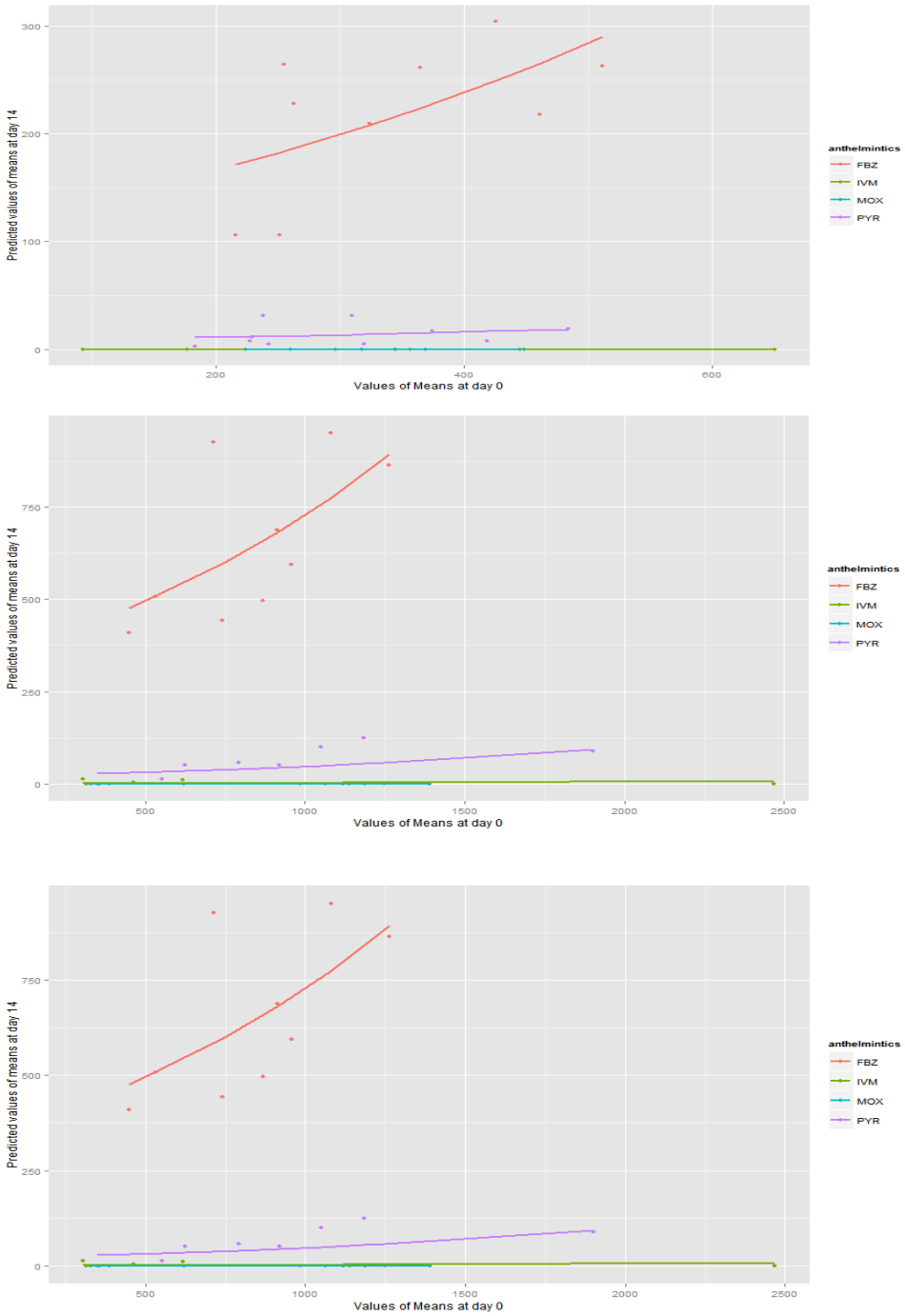


Figure 2.15. Poisson regression with a zero intercept for the Mean Day 0 faecal egg count (FEC) versus the Mean Day 14 FEC when FEC were performed by A. centrifugal flotation (CF), B. McMaster with an egg detection limit of 15 eggs per gram (EPG) and C. McMaster with an egg detection limit of 50 EPG for each anthelmintic (fenbendazole, FBZ; ivermectin, IVM; moxidectin, MOX and pyrantel, PYR)

2.6 Discussion

A single FEC is often used as a basis for making decisions on whether or not to administer anthelmintic to horses (Uhlinger, 1993). If FEC are to form the foundation of evidence-based helminth control, there is a need to ensure that related methodologies and procedures, including pre-analytic factors such as sample collection and storage are optimised. Despite the limitations of FEC analysis, it is currently the only tool available for implementing targeted anthelmintic treatments and for routine monitoring of efficacy (Nielsen et al, 2014; Matthews, 2014). Here, it was demonstrated that strongyle eggs were over-dispersed in horse faeces. Thorough mixing prior to sub-sampling reduced variance and over-dispersion of eggs and the use of a FEC method with a lower egg dl reduced variance between samples/tests. The FEC method used influenced decisions for anthelmintic treatment, with more horses exceeding a 200 EPG threshold when FEC were analysed by the MCM50 method compared to the more sensitive CF method. When determining anthelmintic efficacy, different assumptions were made depending on the FEC method used. When efficacy was high (IVM & MOX) or when it was very low (FBZ), all FEC methods generated the same assumptions, but when anthelmintic efficacy was around the accepted threshold, then the FEC method implemented had an impact and disagreements between classifications were found.

The results presented here clearly show that strongyle eggs are clumped within faeces to varying degrees at entire motion level, bolus level, sample level and cuvette level. The most important level was the entire motion as OD seen at each level contributed to the OD seen throughout the entire motion. However, estimated values for k at each level were >5.4 indicating a Poisson distribution. As k increases, the variance approaches the mean and the distribution approaches the Poisson distribution (Bolker,

2008). The level of OD of eggs within faeces was low but accumulates at several levels leading to supra-Poisson variation. Hence, effective mixing reduces variance arising from multiple levels of low OD within faecal boluses and entire motions. Denwood et al. (2012) investigated sources of variability arising in equine FEC analysis and found that the major sources of variation could be attributed to the aggregation of eggs within faeces and variations in egg concentration between entire faecal motions. The greatest amount of variance could be explained between samples taken from the same animal, but they reported that taking a larger sample and mixing it thoroughly could reduce this variance. These findings are in agreement with the results obtained in the present study, where mixing prior to taking a subsample for analysis reduced variance and OD in egg counts carried out by McM and CF methods. Another study found that mixing composite sheep faecal samples reduced OD; however, the faecal samples were collected from different individuals showing a higher level of OD compared to the individual horse samples analysed in this study (Morgan et al., 2005). A study looking at the distribution of *Ascaris lumbricoides* eggs in human faeces found that mixing faeces prior to taking a subsample for analysis reduced variance in observed FEC, and found that when a smaller amount of faeces was examined, a greater degree of error was observed (Sinniah, 1982). Another, more recent study looking at the distribution of *Shistosoma mansoni* and hookworm eggs in human stool samples found that mixing samples reduced intra-sample variance (Krauth et al., 2012). Conversely, a study examining the distribution of strongyle and *Parascaris equorum* eggs in horse faeces using a combined sedimentation-flotation method found the distribution of these eggs to be randomly distributed, and found that mixing did not improve reproducibility of results (Kuhnert-Paul et al., 2012). A likely reason for the latter observations is that the researchers examined three, 10 g subsamples per entire motion, compared to the present study, where 1 g subsamples were analysed from multiple boli. The large

amount of faeces analysed would therefore account for lower variance, as it has been demonstrated that increasing the amount of faeces examined significantly reduces sample variance (Denwood et al., 2012). In effect, by taking larger subsamples, OD within those subsamples is eliminated by mixing during the FEC process.

It was observed that, when multiplication factors were used (i.e. when egg density was higher), OD was observed at cuvette level. This is likely to be due to eggs not being randomly distributed once in the cuvette. It has been documented that helminth larvae clump in suspension if not well mixed (Schnyder et al., 2011) and, even though the suspension may have been thoroughly mixed prior to being placed in the cuvette, a degree of clumping may have occurred. To test this, the distribution of eggs at cuvette level was investigated. The results found that the eggs were randomly distributed at cuvette level, and no significant OD was observed in the number of eggs seen in each traverse of the cuvette. In terms of the number of eggs seen when counting a third or a ninth of the cuvette, there was no significant difference between the expected and observed egg counts. The Poisson regression models suggest that there was a significant positive relationship between the 1 EPG and 3 EPG and 9 EPG detection limits. As a larger number of eggs are counted, and a multiplication factor used, variance will be inflated (Torgerson et al., 2012). Alternatively, due to the high egg density, errors in counting may occur, the likelihood being that not all eggs are counted, so a lower EPG is estimated compared to when a third or a ninth of the cuvette is counted. In the study conducted by Denwood et al. (2012), the authors concluded that the McM method was not associated with high level of variance, and overall, gave a low CV. However, they counted between 6 and 10 McM chambers per sample. With more independent counts made and a lower detection limit used, the variance would become smoothed and therefore lower. As Morgan et al. (2005) point out, when counting nematode eggs in sheep faeces, the increased precision gained by

filling and counting more slides if offset by increased effort and diminishing returns if more than four chambers are counted. However, in practice, technicians are unlikely to count more than one slide, but reducing variance and increasing diagnostic sensitivity they can be achieved by taking a subsample from a well mixed sample and counting the number of eggs in two chambers opposed to the grids alone.

Variation between replicate samples using the McM method has been observed (Stear et al., 2006). Mes (2003) found that variance was larger in FEC performed by McM compared to a sugar-salt flotation method. In the present study, 3 g of faeces were used for the McM method compared to 10 g in the CF method. Three grams of faeces is the most commonly used amount when using the McM method (MAFF, 1986). However, a number of modifications of the McM method exist. Two recent studies have attempted to determine which of these is the most accurate and reliable (Pereckiene et al., 2007; Vadlejch et al., 2011). Vadlejch et al. (2011) evaluated seven different McM methods by comparing the results of estimated EPG of *Ascaris suum* eggs in 30 pig faecal samples, while the study by Pereckiene et al. (2007) compared the reliability of three methods by preparing sheep faeces that was artificially spiked with *Teladorsagia circumcincta* eggs at three different concentrations (low (20 EPG), intermediate (50 – 200 EPG) and high (500 EPG)). For each concentration, 30 replicates were analysed and the ratio of samples that detected eggs within both a ±10% and ±20% tolerance limit was calculated. Both studies found that McMaster adaptations that use a larger amount of faeces and a lower dilution ratio (i.e. g of faeces/ml of water) and subsequently, a lower multiplication factor when converting the number of eggs seen into an estimation of EPG, give more reliable results and better sensitivity. When small amounts of faeces are examined, the increased multiplication factor renders the EPG estimate less precise (Cringoli et al., 2004; Mes, 2003; Vadlejch et al., 2011). In the present study, the sample size from which the subsample is taken for analysis was observed to impact variance in

FEC in mixed subsamples but not in unmixed subsamples in this study in agreement with results from other studies. These results indicate that taking a larger mixed subsample will decrease OD but in unmixed samples, the degree of OD will not be reduced by increasing sample size.

Previously, FEC derived by the FLOTAC method (which has a 1 EPG detection limit) have been shown to give empirically lower variability compared to McM methods (Presland et al., 2005; Schnyder et al., 2011). Whilst the CF method employed here differs from the FLOTAC method, both offer greater analytical sensitivity. Both utilise more faeces per test and use lower multiplication factors. In agreement with this, it has been observed that when the multiplication factor is low, there is a substantial reduction in errors associated with EPG (Torgerson et al., 2012).

Screening faecal samples analysed by the McM50 method gave consistently higher estimations of the group mean and greater variance compared to the CF method, and gave consistently lower values for *k* indicating greater apparent OD. This is because the samples analysed by the McM method gave more negative results but a higher overall mean, which generate lower values for *k* compared to CF. The CF method gave fewer 0 EPG readings due to the ability to detect eggs at lower density. For this reason, the percentage of horses meeting the threshold for anthelmintic treatment was consistently greater when FEC were analysed by the McM method, resulting in more horses appearing to need treatment, assuming that a threshold of 200 EPG is a reasonable indication for the necessity of treatment. The 200 EPG threshold is an arbitrary number widely used to select adult horses for anthelmintic treatment (Kaplan and Nielsen, 2010). In theory, this threshold could be increased adult horses grazing low risk pastures. For example, if the threshold was increased to 500 EPG, very few adult horses would require treatment in the population of horses screened in this

study using either FEC method rendering the choice of FEC method a moot point and on the flipside, if the threshold was lowered to 100 EPG the number of horses requiring treatment would increase regardless of which FEC method was used. At the current threshold of 200 EPG using McM50 to select horses for treatment, more horses would require treatment, potentially leading to an increased number of unnecessary treatments, theoretically resulting in fewer parasites within the entire population in refugia and increased selection pressure for resistance.

Overall, the outcome of FECRT using each of the FEC methods was in broad agreement, and when an anthelmintic is highly efficacious (MOX/IVM) or not efficacious (FBZ), the FEC method used had no bearing on the interpretation of the FECRT. However, when anthelmintic efficacy approached the threshold of sensitivity/resistance (as in the case of PYR treatment on two yards), the FEC method used impacted the interpretation of the FECRT and disagreements in the classification were observed. Here, the McM15 method was in disagreement with the other two methods when one set of FECRT data were analysed; here indicating PYR to be efficacious compared to the McM50 and CF (1 EPG detection limit) methods. Likewise, the McM50 method indicated one population to be PYR resistant when the other two methods indicated sensitivity to PYR. As controlled efficacy trials were not undertaken, the true PYR sensitivity status of these strongyle populations remains unknown. In practice, retesting would be recommended to monitor efficacy of PYR if the compound is used subsequently. Efficacy studies performed around the time of licensing of PYR indicated that in drug-sensitive populations reductions in FEC ranged between 94 - 100% after treatment, with substantial variability in efficacy observed amongst farms (Lyons et al., 1975; Nielsen et al., 2013). As such, PYR FECR data should be interpreted with care (Nielsen et al., 2013). However, since 95% confidence intervals were not calculated here, conclusions about classifying resistance can't be made. Previous studies have alluded that using

McM for assessing anthelmintic efficacy may lead to erroneous results when interpreting FECRT data, particularly when animals are shedding a low number of eggs (El-Abdellati et al., 2010; Levecke et al., 2012a; Levecke et al., 2012b) resulting in an increased likelihood of falsely declaring anthelmintic efficacy (false negative result). A recent study compared three FEC techniques (Cornell-Wisconsin (1EPG detection limit), FLOTAC (1 EPG) and McM (10 EPG)) for bias, accuracy and precision of FECRT results in cattle and found that methods with the same detection limit gave different levels of accuracy and precision (Levecke et al., 2012b). The McM and Cornell-Wisconsin techniques were less accurate compared to FLOTAC and as a result significantly underestimated FECR where baseline FEC were low and more efficacious drugs were used. However, for all FEC methods, the precision and accuracy of FECRT increased when the pre-treatment mean increased and FEC methods utilising a low egg dl were used. The authors concluded that the precision of FECRT is affected by the FEC methodology used and that levels of egg excretion should be considered when interpreting the FECRT (Levecke et al., 2012b).

In the present study, when IVM and MOX were administered there was no relationship between Day 0 mean FEC and Day 14 mean FEC. The probable reason for this observation being that these products are still highly efficacious. When FBZ and PYR were administered, the Day 0 14 FEC increased with the Day 0 FEC regardless of the FEC method used, indicating that the higher the pre-treatment mean, the lower the FECR will be. However, the effect was lower when CF and McM15 were used compared to McM50. The same observation was made in a recent study, where a hierarchical model was used for evaluating PYR efficacy data. Here, the authors found that as the group mean pre-treatment FEC increased, PYR efficacy decreased. They concluded that this was likely to be due to the FEC method used rather than underlying biological issues; for example, using a FEC method with a detection limit of 20 EPG, on a horse

with a pre-treatment FEC of 200 EPG, that was then treated with PYR with a known efficacy of 90%, the post-treatment FEC is likely to be 0 EPG resulting in an observed FECR of 100%, compared to a horse with a pre-treatment FEC of 1000 EPG, which, when treated with the same drug is likely to show a positive FEC post-treatment, giving a lower estimated % FECR (Nielsen et al., 2013). The statistical reasoning behind such findings has recently been discussed by Torgerson et al. (2012), and the Poisson process can describe variance observed in FEC. Even if faeces have been well mixed to ensure that eggs are evenly distributed, using a FEC method offering an analytical sensitivity of 1 EPG, when FEC is 1 or 2 EPG, diagnostic sensitivity will be 63% and 86%, respectively, due to the Poisson error (Torgerson et al., 2012). This demonstrates that erroneous declarations of efficacy are more likely when using a single McM count at an analytic sensitivity of 50 EPG. When an observed egg count of 0 is derived by McM with a sensitivity of 50 EPG, 95% lower and upper confidence limits of 0 and 184.5 EPG, respectively, are given (Torgerson et al., 2012). The confidence bounds decrease with increased analytical sensitivity such that a 0 EPG count derived using a multiplication factor of 10 gives lower and upper 95% CLs of 0 and 36.9 EPG, respectively (Torgerson et al., 2012). This has implications when assessing efficacy, as negative counts on Day 14 post-treatment may not actually be negative, thus falsely declaring an anthelmintic to be efficacious.

Although the McM technique is the most widely used method for FEC analysis (Presland et al., 2005; Pereckiene et al., 2010; Denwood et al., 2012), it would appear that there is little appreciation of statistical processes underlying variation in this method. Many studies report FEC data using the McM method at a detection limit of 50 EPG, but counting a larger volume of faecal suspension, which will achieve an egg dl of 15 EPG, and provides a simple way to increase analytical sensitivity, if laboratories are unwilling to change to other more sensitive methodologies such as the CF method.

The volume of faecal suspension examined in a McM slide will affect the reliability of the EPG estimate (Cringoli et al., 2004). In addition, researchers have suggested that multiple counts be performed per sample to reduce variability (Vidyashankar et al., 2007; Denwood et al., 2012). By performing more counts and taking an average, variation is smoothed (Morgan et al., 2005). However, this is often impractical. There are a number of biological factors that can influence FEC. These include; parasite biology including fecundity, parasite density and prepatent period and host biology, including physiological status, immunity, management factors, pre-exposure to parasite challenge, nutrition, anthelmintic treatments (Gasbarre et al., 1996; Pereckiene et al., 2007; Gates and Nolan, 2009; Vadlejch et al., 2011).

The *k* values obtained from the screening FEC between horses on the same yard were lower (0.03 – 0.26) compared to the *k* values obtained in the faecal motion (6.3 – 12.4), between boli (10.6 – 84) or between samples (10.6 – 28.5), indicating that the greatest degree of variation is observed between horses than at any of the other levels investigated. Egg excretion between horses is highly aggregated (Relf et al., 2013) as a host population is unlikely to be homogeneous, and will differ in terms of age, sex, immune status and susceptibility (Morril and Forbes, 2012) as such, groups of hosts that are not uniform are likely to exhibit higher levels of FEC aggregation (Morgan et al., 2005). There are other factors related to parasite biology and epidemiology, such as the distribution of the infective stages in the environment (Quenouille, 1949; Shaw et al., 1998), seasonality, climatic and temperature dependent effects on parasite development both in the environment and within the host (Shaw et al., 1998) and density dependent effects within the host (Anderson and May, 1985). To reduce the degree of aggregation between horses, in terms of the impact of variation on FEC and FECRT, selection criteria are applied, i.e. only horses with a FEC of ≥200EPG are

included in a FECRT. While these factors need to be considered, the purpose of the present study was to evaluate the factors affecting variance that arise in FEC.

A limitation to the study was that the horses selected for the different investigations were selected opportunistically and were not consistent between studies. As such, between horse variation was not accounted for and this could have impacted on the variability of the observed results. However, since the studies into the variability arising in equine FEC were focussed on technical factors and the results demonstrated that strongyle eggs in faeces followed a Poisson distribution in well mixed faeces, the fact that samples were collected from different horses was unlikely to have impacted the results. The results did however show that the greatest degree of aggregation was observed between individual horses.

2.7 Conclusions

From these findings, the following acronym DISMIS is suggested giving practical solutions to decrease variability in FEC:

Decrease variability by:

Increasing sample size - Take at least three individual boli and send for analysis (Denwood, 2010; Vidyashankar, 2012).

Store samples correctly - Place in a zip-lock bag, expel the air before sealing, refrigerate at 4˚C and process within 4 days (Nielsen et al. 2010).

Mix samples thoroughly - Before measuring out the subsample thoroughly mix (Section 2.5.3).

Increase **S**ensitivity - Use a FEC method with a low egg detection limit especially when assessing anthelmintic efficacy. If using the McM method, count more of the faecal suspension by counting all of the eggs present in the two chambers and multiply by 15 (Sections 2.5.5 – 2.5.6)

It is important to identify, understand and quantify the inherent sources of variation to better understand the underlying statistical processes that underpin FEC when making decisions for anthelmintic treatment and assessing the efficacy of these products. This research has answered important questions about sources of variation in FEC methodology and provides a framework for further analysis by exploring the impact of variation on FEC and subsequent decisions for targeted treatment and efficacy through statistical modelling. The challenge remains to fully recognise the sources of variation, which inevitably arise from the Poisson process during FEC analysis and the OD that occurs between horses and individual samples to make accurate estimations of FEC. Data such as those presented in this Chapter provide a stronger basis for modelling the statistical processes impacting FEC results, and hence designing FEC protocols that are fit for purpose and properly interpreted.

CHAPTER 3: Efficacy of anthelmintics against small strongyles

3.1 Introduction

Virtually all grazing horses are at risk of infection from helminths and as such, appropriate control measures are necessary. The long term frequent use of anthelmintics has contributed to the development and spread of anthelmintic resistance (Kaplan, 2002; Kaplan, 2004; Matthews, 2008). However, defining resistance is complicated by the fact that there are no published guidelines for the conduct of faecal egg count reduction tests (FECRT) or for the interpretation of FECRT results in horses, and published studies have used different protocols and different thresholds to classify resistance, making comparisons between studies difficult (Stratford et al., 2014). There are no agreed guidelines regarding optimum group size, the pre-treatment faecal egg count (FEC) and FEC distribution among the group or which FEC method should be used. Most commonly, resistance is defined as a FECRT test <95% for the macrocyclic lactones (ML) and <90% for fenbendazole (FBZ) and pyrantel (PYR) (Kaplan and Nielsen, 2010), however, these thresholds have been arbitrarily chosen and have not been validated against the 'gold standard' controlled efficacy test, whereby infected horses are dosed with an anthelmintic then slaughtered 10 – 21 days later to recover the immature and adult worm burden (Duncan et al., 2002). To account for the range of FECRT data, the calculation of lower 95% confidence limits (LCL) have been suggested (Vidyashankar et al., 2007; 2012). The suggested thresholds to indicate acceptable efficacy are FECR >95% and LCL >90% for the MLs and FECR >90% and LCL >80% for FBZ and PYR (Kaplan and Nielsen, 2010; Vidyashankar et al., 2007; 2012). Regardless of the limitations of the FECRT, it remains the only tool for the evaluation of anthelmintic efficacy in horses under field conditions (Matthews, 2014).

FBZ resistance in cyathostomins is widespread, (Kaplan et al., 2004; Osterman-Lind et al., 2007; Traversa et al., 2012) and reduced sensitivity of cyathostomins to pyrantel

salts is very common in some geographic locales (Kaplan et al., 2004; Comer et al., 2006; Lind et al., 2007; Traversa et al., 2007). In addition, single cyathostomin populations have been identified that exhibit both FBZ and PYR resistance (Kaplan, 2004; Traversa et al., 2007). A reduction in egg reappearance period (ERP) has also been suggested as an early indicator of resistance (Sangster, 1999). A reduction in strongyle ERP from over 8 weeks (Borgsteede et al., 1993) down to 4 weeks after IVM administration has been described (Lyons et al., 2008b; Molento et al., 2008; von Samson-Himmelstjerna et al., 2007) and a shortened strongyle ERP after MOX administration has been reported in Kentucky (Rossano et al., 2010). Concurrent reduced efficacy of all three broad-spectrum anthelmintic classes has also been identified in Brazil (Molento et al., 2008; Canever et al., 2013).

Encysted larvae (EL3), and play an important role in cyathostomin epidemiology as the gradual maturation of these stages in the spring can lead to a significant amount of pasture contamination (Herd, 1986), and most importantly are responsible for larval cyathostominosis, which can be fatal in up to 50% of cases (Love et al., 1999; Lyons et al., 2000). In temperate climates, the disease is most common in winter and spring, and in horses less than six years of age (Reid et al., 1995). Stress-induced immunosuppression may also be a contributory factor (Mair, 1993), while recent administration of an anthelmintic that primarily targets luminal stage cyathostomins may precipitate clinical disease (Reid et al., 1995). For these reasons, it is important that control strategies are aimed at cyathostomin EL3. Cyathostomin EL3 present a considerable challenge when planning control protocols as their identification and quantification is problematic. In the UK, there are two anthelmintics licensed for use in the treatment of cyathostomin EL; MOX administered as a single dose of 0.4 mg/kg has claimed 90.8% efficacy against cyathostomin EL3 and 99.9% against other developing larval stages (Bariden et al., 2001) and FBZ administered at 7.5 mg/kg on five

consecutive days (5d FBZ) with claimed 91.5% efficacy *versus* inhibited EL3 and 99.4% efficacy *versus* developing mucosal larval stages (Duncan et al., 1998). These studies were conducted over 13 years ago in only 8 – 10 horses and, since then, there have been many reports of resistance in cyathostomin populations to FBZ (Fisher et al., 1992; Kaplan 2004; Osterman-Lind et al., 2007). It has been proposed that where low level resistance exists to a single dose of FBZ, a 5d FBZ course might still prove efficacious (Blanek et al., 2006), as prolonged exposure of the parasites to an elevated dose of FBZ is likely to produce greater efficacy. However, in horses that had been previously identified as harbouring FBZ-resistant cyathostomins following a single dose of FBZ, continuous egg shedding was observed when the same horses were treated with a 5d FBZ course (Chandler et al., 2000; Chandler and Love, 2002; Rossano et al., 2010). Despite the high prevalence of FBZ resistance and evidence to suggest that 5d FBZ is likely to be ineffective at eliminating FBZ-resistant cyathostomins, 5d FBZ is still used in the UK to remove EL, especially in debilitated animals in cases of larval cyathostominosis, and as a standard quarantine measure (*personal communication*, Claire Stratford, 2012). If 5d FBZ is used and is not effective against luminal or mucosal stages, then significant egg shedding will continue leading to pasture contamination and reinfection.

3.2 Aims of Chapter

The main aim here was to determine efficacy of all three classes of anthelmintic against strongyle infections in horses based on yards across Eastern Scotland and Southern England as up to date information on the performance of these anthelmintics will inform the decision support tool and guide control programmes. Secondly, the efficacy of 5d FBZ and MOX against strongyles in two populations of horses was compared because these are the two anthelmintics licensed for use versus cyathostomin EL, and if

significant egg shedding is observed post-treatment, this would indicate that the drug had not been effective against the adult worm population, which in turn may indicate a lack of efficacy versus the refractory larval stages which are more difficult to kill (Matthews, 2014). Again, these data will help to inform the decision support tool and guide recommendations for the control of cyathostomin EL.

3.3 Materials and Methods

3.3.1 Scottish and English study populations

British Horse Society (BHS) approved livery yards were approached using the BHS website (www.bhs.org.uk), contacted via email and asked to participate. In addition, boarding stables, competition and welfare yards that were clients of the Bell Equine Veterinary Clinic (Kent, UK) and House and Jackson Veterinary Clinic (Essex, UK) were recruited onto the study. All horses had access to grazing and had been treated with a ML within the last 6 months. Each yard was supplied with a questionnaire to complete, which provided information on the yard (yard type, acreage, number of horses), anthelmintic usage (frequency, last product used, and type of deworming programme) and management practices (Appendix 1).

3.3.2 Study populations used for investigations into the efficacy of 5d FBZ and MOX

Two studies were conducted to investigate the efficacy of 5d FBZ in two populations of horses. For Experiment 1, all equids recruited belonged to a welfare charity and for Experiment 2, all equids were residents at the (R(D)SVS) equine hospital.

3.3.3 Sample collection

All faecal samples were collected and processed in the same manner. Horse owners/yard managers were asked to collect freshly voided from all horses resident on each yard. Pre-labeled zip-lock bags were provided for sample collection, identification and submission of samples. On the first sampling occasion, the pre-labeled bags were left blank for the horse owners to write the name and age of each horse on each bag. On subsequent sampling occasions, pre-labeled bags with the horses' names were supplied. Horse owners were asked to collect at least three faecal boli from a freshly voided motion and to place these into a zip-lock bag, expelling the air before sealing. The samples were sent immediately to Moredun Research Institute and stored at approximately 4˚C. All samples were processed within 4 days of collection. Prior to processing, all samples were logged onto data capture forms. The date of sample arrival, the date of processing, each horse's name and age were recorded. Samples were recorded in batches according to which yard they came from.

3.3.4 Faecal egg count methodology

A modification of the salt flotation method (Christie and Jackson, 1982), sensitive down to 1 egg per gram (EPG), was used as described previously (Section 2.3.2.2).

3.3.5 Larval culture

For 11 of the 16 English yards, positive FEC samples from the first screening occasion were pooled and the eggs cultured to third stage larvae (L3). In brief, faecal samples from individual horses were formed into fist-sized balls and placed into a 500 ml container lined with a polythene bag. A further polythene bag was used to cover the container, which was pierced several times before incubation at 22˚C. After 14 days,

the container was removed and flooded with tepid water and allowed to stand for 4 h. The contents of the container were poured over a filter made from two layers of filter paper, the filter then placed on top of a 200 ml jar filled with tepid water, so that the filter paper was flush with the water and left overnight so that larvae could migrate through the filter paper into the jar. The filter was removed and the contents of the jar were siphoned off until approximately 2 cm of liquid remained. This was poured into a flask, where it was stored at approximately 4˚C until L3 in the sample were enumerated. Strongyle larvae were identified according to MAFF, 1986. On three yards, the FEC were too low for culture to be performed and on two other yards, eggs were not cultured to L3 due to time constraints. No larval cultures were performed on faeces obtained from the Scottish yards.

3.3.6 Faecal egg count reduction tests (FECRT) Scottish and English yards

Faecal samples were obtained from all equids at each site once the minimum ERP of the previously administered anthelmintic had passed. The standard minimum ERP used were 6 weeks for FBZ and PYR, 8 weeks for IVM and 13 weeks for MOX (Stratford et al., 2011b). Horses with strongyle FEC of ≥50 EPG were included in the FECRT and administered *per os* with anthelmintic on Day 0 at the following dose rates; FBZ (Panacur® equine paste)[a] (7.5 mg/kg), PYR (Strongid-P™)[b] (19 mg/kg), IVM (Eqvalan® oral paste for horses)[c] (0.2 mg/kg) or MOX (Equest®)[d] (0.4 mg/kg). Each horse received a dose appropriate for 110% of each individual's body weight, as estimated by weigh tape to minimise the risk of under-dosing (Stratford et al., 2014b). Each individual was administered with the appropriate anthelmintic by their owner and monitored immediately after to ensure that the paste/gel was swallowed. In addition, for individuals with a faecal egg count reduction (FECR) indicative of resistance (i.e. a

FECR reduction below the designated threshold for efficacy) in populations where the mean FECR was above the accepted efficacy threshold, the test was repeated in those individuals using the same anthelmintic. This was performed to exclude administration or sampling error. In cases where the yard mean FECR indicated anthelmintic resistance, all horses with FEC ≥50 EPG at Day 14 were enrolled onto a FECRT using a different anthelmintic class. All animals on the same yard received the same class and batch of anthelmintic. Faecal samples were collected on Day 0 immediately prior to anthelmintic administration and at 14 days post-treatment. Testing of anthelmintic classes was performed in succession in most populations, starting with FBZ, followed by PYR, then IVM and finishing with MOX. Because of its effect on cyathostomin EL (Bairden et al., 2006), which in the UK are thought to be in highest proportions in the autumn/winter months (Ogbourne, 1976), MOX was preferentially tested in these seasons.

3.3.7 FECRT using 5d FBZ in welfare horses and in horses residing at an equine hospital

In the first experiment, equids resident at a welfare charity in Scotland were screened for the presence of strongyle eggs in faeces. Individual equids with a strongyle FEC of ≥50 EPG were selected for a 5d FBZ FECRT. FBZ (Panacur Equine Guard, MSD) was administered *per os* at 7.5 mg/kg for five consecutive days at a dose appropriate for 110% body weight as estimated by weigh tape. Faecal samples were collected prior to the first administration (Day 0) and at 14 days after the final FBZ administration (Day 14). Equids with a FEC of ≥50 EPG at Day 14 of the FECRT were subsequently administered with MOX and a FECRT performed. Faecal samples were collected on Day 0 prior to administration of 0.4 mg/kg MOX and on Day 14. In a second study, horses (*n* = 11) based at the R(D)SVS equine hospital were screened for the presence of strongyle

eggs. Those with a FEC of ≥50 EPG were selected and randomly allocated to one of two treatment groups; Group 1 received FBZ (Panacur Equine Guard, MSD) administered *per os* at 7.5 mg/kg for five consecutive days and Group 2 received MOX (Zoetis Animal Health) *per os* at a dose of 0.4 mg/kg on Day 4. Doses were appropriate for 110% body weight as estimated by weigh tape. Faecal samples were collected prior to the first administration (Day 0 and Day 4) and at 14 days after the final FBZ administration (Day 19). For both studies, a mean FECR of >90% for 5d FBZ and >95% for MOX was noted as acceptable efficacy (Kaplan and Nielsen, 2010a). FECR for both 5d FBZ and MOX were calculated using arithmetic means. Estimates of the 95% CL were derived from these figures. Eggs were not cultured to third stage larvae.

3.3.8. Data analysis

3.3.8.1. FECRT

Microsoft® Office Excel® 2007 was used for recording data and analysis. Summary data for each yard included median age, age range, interval since last anthelmintic treatment, percentage of horses recruited onto the FECRT study, % of horses with FEC of ≥200 EPG, mean FEC and range of FEC. FECR was calculated for each group of horses using the following formula recommended by the WAAVP (Coles et al., 1992), where arithmetic group mean FEC for Day 0 and Day 14 were used to estimate the group FECRT (Section 2.4.5, Equation 2.5).

There are no agreed guidelines regarding appropriate cut-off limits for determining efficacy for the anthelmintic classes in horses (Vidyashankar et al., 2012). The methodology here followed recently published recommendations; i.e. thresholds chosen for establishing appropriate efficacy were arithmetic mean FECR of >95% for ML and >90% for BZ/PYR (Kaplan and Nielsen, 2010). In addition, 95% lower

confidence limits (LCL) were included to give a more accurate indication of the range of the data (Vidyashankar et al., 2007). Non-parametric bootstrapping was used to sample with replacement from the observed FECR, and upper and lower 2.5-percentiles of 10,000 simulations were taken as the 95% confidence limits (Efron, 1979; Hilborn and Mangel, 1997). PopTools software (CSIRO, Australia) was used for bootstrapping (Hood, 2010) and LCL of 90% and 80% were selected for classifying resistance for ML and FBZ/PYR, respectively. If % mean FECR *and* LCL fell below the designated cut-offs, anthelmintic resistance was *indicated*, and if either the % mean FECR *or* the LCL fell below these cut-offs, resistance was *suggested*. The cut-offs were selected on the basis of the differing original efficacies in anthelmintic-sensitive strongyle populations of the various active ingredients when they were first registered as veterinary medicines (Colglazier et al., 1977; Cornwell and Jones, 1968; Xiao et al., 1994).

3.3.8.2. Factors affecting Day 14 FEC

Factors impacting Day 14 FEC were investigated using generalised linear mixed models (GLMM) with a negative binomial distribution to account for the aggregation of FEC on Day 14. A mixed modelling approach was selected to allow random effects to be fitted alongside fixed effects to account for the variation between yards and psuedoreplication. All GLMM were run in RStudio 2.15.1 using the 'glmer.nb function in the 'lme4' package (Bates et al., 2015). The different factors investigated are outlined in Table 3.1.

Table 3.1. A summary of the different factors and responses included in the analyses to determine factors affecting strongyle egg shedding

Factor	Responses
Yard type	Livery, sanctuary, stud
Country	England/Scotland
Age*	Years
Anthelmintic treatment	FBZ, IVM, MOX, PYR
Day 0 FEC*	Eggs per gram

* continuous variable

Initially, the multivariate model was populated with all explanatory variables; non-significant factors were removed and the factor with the lowest *z* value (the ratio of the estimated slope to its standard error) was dropped from the models in turn until only significant factors ($p \leq 0.05$) remained and the minimal adequate model was achieved. The significance of removing factors from the model was evaluated using log-likelihood ratio tests (LRTs, Zuur et al., 2010; Crawley, 2013). To confirm that the minimal adequate model was reasonable, all dropped terms were sequentially reintroduced back into the final model to ensure a lack of significant change in explanatory power as confirmed by LRTs. Inspecting the model residuals and plotting them against the fitted values and against each significant factor allowed model checking. Yard and individual were fitted as a random effect to account for the heterogeneity between yards and to account for pseudoreplication associated with repeated observations from the same individuals.

3.4 Results

3.4.1 Study population; profile of horses and yards

3.4.1.1. Scottish population

FEC analysis was initially performed on 1,359 samples from 15 yards, from March 2010 to May 2012. Thirteen yards were leisure horse livery yards located in Midlothian (Yards 1s - 12s, 15s), and two were welfare establishments located in the Scottish Borders (Yard 13s) and Aberdeenshire (Yard 14s) (Figure. 3.1). The majority of equids sampled were of mixed breed and were used for pleasure riding or were retired. In general, the premises tested had used anthelmintics fairly infrequently in the preceding year, with the majority of equids receiving an IVM- or MOX-based anthelmintic every 2 - 6 months. All premises, particularly the welfare establishments, reported regular introduction of new equids, often with an unknown worming history, which could lead to pasture contamination if the new arrivals were not quarantined appropriately, or, more importantly, they could harbour resistant worm populations and subsequently contaminate the pasture. Efficacy testing with multiple anthelmintic classes was performed in succession on a number of these populations. Group sizes for FECRT ranged from 3 to 64 equids (median 12), representing 5.3 - 88.9% of each yard population tested. Precise ages were provided for 238 equids; 82% of the population tested (median 9 years, range 1 to 33). Of the remaining 54 equids, 20 were classified as 'aged' by the yard owner, meaning greater than 12 years, and 34 had no age specified. The average interval between last anthelmintic administered and FEC screening was 136 days (range 81 to 240 days) (Table 3.2).

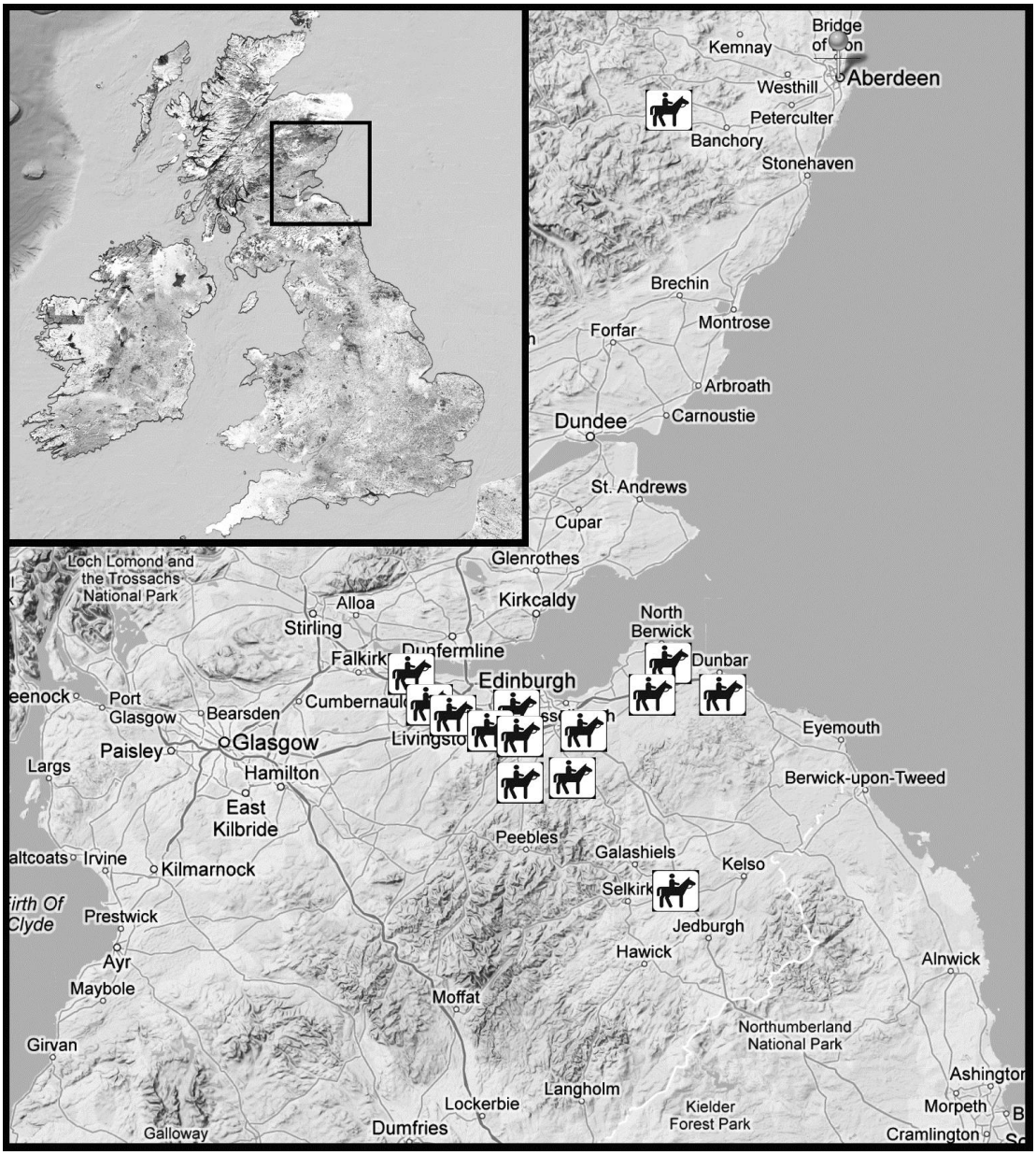


Figure 3.1. Map illustrating the geographical locations of the Scottish yards recruited for the study (adapted from http://maps.google.co.uk/)

Table 3.2. Summary data for each Scottish yard (Yards 1s -15s) including yard type, median age, the last anthelmintic administered (AM) and interval since last treatment

	Yard															
	1s	2s	3s	4s	5s	6s	7s	8s	9s	10s	11s	12s	13s	14s	15s	Totals
Type of yard	L	L	L	L	L	L	L	L	L	L	L	L	S	S	L	
No. of horses	17	79	19	45	35	30	26	21	9	57	28	29	72	57	29	553
Median age	5	10	15	13	10	14	13	10	12	NS	5	NS	7	7	13	10
Range	(2 -17)	(4-20)	(4-28)	(3-23)	(1 -21)	(2-22)	4 to 13	(1- 28)	(7-25)	NS	(1-12)	NS	(1-34)	(0.4-23)	(5-28)	(0.4-34)
Last AM	PYR	MOX	MOX	MOX	MOX	MOX/PRZ	IVM/PRZ	NS	MOX	NS	MOX	MOX	NS	IVM	MOX	
Interval[a] (days)	240	180	90	150	81	104	157	NS	204	NS	102	104	NS	93	121	136

L = Livery, S = Sanctuary, IVM = ivermectin, MOX = moxidectin, PYR = pyrantel

NS = Data not supplied

[a] Interval since last anthelmintic treatment

3.4.1.2. English population

A total of 16 yards were recruited; all were located in the south of England (Figure. 3.2). Eleven were general boarding stables, two (Yards 4e and 15e) were equine welfare/rescue/rehabilitation premises, one (Yard 7e) was a sport horse yard, one (Yard 9e) was a non-Thoroughbred stud and one (Yard 10e) was a private yard where all horses were owned by one person (Table 3.3). FEC analysis was performed on each yard on a minimum of three occasions. In total, 928 faecal samples were collected from 368 horses between March and December 2012. Ages were provided for 313 horses; 85% of the population tested (median 12 years, range 1 to 34), with 55 having no age specified (Table 3.3). The average number of horses per yard was 23 (range 8 - 48). A total of 13 respondents stated that they had previously administered an ML as the last treatment to all animals. One (Yard 13e) had stated that they administered a five-day course of FBZ (Panacur® Equine Guard, MSD Animal Health) and two (Yards 3e and 12e) did not provide this information. Eleven (69%) respondents had used an anthelmintic with licensed efficacy against encysted larvae (EL); 10 had used MOX and one a five-day course of FBZ. The average interval between last anthelmintic administered and FEC screening was 129 days (range 76 to 227 days) (Table 3.3).

Figure. 3.2. Map illustrating the geographical locations of the English yards recruited for the study

Table 3.3. Summary data for each English yard (Yards 1e -16e) including yard type, median age, the last anthelmintic administered (AM) and interval since last treatment

	Yards																
	1e	2e	3e	4e	5e	6e	7e	8e	9e	10e	11e	12e	13e	14e	15e	16e	Totals
Type of yard	L	L/C	L	S	L	L	C	L	NTBS	P	L	L	L	L	R	L	
No. of horses	32	14	18	14	17	14	25	8	23	18	26	43	22	32	48	14	368
Median age	17	NS	NS	7.5	11	15	8	19	NS	17	11	10	12	14	4	12	12.1
Range	3 to 31	NS	NS	3 - 34	4 - 19	9 - 21	3 – 15	16 - 28	NS	3 - 25	4 - 25	5 - 19	3 - 30	3 - 29	1 - 17	4 - 19	
Last AM	MOX	IVM/PRZ	NS	MOX	MOX	IVM/PRZ	MOX	MOX/PRZ	IVM	MOX/PRZ	MOX	NS	5DFBZ	MOX/PRZ	MOX/PRZ	MOX	
Interval[a] (days)	130	148	NS	194	129	171	139	85	89	120	120	NS	76	88	83	227	

C = Competition, L = Livery, NTBS = Non-Thoroughbred Stud, P = Private, R = Rescue, S = Sanctuary
IVM = ivermectin, MOX = moxidectin, PRZ = praziquantel
NS = Data not supplied
[a] Interval since last anthelmintic treatment

3.4.2 Faecal egg count reduction test results

3.4.2.1 Scottish FECRT data

A total of 447 FECRT were performed on 292 equids (Table 3.4) and this was divided as follows: FECRT for one (*n* = 194), two different (*n* = 55), three different (*n* = 29) or four different (*n* = 14) anthelmintics.

Table 3.4. Faecal egg count reduction test (FECRT) summary data for Scottish yards indicating number of individual yards and equids tested per yard. The efficacy range details the yard arithmetic mean percentage reductions in faecal egg counts (FEC) at 14 days post-treatment for each anthelmintic tested. The range of 95% Lower Confidence Limits (LCL) for all yards for each anthelmintic is also shown. NC = not calculable because some Day 14 FEC exceeded Day 0 FEC precluding meaningful calculation of 95% LCL

	FBZ	**PYR**	**IVM**	**MOX**
Total yards	7	8	13	10
Total equids	55	111	163	118
*FECR range (%)	15.8 - 83.4	90.4 - 99.6	99.5 - 100	99.4 – 100
95% LCL range (%)	NC	80.9 - 99.4	94.3 - 100	96.1 – 100

FBZ = fenbendazole, IVM = ivermectin, MOX = moxidectin, PYR = pyrantel
*calculated from all individual horses from each yard

FBZ efficacy was examined on 7 yards with samples obtained from 55 individuals (Table 3.5). On all premises, reduced efficacy was recorded, with mean percentage FECR ranging from 15.8 to 83.4% (Figure. 3.3a). For 18% (10/55) of the individual horses or ponies tested, Day 14 FEC exceeded Day 0 FEC, precluding calculation of meaningful LCL. PYR efficacy was examined on 8 yards using samples derived from 111 individuals (Table 3.5). On all premises, mean percentage FECR was above the designated 90% cut-off, ranging from 90.4 to 99.6% (Figure. 3.3b). The 95% LCL

ranged from 80.9 to 99.4%, providing an early indication of resistance in some populations.

IVM efficacy was investigated on 13 yards using samples derived from 163 individuals (Table 3.6). On all premises, the mean FECR was above the 95% efficacy cut-off, ranging from 99.5 to 100%, indicating acceptable efficacy (Figure. 3.3c). The 95% LCL ranged from 94.3 to 100%. MOX efficacy was examined on 10 yards using samples from 118 individuals (Table 3.6). On all premises, the mean FECR exceeded the designated 95% efficacy cut-off, ranging from 99.4 to 100 % (Figure, 3.3d). The 95% LCL ranged from 96.1% to 100% after treatment with MOX.

Table 3.5. Faecal egg count reduction test data for fenbendazole (FBZ) and pyrantel (PYR) on individual Scottish yards detailing the number of equids recruited (i.e. equids with faecal egg counts (FEC) ≥50 eggs per gram (EPG) at screening). Mean strongyle FEC (EPG) are shown for Day 0 and Day 14. Arithmetic mean faecal egg count reduction (FECR) (%) and 95% lower confidence limit (95% LCL) (%) are displayed for each population. NA = not applicable because Day 14 FEC exceeded day 0 FEC precluding meaningful calculation of 95% LCL. NP = not performed

	FBZ					PYR				
Yard No.	**No. recruited**	**Day 0 FEC [epg] Mean (range)**	**Day 14 FEC [epg] Mean (range)**	**FECR (%)**	**LCL (%)**	**No. recruited**	**Day 0 FEC [epg] Mean (range)**	**Day 14 FEC [epg] Mean (range)**	**FECR (%)**	**LCL (%)**
1s	5	494 (198-851)	287 (147-426)	41.9	NA	8	474 (90-1341)	45 (0-239)	91.0	90.4
2s	14	227 (125-747)	166 (32-405)	27.0	NA	36	253 (2-1472)	1 (0-7)	99.6	95.0
3s	5	269 (30-693)	227 (8-590)	15.8	NA	9	113 (84-207)	2 (0-5)	98.7	98.1
4s	11	261 (57-545)	70 (2-213)	67.6	NA	11	107 (15-548)	2 (0-9)	97.8	90.7
5s	10	259 (85-495)	108 (26-393)	58.3	NA	17	193 (12-563)	1 (0-7)	99.6	99.2
6s	6	340 (62-738)	56.3 (23-125)	83.4	NA	5	388 (47-896)	2 (0-8)	99.5	99.4
7s	4	166 (63-246)	131 (39-284)	21.1	NA	14	254 (2-851)	3 (0-25)	98.7	94.5
8s	NP	NP	NP	NP	NP	11	243 (21-605)	13 (0-86)	94.9	80.9
Total	55					111				

Table 3.6. Faecal egg count reduction test data for ivermectin (IVM) and moxidectin (MOX) on individual Scottish yards detailing the number of equids recruited (i.e. equids with faecal egg counts (FEC) ≥50 eggs per gram (EPG) at screening). Mean strongyle FEC (EPG) are shown for Day 0 and Day 14. Arithmetic mean FECR (%) and 95% LCL (%) are displayed for each population. NP indicates not performed.

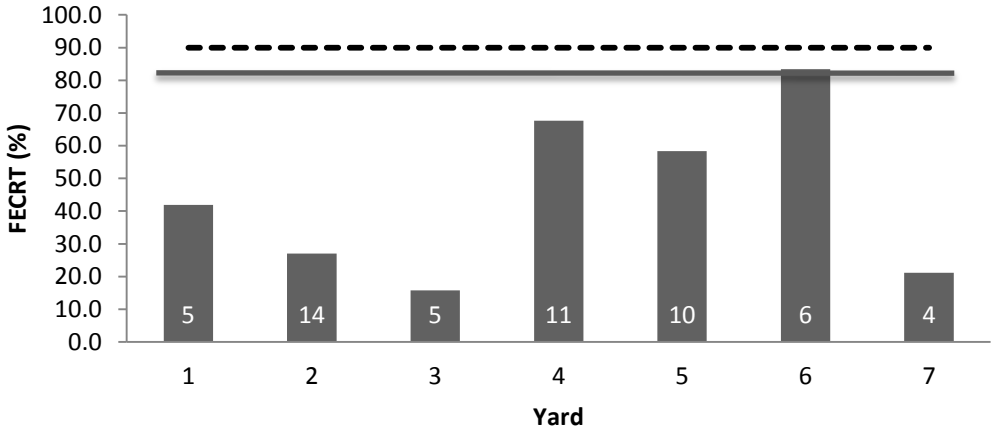
Yard No.	No. recruited	Day 0 FEC [EPG] Mean (range)	Day 14 FEC [EPG] Mean (range)	FECR (%)	LCL (%)	No. recruited	Day 0 FEC [EPG] Mean (range)	Day 14 FEC [EPG] Mean (range)	FECR (%)	LCL (%)
1s	5	222 (59-403)	0 (0-0)	100	100	5	145 (77-287)	0 (0-0)	100	100
2s	39	415 (3-1841)	0.7 (0-4)	99.8	96.9	45	200 (11-675)	0.1 (0-2)	100	99.8
3s	5	162 (40-282)	0 (0-0)	100	100	8	370 (135-801)	2.4 (0-16)	99.4	98.6
4s	10	156 (77-296)	0 (0-0)	100	100	13	237 (6-752)	0.2 (0-1)	99.9	96.1
5s	11	264 (101-518)	0.6 (0-3)	99.8	99.0	9	93 (48-209)	0.3 (0-3)	99.6	99.5
6s	3	212 (137-261)	0 (0-0)	100	100	3	166 (30-338)	0 (0-0)	100	100
7s	4	106 (48-189)	0.5 (0-1)	99.5	98.4	10	303 (29-563)	0 (0-0)	100	100
8s	7	676 (216-1742)	1.9 (0-6)	99.7	98.7	NP	NP	NP	NP	NP
9s	3	196 (86-374)	0 (0-0)	100	100	8	89 (35-191)	0 (0-0)	100	100

Table 3.6. continued. Faecal egg count reduction test data for ivermectin (IVM) and moxidectin (MOX) on individual Scottish yards detailing the number of equids recruited (i.e. equids with faecal egg counts (FEC) ≥50 eggs per gram (EPG) at screening). Mean strongyle FEC (EPG) are shown for Day 0 and Day 14. Arithmetic mean FECR (%) and 95% LCL (%) are displayed for each population. NP indicates not performed.

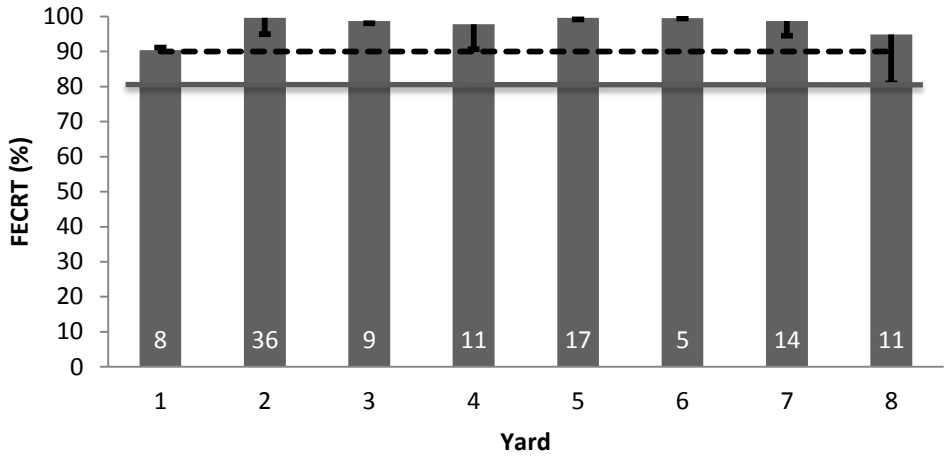
	IVM					MOX				
Yard No.	**No. recruited**	**Day 0 FEC [EPG] Mean (range)**	**Day 14 FEC [EPG] Mean (range)**	**FECR (%)**	**LCL (%)**	**No. recruited**	**Day 0 FEC [EPG] Mean (range)**	**Day 14 FEC [EPG] Mean (range)**	**FECR (%)**	**LCL (%)**
10s	3	83 (67-92)	0 (0-0)	100	100	NP	NP	NP	NP	NP
11s	6	608 (164-1805)	0.4 (0-2)	99.9	99.7	NP	NP	NP	NP	NP
12s	3	112 (89-156)	0 (0-0)	100	100	NP	NP	NP	NP	NP
13s	64	650 (4-2313)	0.4 (0-13)	99.9	94.3	NP	NP	NP	NP	NP
14s	NP	NP	NP	NP	NP	12	525 (29-1067)	0.1 (0-1)	100	99.2
15s	NP	NP	NP	NP	NP	9	154 (48-576)	0 (0-0)	100	100
Total	**101**					**110**				

Figure 3.3. Results of faecal egg count reduction tests (FECRT) (arithmetic mean % reduction) on 15 yards (1 - 15) following; (a) fenbendazole (FBZ), (b) pyrantel (PYR), (c) ivermectin (IVM) or (d) moxidectin (MOX) treatment. Numbers in columns represent number of equids sampled. Dashed lines represent efficacy threshold for the particular anthelmintic (90% for FBZ and PYR, 95% for IVM and MOX). Solid grey lines represent 95% lower confidence limit (LCL) threshold for the particular anthelmintic (80% for FBZ and PYR, 90% for IVM and MOX). Error bars representing the 95% lower confidence limits (LCL) are included for PYR, IVM and MOX. The 95% LCL have been excluded for FBZ as some of the Day 14 FEC exceeded the Day 0 FEC values, preventing meaningful calculation

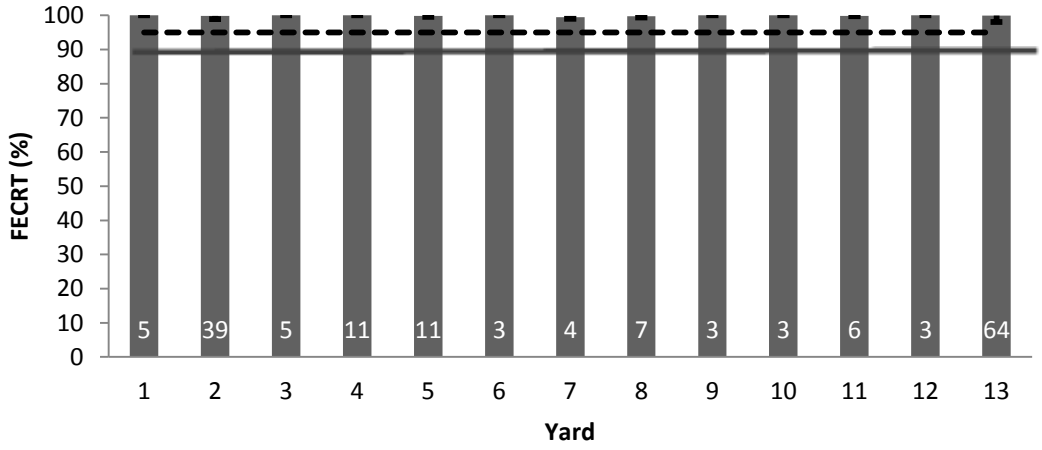
(a) FBZ



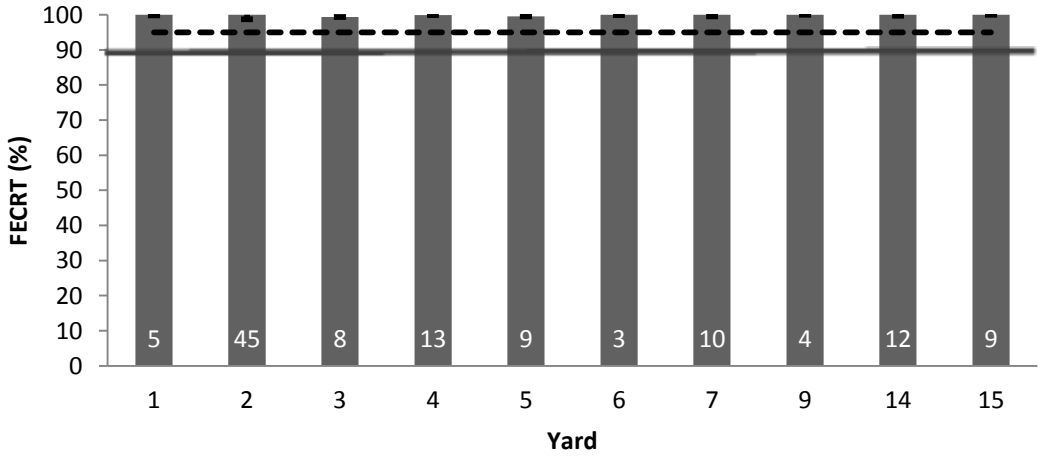
(b) PYR



(c) IVM


100
90
80
70
60
50
40
30
20
10
0
FECRT (%)
5
39
5
11
11
3
4
7
3
3
6
3
64
1
2
3
4
5
6
7
8
9
10
11
12
13
Yard

(d) MOX


100
90
80
70
60
50
40
30
20
10
0
FECRT (%)
5
45
8
13
9
3
10
4
12
9
1
2
3
4
5
6
7
9
14
15
Yard

3.4.2.2. English FECRT data

A total of 404 FECRTs were performed (Table 3.7). All four types of anthelmintic were tested (FBZ, PYR, IVM and MOX) on 8 yards (Yards 1e, 4e, 9e, 10e, 11e, 13e, 14e and 16e); FBZ, PYR and IVM on 2 yards (Yards 7e and 12e); FBZ, PYR and MOX on 2 yards (Yards 3e and 5e) and PYR and MOX on 1 yard (Yard 15). Group sizes for FECRT ranged from 3 - 27 equids (median 7), representing 16% – 76% of each population tested (Table 3.7)

Table 3.7. Faecal egg count reduction test summary data from English yards indicating number of individual yards and equids tested per yard. The efficacy range details the yard arithmetic mean percentage reductions in faecal egg counts (FEC) at 14 days post-treatment for each anthelmintic tested. The range of 95% lower confidence limit (LCL) for all yards for each anthelmintic is also shown

	FBZ	PYR	IVM	MOX
Total yards	12	12	10	12
Total equids	101	110	93	100
*FECR range (%)	-3.4 - 65.8	86.8 - 99.5	96.4 – 100	99.9 - 100
95% LCL range (%)	NC	80.9 - 99.4	94.6 – 100	99.2 - 100

FBZ = fenbendazole, IVM = ivermectin, MOX = moxidectin, PYR = pyrantel
*calculated from all individual horses from each yard

Efficacy of FBZ was examined on 12 yards with samples obtained from 101 individuals (Table 3.7). On all premises, efficacy was below the designated 90% threshold, with mean percentage FECR ranging from 0 - 65.8%. For 19% (*n* = 19) of the individuals tested, Day 14 FEC exceeded Day 0 FEC, precluding calculation of meaningful LCL (Figure 3.4a). PYR efficacy was examined on 12 yards using samples derived from 110 individuals (Table 3.8). On 11 premises, mean percentage FECR was above the designated 90% cut-off, ranging from 90.1 to 99.5%. The 95% LCL ranged from 88.3 to 99.4% (Figure 3.4b). On Yards 4e and 15e, the mean percentage FECR was below the

designated cut-off: i.e. FECR of 86.8 and 87.2% were calculated, respectively. However, for these tests the 95% LCL did not fall below the designated 80% LCL threshold (81 and 83.1%, respectively). Evaluation of individual FECR revealed that, on Yard 4e, three out of eight individual equids had markedly lower FECR compared to the other horses. These horses were retested, and upon retesting the mean FECR was 73.3%, 95% LCL 46%. On Yard 15e, six out of 27 equids had a FECR below 90% (range 53 - 87%). Each horse had a high Day 0 FEC (545 - 1112 EPG). These animals were not retested.

Efficacy of IVM was investigated on 10 yards using samples from 93 individuals (Table 3.9). On all premises, the mean FECR was above the 95% efficacy cut-off, ranging from 96.4 to 100% (Figure 3.4c). The 95% LCL ranged from 94.6 to 100% after treatment with IVM. MOX efficacy was examined on 12 yards using samples from 100 individuals (Table 3.9). On all premises, the mean FECR exceeded the designated 95% efficacy cut-off, ranging from 99.9 to 100% (Figure 3.4d). The 95% LCL ranged from 99.2 to 100% after treatment with MOX.

Table 3.8. Faecal egg count reduction test data for fenbendazole (FBZ) and pyrantel (PYR) on individual English yards detailing the number of equids recruited (i.e. equids with faecal egg counts (FEC) ≥50 eggs per gram (EPG) at screening). Mean strongyle FECs (EPG) are shown for Day 0 and Day 14. Arithmetic mean FECR (%) and 95% lower confidence limit (LCL) (%) are displayed for each population. NA = not applicable because Day 14 FECs exceeded Day 0 FECs precluding meaningful calculation of 95% LCLs. NP = not performed

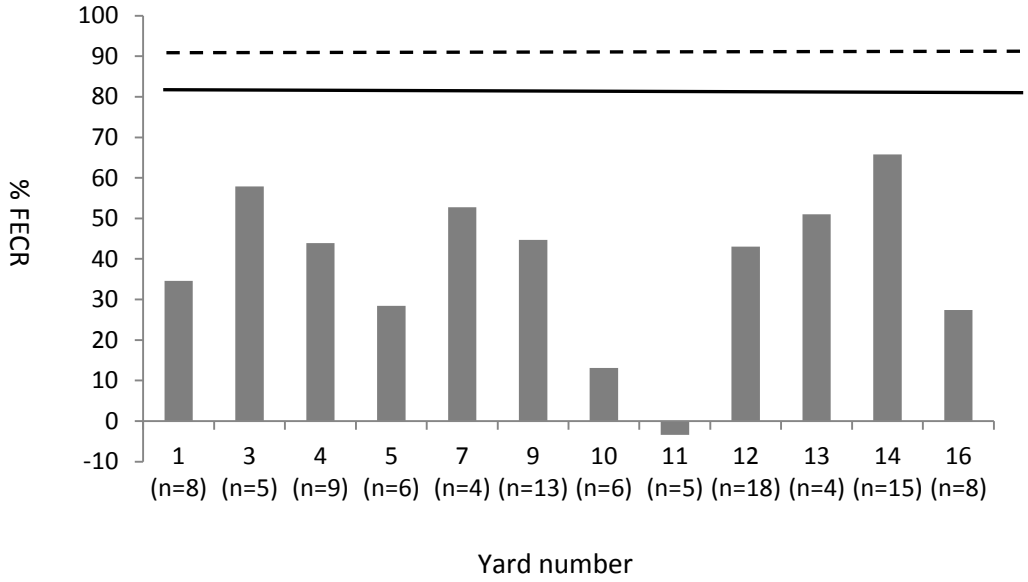
	FBZ					PYR				
Yard No.	**No. recruited**	**Day 0 FEC [EPG] Mean (range)**	**Day 14 FEC [EPG] Mean (range)**	**FECR (%)**	**LCL (%)**	**No. recruited**	**Day 0 FEC [EPG] Mean (range)**	**Day 14 FEC [EPG] Mean (range)**	**FECR (%)**	**LCL (%)**
1e	8	318 (16-1368)	208 (1-585)	34.6	NA	6	311 (30-707)	1 (0-8)	99.5	99.4
3e	5	251 (156-563)	106 (0-282)	57.9	NA	2	189 (125-252)	3 (3-3)	98.4	97.0
4e	9	376 (99-954)	211 (0-936)	43.9	NA	8	238 (48-423)	31 (0-92)	86.8	81.0
5e	6	365 (149-891)	261 (5-743)	28.4	NA	4	246 (56-783)	23 (0-91)	91.0	90.0
7e	4	460 (135-644)	218 (56-324)	52.7	NA	3	370 (273-450)	8 (0-23)	97.9	91.7
9e	13	504 (59-2138)	279 (42-1998)	44.7	NA	13	208 (39-1053)	12 (1-74)	94.1	91.5
10e	6	262 (36-900)	288 (25-612)	13.1	NA	4	484 (239-954)	19 (0-41)	96.1	91.0
11e	5	255 (69-608)	264 (89-725)	-3.4	NA	5	242 (80-666)	5 (1-11)	98.1	97.0
12e	18	645 (33-918)	368 (0-671	43.0	NA	14	319 (41-833)	5 (0-11)	98.5	93.4
13e	4	216 (36-459)	105 (60-227)	51.0	NA	4	226 (16-378)	10 (0-38)	95.5	92.1
14e	15	210 (45-918)	72 (0-279)	65.8	NA	13	98 (21-329)	1 (0-18)	98.5	96.0
15e	NP	NP	NP	NP	NP	27	54 (99-1229)	72 (0-315)	87.2	83.1
16e	8	388 (126-567)	282 (117-549)	27.4	NA	7	374 (194-563)	17 (0-71)	95.5	88.3
Total	101					110				

Table 3.9. Faecal egg count reduction test data for ivermectin (IVM) and moxidectin (MOX) on individual English yards detailing the number of equids recruited (i.e. equids with faecal egg counts (FEC) ≥50 eggs per gram (EPG) at screening). Mean strongyle FECs (EPG) are shown for Day 0 and Day 14. Arithmetic mean faecal egg count reduction (FECR) (%) and 95% lower confidence limit (LCL) (%) are displayed for each population. NP = not performed

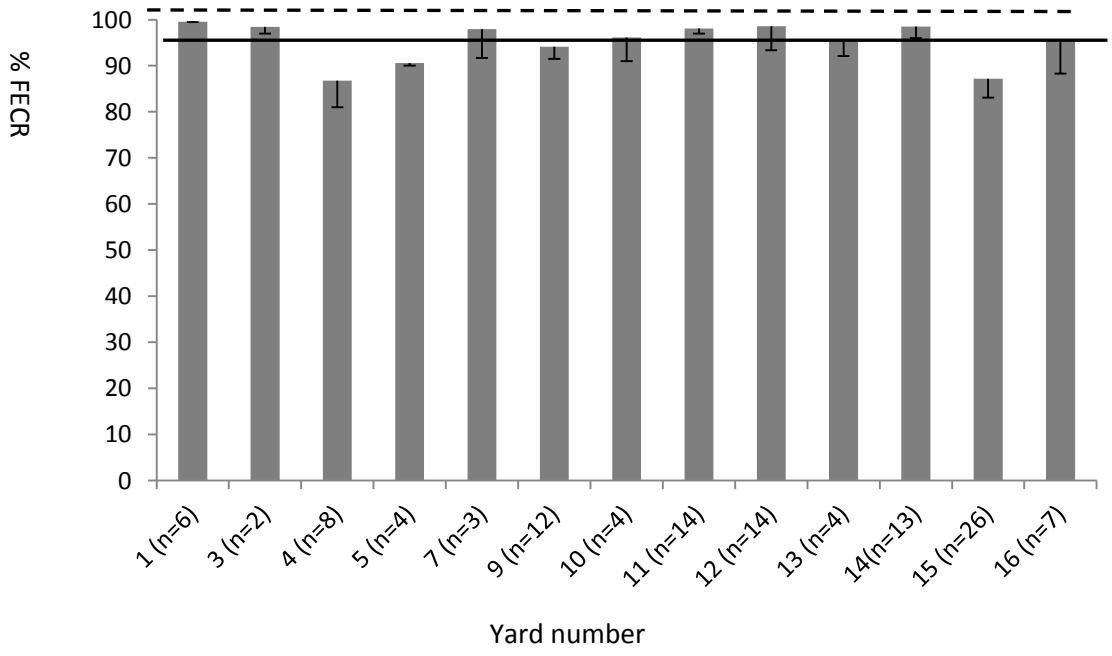
	IVM					MOX				
Yard No.	**No. recruited**	**Day 0 FEC [EPG] Mean (range)**	**Day 14 FEC [EPG] Mean (range)**	**FECR (%)**	**LCL (%)**	**No. recruited**	**Day 0 FEC [EPG] Mean (range)**	**Day 14 FEC [EPG] Mean (range)**	**FECR (%)**	**LCL (%)**
1	5	445 (140-914)	0 (0-0)	100	10	11	344 (59-1094)	0 (0-0)	100	100
3	NP	NP	NP	NP	NP	4	523 (342-657)	0 (0-0)	100	100
4	13	345 (42-824)	0 (0-2)	99.9	99.6	10	221 (63-549)	0 (0-0)	100	100
5	NP	NP	NP	NP	NP	7	318 (148-567)	0 (0-0)	100	100
7	8	175 (68-288)	8 (0-20)	96.4	94.6	NP	NP	NP	NP	NP
9	7	93 (72-131)	0 (0-0)	100	100	14	518 (45-1085)	0 (0-0)	100	100
10	8	1060 (78-2025)	0 (0-0)	100	100	9	369 (35-788)	0 (0-0)	100	100
11	6	296 (107-518)	0 (0-0)	100	100	6	244 (84-410)	0 (0-0)	100	100
12	23	288 (48-585)	0 (0-0)	100	100	NP	NP	NP	NP	NP
13	5	344 (95-887)	0 (0-0)	100	100	5	260 (83-527)	0 (0-0)	100	100
14	18	440 (53-2048)	0 (0-1)	100	99.8	15	335 (42-932)	0 (0-0)	100	100
15	NP	NP	NP	NP	NP	12	231 (45-878)	1 (0-1)	99.9	99.2
16	NP	NP	NP	NP	NP	7	302 (77-671)	0 (0-0)	100	100
Total	93					100				

Figure. 3.4. Results of faecal egg count reduction tests (FECRT) (arithmetic mean % reduction) on 12 yards (1 - 12) following either; (a) fenbendazole (FBZ), (b) pyrantel (PYR), (c) ivermectin (IVM) or (d) moxidectin (MOX) treatment. Numbers on x axis in brackets represent number of equids sampled. Dashed lines represent efficacy threshold for the particular anthelmintic (90% for FBZ and PYR, 95% for IVM and MOX). Solid black lines represent 95% lower confidence limit (LCL) threshold for the particular anthelmintic (80% for FBZ and PYR, 90% for IVM and MOX). Error bars representing the 95% LCL are included for PYR, IVM and MOX. The 95% LCL have been excluded for FBZ as some of the Day 14 FEC exceeded the Day 0 FEC values, preventing meaningful calculation

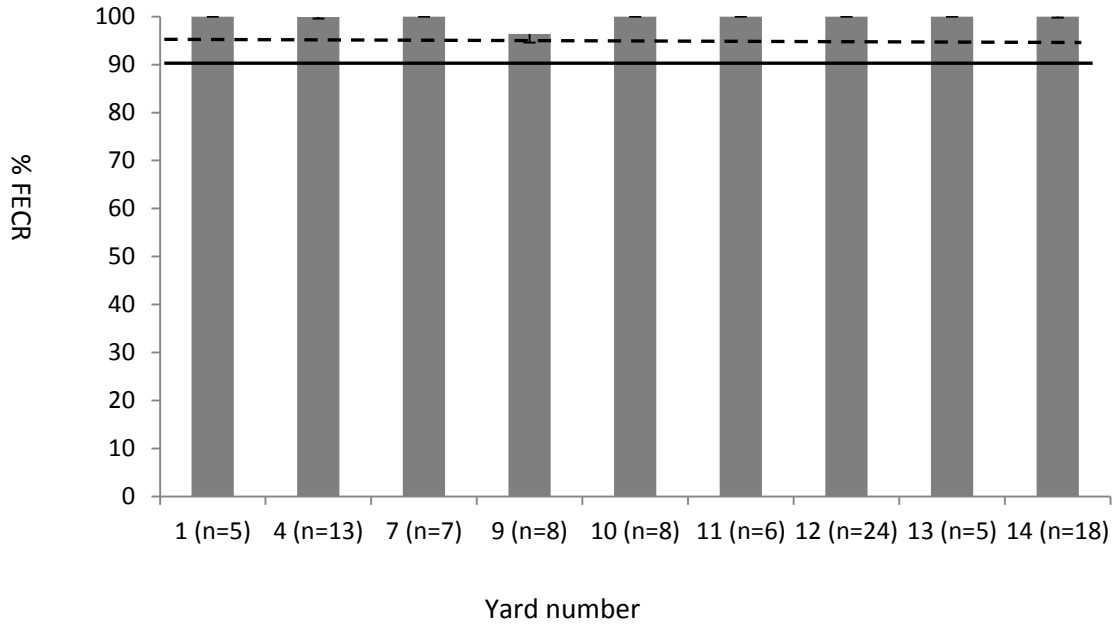
(a) FBZ



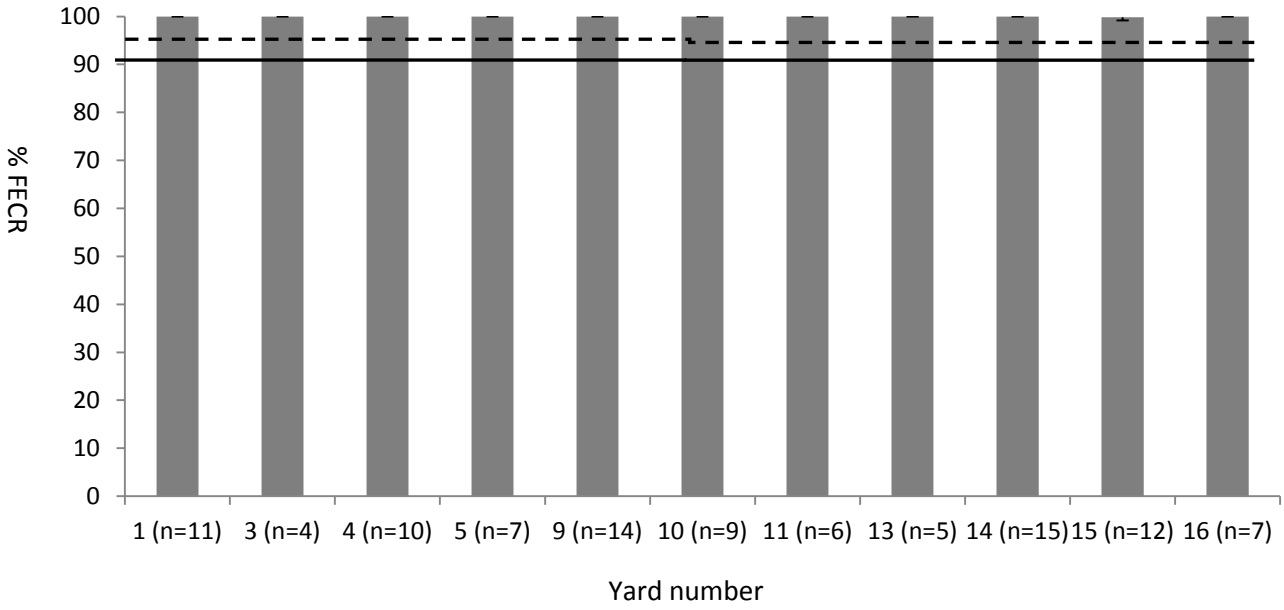
(b) PYR



(c) IVM


100
90
80
70
60
50
40
30
20
10
0
% FECR
1 (n=5)
4 (n=13)
7 (n=7)
9 (n=8)
10 (n=8)
11 (n=6)
12 (n=24)
13 (n=5)
14 (n=18)
Yard number

(d) MOX


100
90
80
70
60
50
40
30
20
10
0
% FECR
1 (n=11)
3 (n=4)
4 (n=10)
5 (n=7)
9 (n=14)
10 (n=9)
11 (n=6)
13 (n=5)
14 (n=15)
15 (n=12)
16 (n=7)
Yard number

3.4.2.3. Factors affecting Day 14 FEC

Plots of Day 0 FEC versus Day 14 FEC (Figure 3.5a) and horse age versus the Day 14 FEC (Figure 3.5b) show that as Mean Day 0 increases Mean Day 14 FEC increases and as horse age increases, Day 14 FEC decreases.

The effect of Day 0 FEC on the Day 14 FEC given treatment (Figure 3.6a) demonstrates that there is a positive trend when FBZ and PYR were administered indicating that the magnitude of the Day 14 FEC is affected by the magnitude of the Day 0 FEC. The effect of horse age on Day 14 FEC given treatment shows a negative trend when FBZ and PYR are administered (Figure 3.6b).

The GLMM analyses revealed that country and yard type did not affect Day 0 FEC (Table 3.10). Age had a negative effect on Day 14 FEC but this was not significant (b = -0.004; p = 0.841). Day 0 FEC had a weak positive effect on Day 14 FEC (b = -0.0008; p = 0.02). Unsurprisingly, the largest negative effect on Day 14 FEC was observed in the MOX treated horses (b = -8.076; p<0.001), followed by IVM (b = -6.407; p<0.001) and PYR (b = -2.852, p<0.001) (Table 3.10).

A.

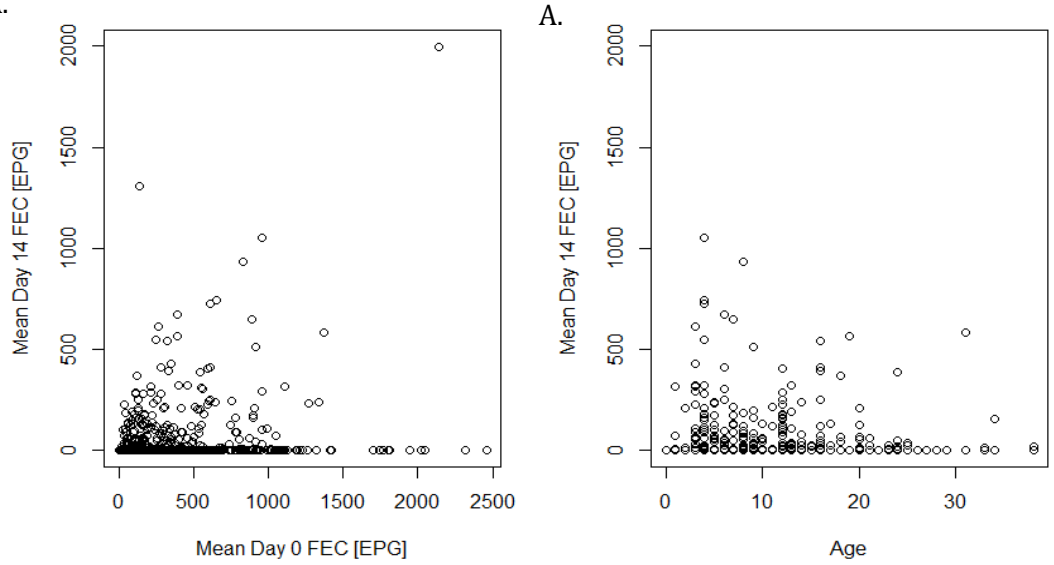
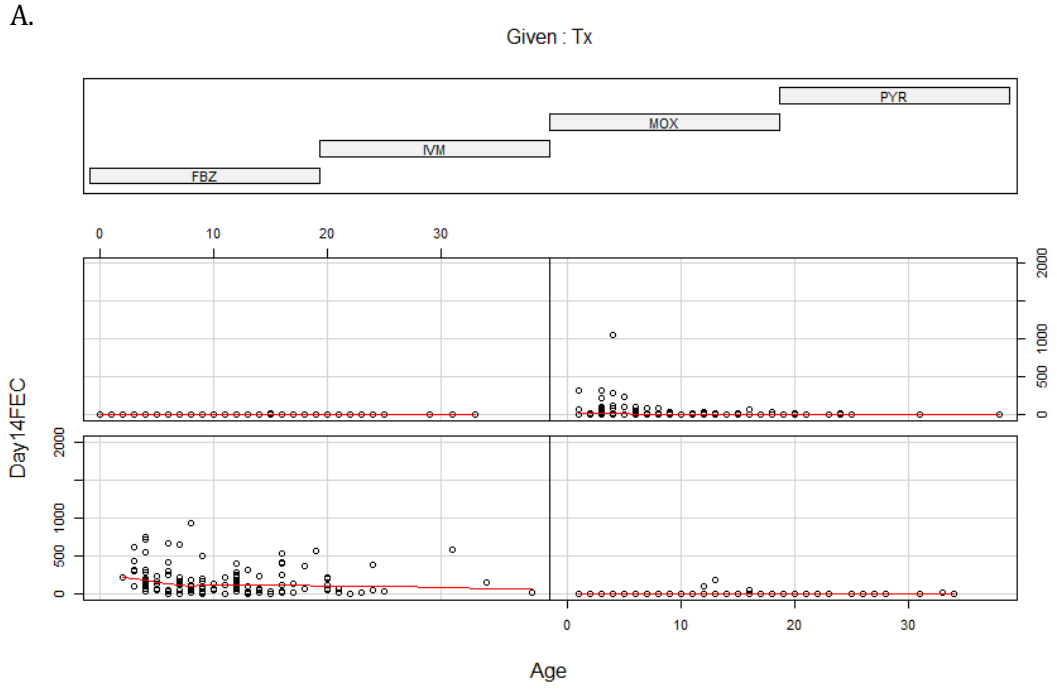


Figure 3.5. Plot of Day 0 faecal egg counts (FEC) measured in eggs per gram (EPG versus Day 14 FEC (A) and Age versus Day 14 FEC (B)

A.



B.

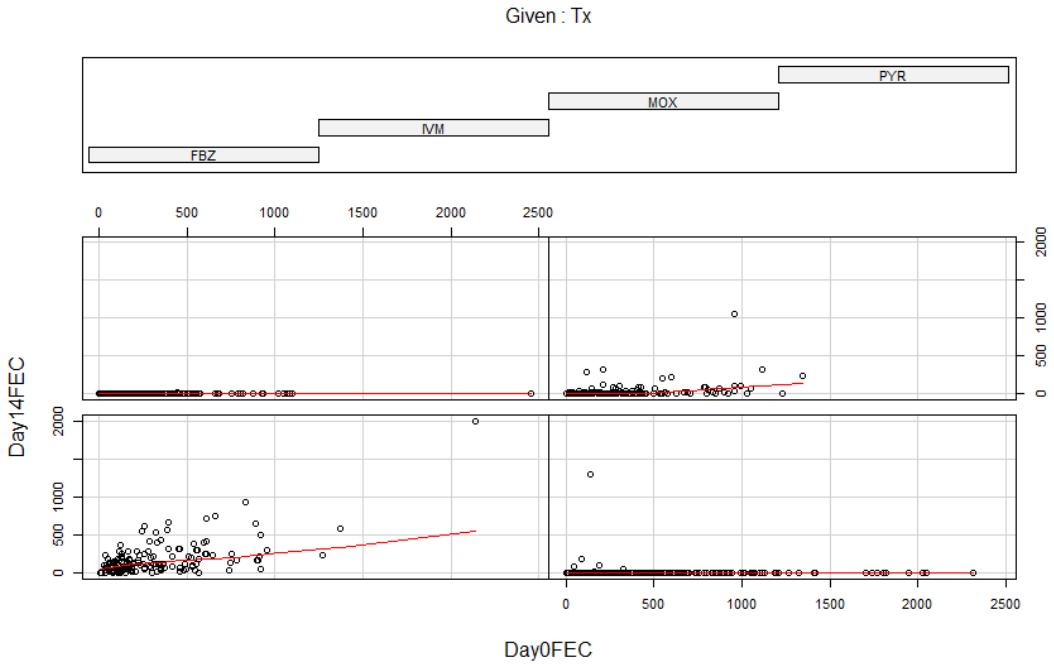


Figure 3.6. Coplot of Mean Day 0 FEC versus Day 14 FEC (A) and Age versus Mean Day 14 FEC (B) given treatment (Tx) for fenbendazole (FBZ; bottom left panel), ivermectin (IVM; bottom right panel), moxidectin (MOX; top left panel) and pyrantel (PYR; top right panel). The red line is a non-parametric smoother to emphasise the contrasting trends in each panel

Table 3.10. Generalised linear mixed models (GLMM) of the Day 14 faecal egg count (FEC), including Yard as a random effect and country, age, treatment (ivermectin, IVM; moxidectin, MOX; pyrantel, PYR) and Day 0 FEC as fixed effects. Included are the estimated effect (*b*), standard error (se) and associated z scores (z), plus the log-likelihood ratio test statistics for the dropped term. The retained terms form the final adequate model

Explanatory variable	Factor	*b*	se	z	*p*	LRT	*p*
<u>Dropped terms</u>							
Country	Scotland	0.324	0.666	0.487	0.626	0.752	0.386
Yard type	Sanctuary	0.714	1.021	0.7	0.484	0.175	0.675
<u>Retained terms</u>							
Age		-0.004	0.018	-0.200	0.841		
Treatment	IVM	-6.407	0.333	-19.241	<0.001		
	MOX	-8.076	0.429	-18.84	<0.001		
	PYR	-2.852	0.294	-9.689	<0.001		
Day 0 FEC		0.0008	0.0003	2.237	0.02		

3.4.2.4. Efficacy *versus Parascaris equorum*

The efficacy of FBZ against *P. equorum* was calculated on two yards (Yards 3e and 9e) where ascarid ova were detected on Day 0. A total of 5 FECRT were performed, the mean Day 0 FEC were 81 and 27 EPG, respectively, and on each occasion the mean FECR was 100%. MOX/PRZ efficacy was examined against *P. equorum* on one yard using five horses (mean Day 0 FEC = 146 EPG, range 5 to 269 EPG). In all horses the FECR on Day 14 was 100% (Table 3.11).

Table 3.11. Faecal egg count reduction test data for fenbendazole (FBZ) and praziquantel against ascarids (A) or tapeworm (T). Mean faecal egg count (FEC) measured in eggs per gram (EPG) are shown for Day 0 and Day 14. Arithmetic mean faecal egg count reduction (FECR) (%) are displayed for each yard

A = Ascarids, T = Tapeworm

AM	Yard number	No. Horses	Day 0 FEC [EPG] mean (range)	Day 14 FEC [EPG] mean (range)	%FECR
FBZ	3e	1	81[A]	0	100
FBZ	9e	4	27[A] (3 - 47)	0	100
PRZ	9e	5	146[T] (5 - 269)	0	100

3.4.2.5. Larval cultures

On 11 of the 16 yards recruited, positive FEC from the first screening occasion were pooled and cultured to generate L3 for classification as large or small strongyles. Of the larvae recovered, 94.3% were identified belonging to the cyathostomin group. The remaining 5.7% L3 were classified as free-living nematodes. No large strongyle larvae were identified in any of the samples examined.

3.4.2.6. Efficacy of 5d FBZ and MOX in two populations of welfare horses and horses residing at an equine hospital

In the first study, 69 equids that were resident on a welfare yard in Scotland were initially screened by FEC, of which 26 (median age 3.5 years, range 3 months to 23 years) were measured as excreting ≥50 EPG and were recruited onto a 5d FBZ FECRT. The population mean % FECR for 5d FBZ was measured as 44.7% (Table 3.12) at Day 14 post-treatment. In six (23.1%) individuals, FEC were higher on Day 14 than on Day 0 of FBZ administration, precluding calculation of 95% LCL. Twelve equids had strongyle FEC ≥50 EPG 14 days after 5d FBZ administration and were enrolled onto a MOX FECRT (median age 3.5 years, range 3 months to 9 years). The population mean FECR after MOX administration was 99.7% (95% LCL = 99.1%) 14 days post-treatment (Table 3.12).

Table 3.12. Population mean percentage faecal egg count reduction (FECR) (%) in strongyle FEC after 5 day fenbendazole (5d FBZ) and moxidectin (MOX) administration. Included are the group mean strongyle faecal egg count (FEC) measured in eggs per gram (EPG) on Day 0 and Day 14, the range of FEC (EPG), the group mean faecal egg count reduction (FECR %) and the lower 95% confidence limit (95% LCL). NC = not calculable because Day 14 FEC exceeded Day 0 FEC precluding meaningful calculation of 95% LCL

	5d FBZ (*n* = 26)		**MOX** (*n* = 12)	
	Day 0	**Day 14**	**Day 0**	**Day 14**
Arithmetic mean strongyle FEC (EPG)	412	217	545	0.1
Range (EPG)	18 - 1701	2 - 1013	29 - 1067	0 – 1
FECR (%)	44.7		99.7	
95% LCL (%)	NC		99.1	

In a further study, 11 horses, resident at the R(d)SVS equine hospital, were screened for the presence of strongyle eggs. The median age of the horses was 12 (range 5 – 16 years). Of the 11 horses, 10 had a FEC of ≥50 EPG and were randomly allocated to one of two treatment groups: Group 1 received 5d FBZ and Group 2 received MOX. On Day 2, one horse from Group 2 was withdrawn from the study as it had to be euthanased. The reason for euthanasia was not attributed to parasitism. The population mean % reduction for Groups 1 and 2 was 59.9% (LCL -3.6%) and 100 % (LCL 100%), respectively (Table 3.13).

Table 3.13. Population mean percentage faecal egg count reduction (FECR) (%) in strongyle faecal egg count (FEC) after 5 day fenbendazole (5d FBZ) and moxidectin (MOX) administration. Included are the group mean strongyle FEC measured in eggs per gram (EPG) on Day 0 and Day 14, the range of FEC (EPG), the group mean faecal egg count reduction (FECR %) and the lower 95% confidence limit (95% LCL)

	Group 1 5d FBZ (*n* = 5)		**Group 2** MOX (*n* = 4)	
	Day 0	**Day 14**	**Day 0**	**Day 14**
Arithmetic mean strongyle FEC (EPG)	292	117	305	0
Range (EPG)	41 - 810	4 – 200	173 – 648	0 - 0
FECR (%)	59.9		100	
95% LCL (%)	-3.6		100	

3.5 Discussion

Here, efficacy of anthelmintics against strongyle nematodes in horses in Scotland and England was investigated by the FECRT. On all premises tested, there was low efficacy of FBZ, consistent with previous reports (Craven et al., 1998; Kaplan et al., 2004; Osterman-Lind et al., 2007). The population arithmetic mean FECR ranged amongst premises (range -3.4% - 83.4%), but tended to be lower on the English yards (-3.4% - 65.8%) compared to the Scottish yards (15.8% – 83.4%). This may reflect differences in historical use of FBZ, particularly previous inappropriate use; for example, repeated under-dosing or high treatment frequency (Stratford et al., 2014b). Alternatively, local climate could account for the differences observed. The climate is wetter in Scotland compared to the south of England, potentially leading to a larger population of infective larvae on pasture in Scotland, thus increasing the population of parasites in refugia, which has been demonstrated to slow the development of resistance in sheep nematodes (Martin et al., 1981). These results indicate that FBZ should no longer be recommended for cyathostomin control on these premises. These results underline high levels of a lack of FBZ efficacy compared to the last study performed in Scotland 20 years ago, when acceptable efficacy (i.e. >90% mean FECR) was identified at 7 out of 9 premises tested (King et al., 1990).

Acceptable efficacy of PYR was found on all Scottish premises, with FECR ranging from 90.4 -99.6%, and LCL ranging from 80.9 to 99.4%. Only on Yard 8s, did the 95% LCL approach the designated 80% cut-off. Evaluation of individual FECR on Yard 8s revealed a single horse with a markedly lower FECR than the yard mean. This individual, who was two years old, was tested on two occasions, and a similar result was obtained in both cases. For the control of *Schistosoma mansoni*, humans often receive two treatments, given 40 days apart (Picquet et al., 1998). This approach has

been shown to be more effective than single treatment alone, particularly in cases where there is high infection intensity. This practice is not routinely adopted in the treatment of cyathostomins, but could be an effective approach in controlling horses with high EL burdens, which would mature after the initial dose, potentially leading to an increase in strongyle egg shedding. However, any such treatment would be 'off-label' and would only be allowed by a veterinary surgeon under the 'cascade'.

Host age may explain why PYR failed to reduce the pre-treatment FEC by >90% as there is previous evidence that anthelmintics are less effective in younger animals (Herd and Gabel, 1990). Furthermore, a recent study that examined anthelmintic efficacy on UK Thoroughbred stud farms, tested PYR on five studs, using 64 horses; in mares, efficacy ranged between 99.4 and 99.8% FECR, while in yearlings efficacy ranged between 8% and 73% FECR (Relf et al., 2014). In agreement with this, a recent study by Nielsen et al. (2013) found that PYR efficacy increased with increasing age and that as pre-treatment FEC increased, PYR efficacy decreased (Nielsen et al., 2013). Here, acceptable PYR efficacy was found on 11 of 13 English premises tested. On two yards, resistance to PYR was suggested; both however, were used for rescue/sanctuary. It is likely that there were frequent movements of animals at these properties. Unless strict quarantine measures were employed, there would be an increased risk of transmission of anthelmintic resistant small strongyles. Resistance on these yards was suspected rather than indicated, and it is possible there were difficulties in administering anthelmintics to some individuals rather than this representing resistance. The findings here contrast with reports from the USA, where PYR resistance was found on 20 - 40.5% farms (Kaplan et al., 2004; Tarigo-Martinie et al., 2001). It is likely that the higher prevalence of PYR resistance in the USA is a result of its availability as a daily in-feed anthelmintic since the 1990s (Slocombe and de

Gannes, 2006; Tarigo-Martinie et al., 2001), likely resulting in a high selection pressure for PYR-resistant genotypes (Brazik et al., 2006).

Acceptable efficacy was demonstrated with both ML anthelmintics; FECR exceeded 95% reduction in FEC on all premises tested. Recent studies have reported a reduction in ERP following IVM treatment in Europe, Brazil and the USA (von Samson-Himmelstjerna et al., 2007; Molento et al., 2008; Lyons et al., 2008; Relf et al., 2014). In a German study, IVM was reported to be efficacious at 14 days post-treatment on multiple sites; however, a reduction in strongyle ERP from 8 to 5 weeks on 2 of 6 farms was noted (von Samson-Himmelstjerna et al., 2007). Recent studies indicate that MOX is still effective at 14 days post-treatment (Becher et al., 2010; Traversa et al., 2009, 2012), in agreement with the findings of this study. However, in the USA, a shortened ERP after MOX administration has been observed (Rossano et al., 2010; Lyons et al., 2011) and concurrent reduced efficacy of all three anthelmintic classes was indicated in Brazil (Molento et al., 2008). In the Brazilian study, only one FECRT was performed for each anthelmintic, with group sizes of six horses. With such a limited sample size, the findings are not likely to reflect the true resistance status of cyathostomins across the whole of Brazil. They failed to mention the age of the horses and the pre-treatment FEC, which, as discussed earlier, could have a bearing on efficacy of the anthelmintics tested (Nielsen et al., 2013). They also failed to mention the FEC method used, which, as reported in Chapter 2 can influence the FECRT. In a more recent study conducted in Brazil, 498 horses from 11 yards were used to assess the efficacy of FBZ, PYR, IVM and MOX. Each FECRT group consisted of eight horses that were >12 months old and had not received anthelmintic treatment in the preceding 60 days (Canever et al., 2013). FBZ resistance was reported on all yards tested. PYR resistance was reported on one yard and reduced efficacy, measured by a LCL <80%, was identified on three yards.

IVM resistance was reported on one yard and resistance to MOX was not observed (Canever et al., 2013). Here again, details on the FEC method used were not included, nor were any details on the mean pre-treatment FEC published. In addition, on the yards where PYR and IVM resistance were observed, the authors did not provide details of whether a low FECR was observed in all or just one horse from the test group or if the horses were monitored to confirm ingestion of the anthelmintic. Further, the animals/groups in which resistance was identified were not retested with the same anthelmintic to confirm resistance (Canever et al., 2013). A recent study looking at anthelmintic efficacy on UK Thoroughbred studs reported a shortened ERP following MOX administration on three yards (*n* = 35 horses), with eggs reappearing in faeces 4 weeks post MOX administration, suggesting that the MLs are not working as effectively as measured previously (Relf et al., 2014). It is not surprising that a reduction in ERP was observed on Thoroughbred stud farms, where populations of small strongyles have been under strong selection pressure to develop anthelmintic resistance due to practices such as frequent and indiscriminate administration of anthelmintics (Relf et al., 2012). Reductions in ERP may be a more sensitive indicator of resistance than FECR (Sangster, 1999) and further investigation into ML ERP is warranted to confirm continued efficacy of anthelmintics in this class.

In the present study, FBZ demonstrated good efficacy (≥90%) against *P. equorum*. In a recent study, conducted on UK Thoroughbred studs, FBZ FECRT were conducted in 16 horses resident on two yards and, on both yards, FBZ demonstrated good efficacy (>95% FECR) compared to FECR observed on a further two farms (*n* = 12 horses) where IVM was administered, and the mean % FECR <90%. There have been several reports of reduced efficacy of the MLs against *P. equorum* (Hearne et al., 2003; Slocombe et al., 2003; Reinemeyer, 2008) therefore; routine use of ML for the control

of *P. equorum* in foals should be questioned, at least on large stud farms in the UK. Further studies are warranted to investigate the efficacy of FBZ against *P. equorum*, as this may be the anthelmintic of choice for use in ascarid control in young horses.

MOX, IVM and PYR had a significant negative effect on Day 14 FEC. MOX had the greatest effect (b = -8.076; p<0.001), followed by IVM (b = -6.407; p<0.001) and PYR (b = -2.852; p<0.001), indicating that MOX is more effective at reducing the Day 14 FEC compared to IVM and PYR. This finding is likely explained by the original efficacy studies, whereby MOX demonstrated 100% efficacy vs. adult cyathostomins, 90.8% efficacy vs. EL3 and 99.9% against all other developing cyathostomin larvae (Monahan et al., 1995; Bairden et al., 2001; 2006; Reinemeyer et al., 2003), compared to reported IVM efficacy of 99% against adults, 98% efficacy against luminal cyathostomin larvae (Klei et al., 1993; Xiao et al., 1994), and reported PYR efficacy of 89 – 96% against adult cyathostomins (Lyons et al., 1974). Furthermore, IVM and PYR have been licensed for longer than MOX, increasing the selection pressure for resistance.

Day 0 FEC had a significant weak positive effect on Day 14 FEC (b = 0.0008; p = 0.02), indicating that the higher the Day 0 FEC, the higher the Day 14 FEC. Age had a weak negative effect on Day 14 FEC but this was not significant (b = -0.004, p = 0.841). Similar findings were reported in a recent study, where a hierarchical model was used for evaluating PYR efficacy data from 64 Danish horse farms (Nielsen et al., 2013). The authors found that the group mean pre-treatment FEC had a significant effect on PYR efficacy and as the pre-treatment mean increased, PYR efficacy decreased and age had a weak negative effect on PYR efficacy with PYR efficacy increasing with age. However, this finding was not significant (Nielsen et al., 2013). There is evidence to suggest that anthelmintics are not as effective when used in younger horses. Early studies conducted on a Thoroughbred breeding farm between 1982 and 1988 found that

oxbendazole (OXB), PYR and IVM were significantly less effective when administered to yearlings compared to adult mares (Herd and Gabel, 1990b). A recent study conducted on UK Thoroughbred studs, reported that PYR was effective in two groups of mares (group mean FECR 98% - 99%), compared to five groups of yearlings in which efficacy ranged from 0 - 73% (Relf et al., 2014). A reason for the observed reduced efficacy in younger horses is the fact that younger horses lack acquired immunity, which allows a greater accumulation of encysted cyathostomins (Lyons et al., 2009). When anthelmintics such as BZ, PYR and IVM are administered, which do not possess high efficacy against these stages, once luminal adult worms have been eliminated, encysted stages may emerge and resume development and eggs are seen in faeces sooner compared to adult horses, which harbour fewer encysted stages (Herd, 1986; Herd and Gabel, 1990b). It is well documented that strongyle FEC are higher in younger horses compared to adult horses (Relf et al., 2013)

Previous studies have used different criteria for determining anthelmintic resistance in horses. Lind et al., 2007 used resistance criteria for IVM and FBZ (FECR 95%, LCL 90%) to describe 'suspected' or 'considered' resistance; and FECR 90%, LCL 80%, for PYR. Tarigo-Martinie et al. (2001), classed an anthelmintic as 'effective' if the FECR was greater than 90%, 'equivocal' if between 80% and 90%, and 'ineffective' if less than 80%. This lack of standardisation makes direct comparison amongst studies difficult, limiting the accurate assessment of temporal or geographical variation in efficacy (Stratford et al., 2014b). Here, the FECR cut-offs chosen reflected differences in efficacy of the anthelmintics in drug-sensitive populations when the products were first licensed (Kaplan and Nielsen, 2010), but further work is required to standardise the way equine FECRT are conducted and reported. To this end, a working group of veterinary parasitologists and equine clinicians have been working towards

standardising the FECRT for use in equids, but to date, are yet to produce ratified guidelines (Nielsen et al., 2011). The guidelines need to define the number of horses to be included in a FECRT, which FEC method to use and the diagnostic sensitivity, what the pre-treatment FEC should be for selecting horses for the FECRT, the statistical method for estimating efficacy and 95% confidence intervals and clear guidelines on how to interpret the results.

The recruitment of yards through two veterinary practices and the BHS website may have introduced a degree of sampling bias, as it could be assumed that BHS yard owners are more aware of good parasite management practices and of anthelmintic resistance. Therefore these findings may not be fully representative of the entire horse population in Scotland and England. That said, there was an element of heterogeneity in the yards recruited (i.e. different uses, breeds present and age range). In addition, four welfare yards were investigated; which were more likely to harbour a population of horses with an unknown, and most likely, infrequent, worming history.

Efficacy was calculated per yard using the group mean pre- and post-treatment counts. This method of analysing the results did not take into account the differences in horse age and immunity, grazing history, worming history and other management factors between yards. These differences will lead to heterogeneity in FEC among and between populations of horses (Morgan et al., 2005), which in turn leads to variation in the outcome of FECRT (Denwood et al., 2010). To counter this, the effects of age, Day 0 mean and treatment on Day 14 FEC were explored through a negative binomial generalised mixed model, in which yards were included as a random effect to account for the variation between yards.

In the face of widespread FBZ resistance, it could be argued that MOX remains the only anthelmintic with high efficacy *versus* encysted cyathostomin larvae. The preliminary

results here also indicated a lack of efficacy of 5d FBZ in reducing strongyle FEC. These results were only capable of determining an effect on egg excretion and previous studies have demonstrated poor correlations between the magnitude of FEC and larval burdens (Nielsen et al., 2010a), so whether 5d FBZ had any effect on EL burden cannot be determined from the data collected here. To definitively assess larvicidal efficacy, terminal studies would be required. The development of techniques to determine *in vivo* EL burden (McWilliam et al., 2010; Clements et al., 2012) may assist evaluation of treatment efficacy in future. The limitations to such a test would be the length of time that IgG(T) circulates post-treatment, which could lead to false positive results. Given the high prevalence of FBZ resistance, targeted MOX use should now be recommended for the treatment of encysted cyathostomin larvae at the appropriate time of year. Presently, some veterinarians prescribe 5d FBZ as a treatment for clinical larval cyathostominosis, for treating horses with a high strongyle FEC and as a quarantine measure. This may reflect concerns surrounding toxicity of MOX in thin or debilitated horses (Johnson et al., 1999; Muller et al., 2005; Schumacher and Taintor, 2008) and may also reflect a lack of appreciation of almost ubiquitous FBZ resistance in cyathostomin populations. Few studies have reported the outcome of larval cyathostominosis cases following MOX treatment. Most publications detailing larval cyathostominosis case management were written prior to licensing of MOX (Love and McKeand, 1997). In one small study with eight horses in each treatment group, both anthelmintics demonstrated adulticidal and larvicidal efficacy evidenced by mucosal and luminal worm counts (Steinbach et al., 2006). The data indicated larvicidal death occurred sooner following treatment with FBZ compared to MOX, with worm death cited as 4 - 6 and 6 - 14 days following treatment, respectively. The different lengths of treatment render the data difficult to interpret. Severe mucosal inflammation, increased plasma globulin and increased granuloma formation around intact larvae

were noted 14 days after 5d FBZ treatment, whereas no substantial intestinal histopathological inflammation was observed in MOX-treated horses (Steinbach et al., 2006). The variations in outcome were thought secondary to differing mechanisms of action of the compounds, or differing immunological responsiveness to damaged parasites. None of the horses displayed signs consistent with larval cyathostominosis prior to treatment, apart from a reduction in weight gain in comparison with a worm-free control group, and potential safety of administration to clinical cases could not be determined (Steinbach et al., 2006). Deprez and Vercruysse, (2003) compared efficacy of IVM *versus* MOX in cyathostominosis cases. Either ML was administered on a minimum of one occasion, at the manufacturers recommended dose rate, followed by repeat treatments at 7 or 14 days if larvae were identified macroscopically in faeces at these time points. Four out of 11 horses in the MOX group received two MOX doses; two receiving an additional treatment 7 days later and a further two at 14 days later following the initial dose. In comparison, 3 out of 9 horses in the IVM group received two MOX doses; three receiving an additional treatment 7 days later and a further two at 14 days later. IVM and MOX treatment resulted in elimination of larvae from faeces within 1 - 2 weeks; however, there were no significant improvements in body weight or clinical parameters over the 3-week observation period and no significant intergroup difference. A longer follow-up period would be required for significant differences in weight and clinical parameters to be observed. Such results indicate; firstly, no significant adverse effects associated with MOX administration in clinical cases, and, secondly, prolonged clinical signs, despite apparent anthelmintic efficacy, indicate that clinical signs are a result of parasite-induced damage, and that supportive care is important. Prior to licensing of MOX in the UK, a treatment regimen comprising oral dosing with 5d FBZ followed by IVM on Day 6 of treatment on at least three occasions at 10-day intervals was recommended (Love and McKeand, 1997).

Considering the high prevalence of FBZ resistance, and the spectrum of activity of MOX, revision of these guidelines may be appropriate. Concomitant supportive care comprising anti-diarrhoeal agent, fluid-, electrolyte-, oncotic- support and analgesics are recommended to reduce intestinal inflammation in cases of colitis (Love and McKeand, 1997; Mair et al., 2002).

3.6 Conclusion

These studies have helped to inform on the current anthelmintic sensitivity status of cyathostomin populations in horses in England and Scotland. The observed widespread lack of FBZ efficacy in reducing strongyle FEC warrants discussion into the future use of this anthelmintic for the treatment and control of cyathostomins. This anthelmintic should still be considered for use against other species such as *P. equorum*, in which there appear to be rising levels of resistance to ML products (Boersema et al., 2002; Hearn and Peregrine, 2003; von Samson-Himmelstjerna et al., 2007b). Further studies are required to look at larger populations of horses in different parts of the UK, and different parasite species such as *Anoplocephala* spp., *Oxyuris equi*, *Strongyloides westeri* and *P. equorum*. This study and evidence from many others now suggests that horse owners must move towards a more targeted evidence-based approach to helminth control.

CHAPTER 4: Assessing the performance of methods for testing anthelmintic efficacy *in vivo*

4.1 Introduction

The faecal egg count reduction test (FECRT) is widely used to estimate anthelmintic efficacy against parasitic helminths *in vivo* (Coles et al., 1992). However, use of the FECRT has not been fully validated for use in equids. There are several caveats to its application, particularly in horses, but in the absence of validated *in vitro* and molecular tests, the FECRT remains the only available option for the routine evaluation and surveillance of anthelmintic efficacy in equids (Kaplan, 2002; Vidyashankar et al., 2012). Recently, studies have aimed to quantify variability arising through the FECRT and have used complex statistical analyses to address the underlying statistical limitations arising from equine faecal egg count (FEC) and FECRT data (Torgerson et al., 2005; Denwood et al., 2010; Torgerson et al., 2012; Torgerson et al., 2014; Paul et al., 2014). However, for many, such computationally and statistically intensive methods for determining FECRT are not accessible, as the statistical analyses are run in R, a complex statistical package requiring the user to be familiar with programming language. As such, there is a need to assess the performance and limitations of the less statistically demanding FECRT method currently recommended by the World Association for the Advancement of Veterinary Parasitology (WAAVP) (Coles et al., 1992;2006) and other less statistically demanding methods cited in the literature (Pook et al., 2002; Vidyashankar et al., 2007; 2012), to determine which method generates the most reliable estimates of anthelmintic efficacy for use in the field.

As previously discussed in Chapter 2, there are a number of underlying factors that can lead to variability in FEC data. The major findings from the studies in Chapter 2 demonstrated that strongyle eggs were over-dispersed in faeces (Section 2.5.1), and the use of a FEC method with a lower detection limit reduced variance (Section 2.5.3).

It was identified that the type of FEC method used also influenced decisions for anthelmintic treatment, with more horses found to exceed a 200 EPG threshold when FEC were analysed by the McMaster (McM) method compared to a more sensitive centrifugal-Flotation (CF) method (Section 2.5.5). When determining levels of anthelmintic efficacy in the aforementioned studies, different assumptions were made depending on the FEC method used (Section 2.5.6). In summary, when efficacy was high (i.e. following IVM or MOX administration) or when it was low (i.e. following FBZ administration), all FEC methods generated the same assumptions. However, when anthelmintic efficacy was around the threshold (i.e. following PYR administration), then the FEC method implemented (McM or CF) had an impact on the classification of resistance/susceptibility, and disagreements between methods were observed.

Results from these empirical datasets warrant further investigation to develop a theoretical framework for understanding the optimum diagnostic conditions for conducting a FECRT. It is important to be able to discriminate between an anthelmintic that is efficacious and one to which there is resistance, and reduce the likelihood of misclassification (i.e. either falsely declaring efficacy or falsely declaring resistance). Falsely identifying an anthelmintic as efficacious when it is not is problematic because, when resistance is identified, an alternative anthelmintic should be administered to reduce potential dissemination of resistant alleles through the survival of adult parasites and subsequent pasture contamination or through animal movements. It is also important to define resistance as early as possible to reduce the potential for clinical disease. Once resistance has been identified, this knowledge must be disseminated within the scientific and end user communities to inform control protocols and potentially incentivise pharmaceutical companies to invest in new anthelmintic development.

The outcome of a FECRT is classified on the basis of arbitrary thresholds. Typically, resistance is declared if the mean percentage reduction in FEC is less than 90% (Coles et al., 1992). In the present study, a threshold of 90% was selected for FBZ and PYR and 95% was selected for IVM and MOX. These were chosen as they reflect differences in original efficacy of these anthelmintics in drug-sensitive helminth populations when the products were first licensed (Kaplan and Nielsen, 2010). The current lack of guidance and rigid thresholds makes the interpretation of FECRT data complicated. For example, if an anthelmintic demonstrates 80% efficacy then should that anthelmintic be used in the future on the same parasite population? It must be acknowledged that such an approach is based on the presumed efficacy of an anthelmintic rather than its 'true' efficacy, which is unknown (Vidyashankar et al., 2007). Using a single threshold for defining efficacy is not a statistically robust approach, as neither the spread of the FECRT data is considered nor the impact of outliers on the subsequent classification. As such, many researchers include upper and lower confidence intervals (Vidyaskankar et al., 2007; Stratford et al., 2014b). Previous studies have used different criteria for determining anthelmintic resistance in horse and this lack of standardisation makes clear comparison amongst studies challenging by limiting accurate assessment of temporal or geographical variation in efficacy (Stratford et al., 2014b).

Factors affecting the FECRT can be broadly split into those that can be controlled and those that cannot. The former include the number of horses included, the FEC method used and the egg detection limit of the FEC method utilised, while the latter include the mean pre-treatment FEC (EPG), the distribution of FEC between groups of horses (*k*), and true efficacy. The mean pre-treatment FEC and the distribution of FEC can in theory be manipulated by modifying selection criteria. Systematic exploration of these factors and how they impact the diagnostic performance of the FECRT can realistically

only be conducted using a statistical modelling approach (Torgerson et al., 2005; Vidyashankar et al., 2007; Dobson et al., 2009, 2012; Calvete and Uriarte, 2013). Recently, a study by Calvete and Uriarte (2013) aimed to improve the detection of anthelmintic resistance in sheep nematodes by simulating variation across a range of parameters including, pre-treatment mean FEC, the distribution of the pre-treatment mean FEC, true efficacy, and FEC detection limit. Monte Carlo simulations of all possible combinations of parameters were explored. The major limitation of this study was that the statistical model was based on prior assumptions and was not validated using empirical data obtained in the field. With any simulation approach, it is important to parameterise and validate the model with robust data to ensure that model output is as accurate as possible and fit for purpose (Sargent, 2013). To this end, the model built for the present study was based on the FEC analysis findings of Chapter 2 and FECRT data collected in Chapter 3. This enabled a generalisable theoretical framework to be developed and validated using a specific set of FEC data collected in support of the allied objectives of this thesis.

4.2 Aims and objectives

Two methods (non-parametric bootstrapping (Vidyashankar et al., 2007) and arcsine transformation of FECRT data (Pook et al., 2002) for estimating anthelmintic efficacy were applied to the equine FECRT data presented in Chapter 3 and compared to the currently recommended method (WAAVP, Coles et al., 1992) to investigate if the method used to estimate mean percentage faecal egg count reduction (FECR) had a bearing on the outcome and interpretation of the data in terms of classifying anthelmintic efficacy. The arcsine transformation and bootstrap methods were chosen as they are better able to deal with FEC data that do not follow a normal distribution, and they can be performed relatively simply in Excel opposed to suggested Bayesian

methods which require knowledge of R. As the method suggested by Coles et al. (1992) is currently the method recommended by the WAAVP, and as it is the easiest and simplest formula available to estimate mean % FECR, its performance was investigated further through a simulation approach. The effect of the FEC method used for pre-treatment and post-treatment samples and the egg detection limit of the method, the underlying mean pre-treatment FEC, the underlying distribution of the Day 0 FEC (negative binomial parameter, *k*), the number of horses included in the test and the true efficacy of the anthelmintic on the performance of the WAAVP method for estimating efficacy was investigated. The overall aim being to produce recommendations for interpreting FECRT data that are practical, that take account of underlying influences of methods on results and are validated by field data.

4.3 Materials and methods

4.3.1 Faecal egg count reduction test methods

Three different methods for estimating anthelmintic efficacy were applied to 18 sets of FBZ FECRT data, 21 sets of PYR data, 16 sets of IVM FECRT data and 19 sets of MOX FECRT data. These datasets were presented in Chapter 3 (Section 3.4.2.2).

4.3.1.1 Method 1 (WAAVP method)

Percentage FECR was estimated for each group of horses using the following formula recommended by the WAAVP (Coles et al., 1992) (Equation 4.1), where arithmetic group mean FEC for Day 0 and Day 14 were used to estimate the group FECRT:

FECRT % efficacy=

$$\frac{Group\ mean\ pre-treatment\ counts-\ Group\ mean\ post-treatment\ counts}{Group\ mean\ pre-treatment}\times 100$$

(Equation 4.1)

In addition, 95% lower confidence limits (LCL) were calculated from the standard error (se) of the mean individual % FECR (Equation 4.2). These calculations were based on the actual number of eggs seen before back transforming to EPG.

95% LCL = mean % FECR - (1.96 x se)

(Equation 4.2)

4.3.1.2 Method 2 (arcsine transformation)

Method 2 was used to estimate the group mean % FECR from arcsine transformed individual proportional reductions (Pook et al., 2002, Method 2). The % FECR was estimated for each individual and the arcsine square root of each individual FECR proportion calculated. The group mean % FECR was calculated using Equation 4.3.

Group mean % FECR = 100 x $(\sin(\text{transformed group mean}))^2$

(Equation 4.3)

The 95% LCL were estimated from the standard error of the transformed mean % FECR

95% LCL = 100 x $(\sin(\text{transformed mean \% FECR - (1.96 x se)}))^2$

(Equation 4.4)

4.3.1.3 Method 3 (non-parametric bootstrapping)

Monte Carlo stochastic simulation (Ripley, 1987) was used for non-parametric bootstrapping as a means of estimating % FECR. The bootstrap approach involved generating new datasets by sampling the original data with replacement (Efron and Tibshirani, 1986). Here, the individual % FECR from each horse was re-sampled with replacement. For example, on a yard of 6 horses, the individual % FECR may be 92%, 88%, 95%, 89%, 90% and 87%. The bootstrap data set was constructed by randomly generating six new % FECR from the original dataset with replacement (for example, 88%, 92%, 88%. 90%, 88%, 87%), and a new average % FECR calculated. The newly generated bootstrap dataset was then used to generate 10,000 simulated % FECR to obtain estimates of the yard mean % FECR, and upper and lower 2.5-percentiles were taken as the 95% confidence limits (Efron, 1979; Hilborn and Mangel, 1997). PopTools software (CSIRO, Australia) was used for bootstrapping (Hood, 2010).

4.3.2 Definition of anthelmintic resistance

The thresholds chosen for defining resistance were mean % FECR of <95% for IVM/MOX and <90% for BZD/PYR (Kaplan and Nielsen, 2010). Also, 95% LCL thresholds of <90% and <80% were selected for classifying resistance for IVM/MOX and FBZ/PYR, respectively. Accordingly, if both % mean FECR *and* the LCL fell below the designated cut-offs, anthelmintic resistance was *indicated*. Alternatively, if either the % mean FECR *or* the LCL fell below these cut-offs, resistance was *suspected* (Table 4.1).

4.3.3 Simulation model

The diagnostic performance of the WAAVP recommended FECRT method was explored through a Monte Carlo simulation approach (Efron and Tibshirani, 1986). The simulation model was used to investigate FECRT factors that can be controlled (see above) and factors that cannot be so easily controlled (see above). In theory, some of these factors can be manipulated by modifying selection criteria (Kaplan and Nielsen, 2010), and the simulation included these factors in order to explore their effect more thoroughly. The effect of each of these factors on the classification of efficacy was investigated to define the application and limitations of FECRT design in detecting anthelmintic resistance.

4.3.3.1. Justification of parasitological parameters: factors that cannot be controlled

Strongyle egg shedding is usually highly over-dispersed amongst horse populations (Relf et al., 2013; Wood et al., 2013). The most widely used distribution chosen to represent parasite count data (nematodes and nematode eggs) is the negative binomial distribution (NBD) and previous studies in other host species have shown that the NBD adequately models helminth FEC aggregation (Grenfell et al., 1995; Shaw et al., 1998; Morgan et al., 2005; Dobson et al., 2009; Calvete and Uriarte, 2013). However, other distributions such as the gamma-Poisson (Wilson and Grenfell, 1997), the lognormal distribution (Elston et al., 2001), the Weibull distribution (Gaba et al., 2005) and zero-inflated distributions (Jell et al., 2008; Walker et al., 2009; Denwood et al., 2010) have all been used for modelling parasite aggregation. Here, the NBD (k) was used because its use in describing the distribution of parasite data is well published and it is relatively straightforward to apply. The model simulated eight levels of k (0.5, 1.0, 1.5,

2.0, 2.5, 3.0, 3.5, 4.0 and 5.0) and nine levels of mean pre-treatment FEC (EPG) (50, 100, 150, 200, 250, 300, 350, 400 and 500). These values were selected on the basis of the range of *k* and mean pre-treatment FEC values estimated from pre-treatment FEC collected from 26 sets of empirical FECRT datasets (these data were originally presented in Chapter 3, Section 3.4.2.2), 13 of which were generated by a CF method, with an egg detection limit (dl) of down to 1 EPG (Section 2.3.2) and 13 generated by the modified McM method, with an egg dl of 50 EPG (McM50) (Section 2.2.1). From these data, estimates for *k* ranged between 0.67 and 6.7 and mean Day 0 FEC between 173 and 1900 EPG (Table 4.2 and 4.3). True efficacy was fixed at 70, 90 or 95%. These values were selected to reflect resistance (70%), borderline resistance/efficacy if PYR or FBZ were used (90%) and borderline resistance/efficacy if IVM or MOX were used (95%).

4.3.3.2 Justification of parasitological parameters: factors that can be controlled

The effect of the FEC method used to generate FECRT data was included in the model as a continuation of the findings reported in Chapter 2. FECRT datasets were compiled from simulated FEC generated by either the CF method or the McM method. For FECRT datasets generated by the McM method, the egg dl could be changed (5, 15, 30, 50 and 100 EPG) based on the volume of faecal suspension examined in the McM slide (Section 2.2.1). The number of horses included in the FECRT was explored and *n* ranged from 1 to 20 horses. These numbers were selected to reflect the empirical FECRT datasets collected, where FECRT group sizes ranged from 3 to 18 horses (Table 4.2). When simulating across the range of method-related parameters, the mean pre-treatment FEC was fixed at 200 EPG, *k* at 2.2, *n* at 10 and the egg dl for McM at 50 EPG. These values were selected as they represented the average mean pre-treatment FEC, *n* and *k*

observed from the empirical FECRT datasets, and the McM egg dl of 50 EPG as recommended by the WAAVP (Coles et al., 1992; 2006).

4.3.4 Simulation of FEC data

4.3.4.1. CF model

A total of 20 individual FEC were simulated from set *m*, *k* and efficacy (*eff*) to give a simulated true range of FEC (EPG_1). Efficacy (*eff*) represented the level at which anthelmintic efficacy was set in the model. Each true FEC (EPG_1) was converted to the actual number of eggs observed (egg_1), by dividing EPG by the egg detection limit (*dl)*. Due to the nature of the CF method, *dl* is determined by egg density (Section 2.3.2.2); as such, if EPG_1 was ≥200 EPG then EPG_1 was divided by 9, if EPG_1 ≥50 EPG, EPG_1 was divided by 3 and if EPG_1 was <50 EPG then no conversion was required. From the values of egg_1, a new value was drawn (egg_2) from the Poisson distribution, to represent different aliquots of suspension so that the number of eggs observed should follow a Poisson distribution. The observed values for egg_2 were multiplied by the relevant *dl* to give the observed EPG (EPG_2). The derived EPG_2 values were used as the pre-treatment FEC. The post-treatment FEC ($PtxEPG_1$) were generated from EPG_1 using the following formula;

$$PtxEPG_1 = EPG_1 * (eff/100)$$

(Equation 4.5)

$PtxEPG_1$ was then converted to the actual number of eggs seen ($Ptxegg_1$) by dividing $PtxEPG_1$ by *dl*. From the values obtained for $Ptxegg_1$, a new value was drawn ($Ptxegg_2$) from the Poisson distribution. $Ptxegg_2$ was then converted back to EPG ($PtxEPG_2$) to give the post-treatment FEC.

Individual %FECR was estimated using the following formula:

$$\text{Individual FECR} = ((EPG_2 - PtxEPG_2) / EPG_2) \times 100$$

(Equation 4.6)

4.3.4.2. McM model

The simulated FEC for generated by the McM method were drawn from the same true FEC (EPG_1) that were generated in the CF model, the difference being that the egg dl used for the McM method was fixed for the pre-treatment and post-treatment counts, whereas with CF, the egg dl was dependent on egg density. The egg dl investigated for McM were 5, 10, 15, 30, 50 and 100 EPG, and were representative of how the egg dl of McM can be manipulated by counting 1 grid of a McM slide (100 EPG dl); counting 2 grids (50 EPG dl); counting 1 chamber (30 EPG dl); counting 2 chambers (15 EPG dl), counting 3 chambers (10 EPG dl) or counting 6 chambers (5 EPG dl).

4.3.5 Simulation of FECRT data

The model was populated with the values of *m*, *k* and *eff* to generate individual values of EPG_2 and $PtxEPG_2$. Depending on *n*, the arithmetic mean EPG_2 and arithmetic mean $PtxEPG_2$ were used to estimate observed mean % FECR (4.8) for FEC generated by CF and McM.

$$\text{Mean \% FECR} = ((EPG_2 - PtxEPG_2) / EPG_2) \times 100$$

(Equation 4.7)

4.3.6 Simulated data generation and evaluation of the diagnostic performance of FECRT depending on which FEC method was used

Initially, Monte Carlo simulations were generated by fixing *m* at 200 EPG, *n* at 10 and efficacy at 70%, but simulating over a range of *k* (0.5 – 5). The simulations were repeated with efficacy fixed at 90% and then at 95%. The effect of mean Day 0 FEC was then explored by fixing *k* at 2.2 and *n* at 10 and simulating across a range of *m* (50 - 500 EPG), with efficacy fixed at 70, 90 and 95%. The effect of *n* was investigated by fixing *m* at 200 EPG, *k* at 2.2 and simulating across a range of *n* (1 - 20 horses) with efficacy fixed at 70, 90 and 95%. Finally, the effect of the egg dl of McM was investigated by fixing *m* at 200 EPG, *k* at 2.2 and *n* at 10, and simulating across a range of egg dl's (5 – 100 EPG), with efficacy fixed at 70, 90 and 95%. In order to investigate co-variation of *m* and *k*, six values of mean Day 0 FEC and Day 0 *k* from empirical FECRT datasets were used to populate the model and simulations using these values were performed over a range of *n* (2 - 20 horses) and efficacies (70, 90 and 95%). For each simulation, 100 sets of simulated FECRT data were generated, and from these, data misclassification rates were calculated. The thresholds used to calculate the misclassification rates are described in Table 4.1 and reflect the thresholds widely used to discriminate between efficacy and resistance (i.e. 90% for FBZ and PYR and 95% for IVM and MOX).

Table 4.1. The classification (false positive or false negative) of the simulated faecal egg count reduction test results when a 90% (i.e. fenbendazole (FBZ)/pyrantel (PYR)) or 95% (i.e. ivermectin (IVM)/moxidectin (MOX)) threshold was applied and efficacy was set at 70, 90 or 95%

Set efficacy (%)	Classification of results for 90% threshold (i.e. FBZ/PYR)	Classification of results for 95% threshold (i.e. IVM/MOX)
70	>90% = False negative	>95% = False negative
90	<90% = False positive	>95% = False negative
95	<90% = False positive	<95% = False positive

The relationship between mean and distribution (*k*) Day 0 FEC, and the number of horses included in the test on the misclassification rate was investigated through a generalised linear modelling (GLM) approach using Poisson regression. All analyses were performed using RStudio 12.15.1.

4.3.7 Model validation

Field validation of the model is not possible in a pure sense, as true efficacy is not known and cannot be measured in living horses using a gold standard. Nevertheless, simulation outputs were compared with field FECRT data to assess whether simulations were able to predict measured FECR. A good match between simulated and observed FECR would show that the simulation adequately captures the most important sources of variation in observed FECR. To examine this, 26 sets of equine FECRT data (Section 3.4.2.2) were used to validate the model; 13 sets of data had been generated using the CF FEC method and 13 using the McM50 method (Tables 4.3 and 4.4). The CF and McM50 generated FECR data were generated from the same animals on the same yard at the same time. For each set of data generated by each FEC method, mean pre-treatment and post-treatment, mean % FECR and *k* were calculated. In total,

four sets of FBZ FECRT data, seven sets of PYR FECRT data and two sets of IVM FECRT data were used. The NBD was fitted to 26 sets of empirical Day 0 FEC data (Yards 1 to 9, 13 sets generated by the CF method and 13 sets by the McM50 method) by maximum likelihood estimation (MLE, Williams and Dye, 1994; Shaw et al., 1998; Morgan et al., 2005) to give estimates of the mean, *m,* and the aggregation factor, *k.* The chi-square goodness of fit test was used to assess whether these data adequately fitted the NBD (Chapter 2, Section 2.4.1). These values were then used to simulate predicted FECRT data as above to compare with the measured FECRT data (Section 3.4.2.2) and hence validate the model. Thus, the empirical mean pre-treatment FEC and corresponding *k* values from FEC performed by the CF method were used to populate the model and generate 100 Monte Carlo simulated predicted mean pre-treatment and post-treatment FEC and expected mean % efficacy as estimated by FECRT. An average of the 100 simulations was taken for the predicted values. The chi-squared (χ^2) test was used to compare predicted (*O*) and measured (*E*) pre-treatment FEC as follows (Equation 2.4, Section 2.4.2). Predicted (*O*) and measured (*E*) post-treatment counts were compared in the same way. The same method was followed using pre-treatment and post-treatment FEC data generated by the McM50 method. A lack of a significant difference was taken to indicate that the error structure in FEC distribution and the FEC process was adequately captured by the simulation and associated assumptions. The χ^2 for FEC performed by CF and McM50 was compared with the chi-squared distribution, a significant difference between the predicted and measured mean pre-treatment and mean post-treatment EPG ($p<0.05$) indicated that the model did not generate realistic FEC based on the empirical FEC data generated by the CF method and McM50 method. The relationship between the predicted mean % FECR and the measured mean % FECR was investigated by linear regression.

4.4 Results

4.4.1. Comparison of three different methods for estimating faecal egg count reduction

For all 18 datasets included in the analysis, the mean % FECR was below 90% (LCL <80%) when FBZ was administered regardless of the method used to estimate FECR (Figure 4.1). For 15 datasets, Method 2 could not be used and for four datasets, Method 3 could not be applied because too many individuals were shedding more eggs at Day 14 than at Day 0 (Figure 4.1). For 14 out of 21 PYR FECRT datasets, Method 2 estimated the mean % FECR to be higher compared to Methods 1 and 3 (Figure 4.2). For one dataset (COO), Method 1 estimated mean % FECR below 90%, LCL >80% (Figure 4.2). For this dataset, Methods 2 and 3 estimated mean % FECR above 90% and LCL <80%. For another (LAS), Methods 2 and 3 estimated mean % FECR below 90%, LCL <80%, compared to Method 1, where resistance was suspected (% FECR >90% LCL <80%). For the WHW dataset, the mean % FECR estimated by Methods 1 and 3 was <90%, LCL >80%, compared to Method 2, where the % FECR and LCL were >90% and 80% respectively (Figure 4.2). In 11 out of 16 IVM FECRT datasets, Methods 1, 2 and 3 generated the same result (mean % FECR 100%) (Figure 4.3). For 3 out of 5 datasets where the % efficacy was not 100%, Method 2 generated the highest estimation of mean % FECR compared to the other methods and for two datasets, Method 3 gave the lowest % FECR compared to the other methods (Figure 4.3). Results from the MOX FECRT demonstrated that for 13 out of 19 datasets, all methods generated the same mean % FECR (Figure 4.4). For four datasets, Method 3 generated the lowest mean % FECR compared to the other two methods, and on two occasions, Method 1 generated the lowest mean % FECR (Figure 4.4). In summary, for FBZ, IVM and MOX, all analytical methods generated the same outcome (Table 4.2). For FBZ, all methods classified the

test populations as '*resistant*' and for IVM and MOX, all methods classified the test populations as '*sensitive*'. For the PYR FECRT dataset for 3/21 (14%) tests, there was disagreement between methods in the interpretation of the data (Table 4.2).

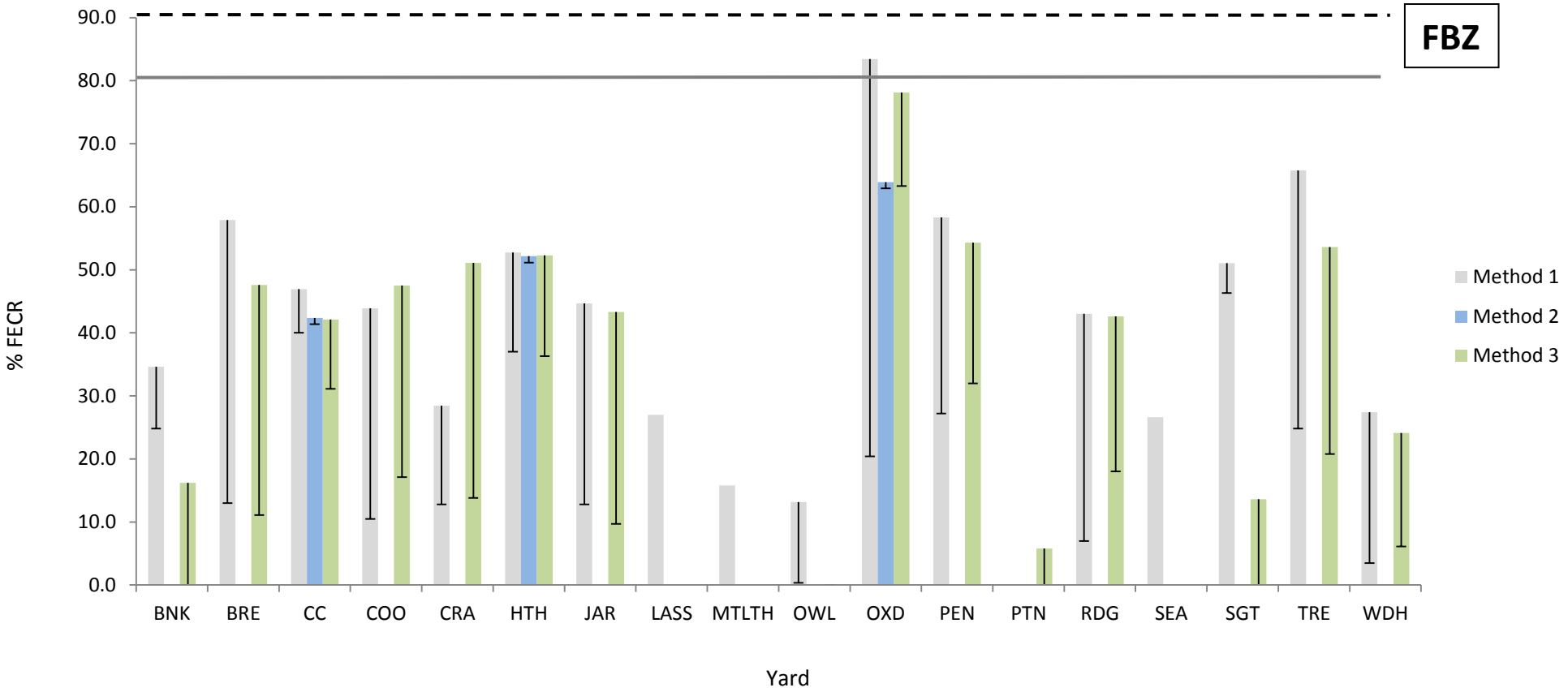


Figure 4.1. The estimated mean percentage faecal egg count reduction (% FECR) and lower 95% confidence limits (LCL), (which have been truncated at zero) derived using three different methods for estimating FECR when applied to 18 sets of fenbendazole (FBZ)-FECR data. The black dashed line represents the 90% threshold for acceptable efficacy and the solid grey line represents the 80 % threshold set for the lower confidence interval. For 15 datasets, Method 2 could not be applied as the Day 14 faecal egg count (FEC) exceeded those calculated on Day 0. For three datasets, only Method 1 was applied as the FEC data precluded the application of Methods 2 and 3. For all datasets and each method, the % FECR and LCL fell below the designated thresholds, suggesting that resistance was present

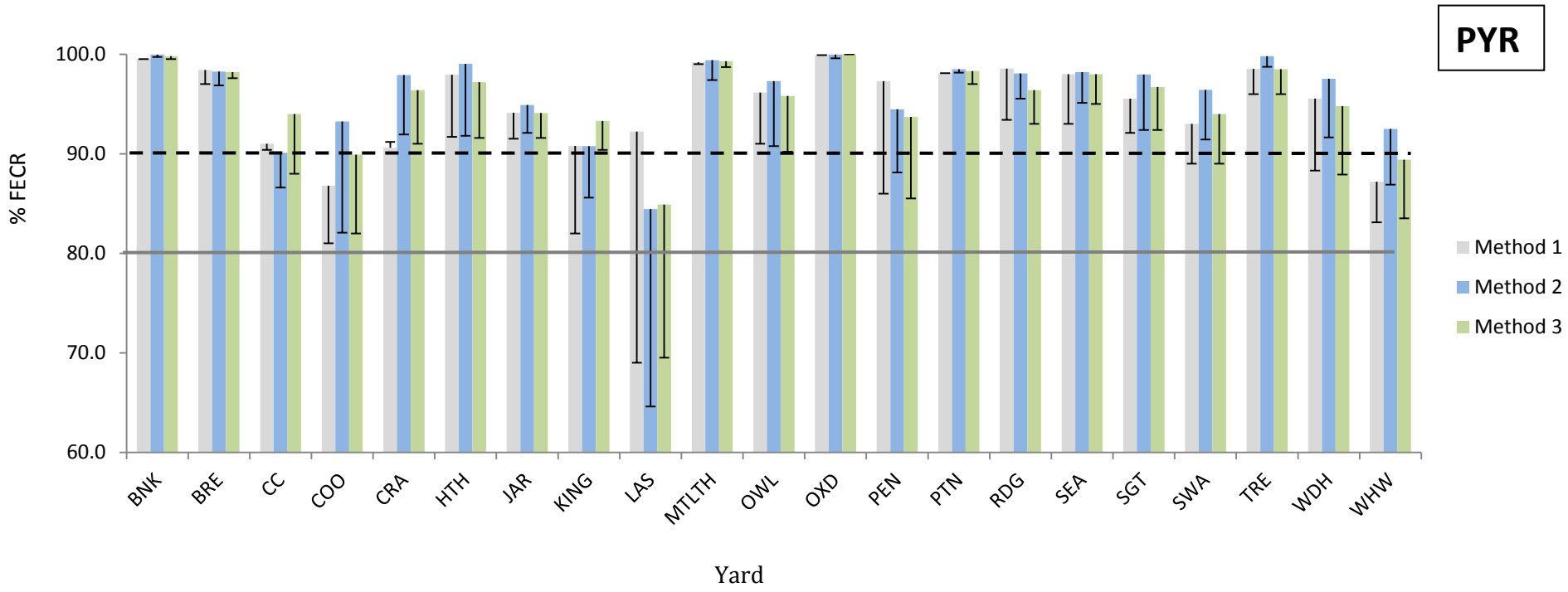


Figure 4.2. The estimated mean percentage faecal egg count reduction (% FECR) and lower 95% confidence limit (LCL) derived from three different methods for estimating FECR when applied to 21 sets of pyrantel (PYR) FECR data. The black dashed line represents the 90% threshold for acceptable efficacy and the solid grey line represents the 80 % threshold set for the lower confidence interval. On 18 yards, all methods were in agreement (i.e. mean % FECR ≥90% and LCL ≥80%).

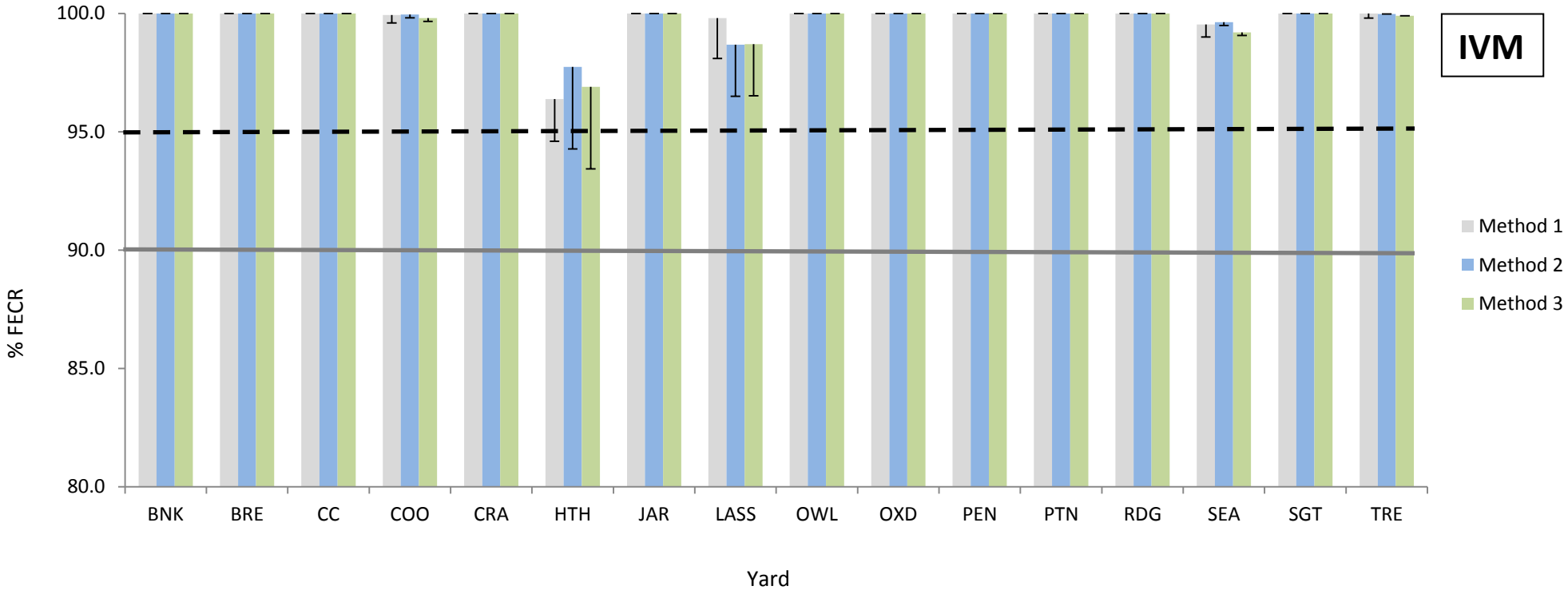


Figure 4.3. The estimated mean percentage faecal egg count reduction (% FECR) and lower 95% confidence limits (LCL) derived from three different methods for estimating FECR when applied to 16 sets of ivermectin (IVM) FECR data. The black dashed line represents the 95% threshold for acceptable efficacy and the solid grey line represents the 90% threshold set for the LCL

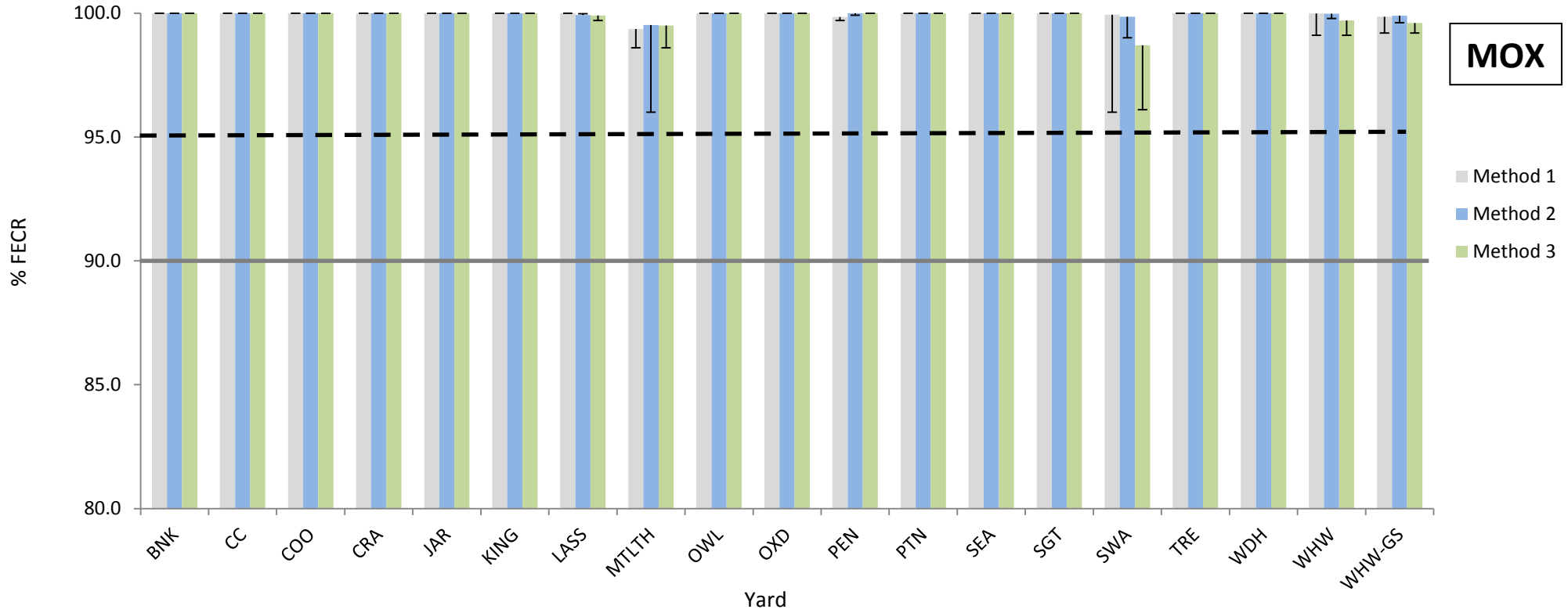


Figure 4.4. The estimated mean percentage faecal egg count reduction (% FECR) and lower 95% confidence limit (LCL) derived from three different methods for estimating FECR when applied to 19 sets of moxidectin (MOX) FECRT data. The black dashed line represents the 95% threshold for acceptable efficacy and the solid grey line represents the 90 % threshold set for the LCL

Table 4.2. A summary the faecal egg count reduction test (FECRT) when three methods of estimating mean percentage faecal egg count reduction (% FECR) were applied to sets of FECRT data collected from yards following administration of either fenbendazole (FBZ), pyrantel (PYR), ivermectin (IVM) and moxidectin (MOX). The outcome of the FECRT was classified as either susceptible (S), suspected resistance (SR) or resistant (R). For FECRT results that were classified as SR or R, the mean % FECR is recorded. Not all anthelmintics were administered on all yards (NP). Tests for which methods disagreed in terms of anthelmintic resistance classification are highlighted by grey shading

	FBZ			PYR			IVM			MOX		
Yard	Method 1	Method 2	Method 3	Method 1	Method 2	Method 3	Method 1	Method 2	Method 3	Method 1	Method 2	Method 3
BNK	R	R	R	S	S	S	S	S	S	S	S	S
BRE	R	R	R	S	S	S	S	S	S	NP	NP	NP
CC	R	R	R	S	S	S	S	S	S	S	S	S
COO	R	R	R	SR (86.8%)	S (93.2%)	SR (89.9%)	S	S	S	S	S	S
CRA	R	R	R	S	S	S	S	S	S	S	S	S
HTH	R	R	R	S	S	S	S	S	S	NP	NP	NP
JAR	R	R	R	S	S	S	S	S	S	S	S	S
KING	NP	NP	NP	S	S	S	NP	NP	NP	S	S	S
LAS	R	R	R	SR (92.2%)	R (84.5%)	R (84.9%)	S	S	S	S	S	S
MTL	R	R	R	S	S	S	NP	NP	NP	S	S	S
OWL	R	R	R	S	S	S	S	S	S	S	S	S
OXD	R	R	R	S	S	S	S	S	S	S	S	S
PEN	R	R	R	S	S	S	S	S	S	S	S	S
PTN	R	R	R	S	S	S	S	S	S	S	S	S
RDG	R	R	R	S	S	S	S	S	S	NP	NP	NP
SEA	R	R	R	S	S	S	S	S	S	S	S	S
SGT	R	R	R	S	S	S	S	S	S	S	S	S
SWA	NP	NP	NP	S	S	S	NP	NP	NP	S	S	S
TRE	R	R	R	S	S	S	S	S	S	S	S	S
WDH	R	R	R	S	S	S	NP	NP	NP	S	S	S
WHW	NP	NP	NP	SR (87.2%)	S (92.5%)	SR (89.4%)	NP	NP	NP	S	S	S
WHW-GS	NP	NP	NP	NP	NP	NP	NP	NP	NP	S	S	S

4.4.2. Simulated FECRT results to assess the performance of Method 1(WAAVP) when FECRT data were generated using CF and McM50

4.4.2.1. Model validation using raw FECRT data

The FECRT data obtained using CF methodology and used for model validation covered a range of mean pre-treatment EPG (177 - 512 EPG) and *k* (0.67 - 4.4), and the number of horses included in the test (3-18 horses) (Table 4.3). The FECRT data obtained using McM50 methodology also covered a range of mean pre-treatment EPG (462 - 1900 EPG) and *k* (0.69 -6.7), the number of horses included was the same as with the CF method (Table 4.4). The measured mean pre-treatment EPG, *k*, *n* and mean % FECR from the actual FECRT dataset were used to populate the model. The pre-treatment EPG, post-treatment EPG and mean % FECR from the measured results were compared to the predicted pre-treatment EPG, post-treatment EPG and mean % FECR generated by the model for both the CF and McM50 generated counts. The chi-squared test statistic (χ^2) was used to measure how close the measured mean pre-treatment EPG and mean post-treatment EPG were to the predicted pre-treatment EPG and mean post-treatment EPG when FEC were generated by the CF, and no significant difference between measured mean pre-treatment EPG and predicted mean pre-treatment EPG (p = 0.83) was observed and no significant difference between the observed and expected mean post-treatment EPG (p = 0.91) were noted, indicating that the model was able to generate adequate mean pre- and post-treatment FEC in comparison to measured data generated by the CF method (Table 4.3). The same process was followed to assess FEC generated by McM50, and there was no significant difference between the measured and predicted mean pre-treatment EPG (p = 0.36) and no significant difference between the measured and predicted mean post-treatment EPG (p = 0.69) (Table 4.4). The relationship between measured and predicted % efficacy when FEC were

performed by the CF method was explored by linear regression (Figure 4.5). Here, there was a significant positive relationship between measured and predicted % efficacy (Pearson – r^2=0.99, p=<0.001). The regression equation was: measured % efficacy = 0.7 + 0.99 x predicted % efficacy. The intercept was not significantly different from 0 and the slope was not significantly different from 1 (Figure 4.5a). For FECRT performed using the McM50 method, there was a significant positive relationship between measured and predicted % efficacy (Pearson – r^2=0.99, p=<0.001). The regression equation for McM50 was: measured % efficacy = -0.67 + 1 x predicted % efficacy. The intercept was negative, but not significantly so, suggesting that predicted efficacy may be lower than measured efficacy (Figure 4.5b).

Table 4.3. The measured and predicted mean pre-treatment (pre-tx) and post-treatment (post-tx) faecal egg count (FEC) and measured and predicted mean % efficacy derived from 13 sets of faecal egg count reduction test (FECRT) data collected from Yards 1 to 9 when FEC performed by centrifugal-flotation (CF). For each yard dataset, pre and post-treatment FEC were performed by CF. The measured mean pre-treatment eggs per gram (EPG), *k* and the % efficacy were used to populate the model. The chi-squared test statistic (χ^2) was used to measure how close the predicted (*E*) mean pre- and post-treatment EPG was to the measured (*O*) pre- and post-treatment EPG for each yard dataset. The χ^2 for CF was compared with the chi-squared distribution, a significant difference between the predicted and measured number of eggs was observed if $p<0.05$

Yard	Drug	*N*	Mean Day 0 FEC (EPG)	*k*	Measured mean pre-tx FEC (EPG)	Predicted mean pre-tx FEC (EPG)	$\frac{(\boldsymbol{O}-\boldsymbol{E})^2}{\boldsymbol{E}}$	Measured mean post-tx FEC (EPG)	Predicted mean post-tx FEC (EPG)	$\frac{(\boldsymbol{O}-\boldsymbol{E})^2}{\boldsymbol{E}}$	Measured % efficacy	Predicted % efficacy
1	FBZ	8	324	0.67	324	332	0.198	209	218	0.388	35.5	34.3
1	PYR	6	262	0.81	227	229	0.018	2	3	0.500	99	98.6
2	FBZ	4	173	0.96	251	265	0.781	106	118	1.358	57.8	55.6
2	PYR	3	310	0.89	183	178	0.137	3	2	0.333	98.4	98.9
3	PYR	8	238	2.5	238	258	1.681	31	40	2.613	87	84.5
3	IVM	13	345	2	345	347	0.012	0	0	0.000	100	100
4	FBZ	6	365	1.8	365	364	0.003	261	256	0.096	28.5	29.7
5	IVM	8	177	4.4	177	154	2.989	0	0	0.000	100	100
6	PYR	4	484	3.7	484	477	0.101	19	18	0.053	96.1	96.2
7	PYR	5	252	1.8	242	253	0.500	5	6	0.200	98	97.6
8	FBZ	18	512	2.3	512	516	0.031	263	259	0.061	48.6	49.8
8	PYR	15	319	2.1	319	320	0.003	5	4	0.200	98.5	98.8
9	PYR	4	229	0.92	229	244	0.983	11	9	0.364	95.2	94.9
						χ^2	7.435		χ^2	6.165		
						p	0.830		*p*	0.910		

Table 4.4. The measured and predicted mean pre-treatment (pre-tx) and post-treatment (post-tx) egg counts and measured and predicted mean % efficacy derived from 13 sets of faecal egg count reduction test (FECRT) data collected from Yards 1 to 9 when faecal egg counts (FEC) performed by McMaster with an egg detection limit of 50 eggs per gram (EPG) (McM50). For each yard dataset, pre and post-treatment egg counts were performed by McM50. Measured mean pre-treatment FEC, *k* and % efficacy were used to populate the model. The chi-squared test statistic (χ^2) was used to measure how close the predicted mean pre- and post-treatment FEC was to the measured pre- and post-treatment FEC for each yard dataset. The χ^2 for centrifugal-flotation (CF) was compared with the chi-squared distribution, a significant difference between the measured and predicted number of eggs was observed if $p<0.05$

Yard	Drug	*n*	Mean Day 0 FEC (EPG)	*k*	Measured mean pre-tx FEC (EPG)	Predicted mean pre-tx FEC (EPG)	$\frac{(\boldsymbol{O}-\boldsymbol{E})^2}{\boldsymbol{E}}$	Measured mean post-tx FEC (EPG)	Predicted mean post-tx FEC (EPG)	$\frac{(\boldsymbol{O}-\boldsymbol{E})^2}{\boldsymbol{E}}$	Expected % efficacy	Observed % efficacy
1	FBZ	8	1263	0.69	1263	1200	3.143	863	860	0.010	31.7	28.3
1	PYR	6	1150	0.75	968	960	0.066	0	0	0.000	100.0	100.0
2	FBZ	4	563	1.38	450	455	0.056	410	404	0.088	8.9	11.2
2	PYR	3	1050	0.88	350	350	0.000	0	0	0.000	100.0	100.0
3	PYR	8	1183	1.1	1183	1255	4.382	125	144	2.888	89.4	88.5
3	IVM	13	462	3.5	462	450	0.312	4	0	0.000	99.1	100.0
4	FBZ	6	742	1.1	742	771	1.133	442	490	5.213	40.4	36.4
5	IVM	8	306	2.2	306	318	0.471	13	14	0.000	95.8	95.6
6	PYR	4	1900	6.7	1900	1973	2.805	88	94	0.409	95.4	95.2
7	PYR	5	920	2.7	920	938	0.352	50	55	0.500	94.6	94.1
8	FBZ	18	867	2.4	867	877	0.115	497	490	0.099	42.7	44.1
8	PYR	15	553	1.4	553	560	0.089	13	13	0.000	97.6	97.7
9	PYR	4	625	0.99	625	637	0.230	50	50	0.000	92.0	92.2
						χ^2	13.153		χ^2	9.207		
						p	0.36		*p*	0.69		

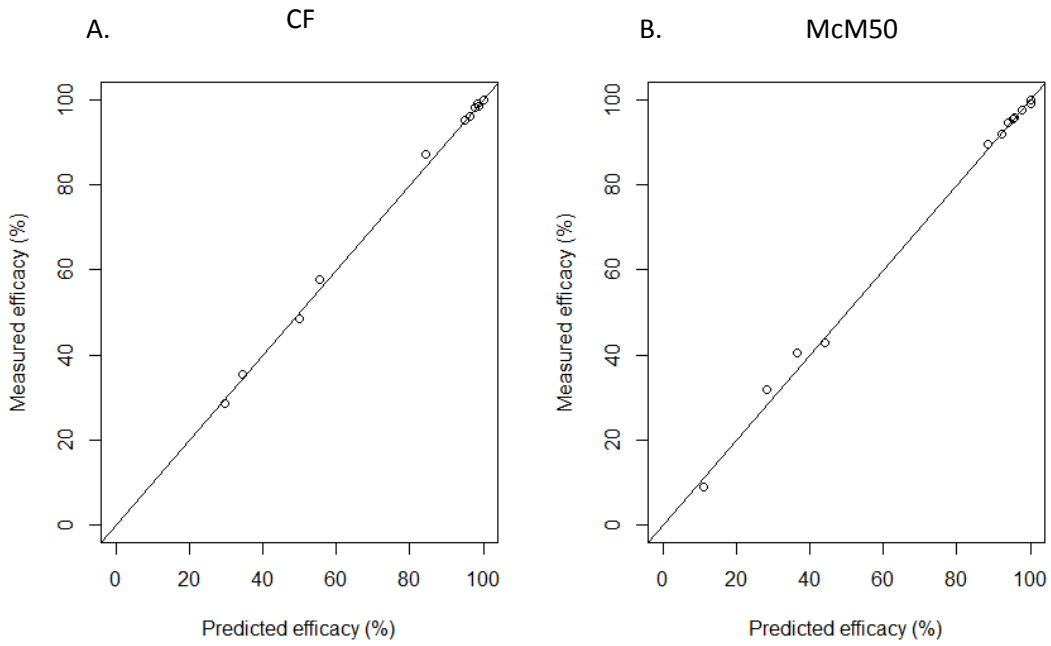


Figure 4.5. Measured and predicted % efficacy when faecal egg counts (FEC) were performed by (a) centrifugal-flotation (CF) (Pearson – r^2=0.99, p=<0.001) or (b) McMaster with an egg detection limit of 50 eggs per gram (McM50) (r^2=0.99, p=<0.001). Measured efficacy values were derived from 13 sets of faecal egg count reduction test datasets for which pre- and post-treatment egg counts were generated from both CF and McM50. Trend lines represent a linear regression with a zero intercept between the predicted and measured % efficacies. The regression equations for CF and MCM50 were measured % efficacy = 0.7 + 0.99 x predicted % efficacy and measured % efficacy = -0.67 + 1 x predicted % efficacy, respectively

4.4.2.2. Simulated FECRT results: the effect on the underlying pre-treatment FEC distribution (*k*) on misclassification

There were no false negative results when the CF method was used and efficacy was set at 70% (Figure 4.6 a-b, Table 4.5), compared to the McM50 method where the percentage of FECRT results that were >90% efficacy ranged from 5 – 9% (Figure 4.6a, Table 4.5). There was no obvious trend and the % misclassification (>90%) did not decrease as *k* increased across the tested range (Table 4.7). The percentage of simulated FECRT results that exceeded 95% efficacy ranged from 0 - 5% and tended to decrease as *k* increased (Figure 4.6b).

When efficacy was set at 90%, the percentage of false positive results (<90%) ranged between 26 - 35% by the CF method and the % misclassification tended to decrease as *k* increased (Figure 4.6c, Table 4.5) but this was not significant (Table 4.6). There were no false negative results and none of the simulated FECRT results exceeded >95% efficacy (Figure 4.6d). with McM50, the percentage of FECRT results that were <90% ranged between 23 – 37% and the % misclassification tended to decrease as *k* increased (Figure 4.6c, Table 4.5) and the percentage of simulated FECRT that exceeded 95% ranged from 33 - 54%. Generally, the false negative misclassification rate increased as *k* increased when simulated FEC were generated by McM50 but this was not significant (Figure 4.6d, Table 4.7).

At 95% efficacy, there were no false positive results (<90%) by the CF method (Figure 4.6e, Table 4.5) and the percentage of false positive results (<95%), ranged between 14 - 25% (Figure 4.6f, Table 4.5) and increasing *k* did not reduce the misclassification rate (Table 4.6). The results generated by McM50 showed that the percentage of FECRT results that were <90% ranged between 7 – 1% (Figure 4.6e, Table 4.5) and the percentage of results the percentage of simulated FECRT results that were <95%

ranged from 2 - 28% (Figure 4.6f, Table 4.5). Generally the misclassification rate decreased as *k* increased but this was not significant (Table 4.7).

Overall, CF tended to produce fewer misclassified results compared to McM50. However, for both CF and McM50 when drug efficacy and the threshold for determining efficacy/resistance were the same misclassification was observed. While increasing *k* generally reduced the misclassification rate for CF and McM50 this was not significant and was not the case for McM50 when efficacy was set at 90% the false negative misclassification rate increased as *k* increased, indicating that as *k* increased, the number of FECRT results that were >95% increased.

Table 4.5. The effect of the pre-treatment faecal egg count (FEC) distribution (*k*) on the misclassification rate (% of false positives or negatives) using 90% and 95% efficacy thresholds to classify resistance when efficacy was set at either 70%, 90% or 95% and when simulated FEC were generated by either the centrifugal-flotation method (CF) or the McMaster method with an egg detection limit of 50 eggs per gram (McM50)

			Pre-treatment FEC distribution (*k*)							
Drug efficacy	FEC method	Misclassification rate	0.5	1	1.5	2	2.5	3	4	5
70%	CF	False negative (>90%)	0	0	0	0	0	0	0	0
		False negative (>95%)	0	0	0	0	0	0	0	0
	McM50	False negative (>90%)	6	8	5	6	7	9	6	8
		False negative (>95%)	4	5	4	2	3	4	1	0
90%	CF	False positive (<90%)	35	31	29	26	29	30	26	27
		False negative (>95%)	0	0	0	0	0	0	0	0
	McM50	False positive (<90%)	33	27	37	33	33	27	29	23
		False negative (>95%)	33	33	34	41	39	34	43	54
95%	CF	False positive (<90%)	0	0	0	0	0	0	0	0
		False positive (<95%)	21	17	25	20	21	18	20	14
	McM50	False positive (<90%)	7	6	5	2	2	4	2	1
		False positive (<95%)	28	20	15	10	5	7	5	2

Table 4.6. The effect of *k* on the Faecal Egg Count Reduction Test (FECRT) misclassification rate when faecal egg counts (FEC) were performed by centrifugal-flotation using a Poisson generalised linear model. Presented are the slope estimates (*b*) and the associated *p* values (*p*)

Misclassification	Efficacy	Factor	*b*	*p*
False Negative >90%	70%	*k*	na	na
False Negative >95%	70%	*k*	na	na
False Negative >95%	90%	*k*	na	na
False Positive <90%	90%	*k*	3.5553	<0.001
		1.0	-0.1214	0.62
		1.5	-0.1881	0.45
		2.0	-0.297	0.251
		2.5	-0.1881	0.46
		3.0	-0.154	0.54
		4.0	-0.297	0.251
		5.0	-0.256	0.311
False Positive <90%	95%	*k*	na	na
False positive <95%	95%	*k*	0.0345	<0.001
		1.0	-0.0211	0.517
		1.5	0.0174	0.556
		2.0	-0.04879	0.879
		2.5	0.01245	1
		3.0	-0.0542	0.631
		4.0	-0.04879	0.876
		5.0	-0.04055	0.24

Table 4.7. The effect of *k* on the Faecal Egg Count Reduction Test (FECRT) misclassification rate when faecal egg counts were performed by McMaster using a Poisson generalised linear model. Presented are the slope estimates (*b*) and the associated *p* values (*p*)

Misclassification	Efficacy	Factor	*b*	*p*
False Negative >90%	70%	*k*	0.00179	<0.001
		1.0	0.0287	0.594
		1.5	-0.182	0.763
		2.0	0.05294	1
		2.5	0.01542	0.783
		3.0	0.0455	0.442
		4.0	0.0145	0.752
False Negative >95%	70%	*k*	0.0138	0.006
		1.0	0.0223	0.739
		1.5	-0.0024	1
		2.0	-0.0693	0.423
		2.5	-0.0288	0.706
		3.0	-0.0026	1
		4.0	-0.021	0.06

Misclassification	Efficacy	Factor		*b*	*p*
False Negative >95%	90%	*k*		0.03497	<0.001
			1.0	0.0014	1
			1.5	0.0299	0.9
			2.0	0.0217	0.35
			2.5	0.0167	0.48
			3.0	0.0296	0.903
			4.0	0.0358	0.06
False Positive <90%	90%	*k*		3.553	<0.001
			1.0	-0.121	0.623
			1.5	-0.1881	0.454
			2.0	-0.2973	0.251
			2.5	-0.1881	0.454
			3.0	-0.1542	0.536
			4.0	-0.2973	0.251
			5.0	-0.2595	0.311
False Positive <90%	95%	*k*		1.9459	<0.001
			1.0	-0.154	0.782
			1.5	-0.3365	0.566
			2.0	-1.2528	0.118
			2.5	-1.2528	0.118
			3.0	-0.56	0.372
			4.0	-1.2528	0.118
			5.0	-1.9459	0.07
False positive <95%	95%	*k*		0.3045	<0.001
			1.0	-0.2113	0.517
			1.5	0.1744	0.556
			2.0	-0.0487	0.876
			2.5	0.01245	1
			3.0	-1.542	0.631
			4.0	0.04879	0.876
			5.0	-0.0405	0.24

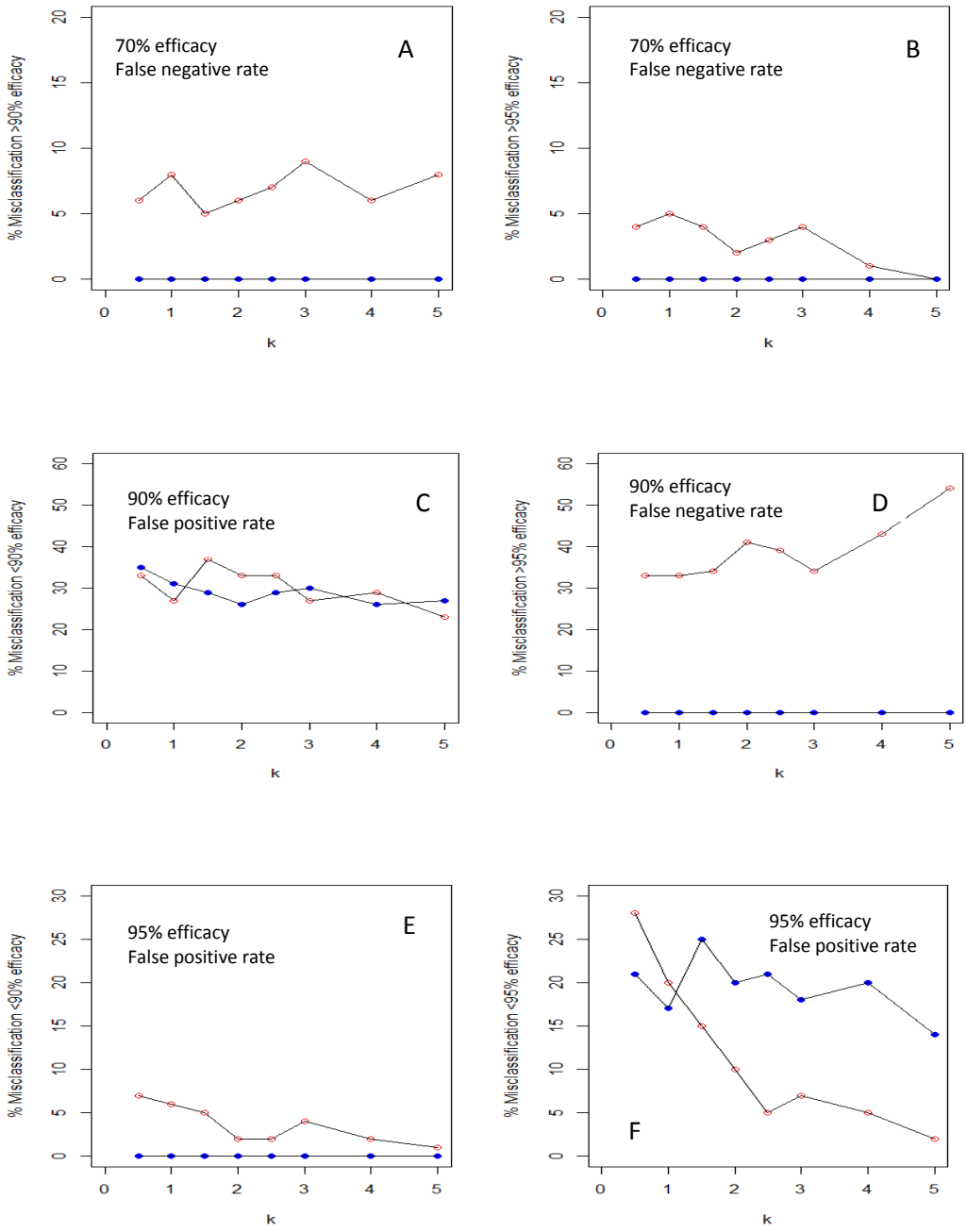


Figure 4.6. Effect of *k* on the misclassification rate (%) when mean eggs per gram (EPG) was set at 200 EPG, *n* =10, and when egg counts were performed by centrifugal-flotation (CF) (blue dots) and McMaster using an egg detection limit of 50 EPG (McM50) (red dots). A and B represent the % of false negative simulated faecal egg count reduction test (FECRT) results (i.e. the % of simulated FECRT results that were >90% efficacy and >95% efficacy, respectively) when drug efficacy was set at 70%. C and D represent the % of false positive simulated FECRT results (<90% efficacy) and false negative results (>90% efficacy), respectively, when efficacy was set at 90% and E and F the % false positive simulated FECRT results (<90% and 95%, respectively) when drug efficacy was set at 95%

4.4.2.3. Simulated FECRT results: the effect of the underlying mean pre-treatment EPG on misclassification (%) when FEC performed by CF or McM50

At 70% efficacy there were no false negative results and none of the simulated FECRT exceeded 90% or 95% efficacy when simulated FEC were generated by the CF method (Figure 4.7 a-b, Table 4.8). With McM50, the percentage of FECRT results that were >90% efficacy ranged between 0 – 31%; the false negative misclassification rate decreased as mean FEC increased (Table 4.10) and there was no misclassification when mean FEC was 350 EPG or more (Figure 4.7a, Table 4.8). When simulated FEC were generated by McM50 the false negative misclassification rate decreased as mean FEC increased (Table 2.10) and there was no misclassification when mean FEC was 250 EPG or more (Figure 4.7b, Table 4.8).

At 90% efficacy, the false positive rate for CF and McM50 ranged between 25 - 36% and 18 - 37%, respectively (Figure 4.7c and d, Table 4.8). Increasing the mean did not significantly reduce the misclassification rate (Table 4.9). For McM50, the false positive rate decreased between 50 and 150 EPG then increased (Figure 4.7c). There were no false negative (>95%) results for CF when mean EPG was ≥100EPG, compared to McM50, where the false negative rate decreased as mean increased (Table 4.10) but there were still 8% false negative results when mean EPG was 500 EPG (Figure 4.7d, Table 4.8).

When efficacy was set at 95% there were no false positive results (<90%) for CF. For McM50, the false positive rate (<90%) decreased from 63% to 10% between 50 and 100 EPG and was less than ≥3% between 150 – 500 EPG (Figure4.7e, Table 4.8). Increasing mean did not significantly reduce the false positive rate (<95%) for CF (Table 4.9), and at 500 EPG 11% of results were false positive, compared to the McM50

results where the false positive rate decreased between 50 and 200 EPG then increased with the incremental mean increases (Figure 4.7f, Table 4.10)

Overall, CF tended to produce fewer misclassified results compared to McM50. However, for both CF and McM50 when drug efficacy and the threshold for determining efficacy/resistance were the same misclassification was observed. While increasing the mean generally reduced the misclassification rate for CF this was not the case for McM50, whereby the misclassification rate increased.

Table 4.8. The effect of the mean pre-treatment faecal egg count (FEC) measured in eggs per gram (EPG) on the misclassification rate (%) using 90% and 95% efficacy thresholds to classify resistance when efficacy was set at either 70%, 90% or 95% and when simulated FEC were generated by either the centrifugal-flotation method (CF) or the McMaster method with an egg detection limit of 50 EPG (McM50)

			Pre-treatment mean (EPG)								
Efficacy	FEC method	Misclassification rate	50	100	150	200	250	300	350	400	500
70%	CF	False negative (>90%)	0	0	0	0	0	0	0	0	0
		False negative (>95%)	0	0	0	0	0	0	0	0	0
	McM50	False negative (>90%)	31	23	13	11	4	3	0	0	0
		False negative (>95%)	28	18	6	2	0	0	0	0	0
90%	CF	False positive (<90%)	35	25	34	35	36	28	25	29	29
		False negative (>95%)	3	0	0	0	0	0	0	0	0
	McM50	False positive (<90%)	57	18	18	31	35	38	38	38	46
		False negative (>95%)	43	79	57	47	24	25	11	16	8
95%	CF	False positive (<90%)	0	0	0	0	0	0	0	0	0
		False positive (<95%)	30	14	22	21	18	23	19	14	11
	McM50	False positive (<90%)	63	10	3	1	2	1	3	1	2
		False positive (<95%)	63	11	4	5	14	14	19	24	25

Table 4.9. The effect of Mean eggs per gram (EPG) on the Faecal Egg Count Reduction Test misclassification rate when faecal egg counts were performed by centrifugal-flotation using a Poisson generalised linear model. Presented are the slope estimates (*b*) and the associated *p* values (*p*).

Misclassification	Efficacy	Factor	*b*	*p*
False Negative >90%	70%	Mean	na	na
False Negative >95%	70%	Mean	na	na
False Negative >95%	90%	Mean	na	na
False Positive <90%	90%	Mean	0.03219	<0.001
		100	0.0365	0.2
		150	0.0307	0.24
		200	0.0365	0.19
		250	0.0346	0.16
		300	0.0133	0.68
		350	0.0102	1
		400	0.0148	0.59
		500	0.0145	0.59
False Positive <90%	95%	Mean	na	na
False positive <95%	95%	Mean	0.0269	<0.001
		100	-0.0621	0.019
		150	0.0452	0.186
		200	0.0455	0.2399
		250	0.2513	0.481
		300	0.0496	0.143
		350	0.0354	1
		400	0.0249	1
		500	-0.0241	0.55

na = model not performed as there were no misclassifications

Table 4.10. The effect of *k* on the Faecal Egg Count Reduction Test (FECRT) misclassification rate when faecal egg counts were performed by McMaster using a Poisson generalised linear model. Presented are the slope estimates (*b*) and the associated *p* values (*p*). Significant effects are highlighted in grey

Misclassification	Efficacy	Factor	*b*	*p*
False Negative				
>90%	70%	Mean	3.1355	<0.001
		100	-0.129	0.1
		150	-0.571	0.1
		200	-0.736	0.04
		250	-1.749	0.001
		300	-2.034	<0.001
False Negative				
>95%	70%	Mean	0.0289	<0.001
		100	-0.0482	0.02
		150	-0.0199	0.01
		200	-0.0217	0.003
False Negative				
>95%	90%	Mean	4.3694	<0.001
		100	-0.608	0.001
		150	-0.326	0.06
		200	-0.5193	0.005
		250	-1.1914	<0.001
		300	-1.1506	<0.001
		350	-1.9716	<0.001
		400	-1.5969	<0.001
		500	-2.29	<0.001
False Positive				
<90%	90%	Mean	0.0321	<0.001
		100	-0.0336	0.04
		150	-0.0336	0.04
		200	0.365	1.99
		250	0.364	0.0161
		300	0.0113	0.68
		350	0.01017	1
		400	0.01484	0.587
		500	0.01484	0.587
False Positive				
<90%	95%	Mean	2.3026	<0.001
		100	-1.841	<0.001
		150	-1.204	0.05
		200	-2.3026	0.03
		250	-1.609	0.04
		300	-2.3026	0.03
		350	-1.204	0.05
		400	-2.306	0.03

Misclassification	Efficacy	Factor	*b*	*p*
		500	-1.609	0.04
False positive				
<95%	95%	Mean	0.0264	<0.001
		100	-0.7621	0.02
		150	-0.452	0.186
		200	0.4055	0.2399
		250	0.2513	0.4807
		300	0.4964	0.1431
		350	0.3054	0.3859
		400	0.7621	1
		500	0.2412	0.5495

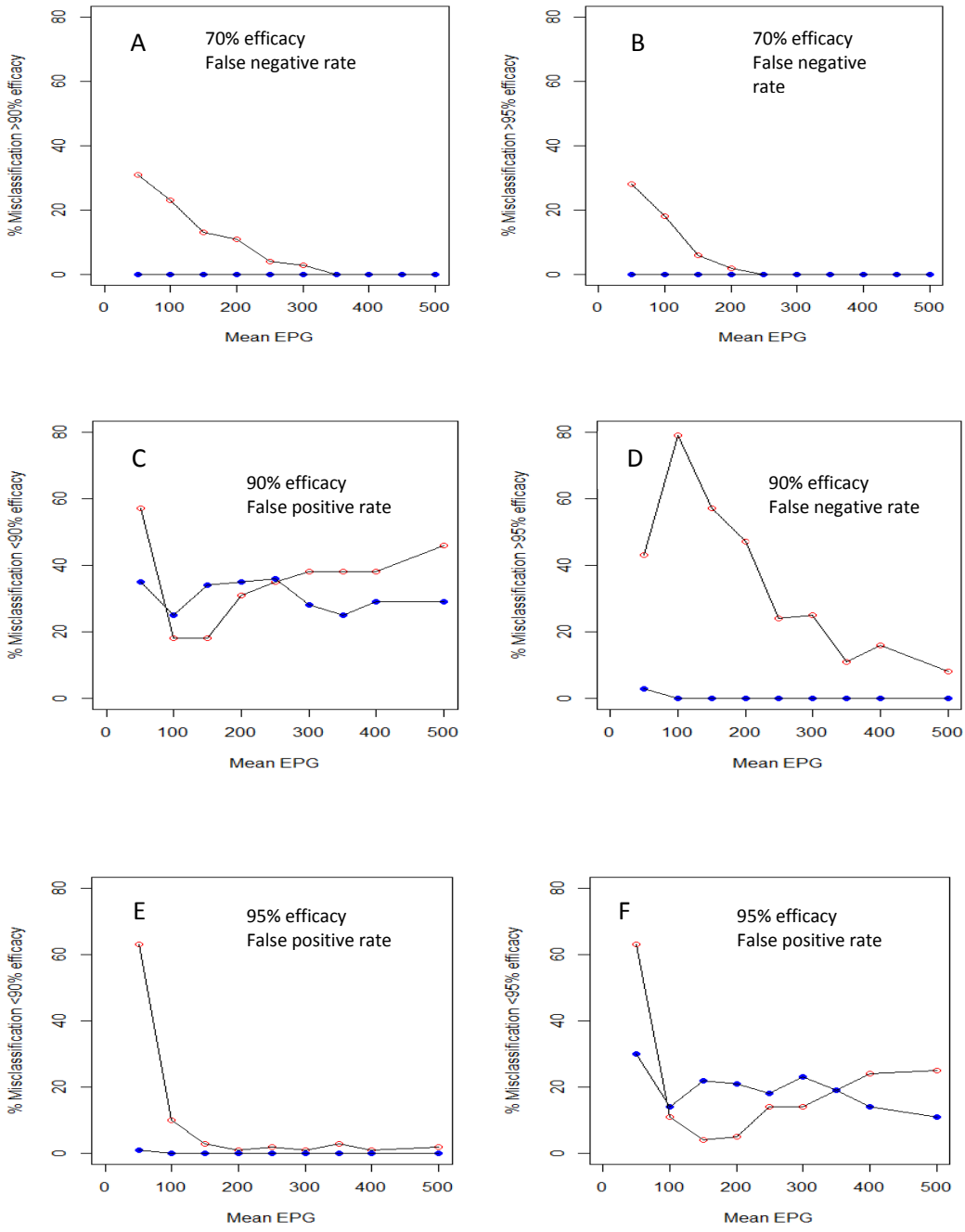


Figure 4.7. Effect of mean pre-treatment eggs per gram (EPG) on the misclassification rate (%) when *k* was set at 2.2 and n at 10, and when egg counts were performed by centrifugal-flotation (CF) (blue dots) and McMaster using and egg detection limit of 50 EPG (McM50) (red dots). A and B represent the % of false negative simulated faecal egg count reduction test (FECRT) results (i.e. the % of simulated FECRT results that were >90% efficacy and >95% efficacy, respectively) when efficacy was set at 70%. C and D represent the % of false positive simulated FECRT results (<90% efficacy) and false negative results (>90% efficacy), respectively, when efficacy was set at 90% and E and F the % false positive simulated FECRT results (<90% and 95%, respectively) when drug efficacy was set at 95%

4.4.2.4. Simulated FECRT results: the effect of number of horses included in the FECRT on misclassification (%) when FEC were performed by CF or McM50

The results indicate that the number of horses included in a FECRT affects the misclassification rate. At 70% efficacy there were no false negative results and none of the simulated FECRT exceeded 90% or 95% efficacy when simulated FEC were generated by the CF method (Figure 4.8 a-b, Table 4.11). With McM50, the percentage of FECRT results that were >90% efficacy ranged between 16 – 1%; the false negative misclassification rate decreased as *n* increased (Figure 4.8a, Table 4.13). The false negative rate (>95%) decreased as *n* increased and there was no misclassification when ≥10 horses were included (Figure 4.8b, Table 4.13).

At 90% efficacy, the false positive rate (<90%) for CF and McM50 ranged between 24 - 53% and 27 - 68%, respectively (Figure 4.8c and d, Table 4.11). Generally, the false positive rate significantly decreased (Table 4.12) as *n* increased but even when 20 horses were included the false positive rate for CF was 24% (Figure 4.8c). There were no false negative (>95%) results for CF, compared to McM50, where the false negative rate did not significantly decrease as *n* increased (Table 4.13) and there were still 26% false negative results when 20 horses were included (Figure 4.8d).

When efficacy was set at 95% there were no false positive results (<90%) for CF when ≥2 horses were included. For McM50, the false positive rate (<90%) decreased from 67% to 10% between 1 and 10 horses and was 0% when 15 or more horses were included. (Figure4.8e, Table 4.11). The false positive rate (<95%) significantly decreased as *n* increased for CF (Table 4.12) and McM50 (Figure 4.8f, Table 4.13).

CF tended to produce fewer misclassified results compared to McM50. However, for both CF and McM50 when drug efficacy and the threshold for determining efficacy/resistance were the same misclassification was observed.

Table 4.11. The effect of the number of horses (*n*) included in the faecal egg count reduction test (FECRT) on the misclassification rate using 90% and 95% efficacy thresholds to classify resistance when drug efficacy was set at either 70%, 90% or 95% and when simulated faecal egg counts (FEC) were generated by either the centrifugal-flotation method (CF) or the McMaster method with an egg detection limit of 50 eggs per gram (McM50)

			Number of horses (*n*)					
Efficacy	FEC method	Misclassification rate	1	2	5	10	15	20
70%	CF	False negative (>90%)	0	0	0	0	0	0
		False negative (>95%)	0	0	0	0	0	0
	McM50	False negative (>90%)	16	11	10	5	2	1
		False negative (>95%)	14	8	6	0	0	0
90%	CF	False positive (<90%)	53	41	27	25	24	24
		False negative (>95%)	0	0	0	0	0	0
	McM50	False positive (<90%)	68	55	33	27	37	29
		False negative (>95%)	30	41	45	36	23	26
95%	CF	False positive (<90%)	15	2	0	0	0	0
		False positive (<95%)	53	37	26	22	14	12
	McM50	False positive (<90%)	57	31	6	3	0	0
		False positive (<95%)	58	33	10	6	5	4

Table 4.12. The effect of the number of horses (*n*) on the Faecal Egg Count Reduction Test (FECRT) misclassification rate when faecal egg counts were performed using centrifugal-flotation using a Poisson generalised linear model. Presented are the slope estimates (*b*) and the associated *p* values (*p*). Significant effects are highlighted in grey

Misclassification	Efficacy	Factor	*b*	*p*
False Negative >90%	70%	*n*	na	na
False Negative >95%	70%	*n*	na	na
False Negative >95%	90%	*n*	na	na
False Positive <90%	90%	*n*	3.9703	0.001
		2	-0.257	0.21
		5	-0.675	0.004
		10	-0.75	0.002
		15	-0.7922	0.001
		20	-0.7922	0.001
False Positive <90%	95%	*n*	na	na
False positive <95%	95%	*n*	3.9703	<0.001
		2	-0.3994	0.09
		5	-0.7122	0.003
		10	-0.897	<0.001
		15	-1.3312	<0.001
		20	-1.485	<0.001

Table 4.13. The effect of the number of horses (*n*) on the Faecal Egg Count Reduction Test (FECRT) misclassification rate when faecal egg counts were performed using McMaster using a Poisson generalised linear model. Presented are the slope estimates (*b*) and the associated *p* values (*p*). Significant effects are highlighted in grey

Misclassification	Efficacy	Factor	*b*	*p*
False Negative >90%	70%	*n*	2.7726	<0.001
		2	-0.3747	0.34
		5	-0.47	0.244
		10	-1.1632	0.02
		15	-2.079	0.006
		20	-2.773	0.007
False Negative >95%	70%	*n*	2.6391	<0.001
		2	-0.559	0.02
		5	-0.847	0.04
False Negative >95%	90%	*n*	3.4012	<0.001
		2	-0.312	0.461
		5	-0.405	0.09

Misclassification	Efficacy	Factor	*b*	*p*
		10	-0.182	0.46
		15	0.26	0.336
		20	-0.1431	0.59
False Positive <90%	90%	*n*	3.9703	<0.001
		2	-0.257	0.217
		5	-0.6745	0.004
		10	-0.7514	0.002
		15	-0.7922	0.001
		20	-0.7922	0.001
False Positive <90%	95%	*n*	4.043	<0.001
		2	-0.6091	0.006
		5	-2.2513	<0.001
		10	-2.944	<0.001
False positive <95%	95%	*n*	3.9703	<0.001
		2	-0.3594	0.09
		5	-0.7122	0.003
		10	-0.8792	<0.001
		15	-1.3312	<0.001
		20	-1.4854	<0.001

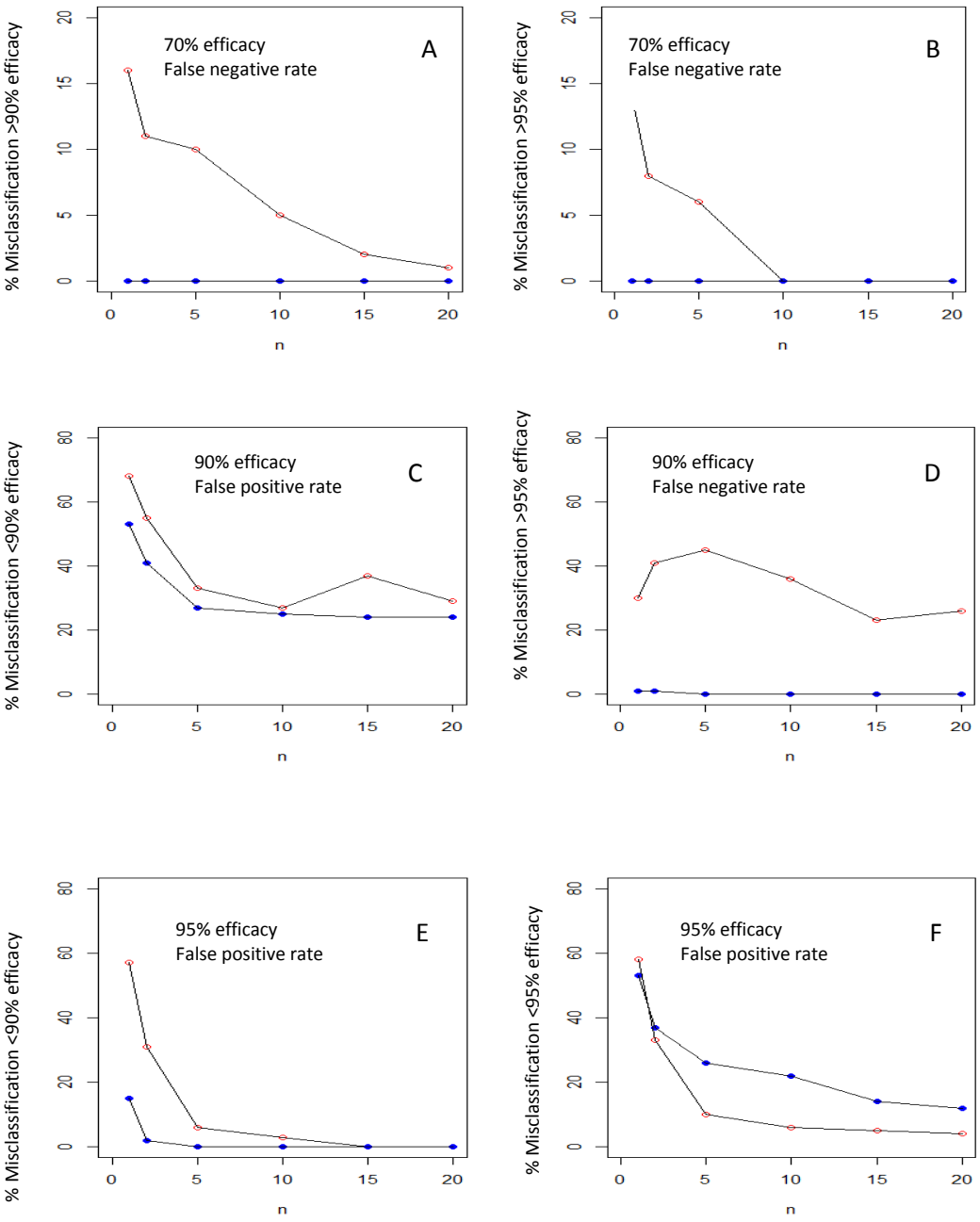


Figure 4.8. Effect of *n* on the misclassification rate (%) when *k* was set at 2.2 and mean pre-treatment eggs per gram (EPG) at 200 EPG, and when egg counts were performed by centrifugal-flotation (CF) (Blue dots) and McMaster using an egg detection limit of 50 EPG (McM50) (red dots). A and B represent the % of false negative simulated faecal egg count reduction test (FECRT) results (i.e. the % of simulated FECRT results that were >90% efficacy and >95% efficacy, respectively) when efficacy was set at 70%. C and D represent the % of false positive simulated FECRT results (<90% efficacy) and false negative results (>90% efficacy), respectively, when efficacy was set at 90% and E and F the % false positive simulated FECRT results (<90% and 95%, respectively) when efficacy was set at 95%

4.4.2.5. Simulated FECRT results: the effect the egg detection limit (dl) on misclassification (%) when simulated FEC were generated by the McM method

The egg detection limit affected the misclassification rate for McM50. When efficacy was set at 70%, the >90% and >95% false negative rate increased as the egg detection limit (dl) increased (Table 4.14, Figure 4.9). When the efficacy and the threshold for classifying efficacy/resistance were the same, the false positive misclassification rate increased as the egg detection limit increased (Table 4.14, Figure 4.9).

Table 4.14. The effect of the egg detection limit (*dl*) of the McMaster method on the misclassification rate using 90% and 95% efficacy thresholds to classify resistance when efficacy was set at either 70%, 90% or 95%

		Egg detection limit (*dl*)					
Efficacy	Misclassification rate	5	10	15	30	50	100
70%	False negative (>90%)	0	0	1	2	2	18
	False negative (>95%)	0	0	0	1	2	4
90%	False positive (<90%)	28	38	42	38	33	10
	False negative (>95%)	2	6	11	30	37	79
95%	False positive (<90%)	0	2	6	10	3	0
	False positive (<95%)	34	30	52	27	10	0

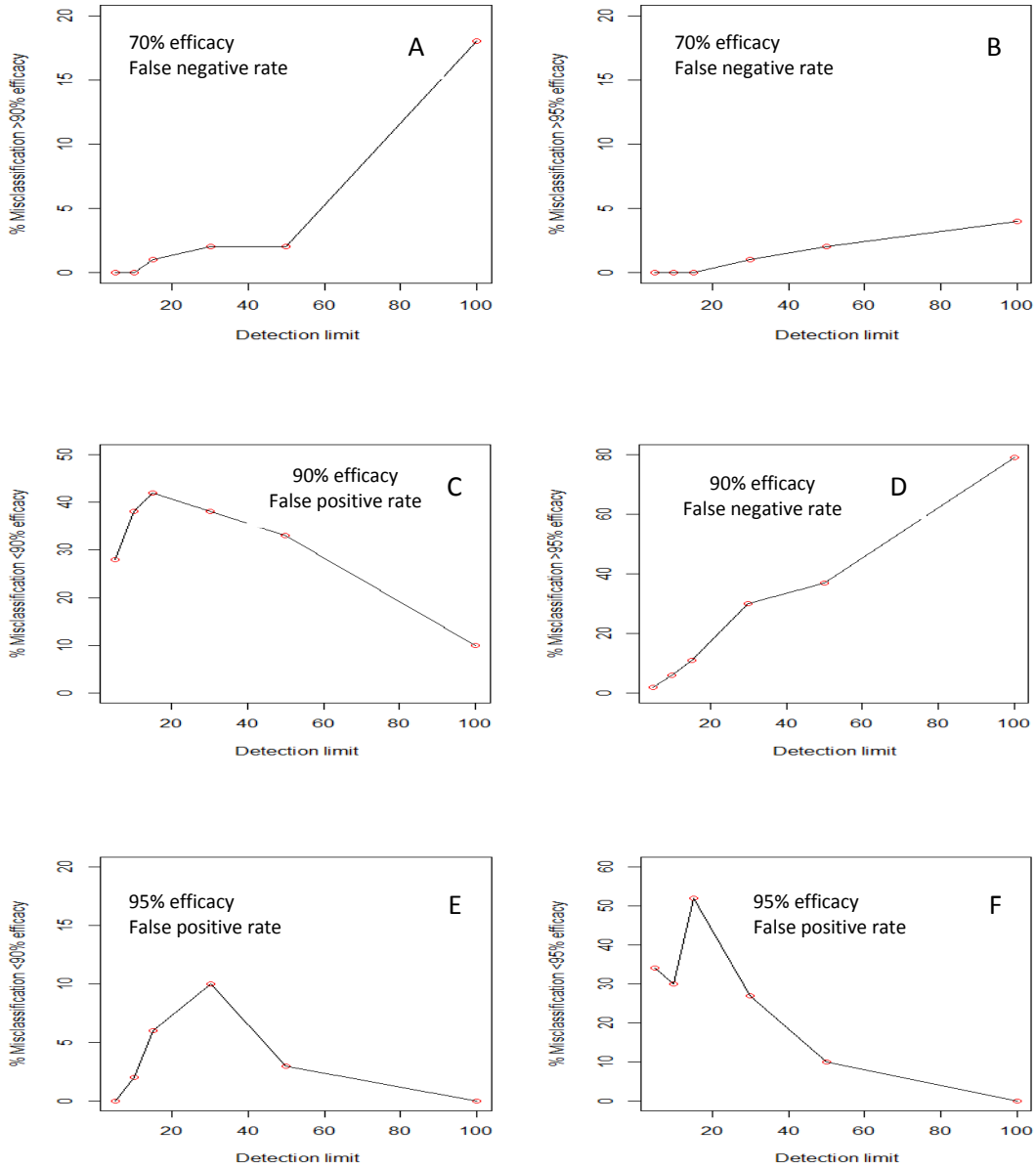


Figure 4.9. Effect of the egg detection limit (*dl*) on misclassification rate (%) when *k* was set at 2.2 and mean pre-treatment egg per gram (EPG) at 200 EPG, and when egg counts were performed by McMaster (McM). A detection limit of 100 EPG represents counting 1 grid of a McMaster slide; 50 EPG represents counting 2 grids; 30 EPG represents counting one chamber; 15 EPG represents counting two chambers, 10 EPG represents counting chambers and 5 EPG represents counting six chambers. A and B represent the % of false negative simulated faecal egg count reduction test (FECRT) results (i.e. the % of simulated FECRT results that were >90% efficacy and >95% efficacy, respectively) when drug efficacy was set at 70%. C and D represent the % of false positive simulated FECRT results (<90% efficacy) and false negative results (> 90% efficacy), respectively, when drug efficacy was set at 90% and E and F the % false positive simulated FECRT results (<90% and 95%, respectively) when drug efficacy was set at 95%

4.4.2.6. Simulated FECRT results: the effect of the underlying mean pre-treatment FEC, the distribution of FEC (*k*), the number of horses included in the FECRT on the misclassification of resistance when simulated FEC were generated either by the CF method or the McM50 method

When efficacy was fixed at 70%, there were no false negative results and none of the simulated FECRT results exceeded >90% or 95% efficacy when FEC were generated by the CF method and all simulated FECRT results were identified as *resistant* regardless of mean, *k* and *n* (Figure 4.10). When FEC were generated by the McM50 method, the number of simulated FECRT that were >90% (false negative rate) ranged from 1 to 17% (Figure 4.10). Generally, the false negative misclassification rate decreased as mean, *k* and *n* increased. When the mean FEC was >300 EPG and 5 or more horses were included, the false negative misclassification rate was less than 5% (Figure 4.10). The percentage of simulated FECRT results that were >95% (false negative rate) ranged from 1 to 14% when FEC were generated by the McM50 method (Figure 4.10). When pre-treatment mean FEC was between 319 EPG and 340 EPG and if 10 horses were included, there were no false negative misclassifications (>95% efficacy) and when pre-treatment mean was >388 EPG there were no false negative misclassifications (>95% efficacy) when 5 or more horses were included (Figure 4.10).

When efficacy was fixed at 90% and the simulated FECRT were generated by the CF method, the false positive misclassification rate (<90% efficacy) ranged between 22 and 55% (Figure 4.11). The false positive misclassification rate (<90%) decreased with FECRT results generated by the CF method, as *m, k* and *n* increased, but even when *m* = 512 EPG, *k* = 2.3 and *n*=20, 30% of simulated FECRT results were <90% (i.e. falsely identified resistance) (Figure 4.11). The percentage of simulated FECRT results that were >95% (false negative) when FECRT results were generated by the CF method was 0% across all *m, k* and *n* values investigated when more than two horses were included

(Figure 4.11). When FECRT results were simulated using the McM50 method, the % of FECRT results that were < 90% (false positive rate) ranged from 25 to 68% across the range of *m*, *k* and *n* values investigated (Figure 4.11). The % of FECRT results that were >95% FECR (false negative rate) ranged between 3 and 55% and tended to decrease as *m* and *n* increased (Figure 4.11).

When efficacy was fixed at 95% and the simulated FECRT results were generated by the CF method the percentage of FECRT results that were <90% (false positive) ranged from 0 to 18% (Figure 4.12). When the mean Day 0 FEC was 120 EPG, *k* was 1.2 and 2 horses were included, 18% of the simulated FECRT results were <90% and therefore falsely identified resistance. This decreased to 1% when 5 horses were included and there was no misclassification observed when 10 or more horses were included (Figure 4.12). As *m*, *k* and *n* increased, the percentage of FECRT that were <90% (false positive) decreased, and when the mean pre-treatment FEC was >388 EPG, there was no misclassification (Figure 4.12). The % of simulated FECRT that were <95% (false positive) when FECRT results were generated by the CF method ranged between 5 and 45% (Figure 4.12). The percentage of FECRT that were <95% generally decreased as *m* and *n* increased, but even when the pre-treatment FEC was 512 EPG and 20 horses were included, 10% of the simulated FECRT results were <95% (Figure 4.12). When simulated FECRT data were generated by McM50, the % of results that were <90% (false positive) ranged between 0 and 62%. Generally, the false positive misclassification rate decreased as *m*, *k* and *n* increased (Figure 4.12). When more than 10 horses were included, the percentage of simulated FECRT results that were <90% was <5%, with the exception of when the mean pre-treatment FEC was 319 EPG and *k* was 0.67. Here, the percentage of FECRT results that were <90% increased when 10 horses were included (Figure 4.12). The percentage of simulated FECRT results that

were <95% (false positive) when the McM50 method was used to generate the simulated FEC, ranged from 2 to 64% (Figure 4.12). When the mean pre-treatment FEC was 120 EPG, *k* was 1.5 and 2 horses were included, 64% of the simulated FECRT were <95% (false positive). This decreased when 20 horses were included, and the false positive misclassification rate was less than 5% (Figure 4.12). However, when the mean pre-treatment FEC was 512 EPG, *k* was 2.3 and 20 horses were included, 30% of the simulated FECRT results fell below 95% (Figure 4.12).

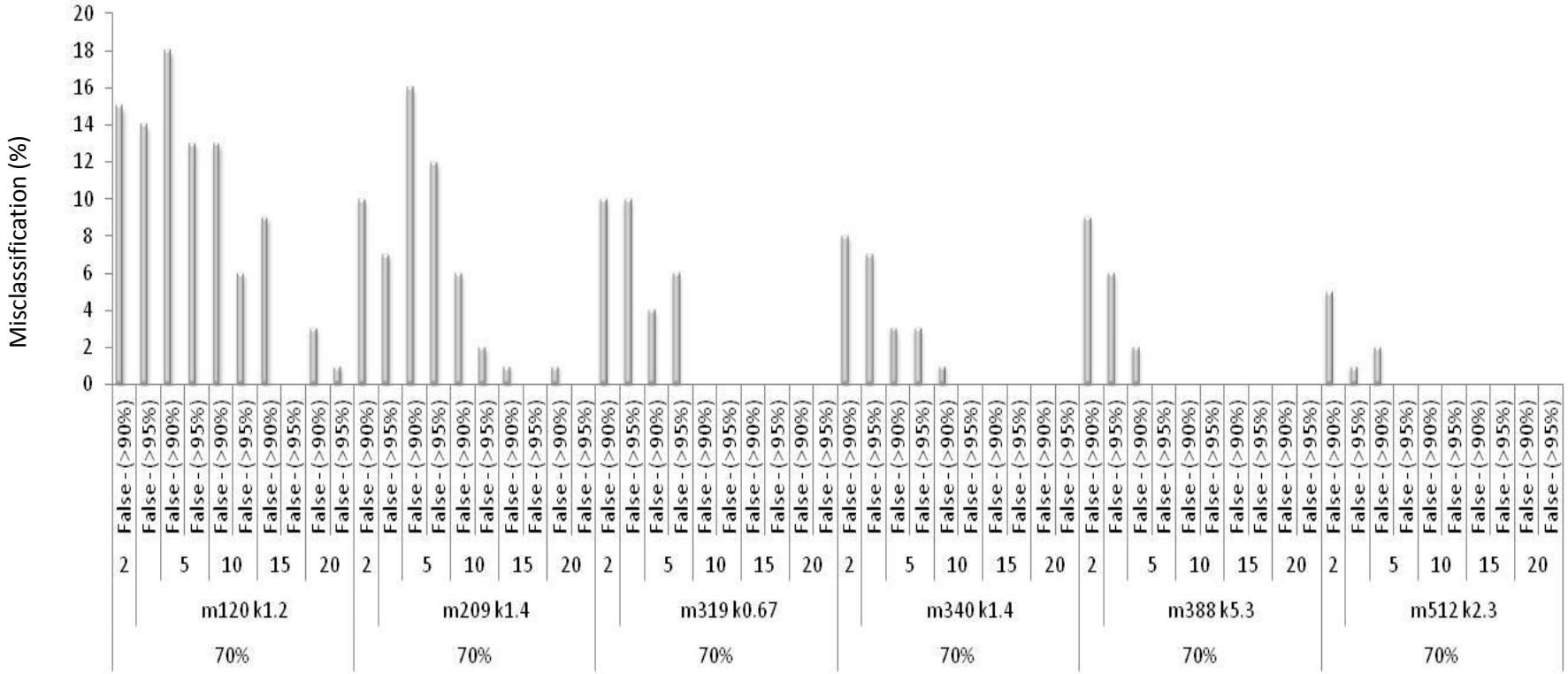


Figure 4.10. The effect of the mean pre-treatment faecal egg count (FEC) measured in eggs per gram (EPG), the pre-treatment FEC distribution (*k*) and the number of horses included (*n*) on the false negative misclassification rate (%). Simulated FECRT results that were >90% and 95% efficacy of simulated FECRT results when efficacy was set at 70%. The grey bars represent the simulated faecal egg count reduction test (FECRT) results when FEC were generated by the McMaster method using an egg detection limit of 50 EPG (McM50). None of the simulated FECRT results exceeded 90% or 95% when FEC data were generated by the centrifugal-flotation (CF) method

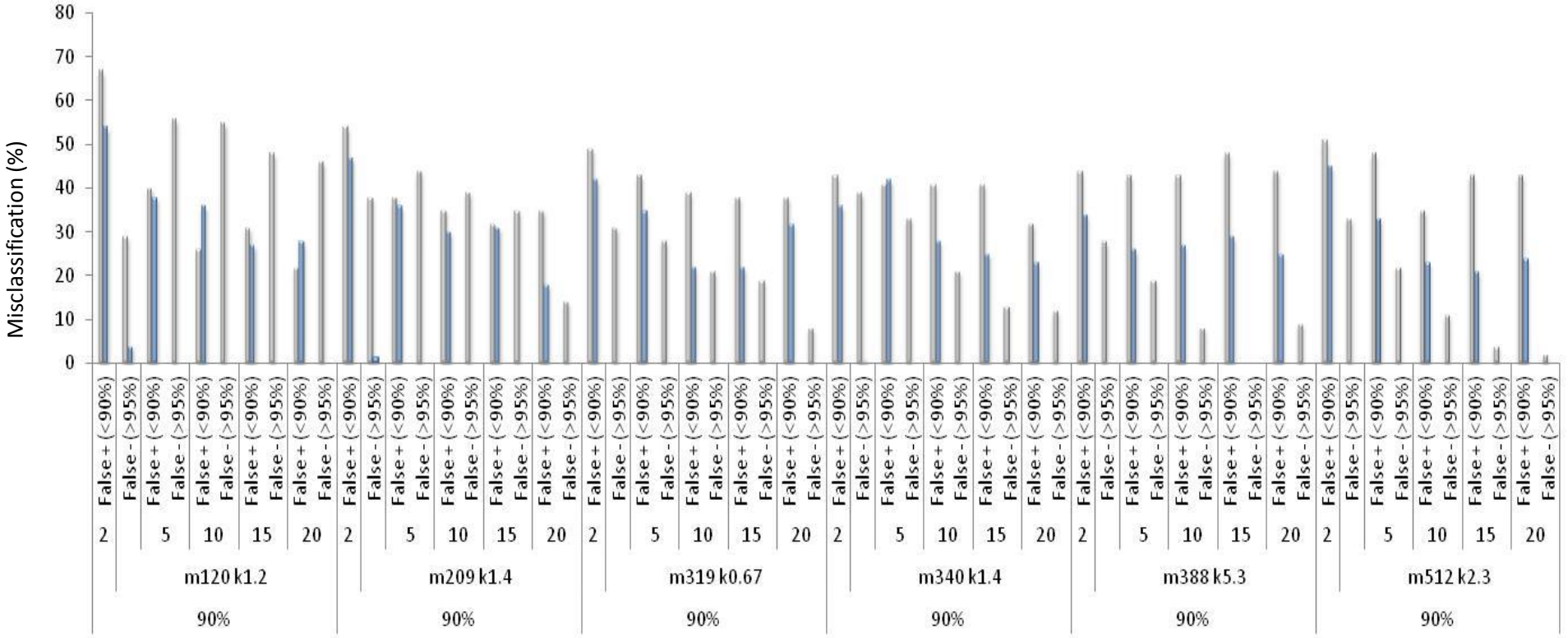


Figure 4.11. The effect of the mean pre-treatment faecal egg count (FEC) measured in eggs per gram (EPG), the pre-treatment FEC distribution (*k*) and the number of horses included (*n*) on the false positive misclassification rate (%) (<90%) and the false negative misclassification rate (>95%) of simulated faecal egg count reduction test (FECRT) data when drug efficacy was set at 90%. The grey bars and the blue bars represent simulated FECRT results that were generated by the McMaster method (50 EPG detection limit) (McM50) method and the centrifugal-flotation (CF) method, respectively

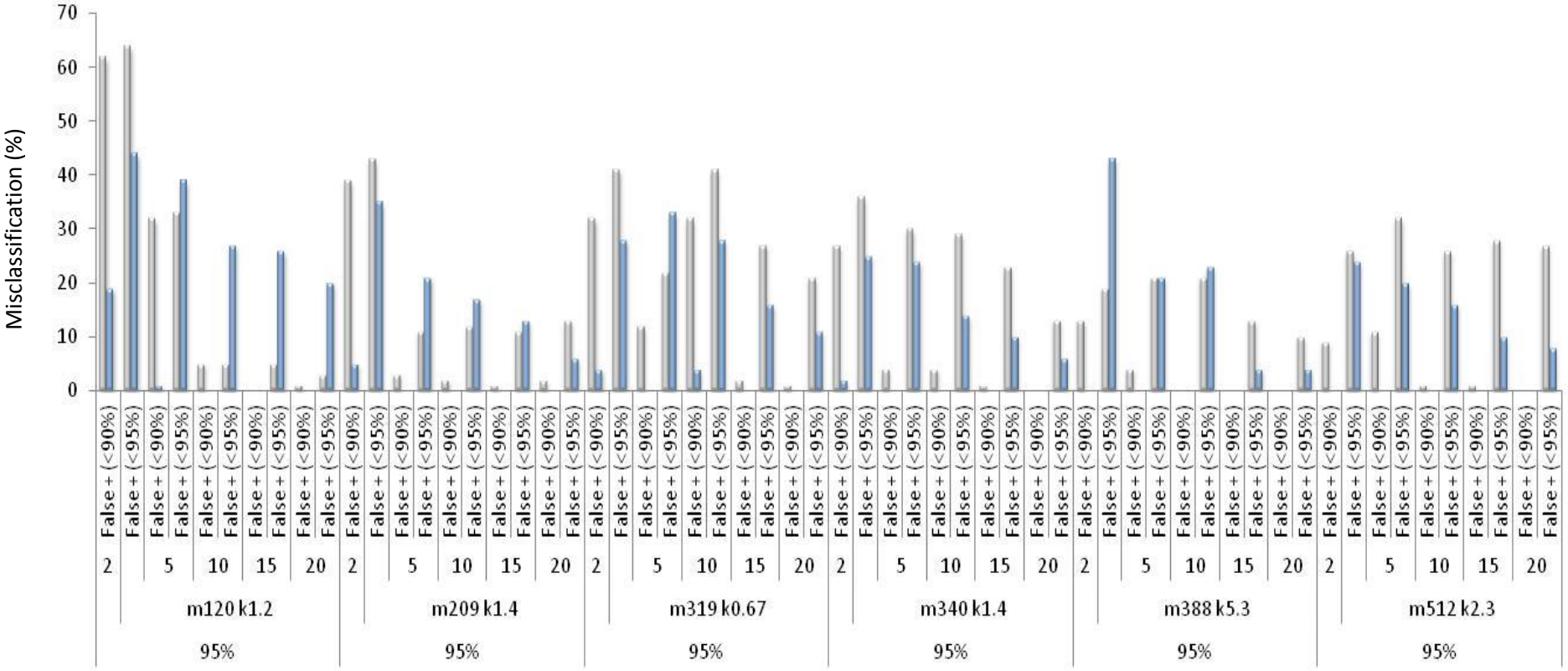


Figure 4.12. The effect of the mean pre-treatment faecal egg count (FEC) measured in eggs per gram (EPG), the pre-treatment FEC distribution (*k*) and the number of horses included (*n*) on the false positive misclassification rate (%) (<90%) and the false negative misclassification rate (>95%) of simulated faecal egg count reduction test (FECRT) data when drug efficacy was set at 90%. The grey bars and the blue bars represent simulated FECRT results that were generated by the McMaster method (50 EPG detection limit) (McM50) method and the centrifugal-flotation (CF) method, respectively

4.5 Discussion

Despite the limitations of the FECRT, it currently remains the most practical method for assessment of anthelmintic efficacy in equids. The work presented here compared three different statistical methods for estimating anthelmintic efficacy using the FECRT data presented in Chapter 3 to investigate if the method used to estimate mean FECR had any bearing on the outcome and interpretation of the data in terms of classifying resistance. Secondly, the performance of the FECRT method currently recommended by the WAAVP (Coles et al., 1992) was investigated through a simulation approach. The effect of the egg counting method used for pre-treatment and post-treatment samples, the egg dl of the FEC method, the underlying mean pre-treatment FEC, the underlying distribution of the Day 0 FEC (negative binomial parameter, *k*), the number of horses included in the test and the true efficacy of the anthelmintic on the performance of the WAAVP method for estimating efficacy were investigated. Such information is essential to help improve understanding of how to best utilise FECRT methodologies to more accurately diagnose anthelmintic resistance.

Three methods of estimating percentage FECR were applied to 18, 21, 16 and 19 sets of FBZ, PYR, IVM and MOX FECRT data, respectively. When efficacy was low (i.e. following FBZ administration), all three statistical FECRT methods generated the same assumption (i.e. mean percentage FECR was <90%, with <80% LCL). When efficacy was high (i.e. following IVM and MOX administration) all FECRT methods generated the same output (i.e. mean % FECR was ≥95 mean % reduction, ≥90% LCL). On 14% of yards where efficacy was close to the 90% threshold (i.e. following PYR administration), the statistical methods generated different results regarding efficacy. Here, Method 2 tended to generate higher estimates of percentage FECR compared to the other methods, and Method 3 was more likely to indicate resistance compared to

the other methods. These data would suggest that when efficacy is high or low, the method used to estimate % FECR is not as critical, but when efficacy is on the threshold of resistance/sensitivity, then the analytical method used is highly relevant to the calculated FECR. A study conducted by Pook et al. (2002) compared three methods of estimating anthelmintic efficacy against strongyles in horses, including Methods 1 and 2 used here. The third method these authors employed was the WAAVP-recommended method that includes an untreated control group (Coles et al., 1992). The authors applied the three methods to seven sets of oxbendazole (OBZ) and morantel (MOR) FECRT data. The chemical OBZ belongs to the benzimidazole class of anthelmintics, and MOR belongs to the tetrahydropyrimidine class. In the Pook (2002) study, the estimated efficacy of OBZ and MOR was comparable to FBZ and PYR, respectively, in the present study, and Methods 1 and 2 calculated different assumptions regarding efficacy. For example, following OBZ administration, on 6/7 yards Method 1 made a calculation of BZ resistance and on 5/7 yards Method 2 calculated resistance. Following MOR administration, 2/7 yards were classed as MOR resistant and on 3/7 yards, resistance was suspected when Method 1 was used to estimate percentage FECR, compared to when Method 2 was used and 1/7 yards were classed as resistant (Pook et al., 2002). These findings are similar to those in the present study and suggest that Method 1 tends to generate lower estimates of percentage FECR compared to Method 2, which tended to generate higher estimates of anthelmintic efficacy. Pook et al. (2002) concluded that, based on a 90% threshold for anthelmintic sensitivity/resistance, Method 2 should be used as it reveals fewer cases of resistance compared to Method 1. However, since the drug efficacy was unknown, Method 1 may be more likely to generate false positive results (i.e. falsely declare resistance) or Method 2 may be more likely to generate false negative results (i.e. falsely declaring efficacy). It could be argued that Method 1 is preferable as it tends to generate more

conservative estimates of efficacy, particularly in the absence of knowledge of the true efficacy of the anthelmintic under study. A conservative estimate could provide an early warning of a lack of efficacy. However, by not including 95% confidence intervals to account for the variance in the FECRT data and not interrogating the FECRT data for outliers using a single threshold to classify the outcome of a FECRT is not a robust approach and misclassifications are likely to occur, leading to falsely classifying resistance and leading to false reports of widespread resistance.

A further study compared the WAAVP method (Coles et al., 1992) to four other methods for estimating anthelmintic efficacy, including two which required untreated control groups and two that utilised geometric means to assess efficacy following treatment with FBZ, PYR or IVM on 56 Danish horse farms (Craven et al., 1998). These authors used the modified McM FEC method with an egg dl of 50 EPG for all pre- and post-treatment FEC. The authors found that the WAAVP method, using a cut-off <95% FECR and <90% LCL, similar to Method 1 used here, classified more FECRT results resistant compared to the other methods (Craven et al., 1998), consistent with the findings here. The problem of using statistical methods which include an untreated control group lies in the fact that equine strongyle egg shedding is highly aggregated between horses and so it is often difficult to find enough horses shedding high enough numbers of eggs to divide them into adequately sized treatment and control groups. This is exacerbated by the fact that usually horses are usually kept together in small groups. Furthermore, leaving horses that are shedding a significant number of eggs in their faeces untreated will lead to significant egg output onto pasture and could lead to clinical disease.

In the present study, estimates of percentage FECR generated by non-parametric bootstrapping (Method 3) were comparable to the estimates generated by Method 1.

Non-parametric bootstrapping is useful when the underlying distribution of the FEC data is unknown, but assumes that the data are fully representative of the distribution describing the population. The latter assumption is potentially violated when a small sample size is used (Denwood et al., 2010). By computing the mean of each bootstrap dataset, this generates an empirical sampling distribution of the mean (Cabaret and Berrag, 2004), so that the mean is not mathematically estimated but empirically reconstructed from the original FECRT data. Vidyashankar et al. (2007) aimed to develop a theoretical framework that was free from the distributional assumptions of egg count distributions. Further, they developed bootstrap-based algorithms to assess efficacy and compared their bootstrap models to a standard t-test, a t-test on FECRT data transformed using arcsine transformation and on FECRT data that had been log transformed using an extensive simulation approach. The performance of the different methods for estimating efficacy was assessed by calculating the Type I error rate (i.e. false positive rate) and power analysis. The authors reported that, for the bootstrap methods, even when the pre-treatment FEC was low (<50 EPG), these yielded lower Type I error rates when true efficacy was less than or equal to 95% compared to the other statistical methods, and that the bootstrap methods demonstrated substantial power to detect changes close to the null hypothesis (i.e. marginal changes in true efficacy) compared to the other statistical methods (Vidyashankar et al., 2007). Their approach differed to that of the present study as the authors constructed two novel bootstrap-based algorithms, which they compared to existing approaches using extensive simulations using different pre-treatment FEC distributions, compared to the present study where a standard non-parametric bootstrap method was applied to sets of empirical data. Also, they did not compare the bootstrap FECRT results to the standard WAAVP FECRT methodology. Further, they compared the results generated by the different methodologies using a t-test to compare the different Type I error rates

for each method. This approach was not applicable for use with the empirical FECRT datasets used here as only one result for each FECRT set was generated using each of the three methods. The other major difference between the Vidyashankar et al. (2007) study and the present study is that, in the former, all the FECRT methods studied require a high degree of statistical knowledge, and none of the approaches would be practical for use in practice, whereas the standard WAAVP recommended method is practical and easy to utilise.

Markov Chain Monte Carlo (MCMC) methods for estimating anthelmintic efficacy have also been suggested (Denwood et al., 2010). This approach takes into consideration many of the statistical caveats arising with equine FECRT data, including the distribution of pre-treatment FEC between horses. The limitation of these methods is that they require an advanced knowledge of statistics and ability to use statistical programmes such as R. Recently a web-interface has been built allowing researchers to enter pre- and post-treatment FEC data, along with the egg dl of the FEC method used to generate the counts, which then estimates percentage FECR using Bayesian hierarchical models via MCMC sampling (http://www.math.uzh.ch/as/index.php?id=calc Torgerson et al., 2014). This approach aims to account for variation that arises through sampling, variation between animals and provides access of the layperson to more robust methods of computing FECR to take into account the likely spread of the FEC dataset. However, currently, the interface is not particularly user-friendly in that there is no simple way to input FEC, and the form needs to be populated by typing in each individual count. This may not be a problem when small numbers of animals are being assessed, but this approach would be laborious if 20 or more animals were being tested.

A study performed in sheep, aimed to gauge the repeatability of the FECRT (Miller et al., 2006) and involved the recruitment of two sheep farms previously recorded as having nematodes present in which there was <95% efficacy after IVM or albendazole (ABZ) administration. From each individual sheep, a faecal sample was collected and analysed by the McM method with an egg dl of 50 EPG. The sheep were then split into three groups of 80 based on their FEC; Group 1 had high FEC, Group 2 had moderate FEC and Group 3 had low FEC. Each group of 80 sheep were further divided into four replicates of 20, each having the same mean FEC. Each group of 20 was split again into two groups of 10, one treated group and an untreated group. Sheep in the treated groups were administered IVM or ABZ. Faecal samples were collected from all sheep pre- and post-treatment, and efficacy estimated using three different methods, all of which used arithmetic mean pre- and post-treatment FEC; Method 1 included an untreated control group (Presidente, 1985), Method 2 was similar to the method described by Coles et al. (1992), but was used in conjunction with results of faecal larval cultures (McKenna, 1990) and Method 3 was the standard WAAVP method (Coles et al., 1992, Method 1 in the present study). The three different methods generated different assumptions regarding efficacy. Across low, moderate and high FEC groups of sheep that received IVM, when efficacy was estimated by Methods 1 and 3, 9/12 populations were classed as resistant, compared to Method 2 where 12/12 populations were classed as resistant. In the groups that received ABZ, 2/12 populations were classed as resistant by Method 1, 5/12 populations were classed as resistant by Method 2 and 0/12 populations classed as resistant by Method 3. In this study, there were many more disagreements in the classification between the different methods compared to the present study. This may be due to the fact that the sheep were infected with multiple helminth species, all of which may have different levels of susceptibility to anthelmintics and different levels of fecundity. However, in the Miller

et al. (2006) study, Method 3 (Method 1 in the present study) classed fewer populations as resistant compared to the other methods, which is comparable to the results here. The results presented by Miller et al. (2006) demonstrated that the FECRT was not highly repeatable within groups of sheep with the same pre-treatment mean, residing on the same farm. Unless efficacy is 100%, there will be variability in the measurement of post-treatment FEC in animals (Vidyashankar et al., 2007). Observed efficacy is not a fixed value, and it has a range of possible outcomes, which are dependent on an array of factors including Poisson associated variability arising in FEC (Vidyashankar et al., 2007), the FEC method used (Levecke et al., 2012), the pharmacokinetic and pharmacodynamics of the anthelmintic within an individual (Kaplan and Nielsen, 2010), the age and immune status of the host (Lyons et al., 2011), the composition of parasite species and their relative susceptibility to the anthelmintic tested (Vidyashankar et al., 2012), all leading to heterogeneity in the data.

Here, the diagnostic performance of the FECRT in terms of misclassifying anthelmintic resistance/susceptibility was investigated using Monte Carlo simulation. The parameters investigated included distribution of pre-treatment FEC between horses, the pre-treatment mean EPG, the sample size and the egg dl of the FEC method used to generate the FEC data. The simulation approach was primarily one-dimensional with each factor investigated in isolation. Further work is required to look at the interaction between sample size, mean, k and the sensitivity of the FEC method on the misclassification rate of FECRT.

Generally, in the present study, the performance of the FECRT in terms of misclassifying resistance increased when the pre-treatment mean FEC and the number of horses included were low and the egg dl was high. When the egg dl was low (i.e. CF method) and efficacy was low (i.e. set at 70%), the level of pre-treatment mean FEC did

not affect the ability to correctly identify resistance, as there were no false negative results regardless of the threshold used to classify anthelmintic resistance/sensitivity. When the egg dl was 50 EPG (standard egg dl used for McM), then the pre-treatment mean did influence the false negative misclassification rate when efficacy was low (70%) and a 90% threshold was used, and the misclassification rate decreased as mean EPG increased and there were no false negative results recorded when the mean FEC was set at 350 EPG or more. However, when true efficacy was set at 90% and a 90% threshold applied, then the false positive misclassification rate ranged between 25% - 36% and 25% - 49%, for CF and McM generated FECRT, respectively, and did not decrease when the mean EPG was increased, which suggests that when the threshold and true efficacy are the same, then the negative misclassification rate increases regardless of the pre-treatment mean, and between 25% - 49% of FECRT results falsely identify resistance regardless of FEC method used. Similarly, when both the efficacy and the threshold were set at 95%, then the false positive misclassification rate for CF and McM was between 11% - 30% and 4% - 63%, respectively. However, the misclassification rate did decrease when mean FEC increased when the CF method was used, but increasing the mean EPG increased the misclassification rate when McM was used to generate the results. These findings are similar to those reported by Levecke et al. (2012a), who set out to empirically assess the impact of sample size, the egg dl of the FEC method used and the level of egg excretion (mean EPG) and distribution (*k*) of the FEC on the interpretation of FECRT data when the WAAVP FECRT method was applied to FECRT data generated using a Monte Carlo simulation approach. Similarly, they used 90% and 95% thresholds to indicate acceptable efficacy. In Levecke et al. (2012a), the authors classified the outcome of the simulated FECRT data as sensitive (i.e. reduced efficacy detected ≥95% of simulated results (true negative)), insensitive (i.e. reduced efficacy detected <95% of simulated results (false negative)), specific (i.e. when true

efficacy was detected in ≥95% of simulated FECRT results (true positive)) and aspecific (i.e. when true efficacy was detected in <95% of simulated FECRT results (false positive)). This differed to the approach of the present study. Here, true efficacy was set at 70, 90 or 95%, and the false positive and negative misclassification rate for simulated FECRT results were calculated for each set of parameter combinations studied, which was a more simple approach compared to the Levecke et al. (2012a) study. However, the both studies aimed to calculate misclassification rates given set thresholds for defining acceptable efficacy when exploring a combination of parameters that influence the outcome of FECRT. In the study by Levecke et al. (2012a), when a 90% threshold was applied, simulated FECRT results were affected by the mean pre-treatment FEC, with lower sensitivity and specificity observed when mean pre-treatment FEC was <200 EPG. However, the impact of mean EPG on the detection of reduced (true efficacy <90%) and normal efficacy (true efficacy ≥90%) decreased as the egg dl of the FEC method decreased, and when the egg dl was 1 or 2 EPG, reduced and normal efficacy could be reliably detected when true efficacy was <87.5% and >95%, respectively, regardless of the pre-treatment mean. The findings described in the Levecke (2012a) study, and those in the present study, suggest that the combination of the egg dl and pre-treatment mean have a profound impact on the outcome of FECRT. In another study conducted by Uriarte and Calvete (2013), their aim was to improve the FECRT by performing an extensive Monte Carlo simulation study across a range of parameters, including pre-treatment mean FEC and FEC distribution, egg dl, the number of animals included and drug efficacy. They used a threshold of 95% to classify resistance/efficacy. These authors reported that as mean pre-treatment FEC increased, the misclassification rate decreased when efficacy was low (i.e. <80%) and a threshold of 95% was applied, but when efficacy and the threshold were the same (95%), then increasing the mean did not reduce the

misclassification rate, which is in agreement with the present study and the findings of Levecke et al. (2012a).

In the present study, the misclassification rate was profoundly affected by the egg dl used to generate the FECRT data. The analysis suggests that, as egg dl increases, the percentage of results that are recorded <90% decreases (false positive rate), but the percentage of results that are greater than 95% increases (false negative rate). This observation may be explained by the Poisson process, which leads to variability in FEC data. Using a FEC method with a high egg dl artificially inflates the variance when transforming the raw egg counts into the EPG estimate (Torgerson et al., 2012). For example, if the number of eggs seen in a McM chamber was 4 and the egg dl was 50, this would give a FEC of 200 EPG. If five further subsamples from the same faecal sample were taken, the actual number of eggs seen could be 2, 0, 7, 3, 2 (these numbers were drawn randomly from a Poisson distribution), giving FEC of 100, 0, 350, 150 and 100 EPG, respectively, demonstrating the range of FEC that theoretically be generated from the same faecal sample due to the Poisson process. The effect of the egg dl and the Poisson process appears to be most apparent when values for efficacy and the threshold for classifying resistance/efficacy are the same. As the egg dl increases, the test is insufficiently sensitive to detect eggs when they are at a low density, therefore, there a fewer false positive results and more false negative results and efficacy is overestimated. It could be argued that false negative FECRT results are of greater concern than false positive results as, if a false positive result is obtained, then from this outcome one would be aware that there may be a possible reduction in efficacy of the anthelmintic tested and one could repeat the FECRT to make sure that the outcome was correct, or chose to use a more sensitive FEC method for counting pre- and post-

treatment FEC. This is opposed to a false negative result, where the outcome of the FECRT would appear to be satisfactory, when in fact it is not.

The results from this study, and those of others (Levecke et al., 2012a; Calvete and Uriarte, 2013) suggest that, to accurately detect anthelmintic resistance in veterinary nematodes using the WAAVP recommended-FECRT protocol, a FEC method with a low egg dl (for example, ≤ 10 EPG) should be used. This is particularly important when the group sizes included in the test are low. Levecke et al. (2012a) found that when the egg dl was 1 or 2 EPG then *m*, *k* and *n* values had a minimal effect on increasing the reliability of detecting resistance when true efficacy was low (i.e. <90%) and a 90% threshold was used to discriminate between resistance/efficacy. These findings are in agreement with those in the present study. Likewise, in other studies where ML efficacy in cattle was tested, it was found that the egg dl was an important factor and unless the error associated with using a multiplication factor in the FEC test is accounted for, then false positive results were more likely to occur (El Abdellati et al., 2010). As discussed in Chapter 2, (Section 2.4) even when faeces have been mixed to ensure that eggs are evenly distributed, a FEC method offering an analytical sensitivity of 1 EPG, when the FEC is 1 or 2 EPG, the test has a diagnostic sensitivity of 63% and 86%, respectively, due to Poisson error (Torgerson et al., 2012). Further, when an observed FEC of 0 is obtained using a McM test with an egg dl of 50 EPG, 95% lower and upper confidence limits of 0 and 184.5 EPG, respectively, are calculated (Torgerson et al., 2012). The confidence intervals decrease when a lower egg detection limit is used, such that a 0 EPG count derived using a multiplication factor of 10 gives lower and upper 95% CL of 0 and 36.9 EPG, respectively (Torgerson et al., 2012). Thus, erroneous declarations of efficacy are more likely when using a single McM count with an egg detection limit of 50 EPG. This has implications when assessing efficacy, as

negative counts of Day 14 post-treatment may not actually be negative, thus falsely declaring an anthelmintic to be efficacious.

Here, the FECRT results were generated across a range of *k* values, which were taken from empirical FECRT datasets. In the study by Levecke et al. (2012a), when a 90% threshold was applied, the level of aggregation of pre-treatment FEC amongst animals did not have an effect on increasing the sensitivity or specificity of the FECRT, in agreement with the findings of the present study when true efficacy was set at 90%. Conversely, in the study by Levecke et al. (2012a), when a 95% threshold was applied to the simulated FECRT data to discriminate between resistance/efficacy, the level of aggregation of pre-treatment FEC did affect the FECRT result. Moreover, when FEC were highly aggregated (k<0.1) and the egg dl was between 50 – 100 EPG, reduced efficacy (true efficacy <95%) and normal efficacy (true efficacy ≥95%) could not be reliably detected (Levecke et al., 2012), which agrees with the findings of the present study.

The number of horses included in the FECRT had a large influence on the misclassification of resistance/efficacy. The results suggest that the effect that a small sample size (n <10) has on false negative misclassification rate can be negated by using a FEC method with a lower egg dl (i.e. <10 EPG). This was not the case when true efficacy and the value for the threshold used to classify an anthelmintic as efficacious were set at the same value. In this scenario, increasing sample size did reduce false negative misclassification rate but did not eliminate it. These findings are similar to those reported by Levecke et al. (2012a), where they found that when the sample size was small (6 – 10 animals), the egg dl was the most important factor. A study by Torgerson et al. (2005), looked to test the efficiency of the FECRT (method by Coles et al., 1992) for falsely assigning anthelmintics as effective by a simulation approach

similar to that used here, where FEC data were simulated from a NBD, where *k* was set at 1.5, the mean pre-treatment FEC was 4,600 EPG, which were values drawn from empirical sheep FECRT datasets. The authors simulated these fixed parameters across a range of sample sizes (*n* = 5 – 20), and used a threshold of 95% to classify resistance/efficacy. They found that when the sample size was small (5 animals), there was a 5% chance of falsely declaring the anthelmintic as efficacious when the true efficacy was less than 84.5%, and when 20 animals were included, there was a 5% chance that an anthelmintic would falsely declare efficacy when true efficacy was 90.2%. Thus, when the sample size was large, there was a significant chance of classifying an anthelmintic as effective when it was not, in contrast to the results found here. In the study by Torgerson et al. (2005), they did not consider the egg dl, and the mean from which the pre-treatment FEC were simulated from was higher than that of the present study and the value used for *k* was lower in their study. These variable factors may be the reason for the differences observed.

Vidyashankar et al. (2007) who developed a theoretical framework for equine FECRT used novel bootstrap-based algorithms to estimate efficacy using simulated datasets. These authors reported that it was difficult to distinguish between a true FECR of 95% to 90% when a small number of horses (i.e. *n* = 8) were included, and, even on farms where pre-treatment mean FEC was 100 - 200 EPG, detection of a 5% drop in efficacy in 8 horses lead to a substantial difference between observed and actual efficacy (Vidyashankar et al., 2007). These authors suggest that as many horses as possible are included, even those with a low FEC, and that a FEC method with a low egg dl be used.

The results from the present study, clearly demonstrate that when true drug efficacy is low (i.e. 70%) and a FEC method with a low egg dl is used (<10 EPG) then the WAAVP FECRT is able to accurately identify resistance when a 90% or 95% threshold is

applied. However, when efficacy and the threshold for classifying an anthelmintic as efficacious are similar, then the false positive misclassification rates were high across a range of parameters. These observations can be explained in part by the Poisson process. For example, if the mean pre-treatment FEC was 200 EPG and the anthelmintic used was 90% efficacious, then it would be expected that the post-treatment FEC would be 20 EPG. However, the lower and upper 95% Poisson confidence intervals around 20 EPG are 12 EPG and 31 EPG, respectively, which means that the FECRT result could lie between 84.5% and 94%. Therefore, it could be argued that regular monitoring of FECR would be a more valuable tool than a single test based on a cut-off of 90 or 95%. Further, such thresholds are generally selected as they reflect efficacy of the anthelmintics measured in drug-sensitive populations when the products were first licensed (Kaplan and Nielsen, 2010), but using such an approach is based on the presumed efficacy of the anthelmintic being tested rather than the true efficacy, which is unknown (Vidyashankar et al., 2007). Using a set threshold for defining efficacy is not statistically sound as the spread of the FECRT data is not considered or the impact of outliers on the subsequent classification. Thus, researchers have included upper and lower 95% confidence limits to address this (Vidyaskankar et al., 2007; Stratford et al., 2014b). Lind et al., 2007 used resistance criteria for IVM and FBZ (FECR 95%, LCL 90%) to describe 'suspected' or 'considered' resistance; and FECR 90%, LCL 80% for PYR. Tarigo-Martinie et al. (2001), classed an anthelmintic as 'effective' if the FECR was greater than 90%, 'equivocal' if between 80% and 90%, and 'ineffective' if less than 80%, This lack of standardisation makes direct comparison between studies difficult, therefore, if thresholds and LCLs are to be used for cross-sectional surveys, there needs to be a consensus reached between researchers and sensible values selected and agreed upon and published in up-to-date guidelines.

From the results obtained here and from other published studies, it is clear that when performing a FECRT, increasing the pre-treatment mean and the number of horses and using a more sensitive FEC method will reduce the likelihood of misclassification, but the current thresholds are not fit for purpose and are not able to discriminate between resistance and efficacy. FECRT remain the only method for routine evaluation of anthelmintic efficacy under field conditions and further guidelines for conduct and interpretation are warranted. In the absence of validated in vitro and molecular tests, which could be used in tandem, the FECRT is not fit for identifying resistance but can be used as a tool for identifying efficacy or a suspected lack of efficacy.

4.6. Conclusions

The results presented in this Chapter clearly demonstrate the effect of mean pre-treatment FEC, the distribution of pre-treatment FEC, the number of horses included in the FECRT and the egg dl on the performance and subsequent interpretation of the FECRT. The most critical factors affecting the outcome of the FECRT were the mean pre-treatment mean FEC and the egg dl. The misclassification rate could be reduced by using a FEC method with a low egg dl (<10 EPG). However, when drug efficacy and the threshold for classifying an anthelmintic as resistant/efficacious were close together then the false positive misclassification rates were high, regardless of increasing the pre-treatment mean FEC, the number of horses included or lowering the egg dl, indicating that when a single threshold is applied then there is a greater chance of misclassifying the outcome of a FECRT. As such, current thresholds are not fit for purpose and none of the statistical FECRT methods are good enough to discriminate between resistance and efficacy. To aid interpretation, 95% CL should be included; however, in the absence of validated in vitro and molecular tests, FECRT will not be able to definitively identify resistance.

The results from this Chapter will help to create evidence-based recommendations for conducting equine FECRT to reduce the likelihood misclassification and optimise the usefulness of the FECRT in monitoring anthelmintic efficacy.

CHAPTER 5: Distribution and prevalence of equine parasites, factors affecting shedding and egg shedding consistency

5.1 Introduction

To inform best practice helminth control for horses, it is essential to understand the epidemiology of strongyle infections in terms of parasite distribution and prevalence, and to appreciate factors that influence strongyle egg shedding. Helminths are typically over-dispersed in their host populations, where relatively few individuals harbour a significant parasite burden (Crofton, 1971; Anderson and May, 1978; Shaw and Dobson, 1995; Shaw et al., 1998; Calabrese et al., 2011). In horses, the vast majority of animals within populations shed low numbers or no eggs in their faeces (Relf et al., 2013; Wood et al., 2013). Such distributions underpin the rationale behind targeting anthelmintic treatments at horses that shed a moderate-to-high number of eggs (Gomez and Georgi, 1991; Kaplan and Nielsen, 2010; Lester and Matthews, 2014). As the work published in this area is limited, it is important to undertake research into factors that affect egg shedding, and identify potential risk factors that are associated with high egg shedding. Such are thought to include host-related factors (i.e. age, immunity, breed, sex), parasite-related factors (i.e. stage of lifecycle, age, fecundity, resistance status), management-related factors (i.e. anthelmintics used, treatment frequency, regularity of removing faeces from pasture) and environment-related factors (i.e. temperature, rainfall, location).

Recent studies have aimed to quantify consistency of strongyle egg shedding in individual horses over time (Dopfer et al., 2004; Nielsen et al., 2006; Becher et al., 2010; Wood et al., 2013), with the rationale being, that if horses generally stay within the same shedding category (if not treated with an anthelmintic), they need not need be faecal egg count (FEC) tested as regularly. This could act as an incentive to horse owners to adopt targeted deworming programmes as it may be associated with reduced costs and effort. Past studies investigating strongyle egg shedding consistency

focussed on estimates of strongyle FEC using McMaster (McM) methods with egg detection limits (dl) between 20 and 50 EPG (Dopfer et al., 2004; Nielsen et al., 2006, Lloyd, 2009; Becher et al., 2010; Wood et al., 2013). As observed in Chapter 2, (Section 2.5.5) FEC generated using the McM method tend to generate higher EPG estimates and greater variance compared to those generated using a centrifugal-flotation (CF) method that has an egg dl down to 1 EPG. In addition, using a FEC method with a higher egg dl (i.e. multiplication factor), the methodology will not be particularly sensitive to relatively low changes in egg abundance (Morrison, 2004), and larger multiplication factors will artificially inflate variance, potentially leading to more varied results (Torgerson et al., 2012). This may potentially lead to an observed lower consistency between egg counts from the same individual over time because of the greater degree of variation in FEC.

5.2 Aims of Chapter

Here, using the equine FEC data collected and presented in Chapter 3, the distribution and prevalence of strongyle egg shedding at yard level and individual level was explored, along with factors affecting strongyle egg shedding and the consistency of strongyle egg shedding in individual horses over time were investigated. Such information will underpin the framework presented in Chapter 6 as current knowledge on the prevalence and distribution of strongyles and factors that affect egg shedding will inform control programmes that aim to promote sustainable parasite control.

5.3 Materials and methods

5.3.1 Data collection

Equine FEC data were collected from 28 horse yards across England and Scotland between 2011 and 2012. All samples were collected as previously described in Section 3.3.3 and analysed by the CF technique described in Section 3.3.4.

5.3.2 Questionnaire

Each yard that participated in the study was supplied with a questionnaire (Appendix 1) to collect information on management practices. The questionnaire consisted of 42 questions divided into 5 sections: 1) general yard information, 2) worm control practices in adult horses, 3) worm control practices in foals and horses <2 years old, 4) grazing and pasture management and 5) the use of FEC analysis. The response rate was 100%, but the questionnaire completion rate was relatively poor with several answers omitted, particularly with regards to information about grazing area.

5.3.3 Data analysis

5.3.3.1 Prevalence

Descriptive statistics (mean FEC, and prevalence (%)) were calculated using Microsoft Excel (2007). The prevalence of strongyle egg shedding was calculated using Equation 5.1 at individual level and yard level, where P denotes prevalence (%), n denotes the number of positive yards/individuals and N denotes the sample size (total number of yards/individuals).

$$P = \frac{n}{N}$$

Equation 5.1

For each individual yard prevalence proportion, the exact upper and lower 95% confidence intervals were calculated using Equation 5.2 a and b, respectively, where *N* denotes the sample size, *n*, the number of positive individuals. *Betainv*, represents the inverse of the beta distribution. The upper and lower 95% confidence intervals have been included to determine the statistical uncertainty associated with the given prevalence estimates.

$$95\%\ UCL = \ 1 - Betainv(\frac{1.96}{2}\ , n - k, k + 1)$$

Equation 5.2a

$$95\%\ LCL = \ 1 - Betainv(1 - \frac{1.96}{2}\ , n - k, k + 1, k)$$

Equation 5.2b

For the overall individual prevalence and yard prevalence, stratified 95% intervals were included to account for the heterogeneity between yards (Thrusfield, 2007). Where *P* denotes prevalence, *y* represents the number of yards samples; *N* represents the total number of animals sampled. *V* denotes variability between yards where n represents the number of animals sampled in each yard and m represents the number of animals with a positive FEC:

$$V = P^2(\sum n^2) - 2P(\sum nm) + (\sum m^2)$$

Equation 5.3a

$$95\%\ UCL = P + 1.96\begin{cases} y \\ N \end{cases}\sqrt{\frac{V}{y(y-1)}}$$

Equation 5.3b

$$95\%\ LCL = P - 1.96\begin{cases} y \\ N \end{cases}\sqrt{\frac{V}{y(y-1)}}$$

Equation 5.3c

5.3.3.2 Distribution

The negative binomial distribution (NBD) was fitted to each set of yard FEC data by maximum likelihood estimation (MLE) (Williams and Dye, 1994; Shaw et al., 1998; Morgan et al., 2005) to give estimates of the mean, *m,* and the aggregation factor, *k* (Section 2.4.1, Equation 2.2). The chi-square goodness of fit test was used to assess whether the data adequately fitted the NBD (Section 2.4.1, Equation 2.3). The relationship between *m* and *k* was investigated by a negative binomial generalised linear model (GLM) using the 'MASS' package (Venables and Ripley, 2002) in RStudio 2.15.1 as the mean follows a negative binomial distribution, and the relationship between prevalence and *k* was investigated using a GLM with binomial errors.

5.3.3.3 Factors affecting strongyle egg shedding

To investigate factors affecting strongyle egg shedding, the first screening sample collected from each horse was used. Only horses of known age and yards with more than 10 horses were included in the analysis. Individuals were classified as foals (<2 years), youngsters (≥2 and <5 years), adults (≥5 and <18 years) and geriatric (≥18 years). Response data for the last anthelmintic administered before sampling (ivermectin (IVM), moxidectin (MOX), pyrantel (PYR)), the country (England or Scotland) and region (southeast England (SE), southwest England (SE), northeast Scotland (SNE), southeast Scotland (SSE) and southwest Scotland (SSW)) in which the

yard was based, the month (March-September) in which the sample was collected (2011 or 2012), the yard type (Competition (CTN), Dealers (DLR), Livery (LVY), Sanctuary (STY) and Stud (STD)), the anthelmintic treatment frequency (2 times/year, 4 times/year and based on FEC) applied on the yard, whether or not the yard enforced poopicking (Yes/No) and the quarantine of new arrivals (Yes/No), the number of weeks after the strongyle egg reappearance period (ERP) for the last administered anthelmintic that the sample was collected (the expected ERP used for MOX was 13 weeks, IVM 8 weeks, 5d FBZ and PYR was 6 weeks,) and the area of grazing that each horse had access to (Table 5.1). Information was provided by each yard on all factors with the exception of grazing area, for which on 6 yards (201 horses) these details were missing.

Table 5.1. A summary of the different factors and responses included in the analyses to determine factors affecting strongyle egg shedding

Factor	Responses
Yard type	Competition, dealers, livery, private, sanctuary, stud
Region	NE, SE, SW
Age category	Foal, youngster, adult, geriatric
Last anthelmintic administered	IVM, MOX, PYR
No. of weeks post the expected ERP	Weeks*
Anthelmintic treatment frequency	2/year, 4/year, FEC
Year sample collected	2011, 2012
Month sample collected	March-September
Season sample collected	Spring, Summer, Autumn
Poo pick?	Yes, No
Quarantine new arrivals?	Yes, No
Use FEC for targeted treatment?	Yes, No
Area of grazing/horse	Acres*

* continuous variable

Because these data were collected from multiple yards and each horse was nested within yard, it was necessary to investigate this with the aim of partitioning variance between yards through a mixed-modelling approach. Generalised linear mixed models

(GLMM) are an extension of generalised linear models (GLM) and combine link functions and exponential-family variation with random effects (Bolker, 2008). Random effects quantify the variation of regression intercept or slopes amongst the levels of an explanatory variable by a probability distribution instead of estimating a fixed regression coefficient for each level (Thiele and Markussen, 2012). As counts for equine FEC are typically over-dispersed, a negative binomial GLMM (NBGLMM) was used as the NBD is used most commonly to describe parasite count data (Anderson and May, 1978; Wilson and Grenfell, 1997; Shaw and Dobson, 1995; Shaw et al., 1998). The NBGLMM is a modification of GLMM to include an estimation of theta (k) (Venables and Ripley, 2002). All GLMM were run in RStudio 2.15.1 using the 'glmer.nb function in the 'lme4' package (Bates et al., 2015).

Before any modelling commenced, the distribution of FEC was plotted (Figure 5.2); the resulting histogram revealed that these data were highly aggregated. Next, relationships between variables were explored using violin plots because of the underlying distribution using 'ggplot2' package in R. Univariate analysis of each factor was performed using NBGLMM to identify factors where egg shedding was significantly higher ($p<0.05$).

Initially, the multivariate model was populated with all explanatory variables; non-significant factors were removed and the factor with the lowest z value (the ratio of the estimated slope to its standard error) was dropped from the models in turn until only significant factors ($p\leq0.05$) remained and the minimal adequate model was achieved. The significance of removing factors from the model was evaluated using log-likelihood ratio tests (LRTs, Zuur et al., 2010; Crawley, 2013). To confirm that the minimal adequate model was reasonable, all dropped terms were sequentially reintroduced back into the final model to ensure a lack of significant change in explanatory power as

confirmed by LRTs. Inspecting the model residuals and plotting them against the fitted values and against each significant factor allowed model checking. Grazing area was not included in the model due to the high proportion of incomplete answers. Country was confounded by year, so country was not included. Interactions between covariates were not investigated as the decision was made to follow a simple modelling approach here.

5.3.3.4 Factors affecting strongyle egg shedding consistency

In total, 573 horses were included in the analysis to examine the consistency of egg shedding in individual horses over time (Table 5.2). Only horses that had had an initial screening sample collected on more than one occasion were included.

Egg shedding consistency in individual horses over time was investigated. Each FEC from each horse on each screening occasion (S1 = first screen, S2 = second screen, S3 = third screen and S4 = fourth screen) was assigned to a shedding category ranging from 1 (0 - 49 EPG) to 7 (>500 EPG) (Table 5.3).

Table 5.2. A summary of the number (*n*) of horses screened on each yard, the last anthelmintic administered on the yard, the year and month in which the first faecal egg count (FEC) screening, the yard type (Competition = CTN, Livery = LVY, Stud = STD, Sanctuary = STY) and the number of occasions the yard was screened (S1 – S4)

Yard	*n*	Last treatment	Year first Screening FEC collected	Month first screening FEC collected	Yard type	Screening occasions
3	31	MOX	2012	APR	LVY	S1-S3
5	14	NA	2012	MAY	LVY	S1-S3
6	14	PYR	2011	MAY	LVY	S1-S4
7	17	MOX	2012	MAY	STY	S1-S3
8	15	MOX	2012	APR	LVY	S1-S3
10	8	NA	2011	JUL	LVY	S1-S3
11	13	IVM	2012	APR	LVY	S1-S3
12	21	MOX	2012	JUN	LVY	S1-S3
13	8	MOX	2012	MAY	LVY	S1-S3
14	20	IVM	2012	MAR	STD	S1-S3
15	36	MOX	2011	MAR	LVY	S1-S4
16	72	MOX	2011	MAY	LVY	S1-S3
17	13	MOX	2010	JUL	LVY	S1-S2
18	10	MOX	2011	MAY	CTN	S1-S3
19	15	MOX	2012	APR	LVY	S1-S3
20	35	MOX	2011	JUN	LVY	S1-S3
21	21	MOX	2010	MAY	LVY	S1-S2
22	21	MOX	2012	MAY	LVY	S1-S3
23	19	NA	2012	MAY	LVY	S1-S2
24	27	IVM	2011	AUG	LVY	S1-S3
25	20	MOX	2012	FEB	LVY	S1-S3
27	47	MOX	2010	JUL	LVY	S1-S4
30	31	MOX	2012	MAR	LVY	S1-S3
31	7	MOX	2011	JUL	LVY	S1-S2
33	28	MOX	2012	APR	LVY	S1-S2
34	10	MOX	2012	AUG	DLR	S1-S2

Table 5.3. Strongyle egg shedding categories used for the analysis based on the eggs per gram (EPG) estimate for each faecal egg count (FEC) sample as determined by the centrifugal-flotation (CF) method. For each horse, two FEC were performed and the mean of the two counts was taken as the EPG estimate

EPG range	Strongyle egg shedding category
0 - 49 EPG	1
50 - 99 EPG	2
100 - 199 EPG	3
200 - 299 EPG	4
300 - 399 EPG	5
400 - 499 EPG	6
>500 EPG	7

In addition to shedding category, each sample was assigned a treatment category; <200 EPG (0, no treatment) or ≥200 EPG (1, treatment). To assess shedding consistency, the rank change in shedding category between S1 and S2, S1 and S3 and S1 and S4 samples was calculated. For example, if the shedding category was ranked as 1 for S1 and 6 for S2, the rank change would be 5. Conversely, if the category was measured as 6 for S1 and 3 for S3, the rank change would be -3. If the shedding category remained the same it was assigned as 0. Treatment category consistency was determined across S1 and S2, S1 and S3 and S1 and S4 samples. Similarly, if a category changed from <200 EPG to ≥200EPG between two sampling points, the rank assigned was 1. Conversely, if the category changed from ≥200EPG to <200EPG it was assigned as -1, and if it remained consistent it was assigned as 0. Each horse was assigned to an age category: foals (<2 years), youngsters (≥2 and <5 years), adults (>5 and < 18 years) and geriatric (≥18 years). For each sampling occasion, the number and percentage of horses that fell into

each shedding and each treatment category was calculated. Further, the number and percentage of horses that fell into each rank change category of shedding (-6 to 6) and treatment (-1, 0 or 1) between S1 and each subsequent screening occasion was calculated. The same analysis was performed on 304 horses that had not received any anthelmintic during the entire study to assess if no treatment was associated with rising egg counts in individuals over time. All analyses were performed in Rstudio, version 2.15.1 (The R Foundation for Statistical Computing, 2012). To test if there was a significant difference ($p<0.05$) in the proportion of horses falling into each shedding and treatment category between screening occasions, a binomial test was performed using the prop.test function in RStudio.

The effect of age, last anthelmintic administered and the number of weeks after the expected ERP of each anthelmintic on the likelihood of a horse changing shedding or treatment category was assessed using multivariate binary logistic regression. Regression analyses were performed using the GLM function in R, specifying the family as binomial, linked to logit transformation, g, where P is the probability of a horses changing egg shedding category or treatment category, β_i are the model (slope) coefficients and X_i are the explanatory variables (Equation 5.4).

$$g = \ln[P/(1-P)] = \beta_0 + \beta_1 X_0 + \ldots + \beta_k X_k$$

Equation 5.4

The probability of a change in egg shedding category and treatment category was estimated using Equation 5.5.

$$P=\exp[\beta_0 + \beta_1 X_0 + \ldots + \beta_k X_k]/(1+\exp[\beta_0 + \beta_1 X_0 + \ldots + \beta_k X_k])$$

Equation 5.5

Regression models were initially populated with all potential explanatory variables and the least significant removed in turn until a model with only significant terms remained. The significance of removing factors from the model was evaluated using log-likelihood ratio tests (LRT) (Zuur et al., 2009; Crawley, 2013). *P*-values of ≤0.05 indicated factors that had a significant influence on changing shedding or treatment category in the final model. The Hosmer-Lemeshow test (Hosmer and Lemeshow, 2005) was used to assess overall model fit using the 'ResourceSelection' package (Lele et al., 2009). The Hosmer-Lemeshow test is commonly used to assess the goodness of fit of multivariate logistic regression models. It is similar to the chi-square goodness of fit test, but has the advantage of partitioning observations into groups of approximately equal size, reducing the likelihood of there being groups with very low observed or expected frequencies (Bewick et al., 2005).

5.4 Results

5.4.1 Prevalence and distribution of equine parasites as measured by FEC

The prevalence of strongyle egg shedding at yard level was 100% (88.7 - 100%, 95% CL) (Table 5.4). For *P. equorum* and tapeworm, the yard prevalence was 14.3 (4/28 yards, 4 - 33% CL) and 3.6% (1/28 yards, 0 - 18.4% CL), respectively (Table 5.5). At individual horse level, the prevalence of strongyle egg shedding ranged between 21.1 (11.4 - 34% CI, Yard 3) and 93% (80.9 - 98.5% CI, Yard 24, (Table 5.4)). The mean EPG per yard ranged from 5 to 327 EPG and individual FEC ranged from 0 to 2972 EPG (Table 5.5). Values for aggregation parameter, *k* ranged between 0.05 and 0.38 (Table 5.4). There was no significant relationship observed between mean strongyle EPG and

k (Figure 5.1A, Figure 5.2), but there was a significant positive relationship between strongyle prevalence and *k* (r^2=0.8, p<0.0001) (Figure 5.1B)

Table 5.4. The prevalence of strongyle eggs at individual level for each yard. Included is the number of horses resident at each yard (*n*), the mean eggs per gram (EPG) and range as estimated by faecal egg count (FEC) taken on the first screening occasion, estimates of distribution of FEC (*k*), the prevalence (%) and the lower (LCL) and upper (UCL) exact binomial 95% confidence limits of prevalence

Yard	*n*	Mean EPG	Range [EPG]	*k*	Prevalence [%]	LCL	UCL
1	18	39	0-513	0.08	38.9	17.3	64.3
2	16	270	0-1341	0.19	92.7	48	93
3	57	5	0-107	0.05	21.1	11.4	34
4	28	5	0-126	0.06	25.0	11	45
5	47	59	0-537	0.1	46.8	32.1	61.9
6	28	123	0-1284	0.1	50.0	30.7	69.4
7	23	60	0-378	0.27	87.0	66.4	97.2
8	33	37	0-555	0.06	33.3	18	51.8
9	31	71	0-450	0.17	58.1	39.1	75.5
10	28	153	0-690	0.22	71.4	51.3	86.8
11	28	94	0-1179	0.12	57.1	37.2	75.5
12	44	51	0-474	0.17	63.6	48	77.6
13	29	16	0-155	0.07	31.0	15.3	50.8
14	8	12	0-33	0.4	87.5	47.4	99.7
15	69	199	0-2927	0.19	76.8	65.1	86.1
16	32	95	0-1530	0.1	46.9	29.1	65.3
17	18	130	0-1233	0.2	66.7	41	86.7
18	14	197	0-963	0.16	85.7	57.2	98.2
20	25	84	0-698	0.14	64.0	42.5	82
21	23	327	0-1137	0.2	73.9	51.6	89.8
22	18	90	0-675	0.23	83.3	58.6	96.4
23	26	60	0-927	0.06	23.1	9	43.7
24	43	114	0-513	0.38	93.0	80.9	98.5
25	22	27	0-306	0.1	36.4	17.2	59.3
26	32	102	0-761	0.06	78.1	60	90.7
27	48	46	0-525	0.23	75.0	60.4	86.4
28	14	293	0-1067	0.27	78.6	49.2	95.3
Total	802	472	0-2927	0.16	58.9	51.6	66.2

Table 5.5. The prevalence (%) of strongyle spp., *P. equorum* and tapeworm eggs as identified by faecal egg count at yard level. Included are the lower (LCL) and upper (UCL) stratified 95% confidence limits

Species	No. of yards	No. of yards positive	Prevalence (%)	LCL	UCL
Strongyle spp.	28	28	100	87.7	100
P. equorum	28	4	14.3	4	33
Tapeworm	28	1	3.6	0	18.4

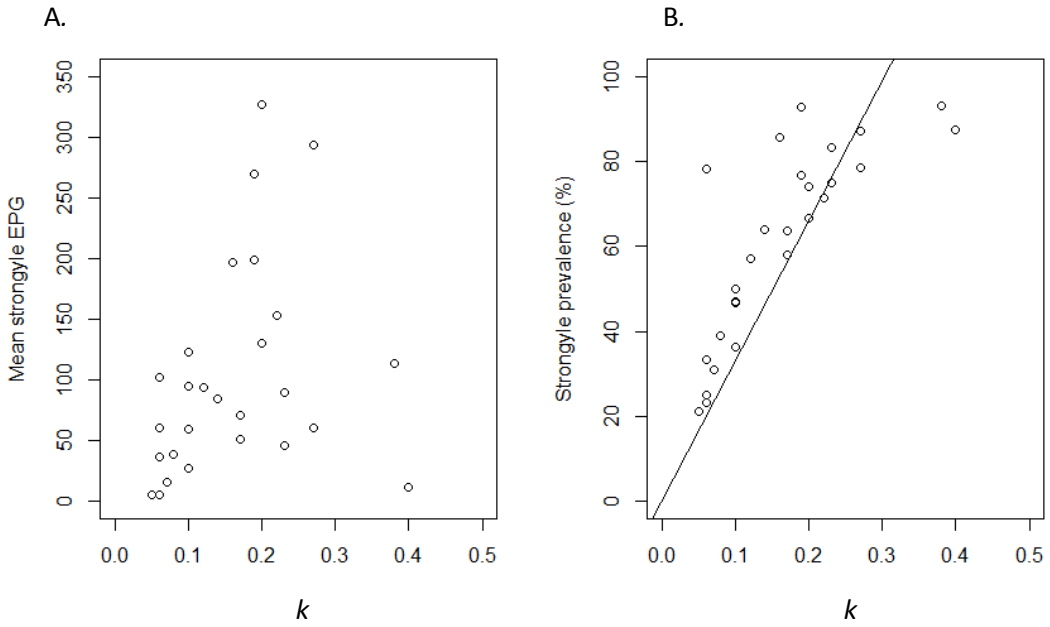


Figure. 5.1. The relationship between strongyle mean eggs per gram (EPG) and *k* (A) and the relationship between strongyle prevalence and *k* (Pearson r^2=0.8 (95% CI: 0.6-0.9), *n*=28, *p* <0.0001) (B)

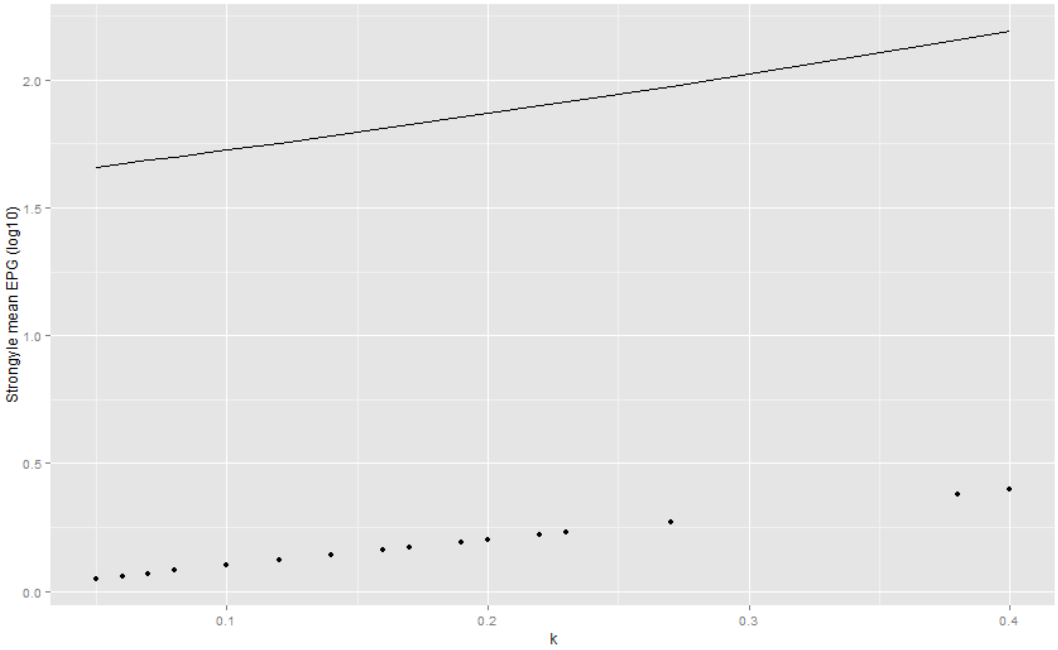


Figure. 5.2. The relationship between log10 strongyle mean eggs per gram (EPG) and *k*. Negative binomial generalised linear model. Model intercept = 0.64, standard error (se) = 0.29, z-value= 1.61, *p*=0.11. The slope for *k* = 0.79, se = 1.49, z = 0.53, *p* = 0.6

The prevalence of *P. equorum* eggs at individual horse level ranged between 2 (1/50 horses, Yard 27) and 23% (23/32 horses on Yard 21, Table 5.6). Mean EPG for *P. equorum* ranged between 1 and 32 EPG, with individual counts ranging between 0 to 269 EPG.

Table 5.6. The prevalence of *P. equorum* eggs at individual level for each yard where *P. equorum* eggs were identified in screening samples. Included is the number of horses resident at each yard (*n*), the mean eggs per gram (EPG) and range as estimated by faecal egg counts (FEC) taken on the first screening occasion, estimates of distribution of FEC (*k*), the prevalence (%) and the lower (LCL) and upper (UCL) stratified 95% confidence limits

Yard	*n*	Mean EPG	Range [EPG]	Prevalence [%]	LCL	UCL
15	69	3	0-86	7.3	2.4	16.1
21	23	32	0-269	23	7.5	43.7
22	18	3	0-47	6	1	27
27	50	1	0-41	2	0	10.7

5.4.2 Factors affecting egg shedding in horses at screening

In total, 27 yards completed the questionnaire and supplied samples for FEC analysis. In total, samples from 728 horses were analysed. Of the yards, three were competition yards, 20 were livery yards, two were non-Thoroughbred studs farms and five were sanctuaries/welfare establishments (Table 5.7). The number of horses per yard ranged between 10 and 69. In total, 12 of the yards were FEC screened in 2011 and 15 in 2012, while 16 were based in England and 11 in Scotland. Thirteen yards were based in southeast England, three in southwest England, one in northeast Scotland, nine in southeast Scotland and one in southwest Scotland (Table 5.7). Of the yards tested, three were first FEC screened in March, seven in April, six in May, three in June and July, four in August and one in September, which, by season, meant that 16 were sampled in the spring, 10 during the summer and one in autumn. On six yards, IVM was the last administered anthelmintic before FEC screening, 20 had last received MOX and one, PYR. Time (at initial FEC sampling) since the expected ERP of the last administered anthelmintic ranged from 0 to 27 weeks. On 18 yards, anthelmintics were administered twice a year, and on six, four times a year. Owners at three yards reported that they followed a FEC directed treatment programme with anthelmintics administered according to individual FEC. Seventeen yard owners said they imposed quarantine measures and 10 did not; while 23 reported that they systematically removed faeces from pasture, four yards did not (Table 5.7).

Table 5.7. A summary of yard information collected by questionnaire, including yard type (CTN=competition; DLR=dealers; LVY=livery; STD=stud; STY=Sanctuary), the year, month and season (1=Spring; 2=Summer; 3=Autumn) the faecal samples were collected, the country (E=England; S=Scotland) and region (SE= southeast England; SW=southwest England; SNE=northeast Scotland; SSE=southeast Scotland; SSW=southwest Scotland) where the yard was, the last anthelmintic administered, anthelmintic treatment frequency (2=2 times/year; 4=4 times/year; Faecal egg count (FEC)= as directed by FEC), the number of weeks after the expected strongyle egg reappearance period (ERP) for each anthelmintic that the sample was collected, whether or not the yard imposed quarantine measures for new arrivals, whether they remove faeces from pasture and the grazing area per horse (1 = < 1 acre/horse; 2 = 1-1.9 acres/horse; 3 = >2 acres/horse; NA= no information provided)

Yard ID	Yard type	Year	Month	Season	Region	Country	*N*	Last anthelmintic	Treatment frequency	Post ERP	Quarantine?	Poopick?	Grazing area/horse
2	STD	2011	JUN	2	SSW	S	18	IVM	4	2	N	Y	1
3	LVY	2012	APR	1	SE	E	32	MOX	4	3	Y	Y	2
4	CTN	2012	AUG	2	SE	E	14	IVM	2	5	Y	Y	2
5	LVY	2012	MAY	1	SE	E	17	MOX	2	4	N	N	NA
6	LVY	2011	APR	1	SSE	S	15	PYR	2	7	Y	Y	1
7	STY	2012	MAY	1	SE	E	14	MOX	2	5	Y	Y	2
8	LVY	2012	APR	1	SE	E	17	MOX	2	3	Y	Y	3
9	STY	2011	JUL	2	SSE	S	63	IVM	2	7	N	N	NA
11	LVY	2012	APR	1	SE	E	14	MOX	2	4	N	Y	3
12	CTN	2012	JUN	2	SE	E	25	MOX	FEC	3	Y	Y	NA
14	STD	2012	MAR	1	SE	E	18	IVM	2	3	Y	Y	1
15	LVY	2011	MAR	1	SSE	S	28	MOX	FEC	2	Y	Y	NA
16	LVY	2011	MAY	1	SSE	S	47	MOX	2	5	Y	N	NA
17	LVY	2011	SEP	3	SSE	S	20	MOX	4	7	Y	Y	2
18	CTN	2011	MAY	1	SW	E	10	MOX	2	1	N	Y	1
19	LVY	2012	APR	1	SE	E	18	MOX	4	3	N	Y	2
20	LVY	2011	JUN	2	SSE	S	30	MOX	2	2	N	Y	2
21	LVY	2011	AUG	2	SSE	S	21	MOX	2	5	Y	Y	2
22	LVY	2012	MAY	1	SE	E	22	MOX	4	2	Y	N	NA

Yard No.	Yard type	Year	Month	Season	Region	Country	*N*	Last anthelmintic	Treatment frequency	Post ERP	Quarantine?	Poopick?	Grazing area/horse
23	LVY	2012	MAY	1	SE	E	43	MOX	4	3	N	Y	2
24	LVY	2011	AUG	2	SSE	S	28	IVM	2	5	Y	Y	2
25	LVY	2012	APR	1	SE	E	24	MOX	2	3	Y	Y	2
27	LVY	2011	JUL	2	SSE	S	44	MOX	FEC	3	Y	Y	2
30	LVY	2012	MAR	1	SW	E	32	MOX	2	1	N	Y	2
32	STY	2011	JUL	2	SNE	S	69	IVM	2	3	Y	Y	2
33	STY	2012	APR	1	SW	E	31	MOX	2	1	Y	Y	3
34	STY	2012	AUG	2	SE	E	14	MOX	2	7	N	Y	1

MOX = moxidectin, IVM = ivermectin, PYR = pyrantel

The distribution of horse age on each yard is shown in Table 5.8. In total, 518 adults, 31 foals, 122 geriatric horses and 73 youngsters comprised the overall population. The number of adult horses per yard ranged from 7 to 45, the number of foals, 0 - 12, geriatric horses, 0 - 17 and youngsters, 0 - 12 (Table 5.8). Yards 32 and 33 had the highest number of youngsters present (*n* = 12) and yard 32, the highest number of foals (*n* = 12). Adult horses were present on every yard, foals on 6 yards, geriatrics on 19 yards and youngsters on 23 yards (Table 5.8).

Table 5.8. The distribution horses by age category; (Foals = <2 years old; Youngsters = ≥2 - <5 years old; Adults = ≥5 - <18 years old; Geriatric = ≥18 years old) in each yard

Yard	Yard	Adults	Foals	Geriatric	Youngster	Total
2	STD	10	0	4	4	18
3	LVY	14	0	16	2	32
4	CTN	14	0	0	0	14
5	LVY	15	0	0	2	17
6	LVY	9	1	0	5	15
7	STY	7	0	5	2	14
8	LVY	13	0	3	1	17
9	STY	41	1	17	4	63
11	LVY	9	0	5	0	14
12	CTN	21	0	0	4	25
14	STD	11	5	0	2	18
15	LVY	20	0	8	0	28
16	LVY	38	0	4	5	47
17	LVY	12	0	7	1	20
18	CTN	10	0	0	0	10
19	LVY	10	0	7	1	18
20	LVY	20	0	8	2	30
21	LVY	27	7	2	1	37
22	LVY	16	0	5	1	22
23	LVY	41	0	1	1	43
24	LVY	25	0	0	3	28
25	LVY	17	0	6	1	24
27	LVY	31	0	10	3	44
30	LVY	21	0	9	2	32
32	STY	41	12	4	12	69
33	STY	14	5	0	12	31
34	DLR	11	0	1	2	14
	Total	518	31	122	73	744

CTN = competition, DLR = dealers, LVY = livery, STD = stud

Details of the distribution of age according to the last anthelmintic administered, the year, month and season in which the sample was collected, the country and region where the horse resided, whether or not quarantine measures were implemented on the yard, anthelmintic treatment frequency, the grazing area for each horse and whether or not there was faecal removal from pasture are presented in Appendix 2 as is the distribution of horses according to yard type by age, last anthelmintic administered, the year, month and season that the samples were taken, the country and region where the horse resided, whether quarantine measures were employed, anthelmintic treatment frequency, grazing area and whether or not the horse came from a yard where there was faecal removal from pasture to check for autocorrelation between covariates. The distribution of strongyle FEC (EPG) between all horses included in this analysis was highly over-dispersed (Figure 5.2). Violin plots and univariate analysis of each explanatory variable revealed that strongyle egg shedding (EPG) was significantly higher in foals (p = 0.001) and youngsters (p = 0.0001) compared to adult and geriatric horses (Figure 5.3 and Table 5.9). Egg shedding was significantly higher in horses for which a greater number of weeks had elapsed between the expected ERP of the previously administered anthelmintic and the time of sampling (p = 0.002) (Figure 5.4 and Table 5.9). Egg shedding was significantly lower in horses that were based on yards that administered anthelmintics four times a year (p = 0.02) and in horses that were based on yards that followed a FEC directed targeted treatment protocol (p = 0.04) compared to horses that were treated twice a year (Figure 5.5 and Table 5.9). Horses from Dealers yards and sanctuaries shed significantly more eggs at the initial sampling time point (p = 0.01 and p = 0.0004, respectively) compared to the other yard types (Figure 5.7 and Table 5.9). Egg shedding was significantly higher in July (p = 0.03) compared to the other months in which samples were analysed (Figure 5.6). There was no significant difference in egg

shedding detected between last treatment (Figure 5.8), years (Figure 5.9), seasons (Figure 5.10), on yards that quarantined their horses (Figure 5.11), on yards that removed faeces from pasture (Figure 5.12) nor the area grazed (Figure 5.13).

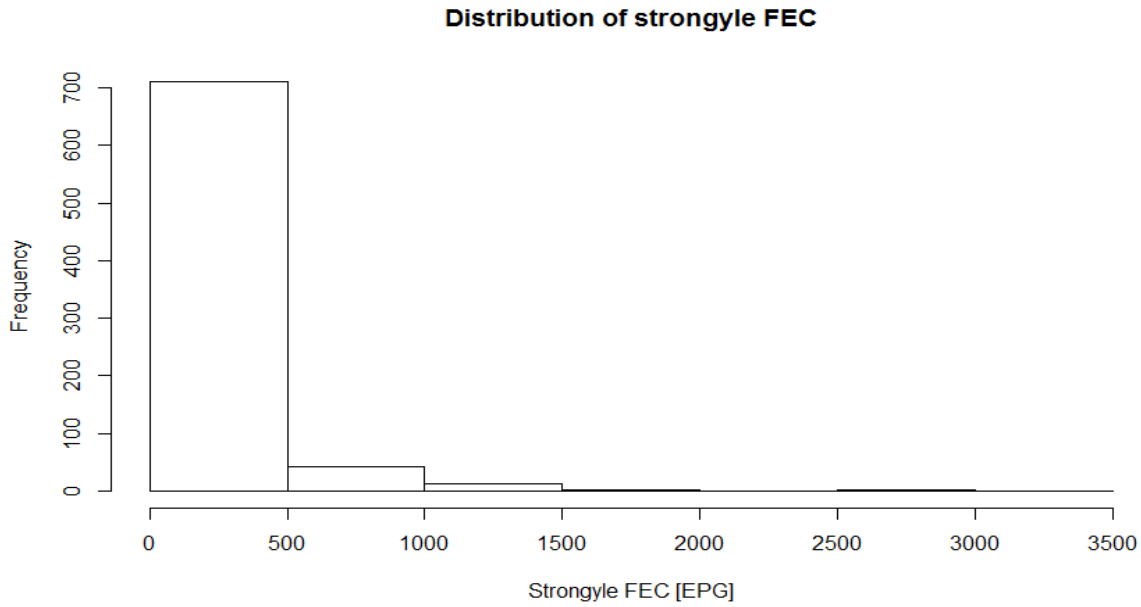


Figure 5.3. A histogram of the distribution of strongyle faecal egg counts as measured in eggs per gram (EPG) in all horses tested at screening

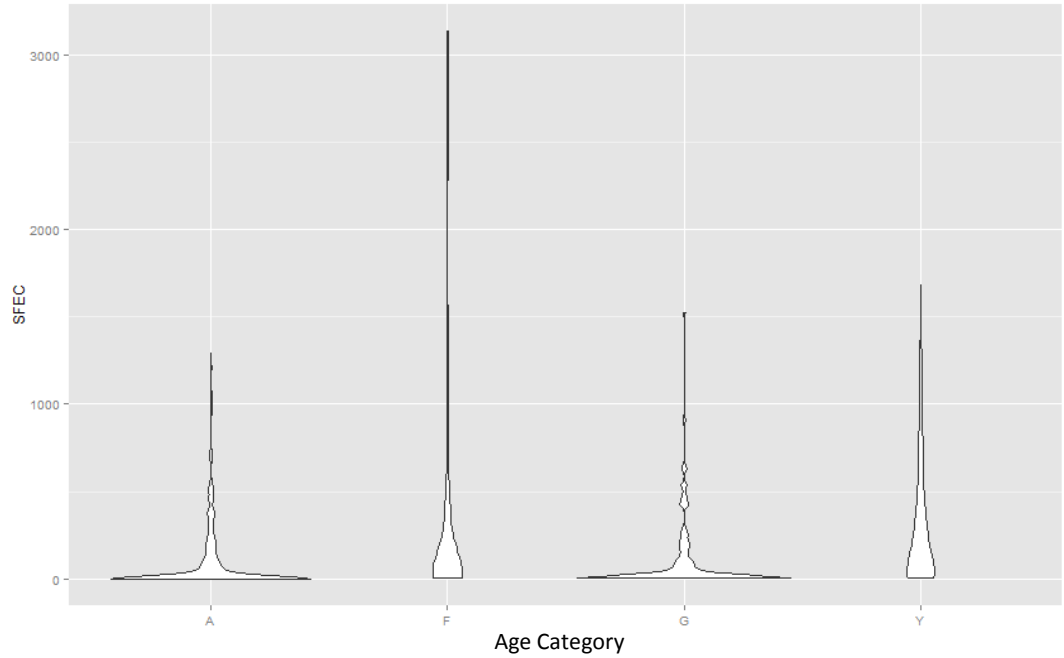


Figure 5.4. Violin plot of strongyle faecal egg count (SFEC) measured in eggs per gram [EPG] by age (A = adults, F = foals, G = geriatrics, Y = youngsters).

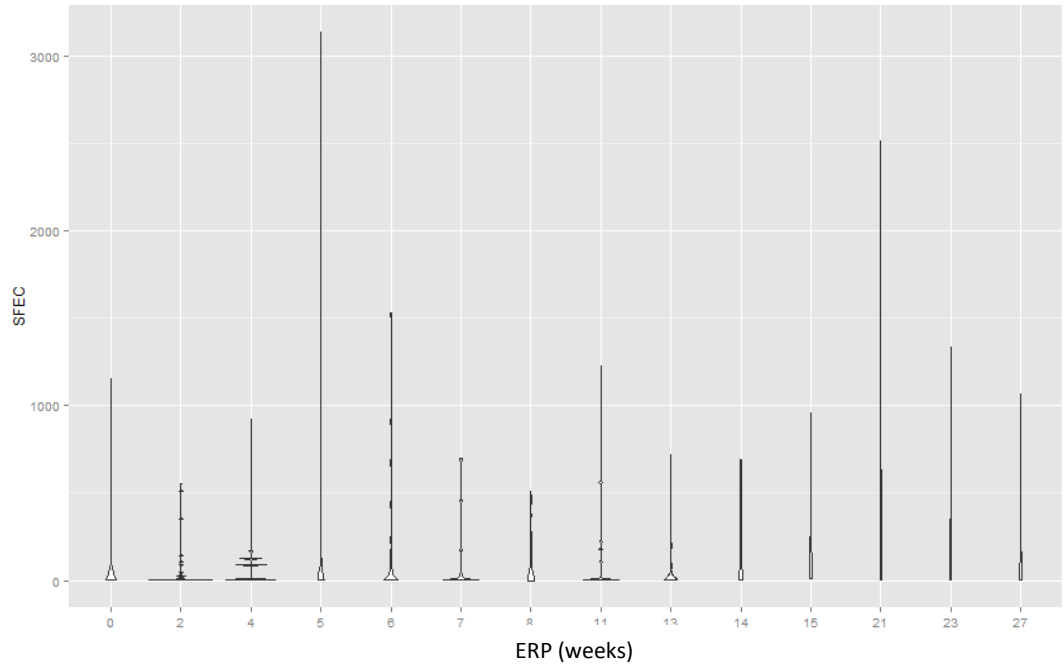


Figure 5.5. Violin plot of strongyle faecal egg count (SFEC) measured in eggs per gram [EPG] by the number of weeks past the expected strongyle egg reappearance period (weeks)

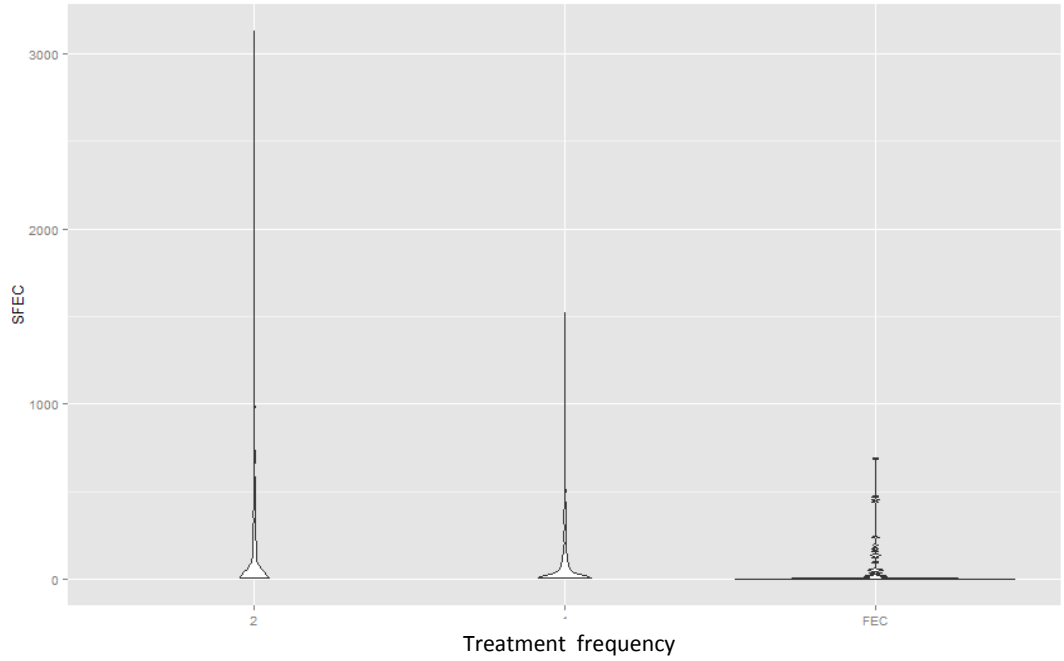


Figure 5.6. Violin plot of strongyle faecal egg count (SFEC) measured in eggs per gram [EPG] by treatment frequency (2 = twice per year; 4 = four times per year; FEC = FEC used to direct treatments)

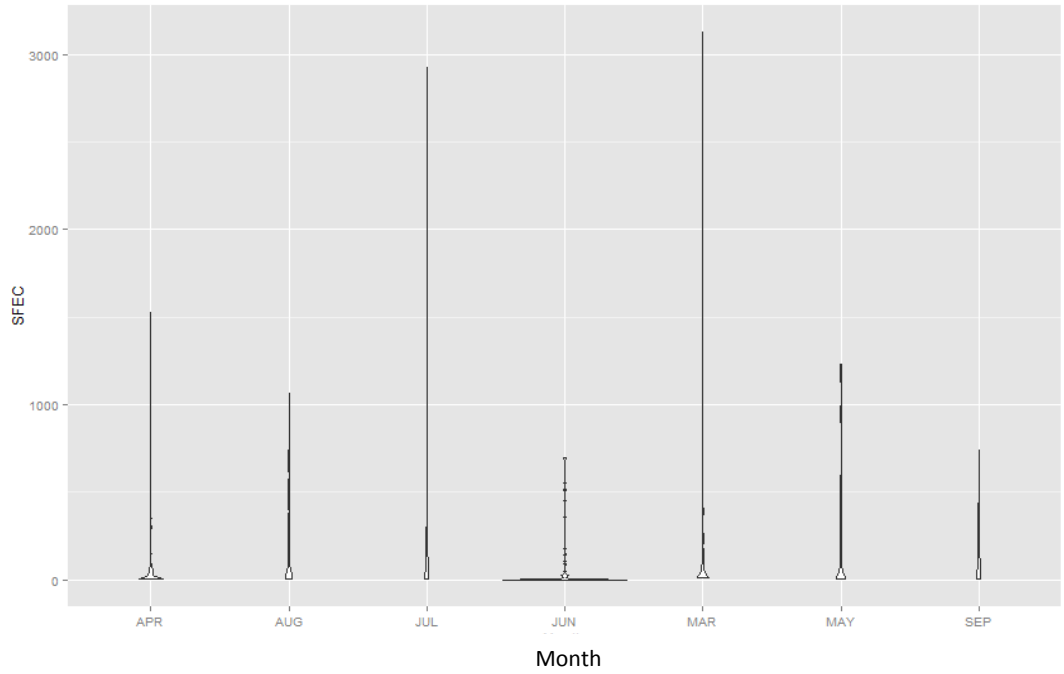


Figure 5.7. Violin plot of strongyle faecal egg count (SFEC) measured in eggs per gram [EPG] by month

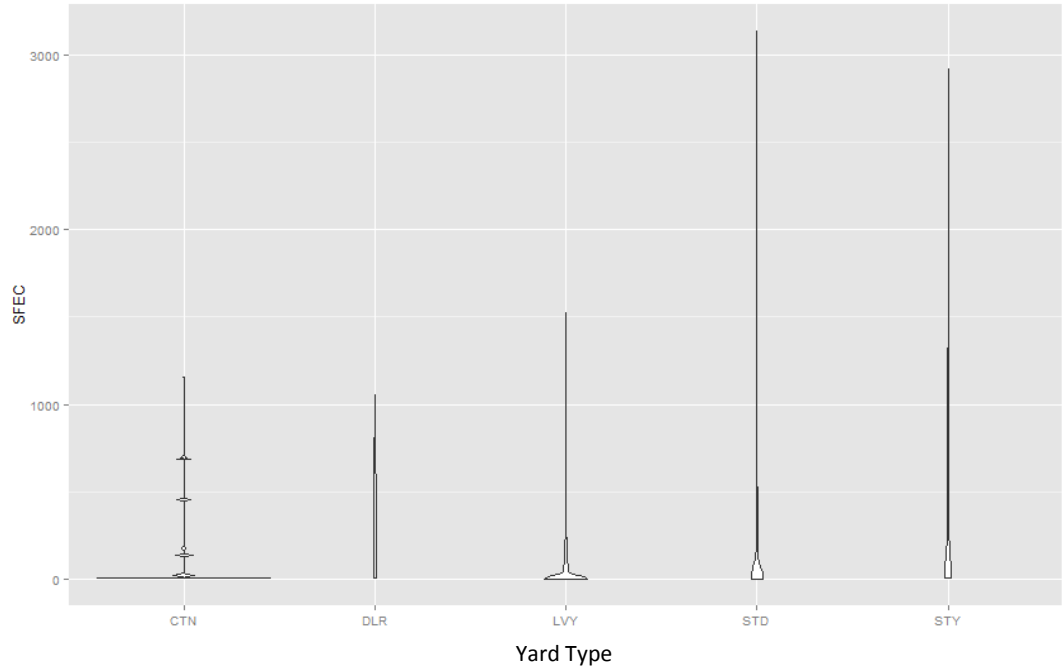


Figure 5.8. Violin plot of strongyle faecal egg count (SFEC) measured in eggs per gram [EPG] by Yard type (CTN = competition; DLR = dealers yard; LVY = livery; STD = stud; STY = sanctuary)

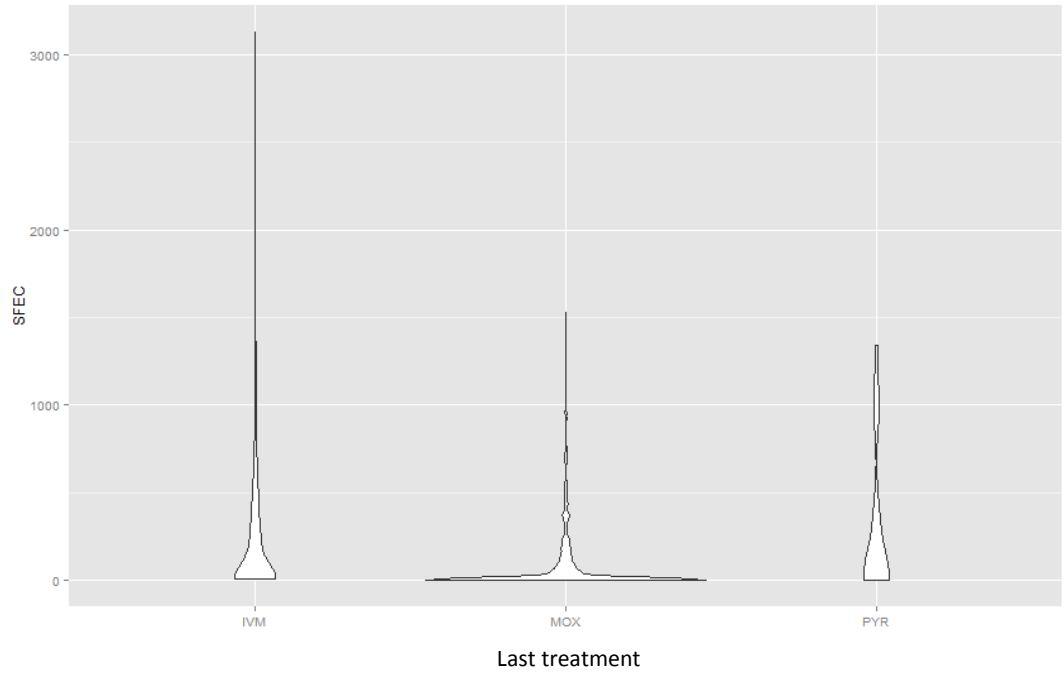


Figure 5.9. Violin plot of strongyle faecal egg count (SFEC) measured in eggs per gram [EPG] by Last treatment (IVM = ivermectin; MOX = moxidectin; PYR = pyrantel)

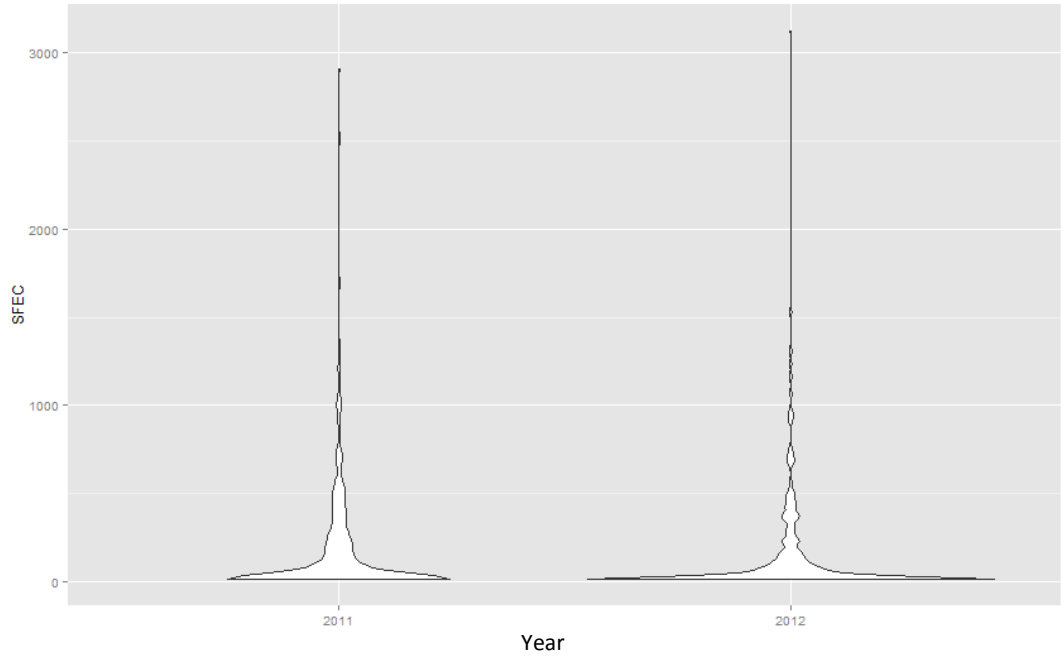


Figure 5.10. Violin plot of strongyle faecal egg count (SFEC) measured in eggs per gram [EPG] by Year

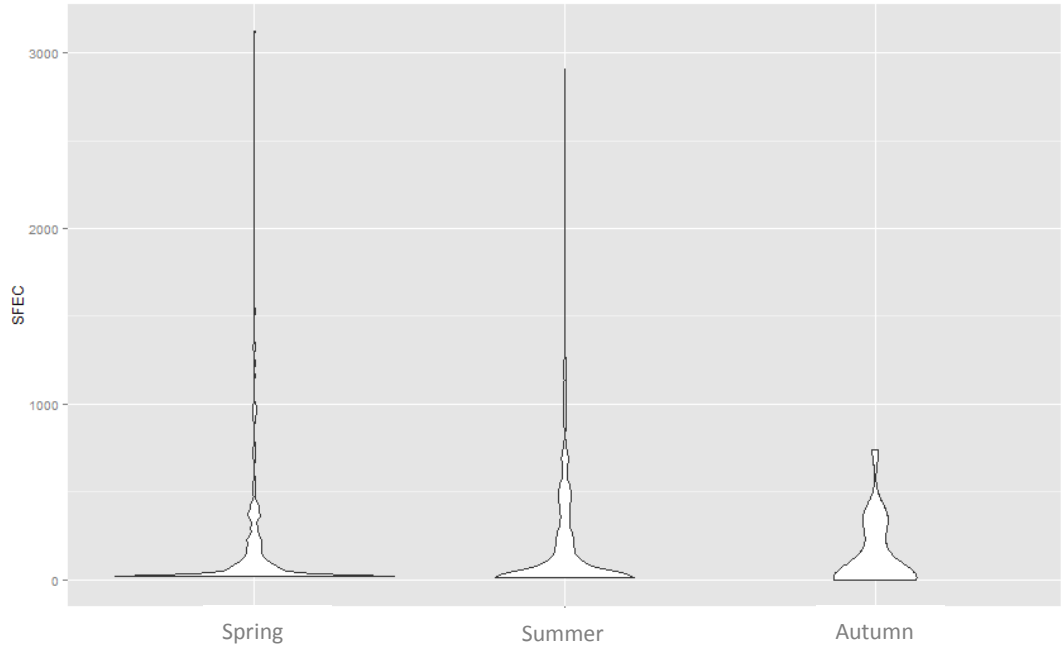


Figure 5.11. Violin plot of strongyle faecal egg count (SFEC) measured in eggs per gram [EPG] by Season

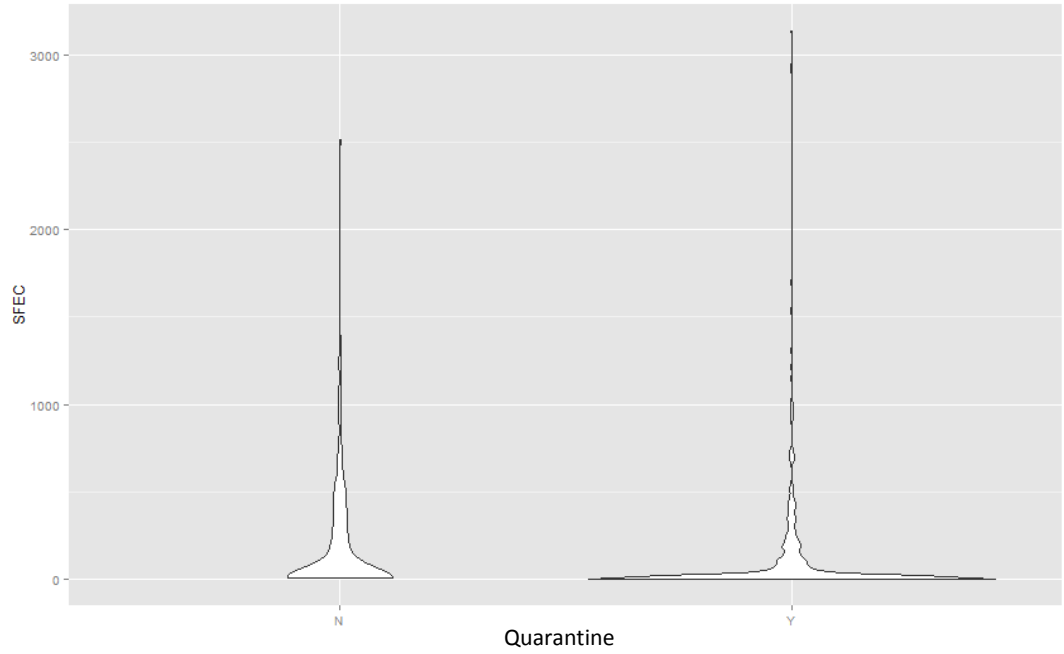


Figure 5.12. Violin plot of strongyle faecal egg count (SFEC) measured in eggs per gram [EPG] by Quarantine (N = no; Y = yes)

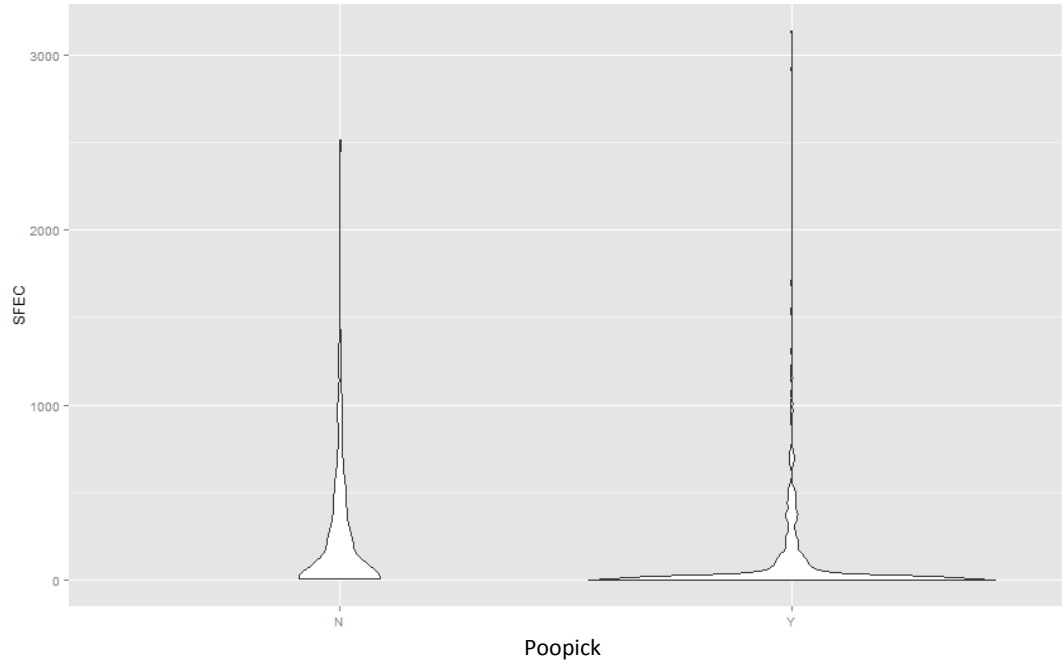


Figure 5.13. Violin plot of strongyle faecal egg count (SFEC) measured in eggs per gram [EPG] by poopick (N = No; Y = yes)

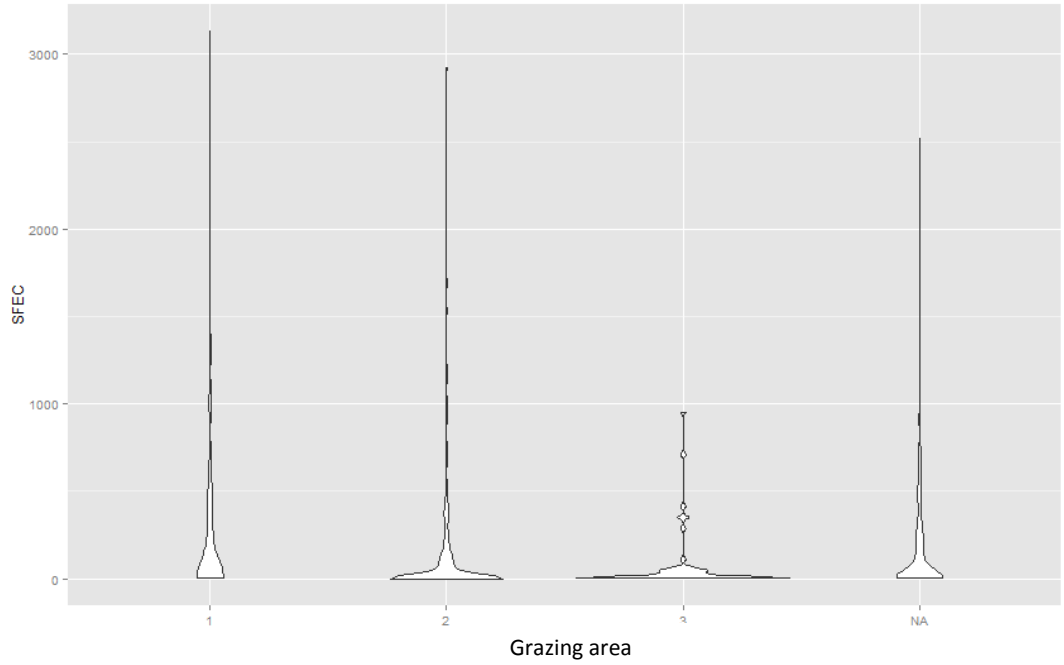


Figure 5.14. Violin plot of strongyle faecal egg count (SFEC) measured in eggs per gram [EPG] by Grazing area per horse (1 = <1 acre/horse; 2 = ≥1 -<2 acres per horse; 3 = > 2 acres per horse; NA = data not provided)

Table 5.9. Results from univariate analysis of each factor using negative binomial generalised linear models (NBGLMM). Included are the estimated effects (*b*), the standard error (se) and associated *z* scores (*z*), the *p*-value for each factor. Significant factors ($p<0.05$) are highlighted in grey

Explanatory variable	Factor	*b*	se	*z*	*p*
Age	Model	0.76	0.32	2.42	0.015
	Foal	1.79	0.56	3.20	0.001
	Geriatric	-0.02	0.31	-0.63	0.95
	Youngster	2.89	0.36	8.03	<0.0001
Last treatment	Model	1.82	0.65	2.81	0.005
	MOX	-1.02	0.74	-1.38	0.17
	PYR	1.08	1.76	0.61	0.54
Post ERP	Model	0.02	0.46	0.04	0.97
	Post ERP	0.12	0.04	3.06	0.002
Year	Model	1.14	0.50	2.30	0.02
	2012	-0.09	0.65	-0.13	0.89
Month	Model	0.69	0.57	1.22	0.22
	March	0.18	1.03	0.18	0.86
	May	0.62	0.84	0.74	0.46
	June	-1.18	1.03	-1.41	0.25

Explanatory variable	Factor	*b*	se	*z*	*p*
	July	2.10	0.99	2.13	0.03
	August	0.48	0.96	0.51	0.61
	September	1.39	1.60	0.87	0.38
Season	Model	0.96	0.42	2.28	0.023
	Summer	0.25	0.67	0.38	0.70
	Autumn	1.13	1.72	0.66	0.51
Yard type	Model	-0.61	0.84	-0.73	0.47
	DLR	4.22	1.65	2.56	0.01
	LVY	1.31	0.90	1.45	0.15
	STD	2.03	1.30	1.57	0.12
	STY	3.80	1.06	3.57	0.0004
Country	Model	1.06	0.43	2.48	0.01
	Scotland	0.09	0.65	0.13	0.90
Region	Model	0.92	0.46	2.00	0.05
	SNE	1.32	1.61	0.82	0.41
	SSE	0.27	0.70	0.39	0.70
	SSW	-1.57	1.74	-0.90	0.37
	SW	-0.73	1.05	-0.63	0.49
Quarantine	Model	1.68	0.49	3.40	<0.001
	Yes	-0.97	0.64	-1.52	0.13
Poopick	Model	1.72	0.74	2.34	0.02
	Yes	-0.77	0.82	-0.94	0.35
Treatment frequency	Model	1.44	0.37	3.86	<0.001
	4 x/year	-0.49	0.74	-0.66	0.51
	FEC	-2.04	0.97	-2.10	0.04
Area	Model	2.30	0.71	3.25	0.001
	2	-1.13	0.79	-1.42	0.16
	3	-1.97	1.07	-1.83	0.07

Factors affecting egg shedding were first explored through NBGLMM, where all eligible explanatory variables were fitted and the least significant removed sequentially until t he minimal adequate model was achieved in which only significant terms remained (p< 0.05). In the first model, slope estimates and associated values were not generated for s outhwest Scotland due to singularities, indicating autocorrelation between covariates. Quarantine and last treatment were dropped from the model as they were not significa nt factors and their removal did not significantly affect the model (Quarantine: χ^2 = 0.45

, p = 0.51; Last treatment: χ^2 = 1.01, p = 0.62) (Table 5.10). The terms retained in the fin al model that had a significant effect on strongyle egg excretion were age, time since sta ndard ERP, year, month, treatment frequency and faecal removal from pasture. Foals (b = 1.84, p = 0.0001) and youngsters (b = 3.02, p<0.0001) were identified as excreting s ignificantly more eggs compared to adult and geriatric horses (Table 5.10). A weak but significant positive effect on strongyle egg excretion was observed between the numbe r of weeks after the expected ERP for each anthelmintic that the sample was collected (b = 0.22, p<0.0001). Strongyle egg excretion levels were significantly higher in 2012 co mpared to 2011 (b = 1.02, p = 0.02). Egg excretion levels were significantly higher in M arch (b = 2.32, p<0.001), May (b = 1.12, p = 0.03) and July (b = 3.37, p<0.0001) with less egg excretion in August (b =-0.66, p = 0.35) and September (b = -2.25, p = 0.09) (Table 5.10). Strongyle egg excretion levels were significantly lower in horses that received an thelmintics according to FEC analysis (b = -2.49, p<0.0001) (Table 5.10). Faecal remova l from pasture was identified as a significant factor with horses that came from yards w here this practice was performed measured as excreting higher levels of strongyle eggs compared to those where this practice was not undertaken (b = 1.57, p = 0.01) (Table 5. 10). The model residuals are presented in Appendix 3.

Table 5.10. Generalised linear mixed model (GLMM) of strongyle faecal egg counts (FEC) including Yard and individual observation as random effects and year and age included as fixed factors. Included are the estimated effect (*b*), standard error (se) and associated z scores (*z*) plus log-likelihood ratio test statistics (LRT) for terms dropped ('dropped terms'). The dropped terms are presented in the order that they were removed. The 'retained terms' form the final adequate model. Significant terms are highlighted in grey

		b	se	*z*	*p*	LRT	*P*
Dropped terms							
Quarantine	Y	-0.38	0.43	-0.87	0.38	0.45	0.51
Last treatment	MOX	-0.55	0.56	0.98	0.33	1.01	0.62
	PYR	1.29	1.54	0.84	0.4		
Retained terms							
Age	Foal	1.84	0.56	3.27	0.0001		
	Geriatric	-0.17	0.32	-0.52	0.60		
	Youngster	3.02	0.36	8.28	<0.0001		
Post ERP		0.22	0.04	6.09	<0.0001		
Year	2012	1.02	0.44	2.32	0.02		
Month	March	2.32	0.62	3.76	<0.001		
	May	1.12	0.50	2.23	0.03		
	June	0.80	0.65	1.24	0.22		
	July	3.37	0.63	5.36	<0.0001		
	August	-0.66	0.71	-0.94	0.35		
	September	-2.25	1.32	-1.70	0.09		
Treatment frequency	4 times/year	0.42	0.45	0.92	0.36		
	FEC	-2.49	0.55	-4.52	<0.0001		
Poo pick	Y	1.57	0.61	2.55	0.01		

5.4.3 The consistency of strongyle egg shedding in individual horses over time

5.4.3.1 The consistency of strongyle egg shedding in horses regardless of anthelmintic treatment status

The analysis of strongyle egg shedding consistency over time revealed that on the first screening occasion (S1), 70% (401/573) of horses were shedding less than 50 EPG. This decreased slightly on S2 and S3: 65 (306/468) and 64% (279/417), respectively (Table 5.11). At S2 and S3, a greater percentage of horses fell into shedding categories

2 and 3 compared to S1, and the proportion of horses in categories 4 to 6 remained at similar levels (Figure 5.15a). The percentage of horses in the highest egg shedding category (7, >500 EPG) decreased on each screening occasion from 5.8 (33/573) on S1 to 0% (0/63) on S4 (Table 5.11). The number of horses sampled at each screening occasion decreased over time from 573 at S1 to 63 at S3 (Table 5.11). This was due to horses leaving yards and the fact that on the majority of yards, horses were only faecal sampled three times during the course of the study. On each occasion, the percentage of horses that were calculated as shedding <200 EPG ranged from 84 (479/573) at S1 to 91.6% (76/83) at S4 (Table 5.12). The proportion shedding <200 EPG increased over time across the study (Figure 5.15b), whilst the percentage shedding ≥200 EPG ranged from 16 (94/573) at S1 to 8.4% (7/83) at S4 (Table 5.12) and decreased over time (Figure 5.15b).

Table 5.11. The number (*n*) and percentage (%) of horses that fell into each strongyle egg shedding category (1-7) as defined by eggs per gram (EPG) on each screening occasion (S1-S4)

		Strongyle egg shedding category							
Screening occasion		1 (0-49EPG)	2 (50-99EPG)	3 (100-199EPG)	4 (200-299EPG)	5 (300-399EPG)	6 (400-499EPG)	7 (500EPG+)	Total
S1	*n*	401	38	40	23	23	15	33	573
	%	70	6.6	7	4	4	2.6	5.8	100
S2	*n*	306	51	37	17	20	13	24	468
	%	65	10.8	7.9	3.6	4.3	2.8	5	100
S3	*n*	276	39	43	20	10	11	18	417
	%	64	9.4	10.3	4.8	2.4	2.6	4.3	100
S4	*n*	65	8	3	3	2	2	0	83
	%	78	9.6	3.6	3.6	2.3	2.3	0	100

No statistical differences ($p<0.05$) in proportions between screening occasions as determined by the binomial test were observed

Table 5.12. The number (*n*) and percentage (%) of horses that were calculated as shedding <200 eggs per gram (EPG) (category 0, no treatment) or ≥200 EPG (category 1) at each screening occasion (S1-S4, treatment required)

		Treatment category		
Sampling occasion		0 (<200 EPG)	1 (≥200 EPG)	Total
S1	*n*	479	94	573
	%	84	16	100
S2	*n*	394	74	468
	%	84.2	15.8	100
S3	*n*	358	59	417
	%	85.9	14.1	100
S4	*n*	76	7	83
	%	91.6	8.4	100

No statistical differences ($p<0.05$) in proportions between screening occasions as determined by the binomial test were observed

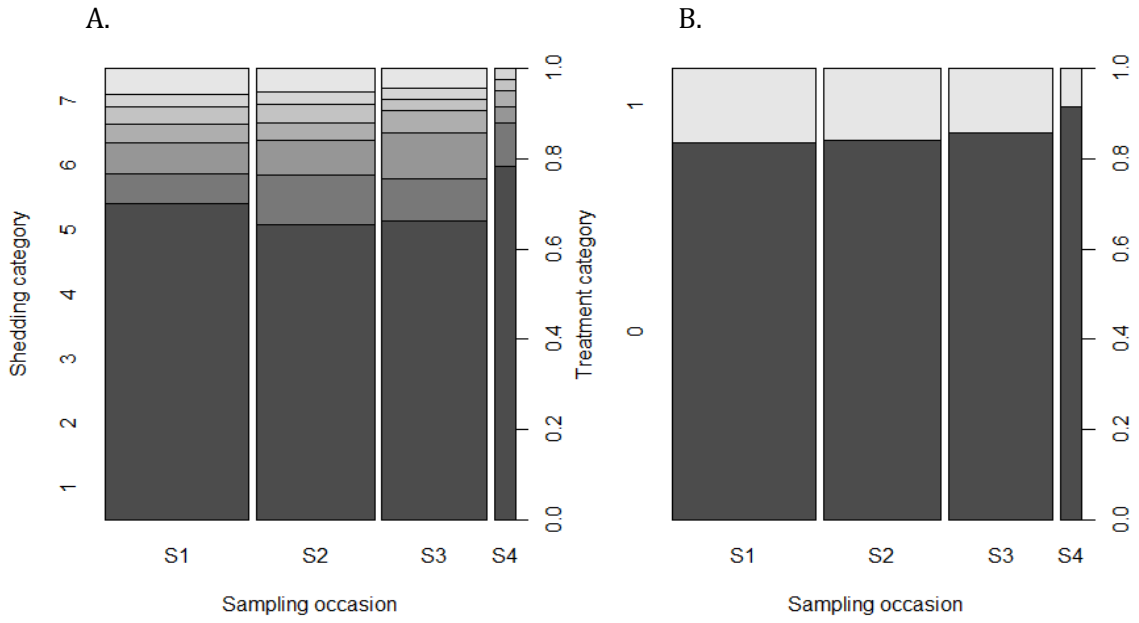


Figure 5.15. The percentage of horses that fell into each strongyle egg shedding category (1-7) on each screening occasion (S1-S4), with category S1 represented by the darkest shade of grey and subsequent categories in lighter shades (A). The percentage of horses that were either shedding <200 eggs per gram (EPG) (Category 0, dark grey) or ≥200 EPG (Category 2, light grey) as measured by faecal egg count (FEC) on each screening occasion (B). The width of the bars is proportional to the number of observations per screening occasion

The majority of horses remained in the same shedding category (Figure 5.16.a) over time. From S1 to S2, 61.5% horses (288/468) remained in the same category (Table 5.13). The percentage decreased to 58.3% (243/417) between S1 and S3, then increased to 73% (61/83) from S1-S4 (Table 5.13). Between S1 and S2, 16.6% (77/468) moved into a lower shedding category and 22% (103/468) to a higher shedding category (Table 5.13). The percentage of horses that moved to a lower shedding category increased at S3 (23.7%; 99/417) compared to S2 and fewer (18%; 75/417) moved to a higher category. Overall, from S1 to S4, 14.5% (12/83) of horses moved into a lower shedding category and 8.3% (10/83) moved to a higher shedding category (Table 5.13).

Similarly, the majority of horses remained in the same treatment category at each sampling occasion; however, more changed from requiring, to not requiring, treatment between S1 and S3 compared to S2 (Figure 5.16b). Between S1 and S2, 81.8% (383/468) remained in the same treatment category, 7.8% (36/468) went from requiring treatment to not and 10.5% (49/468) went from not requiring treatment to exceeding the threshold for anthelmintic administration (Table 5.14). The percentage of horses remaining in the same category increased on S3 to 82.7% (345/417), and the percentage of horses that changed to not requiring treatment increased to 10.1% (42/417), while the percentage of horses changing to requiring treatment decreased to 7.2% (30/417) (Table 5.14). From S1 to S4, 94% (78/83) horses remained in the same treatment category, 8.4% of these moved to the not requiring treatment category, while only 1.2% moved from no treatment required to treatment required (Table 5.14).

Table 5.13. The number (*n*) and percentage (%) rank change in the shedding category (-6 to 6) from the first screening (S1) to the following screening occasion.

	Screening occasion					
	S1-S2		S1-S3		S1-S4	
Rank change in egg shedding category	*n*	%	*n*	%	*n*	%
-6	6	1.3	6	1.4	1	1.2
-5	3	0.6	1	0.2	0	0
-4	9	2	12	2.9	2	0.5
-3	9	2	17	3.6	0	0
-2	19	4.1	26	6.2	6	7.2
-1	31	6.6	37	8.9	3	3.6
0	288	61.5	243	58.3	61	73
1	35	7.5	28	6.7	8	9.6
2	26	5.6	20	4.8	1	1.2
3	12	2.6	8	1.9	1	1.2
4	12	2.6	6	1.4*	0	0*
5	8	1.7	3	0.7*	0	0*
6	10	2.1	10	2.4	0	0
Total	468	100	417	100	83	100

* statistical difference ($p<0.05$) in proportions between screening occasions as determined by the binomial test

Table 5.14. The number (*n*) and percentage (%) rank change in treatment category (-1 (change from ≥ 200 eggs per gram (EPG) to <200 EPG), 0 (stayed in the same category) or 1 (change from <200 EPG to ≥200 EPG)) on each screening occasion

	Screening occasion					
	S1-S2		S1-S3		S1-S4	
Rank change in treatment category	*N*	%	*n*	%	*n*	%
-1	36	7.8	42	10.1	7	8.4
0	383	81.8	345	82.7	78	94
1	49	10.5	30	7.2	1	1.2*

* statistical difference ($p<0.05$) in proportions between screening occasions as determined by the binomial test

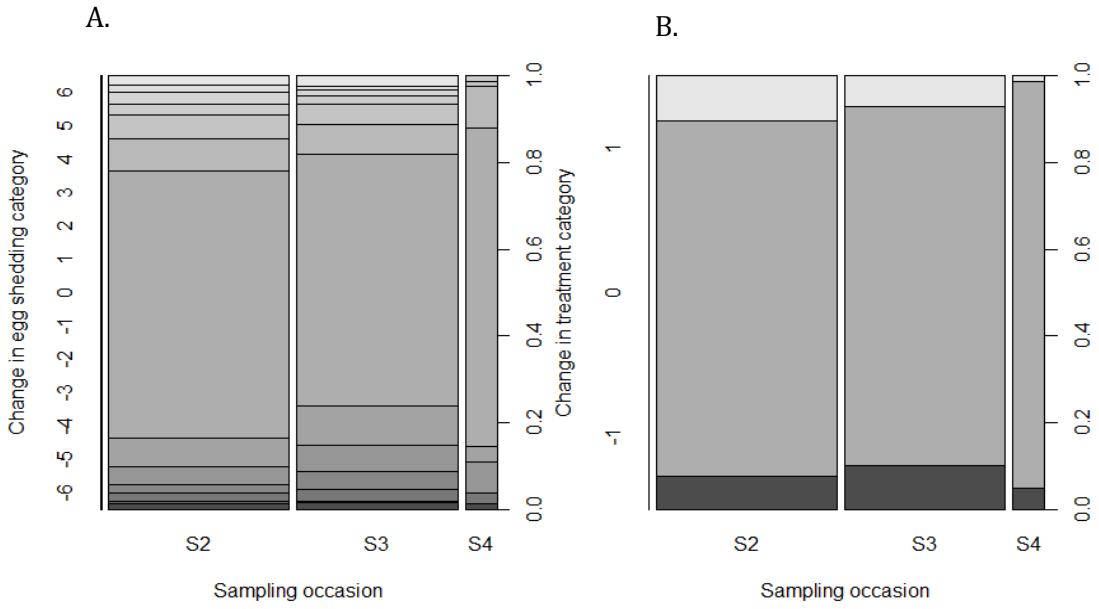


Figure 5.16. The rank change in egg shedding category and treatment category between sampling occasions (S1 and S2, S1 and S3 and S1 and S4). The percentage of horses either remained in the same egg shedding category (0), increased egg shedding category (1 to 6) or decreased egg shedding category (-1 to -6), with a rank change in -6 categories represented by the darkest shade of grey and subsequent ascending categories in lighter shades (A). The percentage of horses that were either remained in the same treatment category (0, mid-grey), increased in treatment category (1, light grey) or decreased in treatment category (-1, dark grey) (B) as measured by faecal egg count (FEC) on each screening occasion. The width of the bars is proportional to the number of observations per screening occasion

5.4.3.2 Consistency of egg shedding in horses that did not receive anthelmintic treatment

Here, the egg shedding consistency in a sub-cohort of horses that did not receive any anthelmintic treatment (*n* =304) during the course of the study was investigated. The majority tested on each screening occasion (S1 - S3) were shedding <50 EPG (Figure 5.17a), with few shedding 200 EPG or more (Figure 5.17b). On the first screening occasion, 97.3% (296/304) horses shed <50 EPG. This dropped to 84.9% (258/304) at the second screening then increased to 85.5% (213/249) at S3 (Table 5.15). A total of 99.3% (302/304), 95.5% (290/304) and 95.1% (248/249) horses tested at S1, S2 and

S3, respectively, shed <200 EPG and fell into the no treatment category (Figure 5.17b). Overall, egg shedding in individuals over time was consistent (Figure 5.18a). Between S1 and S2, 92.1% (280/304) remained in the same shedding category, 0.3% (1/304) decreased shedding category and 7.9% (23/304) increased in shedding category (Table 5.16). Between S1 and S3, the percentage of horses that remained in the same shedding category decreased slightly to 90.4% (225/304) and the percentage of horses that increased shedding category increased to 9.6% (24/304) (Table 5.16). There was a high level of consistency in terms of treatment category in the non-treated horse cohort (Figure 5.18b). Between S1 and S2, 98.7% (300/304) of horses remained in the same treatment category and, between S1 and S3, 95.6% (238/304) remained in the same category. (Table 5.17; Figure 5.18b).

Table 5.15. The number (*n*) and percentage (%) on horses that fell into each shedding category (1 – 7) as defined by eggs per gram (EPG) on each sampling occasion (S2 to S3) in animals that had not received anthelmintic treatment over the course of the study

		Egg shedding category							
Screening occasion		1 (0-49EPG)	2 (50-99EPG)	3 (100-199EPG)	4 (200-299EPG)	5 (300-399EPG)	6 (400-499EPG)	7 (500EPG+)	Total
S1	*n*	296	2	6	0	2	0	0	304
	%	97.3	0.7	2	0	0.7	0	0	100
S2	*n*	258	19	13	4	3	4	3	304
	%	84.9	6.3	4.3	1.3	1.0	1.3	1.0	100
S3	*n*	213	14	10	4	2	3	3	249
	%	85.5	5.6	4.0	1.6	0.8	1.2	1.2	100

No statistical differences ($p<0.05$) in proportions between screening occasions as determined by the binomial test were observed

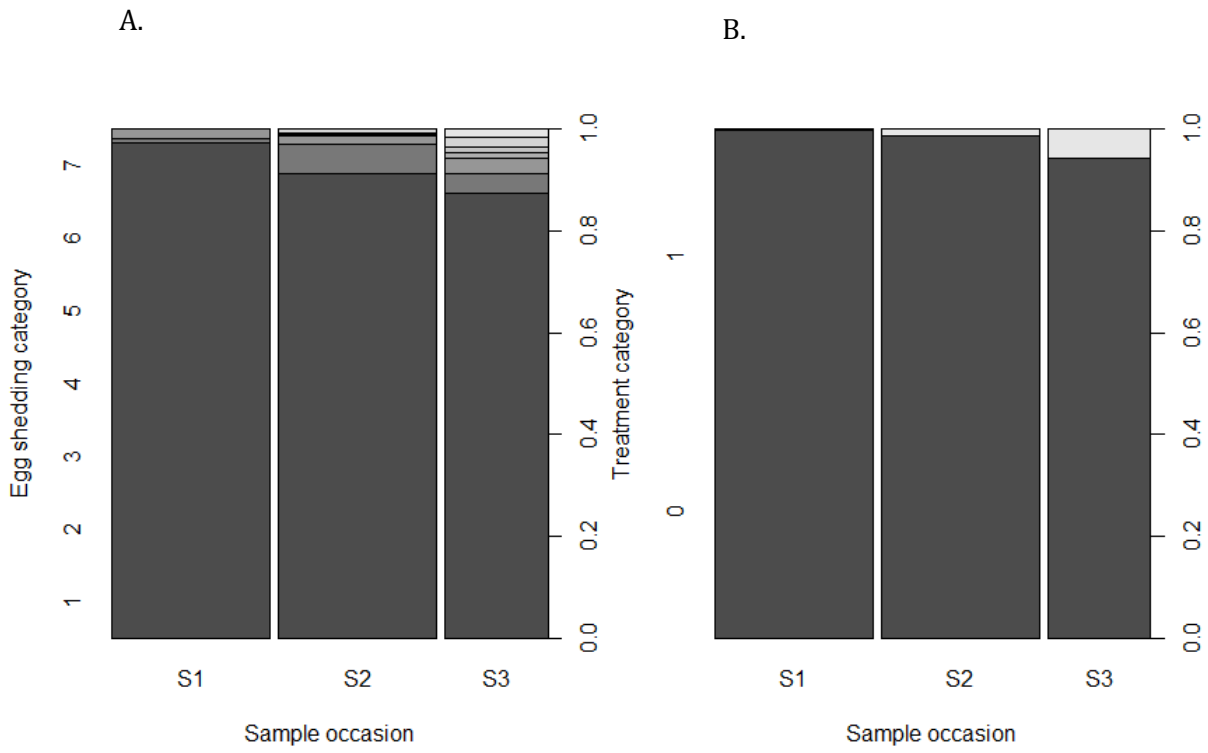


Figure 5.17. The percentage of horses that did not receive anthelmintic treatment that fell into each strongyle egg shedding category (1-7) on each screening occasion (S1-S3), with Category 1 represented by the darkest shade of grey and subsequent categories in lighter shades (A). The percentage of horses that were either shedding <200 eggs per gram (EPG) (Category 0, dark grey) or ≥200 EPG (Category 1, light grey) as measured by faecal egg count (FEC) on each screening occasion (B). The width of the bars is proportional to the number of observations per screening occasion

Table 5.16. The number (*n*) and percentage (%) of horse that changed egg shedding status from one sampling occasion to the next by either increasing egg shedding (1 to 6) or decreasing (-1 to -6) or staying the same (0) in horses that did not receive treatment

	Screening occasion			
	S2		S3	
Rank change in egg shedding category	*n*	%	*n*	%
-6	0	0	0	0
-5	0	0	0	0
-4	0	0	0	0
-3	0	0	1	0.4
-2	3	1.0	1	0.4
-1	3	1.0	7	2.8
0	255	83.9	206	82.7
1	20	6.6	13	5.2
2	11	3.6	10	4.0
3	3	1.0	3	1.2
4	3	1.0	2	0.8
5	4	1.3	3	1.2
6	2	0.7	3	1.2
Total	304	100	249	100

No statistical differences ($p<0.05$) in proportions between screening occasions as determined by the binomial test were observed

Table 5.17. The number of horses that changed treatment category from one sampling occasion to the next by either falling into the no treatment category (-1) or increasing to the treatment category (1). The 0 category represents no change in treatment status

	Screening occasion			
	S2		S3	
Rank change in treatment category	*n*	*%*	*n*	*%*
-1	2	0.5	1	0.4
0	288	95	237	95
1	14	4.6	11	4.6

No statistical differences ($p<0.05$) in proportions between screening occasions as determined by the binomial test were observed

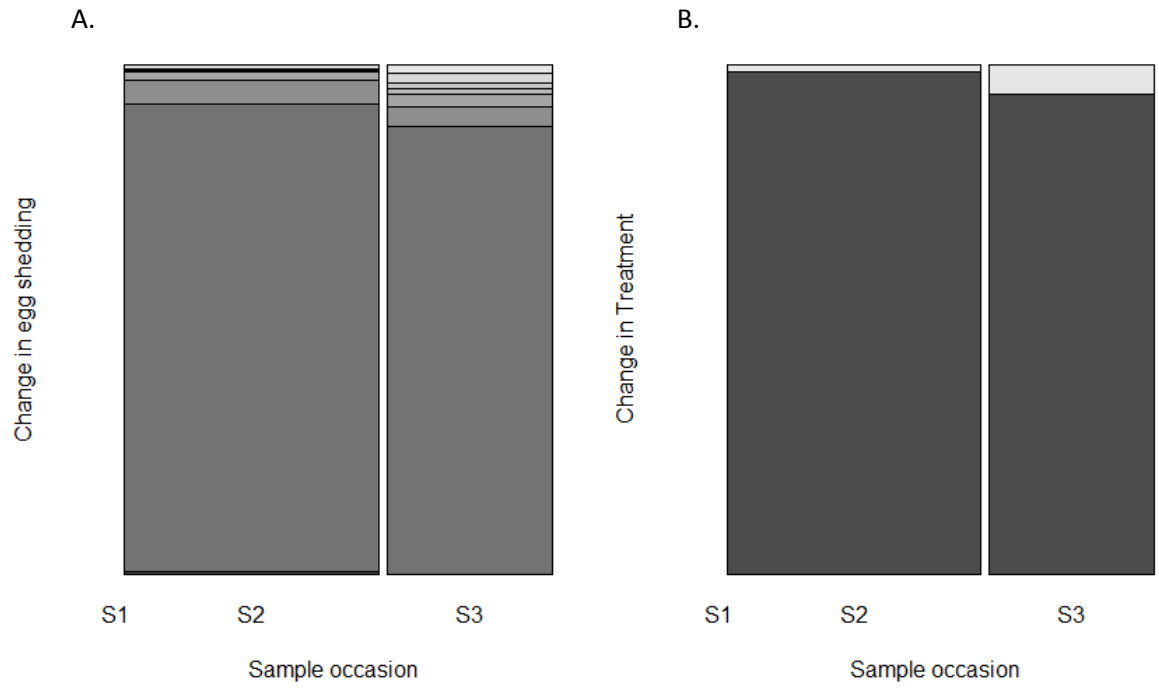


Figure 5.18. The rank change in egg shedding category and treatment category between S1 and S2, S1 and S3. The percentage of horses either remained in the same egg shedding category (0), increased egg shedding category (1 to 6) or decreased egg shedding category (-1 to -6), with a rank change in -6 categories represented by the darkest shade of grey and subsequent ascending categories in lighter shades (A). The percentage of horses that were either remained in the same treatment category (0, mid-grey), increased in treatment category (1, light grey) or decreased in treatment category (-1, dark grey) (B) as measured by faecal egg count (FEC) on each screening occasion. The width of the bars is proportional to the number of observations per screening occasion

5.4.3.3 Factors affecting egg shedding and treatment group consistency; factors associated with the likelihood of a horse changing strongyle egg shedding category or treatment category over time

Only horses whose age and last treatment were known were included to ensure that the analysis was robust. In total, 346 horses were included here. These had been screened at S1 and S3. Whether the horse had changed egg shedding category and treatment category between these two time points was determined and expressed as a binary outcome. Firstly, factors that influenced the likelihood of a change in egg

shedding category were investigated. Initially, the model was populated with all explanatory variables (Model 1, Table 5.18). In the final model, age (youngsters) and last treatment (MOX) were identified as significant explanatory variables (Table 5.18). Young horses were 3.3 times more likely to change shedding category compared to other age categories, while horses that had received MOX as their last anthelmintic treatment were less likely to change shedding category compared to the other treatments (OR=0.9, p = <0.0001)(Table 5.18).

Table 5.18. Significant factors affecting the likelihood horses changing egg shedding category of between sampling occasions (S1 and S3) assessed by logistic regression. Model fit was assessed using the Hosmer and Lemeshow test

Model (fit)	Significant variable	Factor	Logit coefficient (SE)	se	OR (95% CI)	p
1 (0.11)			1.44	0.27	na	<0.0001
	Age	Youngsters	1.20	0.55	3.3 (1.22 – 8.46)	0.02
	Last treatment	MOX	-1.90	0.31	0.15 (0.05 – 0.17)	<0.0001

MOX = moxidectin

Secondly, factors affecting the likelihood of a horse changing treatment category between S1 and S3 was investigated. Initially the model was populated with all explanatory variables (Model 2, Table 5.19). In the final model, age (youngsters) and the last treatment (MOX) were identified as significant explanatory variables (Table 5.19). Young horses were 2.8 times more likely to change treatment category compared to other age categories, while horses that received MOX as their last treatment were significantly less likely to change treatment group (OR = 0.15, p = <0.0001) (Table 5.19).

Table 5.19. Significant factors affecting the likelihood of a horse changing from one treatment group to another from the first screening (S1) to the last screening (S3) assessed by logistic regression. Model fit was assessed using the Hosmer and Lemeshow test

Model (fit)	Significant variable	Factor	Logit coefficient	se	OR (95% CI)	*p*
2 (0.38)			1.48	0.22	na	<0.0001
	Age	Youngsters	1.04	0.47	2.8 (1.1 – 6.3)	0.028
	Last treatment	MOX	-1.89	0.61	0.15 (0.1 – 0.4)	<0.0001

MOX = moxidectin

5.5 Discussion

Here, strongyle eggs were the most prevalent helminth eggs detected, and were present on 100% of yards. At horse level on each yard, prevalence ranged from 21 to 93%, confirming that strongyle eggs are the most abundant helminth eggs shed in equine faeces, thus justifying the deployment of current control strategies focussed at controlling these types of parasite, with the exception of control in foal populations, in which the most important where the most important helminth is *P. equorum*. In the horses tested here, *P. equorum* egg prevalence was 14.3% at yard level and ranged between 0 and 23% at individual level. Tapeworm egg prevalence was measured at 3.6% at yard level.

These findings were consistent with a recent study in the UK where 737 horses from 51 yards were screened for the presence of strongyle eggs in their faeces and 100% yard prevalence and an average 63% prevalence at individual horse level was reported (Traversa et al., 2009). A recent study, which investigated 1221 Thoroughbred horses residing at 22 UK stud farms, recorded a mean prevalence of 56% FEC-positive horses across farms and a farm level prevalence of 100% (Relf et al., 2013). Similar prevalence

data have been reported from other EU countries. In Germany, Hinney et al., (2011) performed FEC analysis on 1407 horses from 126 yards and reported a 98.4% yard level prevalence and a 67.4% horse level prevalence (Hinney et al., 2011). In other German studies, 100% yard prevalence and a mean 48% individual prevalence values were reported (Traversa et al., 2009). Wirtherle et al. (2004) reported a 92% yard prevalence and 62% individual prevalence, while Fritzen at al. (2010) found yard prevalence to be 99% and an average prevalence of 55% at individual horse level (Fritzen et al., 2010). Similar prevalence data have been reported in Sweden, where yard prevalence was observed as 100% and individual horse prevalence as 78% (Osterman-Lind et al., 1999). The prevalence data from the present study and other recent studies vary to a degree at individual horse level, and, to a lesser extent, at yard level. This could be due to factors including FEC method used to estimate strongyle EPG, heterogeneity amongst populations (i.e. individual susceptibility or age of the horses sampled), environmental conditions (i.e. time of year of sampling, local climate conditions), management practices (i.e. extent of faecal removal from pasture, clipping or harrowing), frequency of anthelmintic treatments, anthelmintic products used (and their efficacy) and the time since last treatment to sampling.

P. equorum egg prevalence in the present study were similar to two recent studies conducted in Germany, where Hinney et al., (2011) reported *P. equorum* prevalence as 16.7% at yard level and 2% at horse level and Fritzen et al. (2010) found that *P. equorum* eggs were prevalent on 21% of yards and in 2% of horses. These values are low compared those of Relf et al. (2013) who reported a site prevalence of 58% and an overall mean prevalence of 9% in 1221 UK Thoroughbred horses, with, as would be expected, the highest prevalence seen in horses <1 old (38%). The reason why prevalence was higher in the latter is that the population studied were Thoroughbred

breeding stock, with a higher proportion of animals <2 years old compared to the present study. Protective immunity to *P. equorum* is claimed to develop after 6 months of age (Clayton and Duncan, 1979a), and patent infections are seldom measured in horses >2 years old (Reinemeyer, 2012). However, older horses can harbour patent ascarid infections at a low level (Gawor, 1996; Fritzen, 2010; Kornaś et al., 2007; Hinney et al., 2011). A further reason for the apparent low prevalence could be that ascarid eggs are not reliably detected by the FEC method used here. Recently, a study investigating the prevalence of parasites in horses necropsied in Germany reported a discrepancy between the number of horses harbouring ascarids and the number shedding eggs (Rehbein et al., 2013). The main reason for this is likely to be the proportion of the overall worm burden that comprises adult female worms shedding eggs. If this is low, then fewer eggs will be detected even though there are worms present. Thus, prevalence estimations by faecal examination, especially when using dilution techniques, might underestimate the true level of *P. equorum*, as is the case for all helminth species, emphasing the caveats of FEC analysis.

Here, tapeworms were detected at a low level (3.6% at yard level). This likely underestimates the true prevalence as traditional FEC methods do not reliably detect tapeworm eggs (Nilsson et al., 1995). This is partly because tapeworm eggs are shed intermittently (Dunn, 1978). Counts performed by McM methodology have been reported to provide relatively low sensitivity (8 – 61%) for tapeworm eggs (Proudman and Edwards, 1992; Abbott and Barrett, 2010). Modifications to traditional methods have been suggested; i.e. using a larger volume of faeces (30 - 40 g) (Proudman and Edwards, 1992; Ihler et al., 1995; Nilsson et al., 1995; Meana et al., 1998; Kjaer et al., 2007), or adopting centrifugal-flotation methods (Proudman and Edwards, 1992). The sensitivity of tests using these modifications has been reported to range between 37

and 61%, indicating that even then, the test is not particularly effective at detecting tapeworm infection (Proudman and Edwards, 1992; Ihler et al., 1995; Nilsson et al., 1995; Meana et al., 1998). In the study by Relf et al. (2013), the authors reported a mean prevalence of *A. perfoliata* eggs of 4% at individual horse level and 41% at yard level; markedly higher compared to the present study. The same FEC method was used in both the study by Relf et al. (2013) and the present study. Possible reasons for the difference measured in prevalence could be the time of year that the samples were collected. There is evidence to suggest that the prevalence of *A. perfoliata* varies with season: a recent study on 400 horses at necropsy in Germany reported prevalence as significantly higher ($p<0.001$) in autumn (36.1%) and winter (36.5%) compared to spring (17.3%) and summer (15.9%) (Rehbein et al., 2013). The variation was attributed to the seasonal exposure of horses to orbatid mites, which are the intermediate hosts in the tapeworm lifecycle (Bashkirova, 1941). Another possible explanation for the difference in prevalence between the present study and that of Relf et al. (2013) could be differences in prevalence of orbatid mites between study locations. Orbatid mites are ubiquitous, but their abundance is affected by temperature and moisture (Mitchell, 1979). The mites prefer warmer, drier climatic conditions and open pasture environments (Mitchell et al., 1979). In the present study, the majority of yards were based in southeast Scotland, which is relatively cold and wet, compared to Suffolk, where the majority of the stud farms were based (Relf et al., 2013). Further, Suffolk is a large arable area, with a high density of arable farming compared to southeast Scotland. How the environment influences the abundance of orbatid mites was reported by Proudman et al. (1998), where tapeworm prevalence was found to be lower in horses kept on dry, sandy pastures. A further reason for the differences in prevalence could be variation in praziquantel (PRZ) efficacy against *A. perfoliata*. The population in the Thoroughbred study were administered PRZ frequently:

approximately 60% of stud farm managers had administered IVM combined with PRZ, 50% had administered MOX combined with PRZ and 40% had administered PRZ on its own, in the preceding 12 months, as often as every 6 – 8 weeks (Relf et al., 2012), thus exerting strong selection pressure for resistance. To date, there have been no reports *A. perfoliata* resistance to PRZ, but there have been reports of a suspected lack of PRZ efficacy against the trematode, *Schistosoma mansoni* (Fallon et al., 1995; Ismail et al., 1996; Wang et al., 2012), where PRZ has been used prophylactically for many years. However, the reported lack of efficacy is associated with heavy infections pre-treatment and acceptable efficacy has been achieved by administering two doses (Picquet et al., 1998). This is an area that warrants further investigation; however, testing of PRZ efficacy by use of the FECRT would be difficult due to a lack of sensitivity of standard FEC methods in detecting tapeworm eggs (see above), and the 12-13 kDa antigen ELISA would be insensitive due to the relatively long half-life of antigen-specific IgG(T) in equine serum (Abbott and Barrett, 2010).

Here, strongyle FEC were highly over-dispersed (OD) between horses from the same yard, and the NBD adequately described the observed distribution. The NBD is a discrete probability distribution that is used to describe the amount of aggregation or OD in data, where decreasing values of k correspond to increasing levels of OD (Lloyd-Smith, 2007). In the present study, the mean value for the aggregation parameter k was 0.16. The k values obtained here are comparable to values reported in the recent Thoroughbred study, in which strongyle FEC were highly OD (k = 0.111) (Relf et al., 2013). The k values for both studies were smaller than those observed in horses managed for conservation purposes in England, where values for k ranged between 0.43 and 1.61 (Wood et al., 2013). In the study by Wood et al. (2013), the five populations of ponies sampled were not regularly treated with anthelmintics. It is not

surprising that regular anthelmintic intervention serves to increase aggregation, although there are few published studies on this.

Here, no significant positive relationship between mean FEC and *k* was observed, even when the FEC data were log transformed, in contrast to Relf et al. (2013), who found that *k* tended to track the mean (*m*) FEC, indicating that FEC become less aggregated between individuals with a high mean FEC, and tend to become more clumped between individuals with a low group mean FEC. A possible reason for the findings of the present study may be the fact that horses within yards were different ages, leading to greater heterogeneity in observed FEC. In the analysis of Thoroughbred breeding stock, *k* was estimated for each age group (Relf et al., 2013) and was found to track the mean in all age groups except yearlings (Relf et al., 2013). Here, analysis was performed on the whole population because of low numbers of foals and yearlings, which may have resulted in outliers, explaining why no relationship was observed between mean FEC and *k*. However, a significant positive relationship between mean FEC and prevalence was observed (r^2 = 0.8 (95% CI: 0.6-0.9), n = 28, p<0.0001), with yards with higher mean FEC having a higher prevalence of egg shedding. A similar relationship was observed by Relf et al. (2013) who reported a similar significant positive relationship (r^2 = 0.95, n = 6, p = 0.004).

The factors associated with parasite aggregation among host species have been described (Crofton, 1971; Anderson, 1976; Anderson and May, 1978; Shaw and Dobson, 1995; Shaw et al., 1998; Calabrese et al., 2011; Morril and Forbes, 2012; Poulin, 2013), and have been mostly attributed to heterogeneity amongst hosts, in particular an individual's exposure to parasitic challenge and its susceptibility to infection (Poulin, 2013). Any host population is unlikely to be homogeneous and will differ in age, sex, immune status and susceptibility (Morril and Forbes, 2012) leading to

variation in FEC (Morgan et al., 2005). Factors related to parasite biology and epidemiology may also play a role; for example, the distribution of infective stages in the environment (Quenouille, 1949; Shaw et al., 1998), which is likely to be temporally and spatially uneven relative to hosts (Poulin, 2013), climatic and temperature dependent effects on parasite development (Shaw et al., 1998) and density dependent effects within the host (Poulin, 2013).

The NBGLMM found that age, strongyle ERP, year, month and treatment frequency all had a significant effect on strongyle egg shedding.

Strong associations between shedding levels and horse age were identified in the current study. Strongyle egg shedding was significantly higher in younger horses (under 5 years) compared to adult and geriatric horses and this is consistent with the findings of other studies. The recent study on UK Thoroughbred stud farms used logistic regression analyses to identify significant predictors of strongyle prevalence and strongyle egg shedding (≥200 EPG) and reported that age had a significant effect on strongyle prevalence and that yearlings were three times more likely to be positive for strongyle eggs in their faeces compared to the other age groups (Relf et al., 2013). Hinney et al., (2011) also analysed equine FEC data from 1407 German horses (126 farms in one region) and reported that having young horses on a yard was a significant risk factor for a population of horses to have a high proportion of animals shedding ≥200 EPG. It is likely that these age effects are related to the acquisition of immunity against strongyle infections over time. Here, the number of weeks after the expected strongyle ERP for the last administered anthelmintic that the sample was collected had a significant positive effect on strongyle egg shedding. Wood et al. (2013) also reported that strongyle egg shedding increased significantly beyond each anthelmintics ERP, and found that when the ERP effect and weeks since treatment was included in a

generalised additive mixed effects model (GAMM), this removed the effect of month, which strongly dominated egg shedding (Wood et al., 2013).

The results here showed a clear fluctuation in strongyle FEC depending on month tested and found that strongyle FEC were significantly higher in 2012 compared to 2011. Egg excretion was significantly higher in July with FEC levels lower in August and September, consistent with other studies (Herd, 1985; Lloyd, 2009; Wood et al., 2013). Climatic parameters such as rainfall and temperature play an important role in the epidemiology of cyathostomin infections, leading to seasonal fluctuations in strongyle egg output (Poynter, 1954; Duncan, 1974; Lloyd, 2009; Wood et al., 2013). In the study of Wood et al. (2013), a clear seasonal trend in egg shedding was also observed, where average FEC increased from a minimum in February to a peak in May, after which there was a plateau until September before a decline in autumn and winter (Wood et al., 2013). In that study, the effects of year, month and local rainfall were identified as playing significant roles in the dynamics of strongyle egg excretion and year-to-year variation was attributed to annual variation in climate, while local temperature and rainfall were postulated to be associated with most of the monthly variation in FEC (Wood et al., 2013). The approach by Wood et al. (2013) differed from that of the present study as they investigated the effect of climate, rainfall and age on strongyle egg excretion using longitudinal data from five different populations, where in the present study, climate and rainfall were not considered and only the first screening FEC samples were included in the models. The rationale for the present studies approach was to enable a simple method of capturing the effects of factors that affect egg shedding at one point in time, to reduce the effects of pseudo-replication. Wood et al. (2013) were able to account for pseudo-replication and heterogeneity between yards by using generalised additive mixed models (GAMMs) (Pinheiro and Bates,

2000). Further, in the present study, the effects of rainfall and temperature was not considered, because of the spatial distribution of the yards tested, data would have had to be gathered from many different weather stations.

In the present study, a reduction in strongyle egg shedding was observed in August and September. It has been reported that during autumn, a large proportion of ingested cyathostomin L3 encyst in the large intestinal wall (Ogbourne, 1976; Eysker et al., 1984), leading to a reduction in egg excretion during the winter months. This has been confirmed in horses in France, where 42 post mortem examinations of horses between October and March found that EL represented the majority of the total cyathostomin burden (83%) (Collobert-Laugier et al., 2002). In this the UK, it has been reported that up to 90% of the total cyathostomin burden may exist as EL, with several million present in individual horses in the autumn/winter (Murphy and Love, 1997; Dowdall et al, 2002), serving as a potential reservoir for future transmission. The emergence of larvae from the intestinal wall is thought to be influenced by environmental conditions, host immunity and/or worm population density, and may also coincide with the removal of adults from the lumen following anthelmintic dosing (Gibson, 1953; Smith, 1976). The emergence of encysted stages has been proposed as responsible for the spring rise of FEC observed in April and May in temperate climates (Reinemeyer, 1986). The study looking at factors that that affect helminth egg excretion in Thoroughbreds (Relf et al., 2013) reported that horses that had access to grazing during winter had a were 7.61 times more likely to shed ≥200EPG in their faeces (OR=7.61, p=0) possibly due to milder autumn/winters (van Dijk et al., 2010), which will ensure the survival of eggs and larvae on pasture. This requires further study as it may have implications on levels of infections (and hence disease) and treatment applications.

Lloyd (2009), examined strongyle faecal egg shedding in 267 horses that had previously been managed under three different control programmes; one group had previously received no anthelmintic treatments, another received two anthelmintic treatments/year and the other had been treated 5 - 7 times a year. FEC were performed in February, May, August and November. In all groups, mean EPG peaked in August, slightly later than observed here, which is probably attributable to differences in local temperature and rainfall, which could have influenced the epidemiology. In an earlier study faecal samples were collected every two weeks from 10 untreated ponies in Newmarket, UK reported two peaks in strongyle egg shedding were observed in April and September, both of which led to peak rises in L3 on pasture two weeks later, coincidental with abundant rainfall (Herd, 1985).

In the present study, horses from yards where anthelmintic treatment was directed by FEC were shedding significantly fewer eggs compared to horses that were treated twice or four times per year. The number of yards and horses following a FEC directed worming protocol in the study population was small (three yards; 97 horses) and the questionnaire did not request information on how long the horses had been following a targeted treatment plan. Despite this, a significant effect was seens, indicating that the majority of high egg shedders were being identified and treated when following a FEC directed treatment plan. Further studies are warranted to examine the effect that FEC directed targeted programmes have on strongyle egg shedding and pasture contamination, in particular data should be captured to inform on the optimum testing frequency and proportion of horses to be tested at each sampling. This should be performed using different treatment regimes on horses with access to the same paddock and management practices.

In a study similar to the current one, Hinney et al. (2011) investigated risk factors associated with strongyle egg excretion in 1,407 horses from 126 farms using multivariate logistic regression analyses. These authors found that horses treated with anthelmintic less than three times a year were 3.24 (p = 0.011) times more likely to be shedding >200 EPG (Hinney et al., 2011). In the study by Lloyd (2009) the effect of previous control programmes on strongyle egg shedding was investigated (as outlined above). The yard managers were asked to withdraw anthelmintic treatments during the study period. Unsurprisingly, there was a significant difference in strongyle egg shedding between the yard that had previously administered frequent treatments (mean 99 EPG) and the yard that had not administered any treatments (1200 EPG) (Lloyd, 2009). It is not clear whether the horses grazed the same pastures that they were on before the study started. If they were, then the results are likely explained by the level of pasture infectivity, which one would expect to be much higher on pasture grazed by untreated horses. These results demonstrate that frequent effective anthelmintic treatments reduce parasite transmission via reduced egg excretion, but on the flipside, increased treatment frequency is linked to the selection of anthelmintic resistance (van Wyk, 2001).

In the current study, the last anthelmintic administered prior to the start of the study, did not have a significant effect on the level of strongyle egg shedding. A likely reason for this observation could be that horses were sampled at least two weeks after the expected ERP of each anthelmintic, thus minimising any persistent effects on reducing strongyle egg shedding. It could be hypothesised that egg shedding would be lower in horses that had been previously treated with MOX due to its persistent effect against strongyle infections (Cobb and Boeckh, 2009). MOX has an elimination half-life of 23.11 days compared to IVM (4.25 days) and PYR (13.43 hours) (Cobb and Boeckh, 2009).

This means that parasites are exposed to the active drug for longer periods and underpins the longer strongyle ERP of 13 weeks or more observed after MOX treatment (Cobb and Boeckh, 2009). Furthermore, MOX exhibits high larvicidal activity, whereas, IVM and PYR do not (Xiao et al., 1994; Bairden at al., 2006; Schumacher and Taintor, 2010). Administering an anthelmintic with adulticidal activity will only eliminate the luminal stages (L4 and adults), and this stimulates the EL to mature, leading to downstream contamination of pasture. By eliminating encysted stages, the strongyle ERP in increased (Schumacher and Taintor, 2010). Relf et al. (2013) reported that horses were 31 times more likely to be shedding ≥200 EPG if they had last been treated with FBZ. This was unsurprising given the high level strongyle resistance to FBZ.

Here, horses from yards that imposed appropriate quarantine measures did not have significantly lower strongyle FEC compared to those that did. Possible reasons for this observation could be that the yards do not have new horses arriving frequently; therefore, the effect of quarantine on strongyle FEC would be minimal. However, in a study looking into parasite control practices on 61 UK Thoroughbred studs, 47% of respondents applied quarantine measures (Relf et al., 2011), while 57% of 193 horse yards in Scotland quarantined new arrivals (Stratford et al., 2014a), indicating that quarantine measures are not widely practiced.

The regular removal of equine faeces from pasture has been advocated to reduce contamination (Herd, 1986; Herd and Coles, 1995; Duncan and Love, 1991), by removing parasite eggs before they hatch and develop to L3 (Mathee et al., 2004). In the present study, FEC of horses grazed on pastures from which faeces was removed were significantly higher compared to horses grazing pastures that did not have faeces

removed. This result contrasts to studies carried out at the UK Donkey Sanctuary, where a significant reduction in FEC in donkeys grazing pastures where dung was removed twice weekly (mechanically or manually) compared to pastures where dung was not removed (Corbett et al., 2014) was observed. This conflicting result may highlight the fact that in the questionnaire the response was a binary 'yes' or 'no', and did not provide information of frequency, hence a respondent that ticked 'yes' may have removed faeces from pasture once a year or twice a week. Also, there may be an element of respondent 'prestige' bias, 23/28 respondents stated that they removed faeces from pasture and this may be because it is generally regarded as best practice (Waltner-Toews, 1983; Houe et al., 2004). Future questionnaires should ask specific questions on frequency of dung removal and method of removal (i.e. manually or by machine) to assess if this practice is significantly associated with lower levels of strongyle egg shedding in faeces. Dung removal could reduce levels of refugia, potentially increasing the risk of anthelmintic resistance (Nielsen et al., 2007), therefore further research is required to investigate this phenomenon.

Alternatives to the NBD have been used to describe parasite population distributions. FEC datasets are dominated by zero counts, which can lead to biased parameter estimates and OD (Zuur et al., 2009). Zero-inflated distributions are useful when zero observations arise from either count data (such as Poisson, lognormal-Poisson or gamma-Poisson distributions) or from a truly zero individual (Martin et al., 2005). Zero-inflated models involve predicting the number of true zeros from the distribution of observed counts and it is also possible to use a zero-inflated distribution to approximate a sub-population, which has a very low mean rather than a mean of zero (Martin et al., 2005; Zuur et al., 2009). The application of zero-inflated models to equine FEC would be useful to estimate the true number of zero egg counts. A zero FEC

result could mean that there were truly no eggs shed, or it could mean that eggs were not detected because the FEC method used was not sensitive enough to reliably detect eggs at a low abundance, or that the worm population within the host was comprised of immature stages only, therefore no eggs were detected. To counter these possible scenarios, in the present study, a sensitive FEC method was used, reducing the likelihood of a false negative result and the faecal samples were collected from horses between March and September, when there are likely to be fewer encysted stages in the host. However, it would be useful to explore the use of zero-inflated models for analysing equine FEC data.

Knowledge that horses consistently shed similar levels of strongyle eggs over time can help establish evidence-based control strategies (Nielsen et al., 2014a). Here, horses were screened up to four times a year and shedding consistency in all horses tested was found to be relatively consistent over time, and encouragingly, egg shedding was highly consistent in horses that did not receive anthelmintic treatment during the course of the study. The results also revealed that young horses were more likely to change egg shedding or treatment category compared to the other age groups. A total of 61.5% of horses remained in the same shedding category between the first and second screen, and 58.3% between the first and third screen, while 81.8 and 82.7% remained in the same treatment category between the first and second screen and the first and third screen, respectively. In horses that did not receive anthelmintic treatment during the study, 92.1% remained in the same egg shedding category between the first and second screening and 90.4% between the first and the third screening. In terms of treatment category, 98.7% remained in the same category between the first and second screen and 95.6% between the first and the third screen, suggesting high levels of consistency. These results were similar to those of previous

studies that measured strongyle egg shedding consistency. In one, Nielsen et al. (2006) performed FEC analysis on 424 horses from 10 yards, twice a year for three years. In the first year, horses that shed >200 EPG were treated with anthelmintic chosen by the horse owner, and in the second and third years, horses received PYR and IVM, respectively. The authors reported that if the first two FEC were 0 EPG, there was an 82% probability that the third FEC would be 0 and a 91% chance that it would be <200 EPG. If the first two counts were <200 EPG, there was an 84% chance that the next FEC would be <200 EPG. Finally, if the first two FEC were ≥200 EPG, they reported a 59% probability that the next FEC would be ≥200 EPG (Nielsen et al., 2006). These results suggest a tendency for horses in low egg shedding categories to remain in this category on subsequent occasions. In the study of Nielsen et al. (2006), factors affecting shedding consistency were not examined. In another study, 129 horses from 19 yards were sampled every 4 weeks between March and October (9 samples/horse) (Becher et al., 2010). FEC were analysed by modified McM (30 EPG egg dl). Horses were treated with anthelmintics (PYR on the first occasion, IVM on the second occasion and MOX on the third occasion) when FEC were measured ≥250 EPG. Only horses that had not received anthelmintic treatment were included in the subsequent analysis. The authors reported that if the first two samples were FEC 0 EPG, there was a 62% probability that the maximum FEC of the next seven samples would be 0 EPG, and if the first two samples were FEC 0, there was an 88% probability that the maximum FEC of the next seven counts would be < 200 EPG, and a 92% probability of the next FEC being <250 EPG. They concluded that for individual horses, the magnitude of the initial FEC was significantly correlated to the maximal FEC of the subsequent eight counts (Becher et al., 2010). An earlier study examined strongyle egg shedding consistency over tighter sampling frame (two samples over 6 weeks) in 484 horses from 18 yards (Dopfer et al., 2004). Here, FEC analysis was performed by modified McM (50 EPG egg dl), and horses

were categorised as high egg shedders (HES, ≥100 EPG) or low egg shedders (LES, <100 EPG). A total of 55.2% of horses remained consistently low and 32% remained consistently high and did not move from one category to another. The remaining 12.8% went from low-to-high or high-to-low (Dopfer et al., 2004). As the threshold for a HES was only 100 EPG compared to 200 or 250 EPG in other studies (Nielsen et al., 2006; Becher et al., 2010), this could account for the reported lower level of consistency. One thing in common between these studies and the current one is that all horses received anthelmintic treatments at defined intervals or by FEC directed targeted treatment. A recent study that examined strongyle egg shedding consistency in ponies managed for conservation purposes found that shedding consistency at individual level was generally weak (Wood et al., 2013). In this study, FEC data were collected from the populations up to 11 years and four out of the five populations did not receive anthelmintic treatment. These data were analysed using GAMMs to estimate repeatability of FEC at individual level and to test for differences in mean FEC amongst populations and age classes. Climate and season were found to exert a significant effect on FEC in the populations that did not receive anthelmintic treatments. They reported a strong interaction between age and climate, suggesting that the highest FEC would be expected in young horses living in a warmer and wetter climate (Wood et al., 2013). The lack of individual consistency observed in this study (Wood et al., 2013) compared with others (Dopfer et al., 2004; Nielsen et al., 2006; Becher et al., 2010) could be due to the length of time the data were collected, the absence of anthlemintic treatments and the type of statistical analysis undertaken. In the anthelmintic treated horses, climatic effects were not detectable (Wood et al., 2013), supporting the impact of anthelmintic treatment on parasite distribution, as discussed above. Taken together, the evidence presented here and elsewhere (Dopfer et al., 2004; Nielsen et al., 2006; Becher et al., 2010), it is important to monitor FEC shedding patterns over time, and

not categorise shedding status over a short sampling time as factors such as management, immunity, anthelmintic use and climate that may affect longitudinal egg shedding patterns (Lloyd, 2009; Wood et al., 2013). Here, egg shedding and treatment consistency was higher in adult and geriatric horses compared to youngsters and foals, and youngsters were 3.3 (p=0.03) times more likely to change egg shedding category and 2.8 (p=0.028) times more likely to change treatment category between the first and third screening occasion compared to other age groups. Wood et al. (2013) also found that younger animals were more likely to shed more strongyle eggs compared to older animals and another study reported that mean FEC was negatively correlated to age (r = -0.328, p<0.01), with younger horses more likely to shed more strongyle eggs in their faeces (Becher et al., 2010).

Overall, the results indicate that there is a high probability of a horse measured as having a negative or low FEC having a low FEC on subsequent occasions and a high probability that a horse not requiring treatment based on a 200 EPG threshold, will not on subsequent occasions (Section 5.4.3.1 – 5.4.3.2). However, climatic factors will influence the number of larvae that survive on pasture, so if horses are exposed to a greater parasitic challenge then their strongyle egg shedding potential may increase. Further, changes in management practices such as cessation of regular dung removal from pasture (potentially leading to an increased number of infective larvae on pasture), or the introduction of a new horse that has not undergone quarantine measures and may increase the likelihood of a LES becoming a high egg shedder HES. Another reason for an increase in strongyle egg shedding could be if the horse became immunocompromised due to an underlying illness or pregnancy. By using FEC analysis, horses can be categorised as LES (i.e. if FEC are consistently <50 EPG over three sampling occasions), medium egg shedders (MES, i.e. FEC are consistently ≥50 EPG but

<200 EPG) and HES (i.e. if consistently shedding >200 EPG on three sampling occasions). In practice, this means that LES could be checked less frequently (1 – 2 times per year) saving time and money. Since in this population, only 15% of horses excreted 80% of strongyle eggs over the three sampling points, and on average, 18% of the horses screened would have required anthelmintic treatment on the basis of a 200 EPG threshold for treatment, the majority of adult horses are likely to be LES. However, further analysis revealed that young horses (≥2 - <5 years of age) were 3.3 times more likely to change egg shedding category compared to the other age groups and were 2.8 times more likely to change treatment group. This is likely to be due to younger horses being more susceptible to infection due to a lack of acquired immunity (Klei and Chapman, 1999), so that after treatment they are more likely to become infected and shed strongyle eggs. As their FEC are more likely to change, as well as be higher in count, FEC monitoring of young horses should be carried out more frequently than in older horses. If a horse has three or more consecutive FEC that measure <50 EPG using a FEC method with and egg dl of <10 EPG, the horse may be classified as a LES. However, further studies are warranted to ensure that these thresholds are suitable, and that there is no risk to horse health.

Here, egg shedding and treatment status consistency at individual level was relatively high, especially in adult horses. However, FEC data were collected over one grazing season, so wider temporal effects were not accounted for. There is a lack of published information on long term patterns strongyle egg shedding in populations, especially in those managed under a FEC-driven treatment programme and this should be assessed in future, and include the analysis of various species of nematode.

5.5 Conclusions

These data on helminth prevalence, distribution patterns and factors that affect egg shedding will inform improved methods of parasite control, which are less reliant on regular anthelmintic treatments. The results confirm that strongyle eggs are the most prevalent helminth egg found in the faeces of horses, confirming that strongyle control should be the main objective of most control programmes, and that egg shedding is highly over-dispersed amongst individuals, supporting the application of FEC directed anthelmintic treatments to those individuals shedding moderate-to-high numbers of. In terms of factors that affect strongyle egg shedding, age, time since last anthelmintic treatment, time of year and anthelmintic treatment frequency all affect the level of excretion. Such information informs the basic epidemiology of cyathostomin infections, which will ultimately underpin improved control programmes. Further, there was evidence to suggest that there is a high level of consistency in the level of strongyle egg shedding in individuals over time, which, will help to build guidelines for FEC directed targeted programmes.

CHAPTER 6: A framework for a decision support system for sustainable equine parasite control

6.1 Introduction

The frequent and indiscriminate usage of anthelmintics in horses has contributed to the development of widespread drug resistance in cyathostomins and *Parascaris equorum* (Kaplan, 2004; Kaplan and Nielsen, 2010; Matthews, 2008, 2011, 2014), and recent evidence suggests that resistance in *Oxyuris equi* to macrocyclic lactones (MLs) is emerging (Wolf et al., 2014). With a limited option of effective anthelmintics, and increasing reports of resistance/lack of efficacy in multiple parasite species, chemical options for control are becoming restricted. With no new anthelmintic classes likely to be licensed for use in equids in the short to medium term, it is essential to preserve efficacy of the currently effective anthelmintics; in particular, moxidectin (MOX), which has larvicidal activity against cyathostomin species (Bairden et al., 2001). One primary aim of helminth control programs must be the preservation of anthelmintic-sensitive nematode populations by maximising levels of parasite refugia thus providing a pool of susceptible parasites, coupled with minimising the risk of parasite-associated disease. Such an approach could usefully employ targeted strategic dosing, which relies on administering anthelmintics at the most appropriate time of year, while considering the life cycle and epidemiology of the associated parasites, as well as taking into account variation in nematode egg excretion amongst individuals. This approach involves the measurement of individual faecal egg counts (FEC) of horses at specific times of year to facilitate targeting anthelmintics to only those horses excreting moderate-to-high levels of nematode eggs in their faeces (Duncan and Love, 1991; Gomez and Georgi, 1991). While this was suggested almost a quarter of a century ago, many horse owners in the UK continue to administer anthelmintics prophylactically at defined intervals. For example, a recent questionnaire study conducted on 61 UK Thoroughbred stud farms (Relf et al., 2012), reported that 100% of respondents

administered treatments to all individuals on a treatment interval basis. While 58% said that they had conducted faecal egg counts (FEC), 100% of those said that FEC analysis was not used regularly and usually only if parasite-associated disease was suspected. Further, 68% of respondents were not aware of the faecal egg count reduction test (FECRT, Relf et al., 2012). These figures are concerning, particularly as stud farms contain high numbers of young stock whose helminth burdens are likely to be higher (Relf et al., 2014) and they are known to frequently administer anthelmintics, in particular ML (Comer et al., 2006). Such practices are likely to exert a strong selection pressure for anthelmintic resistance. Another recent questionnaire study looking at control measures on 193 Scottish equine livery/leisure yards (Stratford et al., 2014a) found that 40% of respondents believed that they were following a targeted treatment programme however, this was not associated with delaying treatment beyond the standard strongyle egg reappearance period (ERP), and the pattern of use of products did not specifically 'target' encysted cyathostomin larvae. This indicated that the respondents were unsure of the definition of 'targeted' worming. In total, only 19% of respondents administered anthelmintics based on FEC analysis and 61% were aware of the FECRT, but only 16% had performed a FECRT on their yard (Stratford et al., 2014a). Similarly, results from an earlier questionnaire study conducted in Ireland reported that of 55 yard owners questioned, none of the respondents left any animals untreated (O'Meara and Mulachy, 2002). A total of 72% of the respondents administered anthelmintics based on a treatment interval of <8 weeks, indicating that if they were administering MLs, they would be treating horses within the standard strongyle ERP (O'Meara and Mulachy, 2002). An online study performed in the UK targeting the general horse owning population (Allison et al., 2011) found that <60% of respondents (n = 574) believed that their current deworming programme was not as effective as it could be. In this study, 49% of the horses were kept on livery yards and

the owner's indicated that they followed a deworming regime imposed by the yard manager. In total, 40% of these owners indicated that were 'not happy' with the imposed programme. Further, while 94% of respondents believed their horses were reasonably or well protected from worms, only 25% wanted to reduce the amount of anthelmintic they used and, only 31% followed a FEC directed control programme (Allison et al., 2011). The results suggested that while the horse owners were aware of anthelmintic resistance and felt well informed about targeted treatment, the vast majority were not following a targeted treatment protocol (Allison et al., 2011).

The results from the aforementioned studies demonstrate gaps in knowledge about equine parasite control. Overall, the leisure yard owners appeared to be more aware of evidence-based control methods compared to the Thoroughbred stud farm sector. Generally, the results indicate that horse owners find parasite control confusing, particularly with regard to which species should be targeted at what time of year, and which anthelmintic should be used against which parasite species or stage. The need to take time to define prevalence (i.e. egg shedding status) and drug sensitivity by performing FEC analysis and FECRTs can disincentivise owners to practice evidence-based control. Thus, a decision support system (DSS) aiding evidence-based steps for control would be a useful tool for horse owners, and those that prescribe anthelmintics for horses: veterinary surgeons, veterinary pharmacists and Suitably Qualified Persons (SQPs). Decision support tools have been used to support definitive disease diagnosis in human and veterinary medicine, where they incorporate sets of rules for solving problems, and use details such as clinical signs, laboratory results and opinions of experts to guide decision making (Thrusfield, 1995). A DSS does exist for equine parasites, Parasietenwijzer (www.parasietenwijzer.nl), written by the University of Utrecht. This resource provides decision trees for equine parasite control and is a

passive tool that provides suggestions based on risk. It advocates the use of FEC directed anthelmintic treatments, but does not suggest a plan for taking faecal samples over time, nor does it advocate performing FECRT. The aim of this chapter is to build an evidence-based framework for a more prescriptive DSS, using the results from earlier chapters, as well as existing published expert opinion to provide a relatively simple, informative system to aid horse owners and managers in adopting sustainable approaches to control. Furthermore, as there is a lack of published information on the resource implications of adopting targeted treatments, a cost-comparison analysis was performed to assess the impact of applying targeted anthelmintic treatments in populations of horses in the UK.

6.2 Aims and objectives

The aim of this Chapter is to apply the major findings of the earlier chapters, together with that of other published data, to build an evidence-based framework for helminth control in horses. A small study on the financial implications of adopting targeted based treatment regimes is also presented.

6.3 Methods

6.3.1. The framework for an evidence-based approach to sustainable parasite control

Here, a framework for evidence-based parasite control is developed, based on the information and results generated in the earlier chapters, and using published evidence from other studies. This framework will be used to create rules that will be incorporated into a decision-based process, which will guide users through a set of questions regarding treatment decisions for helminth control. Four frameworks have

been constructed; the first is a simple framework, which considers three factors (last anthelmintic administered, when it was administered and the time of year) and guides the user through a set of questions relating to these factors, each with a set rules and recommendations, which will ultimately advise the user whether or not to perform a FEC and what to do if the FEC is ≥200 EPG. The second builds in the level of strongyle egg shedding in individual horses, includes performing FECRT analysis, surveillance of large strongyle infection and recommendations for tapeworm treatment. The third considers control in horses under the age of three years, and the fourth, control in foals.

6.3.2 A cost-comparison of performing FEC directed targeted treatment protocols compared to interval dosing strategies

Targeted treatment control programmes are underpinned by FEC analysis, which are likely to incur extra costs to the horse owner. The perceived benefit may be outweighed by concerns regarding the cost of performing routine FEC analysis. There is a lack of published information on the financial implications of adopting FEC directed targeted treatments compared to regular (interval) treatments with anthelmintics. Here, a cost-comparison analysis was performed to assess the financial impact of applying targeted treatments in 16 populations of horses across the UK. This analysis was applied to data collected from 368 horses on 16 yards that were part of the anthelmintic efficacy studies described in Chapter 3. Faecal samples from all horses were screened for the presence of strongyle eggs using a modification of the salt flotation method (Christie and Jackson 1982), with an egg detection limit (dl) of down to 1 EPG as described previously in Section 2.2.2.2. Each horse was screened three times (February/March, June/July, and September/October). Horses with a FEC measured at 50 EPG or more were treated in February/March and in June/July with

PYR and with IVM in September/October. Anthelmintics were administered *per os* at the following dose rates; PYR (Strongid-P™, Elanco Animal Health, UK) (19mg/kg), IVM (Eqvalan® oral paste for horses, Merial Animal Health, UK) (0.2mg/kg). Each horse received a dose appropriate for 110% of each individual's body weight, as estimated by weigh tape, to minimise the risk of under-dosing (Stratford et al., 2014b). In December, all horses were treated with moxidectin (MOX)/praziquantel (PRZ)(Pramox™, Zoetis) at a dose rate of 0.4 mg/kg (MOX) and 2.5 mg/kg (PRZ) to target encysted small strongyle larvae and tapeworm, which are most prevalent during the winter. The protocol was designed around findings from a recent questionnaire study, which identified that 92% of respondents had administered MOX or MOX/PRZ anthelmintic combination to their horses in the last 12 months (Stratford et al., 2014a). For the purpose of this study, each yard was asked to provide information regarding management practices; 94% of respondents had used a ML in the previous 6 months (19% IVM, 81% MOX). These products were administered at intervals that varied: 12.5% (2/16) of respondents reported using them every 6 - 7 weeks, 68.8% (11/16) every 2 - 4 months and 18.8% (3/16) every 6-12 months. Since the majority of horse owners administered an anthelmintic every 2 - 4 months, and, with 81% administering MOX, the comparison here was based on each horse receiving two treatments of MOX and two treatments with MOX/PRZ during the course of the year (calculated as £55 per horse per year, assuming each horse received one dose/syringe and based on the average retail price of MOX, which was compared across five different retailers (average £10.62/dose for MOX and £16.65/dose of MOX/PRZ). The average cost of each FEC analysis was estimated to be £7 (based on the average of five commercial online FEC services (£7.29), with the average cost of an adulticidal treatment of PYR, £5 (price compared across five different online retailers (average £5.34)) and IVM, £8 (price compared between five different online retailers (average £8.23)). Included in

the analysis was the cost of two further FECs, using samples collected 14 days following treatment with PYR, after the first FEC screening occasion, and following treatment with IVM, on the third screening occasion, to assess efficacy of the anthelmintic used.

6.4 Results

6.4.1. Development of a framework for sustainable parasite control in equids

Results contained within this thesis and other published studies have been collated into Table 6.1, which inform the recommendations and rules that will be used in the framework. Factors that have not been discussed previously are briefly introduced here.

6.4.1.1. Recommendations for responsible anthelmintic use

It is proposed that PYR and IVM should be used for controlling adult strongyles at the appropriate time of year (i.e. March - November) and reserve MOX for strategic use over the winter months to target cyathostomin EL (Matthews, 2014).

Table 6.1. A list of recommendations for evidence-based control linked to specific areas

Factor	Recommendations	Thesis Section/ Reference
1. Recommendations for faecal sample collection and storage	• Wait a minimum of four weeks after the standard ERP of the last administered anthelmintic before taking performing FEC analysis. • Freshly voided faeces should be used (sample within 12 hours of excretion). • Collect at least three faecal balls to ensure representative material is obtained. • Place samples into a zip-lock bag (or similar) and remove as much air as possible before	Nielsen et al., 2010

	sealing. • If not processed immediately, samples should be refrigerated at the laboratory as soon as possible. • All samples should be processed and eggs counted within five days.	
2. Recommendations for performing faecal egg counts	• Thoroughly mix faecal samples before taking subsamples for analysis. • Use a FEC method with a low egg detection limit (dl) (≤10 EPG). • Ensure specific gravity of medium solution is correct (i.e. 1.2 for saturated NaCl solution).	Sections: 2.4.1, 2.4.3 2.4.5 Lester and Matthews, 2014
3. Recommendations for performing a faecal egg count reduction test	• Collect and handle pre- and post-treatment sample in a consistent manner. • Use a FEC method with an egg dl ≤10 EPG. • Include more than 10 horses. • Perform the FECRT method according to Coles et al. (1992) (as referred to as Method 1 in this thesis). • Calculate 95% lower confidence limits when calculating % FECR. This will take into consideration spread of individual FECR results and give a more accurate indication of sensitivity if especially if % efficacy is close to that of the threshold used to discriminate sensitivity and resistance. • Alternatively, a web-based tool (Torgerson et al., 2014) is freely available to generate FECRT estimates: http://www.math.uzh.ch/as/index.php?id=calc. This accounts for variation arising through sampling and variation between animals and now provides access of the layperson to more robust methods of computing FECR to take into account the likely spread of the FEC dataset. • Examine the FECRT dataset to identify shedding patterns post-treatment. If one or two individuals are shedding eggs post-treatment consider retesting in case of administration error in the first test.	Sections: 4.4.1 4.4.2.3 4.4.2.4 4.4.2.5 4.5
4. Anthelmintic use	• FBZ is not recommended for small strongyle control. • FBZ is recommended to target *P. equorum*. If MLs are used to control this species, test efficacy. • PYR or IVM can be used during the grazing season to target adult strongyles. Efficacy of PYR should be tested. • MOX should be used strategically to target	Section 3.5 Matthews, 2014

	encysted cyathostomin larvae in the autumn/winter. • PRZ should be used annually to target tapeworm in the autumn/winter.	
5. Equine helminth prevalence	• Cyathostomins are the most prevalent helminths, therefore, apart from foals, parasite control programmes should be focussed on this group. *P. equorum* is the major target of control programmes in foals. • If following a FEC directed targeted treatment programme, annual coproculture is recommended for surveillance of *S. vulgaris* or administration of an ML once a year to break the parasite lifecycle. • *O. equi* eggs are not routinely detected by FEC methods. If tail-rubbing is observed, perform a tapetest. • Tapeworm eggs are not reliably detected by common FEC methods; perform a tapeworm ELISA or treat prophylactically with PRZ in autumn/winter. • If co-grazing pastures with ruminants, ensure ruminants are free of liver fluke. Treat with an effective flukicide before turnout.	Section 5.4.1 Matthews, 2014 Nielsen et al., 2014
6. Cyathostomin distribution	• Strongyle egg shedding amongst populations of horses is highly over-dispersed. • Use FEC analysis to identify horses that are shedding ≥200 EPG and treat these animals only.	Section 5.4.1 Matthews, 2014
7. Factors affecting egg shedding	• Young horses (<5 years) shed significantly more strongyle eggs in their faeces compared to adult horses therefore, young horses should be targeted appropriately to reduce pasture contamination. • Strongyle egg shedding is significantly higher during the summer months. • In the spring, target treatments at high egg shedders to reduce the peak observed in the summer months.	Section 5.4.2
8. Consistency of egg shedding	• Here, young horses are more likely to change egg shedding category over the course of a grazing season (i.e. February to November). • If a horse has three or more consecutive FEC that measure >50 EPG and <200 EPG using a FEC method with and egg dl of ≤10 EPG the horse may be classified as a medium egg shedder (MES). • If a horse has three or more consecutive FEC that measure <200 EPG using a FEC method	Section 5.4.3

	with and egg dl of less than 10 EPG the horse may be classified as a low egg shedder (LES). • Once an individual is identified as an LES, perform FEC analysis once or twice per year in spring and in summer to ensure that the FEC has not risen significantly. Treat with MOX/PRZ in late autumn/winter to target encysted small strongyles and tapeworm regardless of egg shedding status. • For MES and HES perform FEC analysis at more regular intervals and treat if FEC >200 EPG. Treat with MOX/PRZ in late autumn/winter to target encysted small strongyles and tapeworm regardless of egg shedding status.	
9. Recommendations for young horses (<3 years)	• For horses > 12 months but < 3 years, perform FEC analysis every 4 – 6 weeks between February and November • Treat all youngsters with a FEC of ≥200 EPG with an ML. • Perform FECRT to ensure treatment efficacy. • Treat with MOX in late autumn/early winter to target encysted cyathostomin larvae. • Perform tapeworm ELISA in autumn and treat if necessary, otherwise treat with PRZ in late autumn/early winter.	Section 5.4.2 Matthews, 2014
10. Recommendations for foal management	• Between 0 and 1 month treat with FBZ to target migrating ascarid larvae. • Between 2 and 4 months perform FEC analysis and treat with FBZ if ascarid eggs are present. Perform FECRT to ensure treatment efficacy. • Perform FEC at 6 months (weaning). Treat with FBZ if only ascarids are present and treat with an ML if strongyles are present. Perform FECRT to ensure treatment efficacy. • Perform FEC at 9 – 12 months. Treat with FBZ if only ascarids are present and treat with an ML if strongyles are present. Perform FECRT to ensure treatment efficacy. Perform tapeworm ELISA or treat with PRZ to target tapeworm.	AAEP,2013

6.4.1.2. Non-cyathostomin species

Here, small strongyles were identified as the most prevalent species in the populations examined (Section 5.4.1), thus, cyathostomins should be the main focus of control programmes in adult horses, but it is important to consider other helminth species

when designing a tool for equine parasite control. In populations of horses that do not receive any broad-spectrum anthelmintic treatments, there is a risk that *Strongylus vulgaris* may re-emerge (Nielsen et al., 2012). Therefore, yard owners that follow a FEC directed targeted programme should be encouraged to perform an annual coproculture to monitor large strongyle prevalence. Alternatively, owners could consider treating with MOX annually in the late autumn. This will reduce the likelihood of *S. vulgaris* infection because MOX is highly effective against all stages of *S. vulgaris* (Monahan et al., 1995) and due to the long prepatent period observed with *S. vulgaris* (6 – 7 months) (Duncan and Pirie, 1972) transmission of this parasite species can be broken. In addition, treating with MOX in late autumn will also target encysted cyathostomins.

O. equi eggs were not detected on any yards or in any horses tested during this study. There is no validated method for the detection of *O. equi* in horse faeces,. An inadequate method for the detection of *O. equi* eggs renders efficacy testing problematic. It has been suggested that once *O. equi* eggs have been detected using the tapetest, an anthelmintic is administered and the perianal area washed with warm soapy water immediately after treatment to remove all eggs. A tapetest should be performed every week for a month after treatment to ensure that no further eggs are shed. If eggs are detected within a month, this may suggest reduced efficacy (Reinemeyer and Nielsen, 2013). However, the use of this test for determining anthelmintic efficacy against *O. equi* has not been validated, and further work is required to ensure that it is fit for purpose. The best prevention is to regularly wash the perianal area with mild detergent using disposable cloths and ensure that stables and grooming equipment are regularly cleaned and are not shared.

Stongyloides westeri eggs were not detected in any horses tested during this study*. S. westeri* is typically found in foals as somatic larvae can become mobilized from the

ventral abdominal wall of the dam and infect the foal through milk (Greer et al., 1974). Since the population studied was biased towards adult horses, this finding is not surprising. Many worming protocols for foals recommend treating with MLs in the first month of life to target *S. westeri*, however, since this is mildly pathogenic at most, this practice has been questioned (Reinemeyer, 2009). For the control of *S. westeri*, it has been suggested that treatment of the mare with IVM within 24 h of parturition significantly reduces vertical transmission (Ludwig et al., 1982).

As tapeworm eggs are not reliably detected by routine FEC analysis (Nilsson et al., 1995), the only way to target anthelmintic treatment is to perform regular ELISA tests, otherwise it is recommended that owners treat their horses prophylactically once a year, in the autumn, as this is when adult tapeworm are thought to be most prevalent (Rehbein et al., 2013).

6.4.1.3. Young horses and foals

Horses under the age of five were measured as having significantly more strongyle eggs in their faeces compared to horses of 5 years and over (Section 5.4.2). Young horses are more susceptible to cyathostomin infection and are at greater risk of cyathostominosis (Reid et al., 1995). On this basis, the AAEP does not recommend targeted treatment programmes for horses under three years of age (AAEP, 2013) and they recommend that foals are treated a minimum of four times during their first year. FBZ is recommended at 2 – 3 months to target ascarids, and a FEC is recommended just before weaning (approximately six months) to determine which species are present (i.e. ascarids or strongyles) and then using an appropriate anthlemintic to target either or both species. The recommendations are then to treat again at nine and 12 months, targeting strongyles and tapeworm with a FECRT performed after each

treatment to ensure efficacy. It is also recommended that newly weaned foals are turned out onto 'clean' pasture and that yearling and two year olds should be treated three to four times a year with ML because of high strongyle egg shedding potential and their increased risk of larval cyathostominosis (Reid et al., 1995). It is feasible that younger horses (<3 years old) could follow a FEC directed treatment plan; however, FEC analysis should be performed at more regular intervals because of the reduced strongyle ERP observed in younger horses (Lyons et al., 2008; Lyons et al., 2009). This would ensure adequate surveillance to identify horses that need treatment. Recommendations for ascarid control suggest not treating foals before 2 months of age and to use FBZ at a dose rate of 10mg/kg (Reinemeyer, 2009) to minimise the use of MLs. However, the larval stages *of P. equorum* are pathogenic as they can cause pathology to the liver and bronchitis during hepato-tracheal migration (Nichols et al. 1987); therefore if *P. equorum* has been detected on a yard in the past, then it would be prudent to treat foals within their first month, as it takes approximately one month from the time the egg is ingested to the arrival of the L4 to the small intestine (Clayton and Duncan, 1977). Further, advice to treat *P. equorum* with FBZ at a dose rate of 10mg/kg is problematic, as the administration of FBZ at this dose rate is not licensed in the UK. Treatment at this dose rate would only be allowed by a veterinary surgeon under the 'cascade' option, a legal flexibility that allows veterinary surgeons to administer a licensed product to a particular target species at an unlicensed dose rate or, an unlicensed product to a particular species in the absence of a suitable licensed product in order to treat a condition or disease to reduce animal suffering (refer to: 'Guidance note on the use of the cascade' available at: www.vmd.defra.gov.uk).

6.4.1.4. The envisaged framework for the decision support system

Figure 6.1 builds on the evidence above, and shows a schematic representation of the factors requiring consideration when arriving at a decision whether to treat or not, and how these factors interact with one another other. In summary, the FEC result of an individual will depend on host age, and host age will have a bearing on which nematode species are more likely to be present. Further, host age will also have an effect of whether or not the horse is a high egg shedder or a low egg shedder. Thus, host age and parasite species present will affect anthelmintic choice. This also depends on season, the anthelmintic sensitivity status of the nematode population, and activity of the anthelmintic. The choice of anthelmintic will have an impact on recommendations for when to perform the next FEC, based on the strongyle ERP (Figure 6.1).

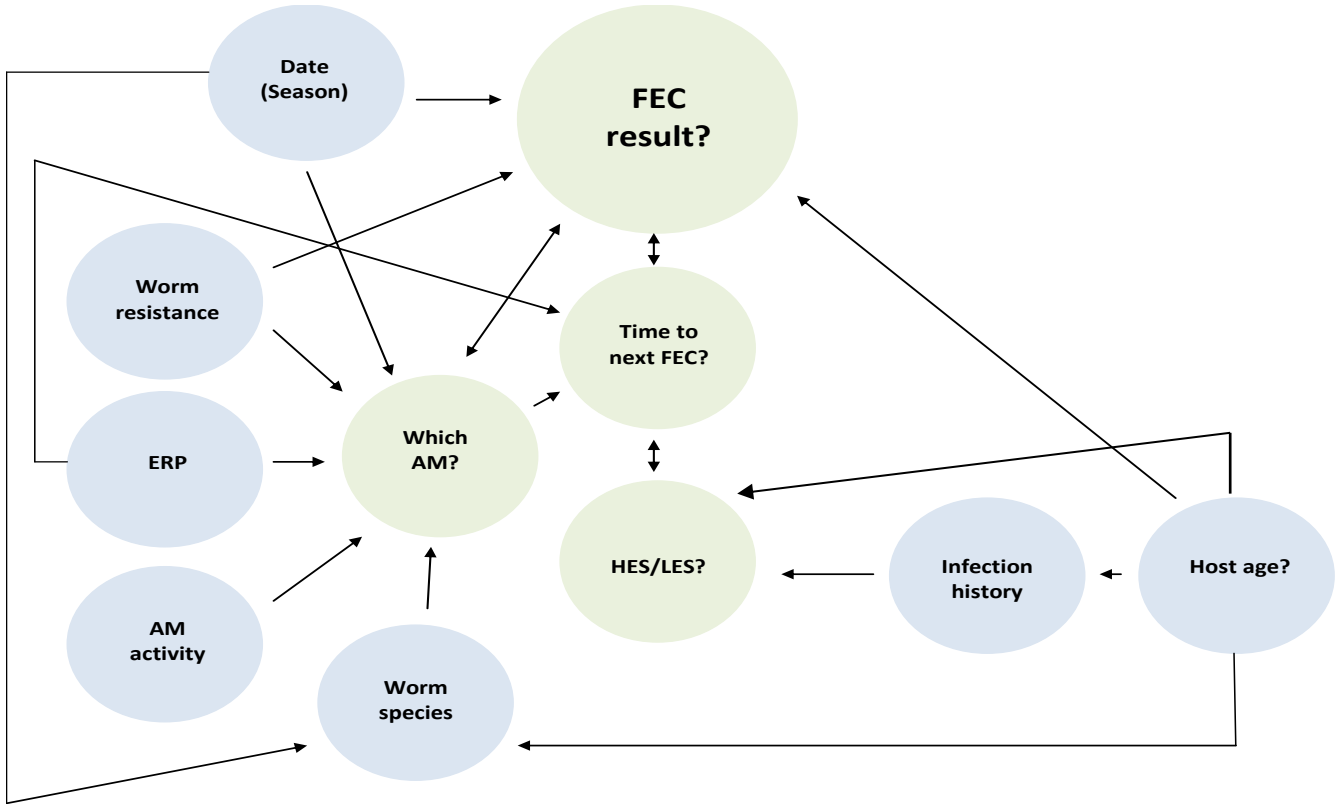


Figure 6.1. A schematic presentation of factors to consider in arriving at a decision of whether or not to treat, and their interaction with one another as represented by the blue circles and the envisaged outputs, represented by the green circles. Host age will affect the faecal egg count result (FEC), the infection history, whether or not the horse is a low egg shedder (LES) or a high egg shedder (HES) and the worm species present. The season in which the sample is taken, the worm species present, anthelmintic (AM) activity, the strongyle egg reappearance period (ERP) and resistance status of the helminth population will determine which anthelmintic (AM) should be administered. The season will also have an effect on the stage and species needing to be targeted and the FEC result

Using the evidence above (Table 6.1), an annual framework is presented (Figure 6.2). This is primarily designed for use with adult horses. Horses should be FEC tested as a group: here, recommended in March, based on the assumption of a MOX treatment in the previous autumn. Typically, in the UK, horses that have been stabled over-winter are grazed for longer periods in spring. Strongyle L3 that have survived on pasture over-winter can act as a source of infection. Further, any cyathostomin EL that are present are likely to resume development (Herd, 1985), leading to pasture contamination with eggs. Horses measured at this test point as having a FEC of ≥200 EPG should be treated with an anthelmintic with licensed (and preferably known) efficacy against adult strongyles. Effective treatment of moderate-to-high FEC shedders will help reduce the build up of strongyle larvae on pasture during the following months. This will be dependent on active ingredient used and the anthelmintic sensitivity of the population of worms present. Horses should then be tested in June and September to ensure that individual FEC have not increased. Again, anthelmintic treatments should be administered to horses with a FEC of ≥200 EPG with the same anthelmintic that was administered earlier in the year (i.e. either IVM or PYR). IVM or PYR, if identified as effective, should be used exclusively over the course of the year, and one FECRT performed to ensure that the drug remains effective. Thus, any reduction in efficacy can be identified and not masked by the use of another class of anthelmintic. In late autumn/early winter, all horses should be treated with MOX and PRZ to target strongyle species (including larval stages) and *A. perfoliata* respectively. Once three FEC samples have been analysed for an individual, the animal can then be categorised as a high egg shedder (HES - >200 EPG), medium egg shedder (MES – ≥50 - ≤200 EPG) or low egg shedder (LES - <50 EPG). These results support categorising horses into strongyle egg shedding categories, and demonstrate that a low egg shedding horse, is highly likely to remain a LES over the course of a grazing season

(February – November), and that young horses (<3 years) should be treated separately because of their tendency to change egg shedding category and treatment category.

Based on the annual framework (Figure 6.2), it is recommended that LES should be FEC analysed at least twice a year (March and June) to ensure that their strongyle egg shedding status remains low and that they do not require anthelmintic treatment. MES and HES should be FEC tested in March as this is the time that egg shedding increases after any EL present in the gut wall will start to mature and adults females will begin to lay eggs (Ogbourne, 1972; 1973; Herd, 1985; Reinemeyer, 1986) and in June/July, as this is the time when peak egg excretion was observed in this study (Section 5.4.2., Tables 5.10 and 5.11) and in August/September to identify any horses that are still shedding ≥200 EPG to reduce pasture contamination with infective larvae, which if ingested at or after this time of year, are likely to become encysted in the gut wall (Ogbourne, 1975; Eysker et al., 1984). The rationale behind treating MES and HES the same here is that more research is required to investigate how consistent strongyle egg shedding is in these groups and that adequate surveillance of the MES is undertaken to ensure that they do not become HES.

The efficacy of each anthelmintic should be examined on an annual basis by performing a FECRT (Table 6.2). The FECRT should be conducted early in the year as this will guide decisions on which anthelmintic to use in the future. Only horses that require treatment (i.e. those with a FEC of ≥200 EPG) should be included in the FECRT.

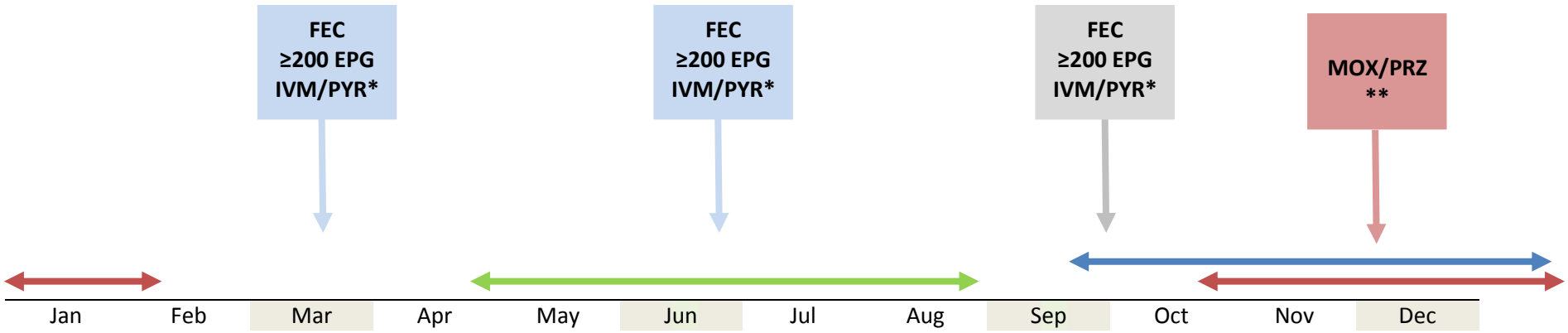


Figure 6.2. Proposed framework for faecal egg count directed strongyle control in adult horses. The blue and grey boxes indicate when faecal egg count (FEC) analysis should be performed. The blue boxes indicate that high egg shedders (HES), medium egg shedders (MES) and low egg shedders (LES) should be tested and the grey box indicates that only HES and MES should be tested. Horses with a strongyle FEC of 200 eggs per gram (EPG) or more at these time points should be treated with either ivermectin (IVM) or pyrantel (PYR) to control the adult strongyle burden. * If PYR is administered, then efficacy should be examined by FECRT – state when and indicate on figure. The red box indicates all horses should be treated with MOX/PRZ** to target strongyles (including encysted stages) and *A. perfoliata*. The green arrow represents the likely seasonal peak in strongyle egg excretion (Herd, 1985; Lloyd, 2009), the blue arrow represents the time of year when adult tapeworm are likely to be most abundant (Rehbein et al., 2013) and the red arrows represent when cyathostomin encysted stages are likely the most abundant (Ogbourne, 1975; Murphy and Love, 1997; Dowdall et al, 2002)

Figure 6.3 presents a simple decision tree. The first step explores which anthelmintic was previously administered, and then asks when the last treatment was administered, followed by the season. This then leads to a recommendation on whether or not to perform FEC analysis. Based on the FEC result, recommendations for treatment are indicated. The decision tree is based on four questions, and their respective responses. For each response there are rules based on current evidence (Table 6.3). For example, if the last treatment was FBZ, the recommendation is to perform a FEC to ensure that the FEC is <200 EPG because of widespread FBZ resistance in small strongyles. If the response is PYR, IVM or MOX, the next question prompts the time elapsed since last treatment. FEC analysis should be performed a minimum of four weeks beyond the strongyle ERP of the last administered anthelmintic (AAEP, 2013). The next step explores season and based on this and/or FEC result data and a treatment recommendation is given.

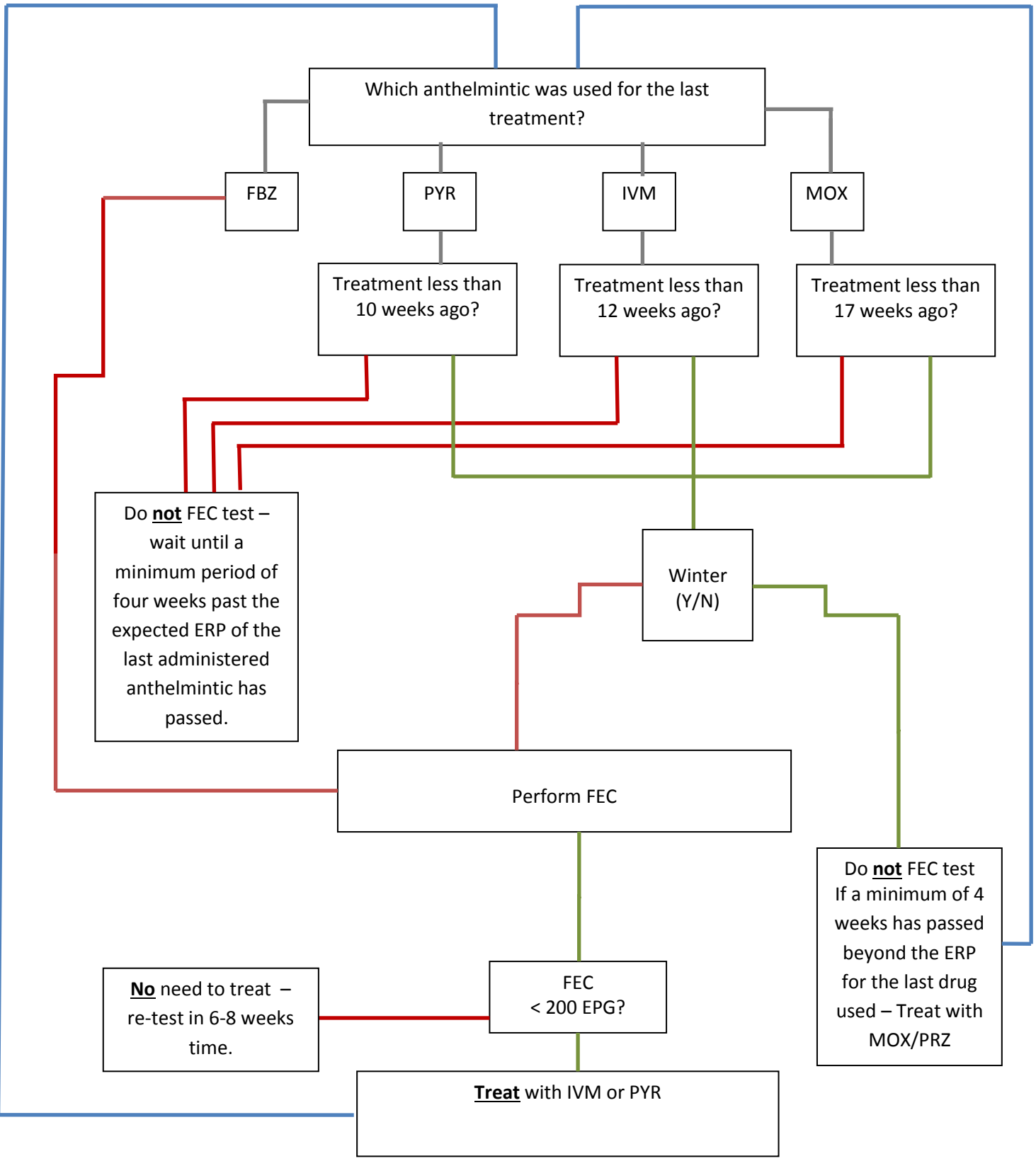


Figure 6.3 Framework for a simple decision support system for strongyle and tapeworm control in adult horses using faecal egg counts (FEC) to guide decisions whether or not to treat with anthelmintic. The red lines represent a 'No' answer, the green lines represent a 'Yes' answer, grey lines represent a multiple choice and blue lines take the user back to the start

Table 6.3. The questions, responses and rules underpinning the simple decision tree for strongyle and tapeworm control in adult horses

Question	Response	Rule
Have all new arrivals been quarantined?	Yes	The risk of a newly introduced horse shedding strongyle eggs of unknown resistance status is minimised
	No	This poses a greater risk as newly introduced horses could be shedding resistant strongyles onto pasture. Perform a FEC immediately and treat with MOX if necessary
When was the horse was last treated?	Date	Used for determining whether horse is within ERP
What was the horse last treated with?	FBZ	Perform FEC to check efficacy and treat with anthelmintic if >200 EPG
	PYR	If <10 weeks do not FEC If >10 weeks recommend FEC depending on season
	IVM	If <12 weeks do not FEC If >12 weeks recommend FEC depending on season
	MOX	If <17 weeks do not FEC If >7 weeks recommend FEC depending on season
What is the current season?	Spring	A minimum of 4 weeks has passed beyond the ERP for the last drug used – perform FEC analysis
	Summer	A minimum of 4 weeks has passed beyond the ERP for the last drug used – perform FEC analysis
	Autumn	A minimum of 4 weeks has passed beyond the ERP for the last drug used – perform FEC analysis
	Winter	A minimum of 4 weeks has passed beyond the ERP for the last drug used – Treat with MOX/PRZ to target encysted larvae and tapeworm
What is the FEC result?	<200 EPG	Do not treat – re-rest in 6 - 8 weeks time
	≥200 EPG	Treat with IVM or PYR to target adult small strongyles

Tables 6.4 and 6.5 provide a more comprehensive framework for a decision support system, incorporating individual strongyle egg shedding status, anthelmintic efficacy testing and surveillance for *S. vulgaris*. The framework outlined in Table 6.4 is based on questions, responses and rules, which guide the user and prompt them to, perform a FEC test or to treat with anthelmintic. It also explores the use of faecal culture for large strongyle identification. Table 6.4 is for horses for which their egg shedding status is unknown and for known HES and MES horses. Table 6.5 is for LES status horses

Table 6.4. Questions, responses and rules for a decision support tool for adult horses that have been identified as high strongyle egg shedders (HES).

Question	Response	Rule
Have all new arrivals been quarantined?	Yes	The risk of a newly introduced horse shedding strongyle eggs of unknown resistance status is minimised
	No	This poses a greater risk as newly introduced horses could be shedding resistant strongyles onto pasture. Perform a FEC immediately and treat with MOX if necessary
When was the horse was last treated?	Date	Used for determining whether horse is within ERP
What was the horse last treated with?	FBZ	Perform FEC to check efficacy and treat with anthelmintic if >200 EPG
	PYR	If <10 weeks do not FEC If >10 weeks recommend FEC depending on season
	IVM	If <12 weeks do not FEC If >12 weeks recommend FEC depending on season
	MOX	If <17 weeks do not FEC If >17 weeks recommend FEC depending on season
What is the current season?	Spring	A minimum of 4 weeks has passed beyond the ERP for the last drug used – perform FEC analysis
	Summer	A minimum of 4 weeks has passed beyond the ERP for the last drug used – perform FEC analysis
	Autumn	A minimum of 4 weeks has passed beyond the ERP for the last drug used – perform FEC analysis
	Winter	A minimum of 4 weeks has passed beyond the ERP for the last drug used – Treat with MOX/PRZ to target encysted larvae and tapeworm
What is the FEC result?	< 200 EPG	Do not treat – re-rest in 6 - 8 weeks time
	≥ 200 EPG	Treat with IVM or PYR to target adult small strongyles

Table 6.5. Questions, responses and rules for a decision support tool for adult horses that have been identified as low strongyle egg shedders (LES)

Question	Response	Rule
1. What is the current season?	Spring	Go to Question 2
	Summer	Go to Question 2
	Autumn	Go to Question 2
	Winter	A minimum of 4 weeks has passed beyond the ERP for the last drug used – Treat with MOX/PRZ to target encysted larvae and tapeworm
2. When was the horse last FEC tested?	Don't know	Perform FEC analysis at least twice a year during the spring and summer months to ensure egg count does not rise
	>4 months ago	Perform FEC analysis at least twice a year during the spring and summer months to ensure egg count does not rise
	<4 months ago	If performed in spring and FEC analysis was <50 EPG test again in summer to ensure egg count does not rise. If performed in spring and summer and both counts below 50 EPG no need to FEC again – treat horse in winter with MOX/PRZ
3. Has a faecal culture been performed this year?	Yes	If large strongyle larvae present treat all horses with IVM in summer or MOX in winter
	No	Perform faecal culture at least annually if horses are following a targeted treatment programme to check for large strongyles

Helminth control in young horses (i.e. >1 year <3 years) and foals (≤1 year) are considered in Tables 6.6 and 6.7, respectively. The decision support framework outlined in Table 6.6 is similar to the framework for the HES. The differences being that the interval for performing FEC analysis in young horses has decreased to every 4 – 6 weeks. This is to ensure that any animals with a high strongyle FEC are treated promptly. Further, whether or not ascarids are present is considered as patent

infections may be seen in young horses under the age of three years (Reinemeyer, 2012). The framework for young horses has been built on evidence presented in Table 6.1. The framework for helminth control in foals (Table 6.7) is based primarily on the age of the foal and on the recommendations made by the AAEP (AAEP, 2013).

Table 6.6. Questions, responses and rules for young horses (<3 years)

Question	Response	Rule
1. What was the horse last treated with?	FBZ	Perform FEC to check efficacy and treat with anthelmintic if >200 EPG
	PYR	If <10 weeks do not FEC If >10 weeks recommend FEC depending on season
	IVM	If <12 weeks do not FEC If >12 weeks recommend FEC depending on season
	MOX	If <17 weeks do not FEC If >17 weeks recommend FEC depending on season
2. What is the current season?	Spring/Summer/Autumn	A minimum of 4 weeks has passed beyond the ERP for the last drug used – perform FEC analysis
	Winter	A minimum of 4 weeks has passed beyond the ERP for the last drug used – Treat with MOX/PRZ to target encysted larvae and tapeworm
3. What is the FEC result?	< 200 EPG	Do not treat – re-rest in 4 - 6 weeks time
	≥ 200 EPG	Treat with adulticidal anthelmintic – go to Question 6
4. Were any ascarid eggs present?	Yes	Treat with an ML to target strongyles. Perform a FECRT
	No	Treat with an ML to target the adult strongyles
5. In the last year, has a faecal egg count reduction test (FECRT) been undertaken to determine efficacy?	Don't know	Treat with IVM or MOX and perform FECRT once a year to ensure de-wormer is working
	No	Treat with IVM or MOX and perform FECRT once a year to ensure de-wormer is working
	Yes	Go to Question 7
6. What was the last FECRT result for:	PYR	If less than 90% do not use; if ≥90% use during summer months
	IVM	If less than 95% do not use; if ≥95% use during summer months
	MOX	If less than 95% do not use; if ≥95% use annually during autumn/winter to target encysted larvae
7. Has a faecal culture been performed this year?	Yes	If large strongyle larvae present treat all horses with IVM in summer or MOX in winter
	No	Perform faecal culture at least annually to check for large strongyles

Table 6.7. Questions, responses and rules for a decision support tool for foals

Question	Response	Recommendation
How old is the foal?	0 -1 month	Treat with FBZ to target migrating ascarid larvae
	3 – 4 months	Perform FEC. Treat with FBZ if ascarid eggs present. Perform follow-up FEC to ensure treatment efficacy
	6 months (or weaning age)	Perform FEC. If only ascarids present, treat with FBZ. If only strongyles present, treat with ML. If both species present, treat with an ML and perform a FECRT to ensure treatment efficacy. If ascarids still present treat with FBZ.
	9 – 12 months	Perform FEC. If only ascarids present, treat with FBZ. If strongyles only present, treat with ML. If both species present, treat with an ML and perform a FECRT to ensure treatment efficacy. If ascarids still present treat with FBZ. For tapeworm, either perform ELISA or treat with PRZ.

6.4.2 Resource implications of FEC directed targeted treatment protocols compared to interval dosing

The average number of horses per yard (n = 16) in the study was 23 (range 10 - 47). The average saving calculated of adopting an anthelmintic-targeted treatment programme was calculated to be £294.44 (range £57 - 568, Table 6.8). On the basis of the analysis here, a financial saving was calculated for every yard included in the study. Costs associated with the time taken and labour used for collecting samples, administering anthelmintic, and postage costs were not included in the analysis. A linear regression model with a zero intercept with savings as the response variable and the number of horses of the yard (n) as the explanatory variable yielded a maximum

likelihood model of Savings = 6.3 + 0.06 x *n* . The r^2 value was 0.76 indicating that the model accounted for 76% of the variance. These findings show a significant positive relationship ($p<0.001$) between the number of horses per yard and the overall saving that could be achieved, indicating that the more horses present on a yard and undergoing targeted treatment, the greater the savings (Figure 6.4), demonstrating the additional value of applying targeted anthelmintic treatments in horses.

Table 6.8. Comparative analysis of using faecal egg count (FEC) to determine anthelmintic treatment requirement compared to an interval dosing protocol on 16 UK yards. [a] the estimated cost of interval dosing was £55/horse/year. [b] the cost of screening all horses/yard based on £7/FEC, [c] total cost of efficacy testing. Horses were treated with pyrantel (PYR) after the first and second screen and received ivermectin (IVM) after the third screen. The cost of anthelmintic used (AM) was [d] PYR, estimated to cost £5/dose and [e] IVM, estimated to cost £8/dose. All horses were administered moxidectin+praziquantel (MOX/PRZ) in December.

	Yard															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Number of horses	30	17	10	32	20	14	14	25	23	12	26	19	14	28	47	36
Estimated cost of interval treatment programme(£)[a]	1650	935	550	1760	1100	770	770	1375	1265	660	1430	1045	770	1540	2585	1980
Cost of FEC screening (£)[b]	630	357	210	672	420	294	294	525	483	252	546	399	294	588	987	756
Cost of efficacy testing (£)[c]	31	31	0	47	31	73	0	12	58	37	15	16	28	0	117	22
Screen 1 - MAR12	210	119	70	224	140	98	98	175	161	84	182	133	98	196	329	252
No. Horses >200EPG	3	3	0	5	3	3	0	12	9	2	1	2	7	0	5	1
FECRT (£)	21	21	0	35	21	21	0	84	63	14	7	14	49	0	35	7
Cost of AM used (£)[d]	15	15	0	25	15	15	0	60	45	10	5	10	35	0	25	5
Screen 2 - JUN12	210	119	70	224	140	98	98	175	161	84	182	133	98	196	329	252
No. Horses >200EPG	0	0	0	11	0	7	0	16	5	7	3	1	4	1	24	3
Cost of AM used (£)[d]	0	0	0	55	0	35	0	80	25	35	15	5	20	5	120	15
Screen 3 - SEP12	210	119	70	224	140	98	98	175	161	84	182	133	98	196	329	252
No. Horses >200EPG	4	4	0	6	4	10	0	0	7	5	2	2	3	0	16	3
FECRT (£)	28	28	0	42	28	70	0	0	49	35	14	14	21	0	112	21
Cost of AM used (£)[e]	32	28	0	48	32	80	0	0	56	40	16	16	24	0	128	24
All horses treated with MOX/PRZ (£)	499	283	167	533	333	233	233	416	383	199	433	316	233	466	783	599
Total cost of targeted Programme (£)	1225	732	377	1355	849	713	527	1085	1079	550	1021	769	656	1054	2070	1412
Overall saving (£)	425	203	173	405	251	57	243	290	186	110	409	276	114	486	515	568

Figure 6.4. Linear regression with zero intercept of the number of horses per yard and the overall saving (£) achieved (r^2 =0.76, p<0.001).

6.5 Discussion

Presented here are four evidence-based frameworks, using results gathered from earlier chapters and existing published opinion, to provide a relatively simple decision support system to aid a sustainable approach to equine parasite control. There is no uniform way to practice FEC directed targeted control (Nielsen et al., 2014a). Young horses need to be considered separately as they may be subject to varying factors that affect the epidemiology of the parasites (Matthews, 2014), as do horses in different geographic areas (Nielsen et al., 2014b). The frameworks here are based on the epidemiology of equine helminths in the UK, using data collected in this study and data from other researchers. The framework presented here is primarily for use in older horses. A preliminary framework is presented for younger horses and foals. For the latter, more data need to be generated to ensure that withholding treatments in young

animals in not associated with an increase in the incidence of parasite-associated disease. Despite these caveats, the evidence available (Section 5.4.3; Menzel et al., 2012) support the use of FEC directed therapy in horses by illustrating that strongyle egg shedding can be reduced substantially in populations despite leaving up to 84% of the population untreated. However, there are no data available on the long term effect of FEC directed targeted treatment protocols on the development of anthelmintic resistance or on the incidence of parasite-associated disease (Nielsen et al., 2014a).

The choice of FEC cut-off value used for deciding whether or not a horse requires treatment is often arbitrarily set at 200 EPG (Nielsen et al., 2006). The majority of studies evaluating FEC directed therapy quote cut-off values in the range of 100 – 300 EPG (Duncan and Love, 1991; Gomez and Georgi, 1991; Krecek et al., 1994; Mathee and McGough, 2004; Nielsen et al., 2006; Hertzberg et al., 2014). Such cut-offs were recommended without prior knowledge of the relationship between FEC and worm burden in horses (Nielsen et al., 2014a). A study by Nielsen et al. (2010) looked at the correlation between strongyle egg counts and adult worm counts obtained at necropsy from 700 horses, and found that there was no direct linear relationship between EPG and worm burden. They found that horses with FEC between 100 – 500 EPG had significantly larger adult strongyle worm burdens, however; some horses with FEC <100 EPG were found to have 300,000 luminal adult strongyles (Nielsen et al., 2006). This suggests that applying a threshold of 200 EPG may miss some horses with a large burden, allowing these animals to serve as a reservoir for egg shedding and pasture contamination. This is an area that warrants further investigation in order to further understand the impact of selecting specific cut-off values for targeted anthelmintic therapy.

The impact of management factors have not been considered in this framework. While there is evidence that regular removal of dung from pastures can reduce strongyle egg shedding (Corbett et al., 2014), there is little information on how dung removal impacts refugia. Similarly, nothing is known of the impact that co-grazing pastures with ruminants may have on levels of refugia. Further research is required to provide quantitative evidence on the utility of these control methods and to provide baseline values on which to build practical recommendations (Matthews, 2014).

An assumption of the proposed framework is that horses spend less time on pasture during the winter months, and that pasture contamination during winter is lower. A recent study looking at helminth egg excretion on UK Thoroughbred stud farms found that horses grazed in December – February shed relatively high levels of strongyle eggs (Relf et al., 2013). Milder autumns and winters that have been observed in the UK may allow development of nematode larvae from eggs over-winter (van Dijk et al., 2010), serving as a source of infection at these times of year. Rainfall and temperature play an important role in the transmission of strongyle infections (Poynter, 1954; Duncan, 1974; Lloyd, 2009; Wood et al., 2013). The results in Chapter 5 showed a fluctuation in strongyle FEC depending on month and demonstrated that strongyle FEC were significantly higher in 2012 compared to 2011. The generalised linear models in Chapter 5 identified significantly higher levels of egg excretion in July, with FEC lower in August and September, consistent with other studies (Herd, 1986; Lloyd, 2009; Wood et al., 2013). In Wood et al. (2013), a clear seasonal trend was observed in FEC shedding in ponies grazed primarily without anthelmintic treatment. In that study, the effects of year, month and local rainfall were identified as playing significant roles in the dynamics of strongyle egg excretion and year-to-year variation in FEC could be attributed to annual variation in climate, while local temperature and rainfall explained

most of the monthly variation in FEC (Wood et al., 2013). The current frameworks presented in this study, rely on the seasons being consistent, and are based on the egg shedding dynamics observed in the general horse population over a two year period. The next step in development of the framework here would be to develop steps that would allow automatic incorporation of local rainfall and temperature data across regions to allow the impact of these parameters to be included in the decision structure.

One of the caveats in FEC directed treatment programmes is that the tests only detect patent infections and do not provide an indication of prepatent infection. The larval stages of large and small strongyles and *P. equorum* (in foals) are associated with pathology and clinical signs. Diagnostic assays, capable of detecting encysted small strongyle larvae and migrating large strongyle and *P. equorum* larvae, would identify horses at risk of developing disease and better aid targeted treatment protocols (Andersen et al., 2013). For this reason, the recommendations here are to treat all adult horses annually with MOX to target small strongyle EL and any migrating large strongyle larvae. If the EL are highly aggregated between horses, then this prophylactic treatment may be unnecessary in all horses. Recently, a diagnostic ELISA based on detection of antibodies to a cocktail of larval cyathostomin proteins has been described (McWilliam et al., 2010). Such an ELISA would help to identify horses harbouring encysted larvae to allow treatments to be targeted at these individuals only, which could reduce selection pressure for ML resistance and refine anthelmintic treatment recommendations. Further, a serum ELISA for detection of migrating *S. vulgaris* larvae may be a useful monitoring tool in the future, but further work needs to be performed to validate the sensitivity and specificity of this test (Andersen et al., 2013).

Another limitation of FEC methods is that they do not reliably detect tapeworm (Nilsson et al., 1995). Further, while there has been a diagnostic tapeworm ELISA available for over ten years, a lack of sensitivity observed due to the long half-life of antigen-specific IgG(T) in serum in response to previous infection, limits usefulness of this test in the field. In the absence of more sensitive tools to identify infected individuals, current recommendations are to treat once a year with PRZ in autumn winter when adult tapeworms are more prevalent (Rehbein et al., 2013). It is likely that tapeworm infections will be highly aggregated in horse populations; therefore, not all horses will need treatment. To date, there have been no reports of tapeworm resistance to PRZ and this is an area of equine parasitology that warrants further investigation. However, testing of PRZ efficacy by use of the FECRT is difficult due to a lack of sensitivity of standard FEC methods in detecting tapeworm eggs and the ELISA (Abbott and Barrett, 2010).

A potential hurdle to widespread adoption of FEC directed targeted treatments by horse owners and managers may be the perceived cost providing be a disincentive to their widespread application. The cost associated with performing regular FEC analysis should be viewed as a necessary expense for maintaining horse health (Kaplan and Nielsen, 2010). The findings from the cost-comparison study here showed that such a strategy has a high chance of reducing the financial cost associated with interval dosing. The evidence available supports the exploitation of FEC directed therapy by illustrating that strongyle egg shedding can be controlled in populations of horses despite leaving up to 84% of the population untreated, and that such a strategy has a chance of reducing the cost associated with interval dosing. As many veterinarians and horse owners still practice interval treatment control programmes (Stratford et al., 2014a), the advantage of more evidence-based programmes need to be promoted to

ensure further uptake (Matthews, 2014). The development of accessible decision support tools based on up-to-date research and utilising available diagnostic tools to aid evidence-based management would facilitate such uptake. The frameworks presented in this Chapter require further development to be transformed into an accessible tool. The envisaged tool would store data for each horse and yard and would be able to send automated reminders to prompt further steps. The tool would be able to categorise horses to shedding status based on the previous FEC results and alert if there were changes in egg shedding status of individuals. Also, the tool could also prompt the need to test for large strongyle larvae should ML treatments be reduced substantially and would provide guidance on efficacy testing. The tool would need to be flexible to account for changes to incorporate new technologies as they were developed and validated. Such a tool could be developed using Bayesian networks. Bayesian networks offer a flexible graphical way to describe the probabilistic relationships between a set of random variables (Yet et al., 2013) allowing predictive or diagnostic inferences to be made, and have been used in human and veterinary medicine to aid clinical decision making and aid diagnosis (McKendrick et al., 2000; Lucas et al., 2004; Geenen and van der Gaag, 2005; Yet et al., 2013). Bayesian networks can be constructed using evidence obtained from expert opinion and/or published data to form a decision framework based on the probabilistic relationship between a particular variable and all possible outcomes (Yet et al., 2013).

6.6 Conclusions

The aim of this Chapter was to build an evidence-based framework for a DSS, using the results gathered from earlier chapters and existing published expert opinion, to provide a relatively simple and informative system to aid in the adoption of a sustainable approach to parasite control, by utilising FEC directed treatments. The

resource implications of following a FEC directed targeted treatment were investigated by performing a cost-comparison study. The findings indicate targeting anthelmintic treatment at individuals with high FEC can help to reduce selection pressure for resistance by reducing treatment frequency but without a substantial increase in financial cost. Taken together, these findings demonstrate the value of applying targeted anthelmintic treatments in horses. Several frameworks are presented and further work is now required to develop these into useable tools.

CHAPTER 7: General Discussion

7.1 Discussion

Gastrointestinal helminths present a serious challenge to the health and welfare of equids worldwide. With virtually all grazing equids at risk of infection (Nielsen et al., 2006), there is a need for control strategies to reduce the threat of clinical disease. Since the early 1900's, a number of anthelmintic compounds, which, in chronological terms, are of increasing spectrum, efficacy and safety, have been developed (Lyons et al., 1999). Currently, there are four broad-spectrum anthelmintics licensed for use in horses in the UK; these are fenbendazole (FBZ), pyrantel embonate (PYR) and the macrocyclic lactones (ML) ivermectin (IVM) and moxidectin (MOX). Praziquantel (PRZ), which is only effective against tapeworm species, is also licensed. The frequent administration of anthelmintics has significantly reduced the prevalence of *Strongylus vulgaris* and associated morbidity (Nielsen et al., 2014); however, it has led to the development of anthelmintic resistance to FBZ, PYR and MLs in a number of species, in particular the cyathostomins, which are now the most prevalent group of equine helminths worldwide (Nielsen et al., 2014; Matthews, 2014). The heavy use of anthelmintics has also contributed to the development of resistance in *Parascaris equorum* (Boersema et al., 2002; Hearne and Peregrine, 2003; Slocombe et al., 2003), and recent evidence suggests that ML resistance in *Oxyuris equi* may be an issue (Wolf et al., 2014). With no immediate prospects for new classes of equine anthelmintics, and increasing levels of resistance in various helminth species, chemical options for control are becoming limited. For these reasons, it is essential to preserve efficacy of the currently effective products (Kaplan, 2004; Kaplan and Nielsen, 2010; Matthews, 2011; Matthews, 2014); in particular, moxidectin, which has larvicidal activity against cyathostomin encysted larvae (EL) (Bairden et al., 2001), which are a major clinical threat to horses (Love et al., 1999).

As helminth infections and worm egg excretion are highly over-dispersed in horses (Kaplan and Nielsen, 2010; Relf et al., 2013), targeted treatment programmes have been advocated to reduce anthelmintic use (Kaplan and Nielsen, 2010; Matthews, 2014). Targeted protocols encompass the measurement of individual FEC within populations at specific times of the year to facilitate targeting of anthelmintics to those horses excreting moderate-to-high levels of nematode eggs in their faeces (Duncan and Love, 1991; Gomez and Georgi, 1991). The approach aims to reduce the selection of anthelmintic resistant strongyles by reducing treatment frequency and maintaining a population of parasites in refugia (van Wyk, 2001; Sangster, 2003). The role of refugia in slowing the development of anthelmintic resistance has been confirmed in part in sheep (Martin et al., 1981; Dobson et al., 2001); however, critically, practical evidence in equids demonstrating the effectiveness of this strategy is limited and therefore the role of maintaining populations of parasites in refugia to delay the development of anthelmintic resistance in equine parasites is currently an unvalidated theory, and warrants investigation. One caveat to the application of targeted treatment programmes is that many horse owners/managers have insufficient knowledge on which to base these protocols. For example, often they have little idea of the relative contribution that individual horses make to pasture contamination or of the true anthelmintic sensitivity status of the associated parasite population. Furthermore, specific advice is infrequently sought from veterinary surgeons and, when it is, the information provided can be based on out-dated concepts such as interval dosing (Stratford et al., 2014a; Matthews, 2014). Currently, there are no standardised guidelines for performing FEC analysis or efficacy testing in horses using the faecal egg count reduction test (FECRT). Since these tools underpin targeted treatment protocols, there is a need to optimise them and create recommendations for their use to ensure that they are fit for purpose. The aim of this thesis has been to build a framework for

sustainable helminth control to help horse owners and people who prescribe anthelmintics to develop control programmes on an evidence basis. Diagnostic tools that will promote the targeted treatment of horses in the field will underpin such a system.

To achieve this, the following areas have been investigated:

1. Sources of variation in equine FEC analysis with the aim of developing guidelines that will help reduce variation and improve the diagnostic accuracy of these tests.
2. Efficacy of commonly used anthelmintics in populations of horses to inform the current status of the prevalence of anthelmintic resistance in the UK.
3. Comparison of different FECRT methodologies to determine which is the most accurate to ensure that resistance is not misclassified.
4. Risk factors associated with nematode egg excretion to inform on when to perform FEC analysis.
5. Studying the practical utility of FEC directed protocols in the field.

The derived information, along with other published research, has been used to build four decision trees which form the framework for a decision support system (DSS) to facilitate evidence-based helminth control in horses.

The findings of this thesis support targeted anthelmintic treatment strategies in horses based on FEC analysis. However, the use of FEC directed treatment protocols and the framework outlined in this thesis need to be validated under field conditions to ensure that withholding treatments is not associated with an increased risk of disease particularly if low egg shedders (LES) are not administered with MOX to target cyathostomin EL. Lack of ML treatments could also be linked to the re-emergence of *S.*

vulgaris (Nielsen et al., 2014), and this needs to considered when following a targeted treatment protocol, particularly as large strongyle eggs cannot be differentiated from small strongyle eggs during routine FEC screening and given the pathogenicity of the large strongyles, this poses a future challenge for equine parasite control.

Further research into the FEC threshold used for determining anthelmintic treatment is required. The current guide of 200 EPG is an arbitrary figure; in younger horses that tend to have higher burdens this threshold should perhaps be lowered to reduce pasture contamination and the risk of larval cyathostominosis, and, for older horses (>5 years), who generally have lower strongyle burdens the threshold could be higher especially on premised where good management practices (i.e. regular dung removal) are deployed. Because young horses are more susceptible to strongyle infection and tend to shed more eggs, animals in this category require closer attention on monitoring programmes than adult horses, particularly as horses aged less than three years have been associated with are a greater risk of larval cyathostominosis (Reid et al., 1995). Thus, FEC can be used to guide treatment options in populations of young horses, but FEC analysis needs to be performed more frequently than when dealing with populations largely comprising adult horses and the FEC threshold for treatment may need to be lowered (e.g. ≥100 EPG) to reduce the infection intensity on pasture. The use of FEC directed treatment programmes needs further study in young horses to establish an evidence basis for FEC test frequency and thresholds for treatment.

Further studies are required to examine the effect that FEC directed targeted programmes have on strongyle egg shedding and effect on pasture contamination; data should be captured to inform on testing frequency and the proportion of horses to be tested at each sampling. Ideally, this should be performed using different treatment regimes on horses with access to the same paddock and management practices. Here

(Chapter 5), practices such as quarantining new arrivals and regular removal of dung from pastures did not appear to have a significant association with low FEC shedding. Nevertheless, appropriate quarantine is an essential component of best practice control programmes and further dissemination of the importance of this is required, through education of veterinary surgeons and horse owners through scientific and lay publications and through webinars and lectures. Likewise, dung removal from pasture is considered an essential component of equine helminth control (Herd, 1986; Herd and Coles, 1995; Duncan and Love, 1991; Mathee et al., 2003; Corbett et al., 2014). Further research into how management factors fit into and complement targeted treatment protocols; in particular how the regular removal of dung impacts on levels of refugia are required.

The ability to categorise horses on the basis of an initial number of FEC tests (taking into account age, season and previous anthelmintic treatment) could act as an incentive to owners to adopt targeted programmes. However, climatic factors will influence the number of larvae that survive on pasture, so if horses are exposed to a greater parasitic challenge then their strongyle egg shedding potential may increase. The results from the egg shedding consistency analysis are encouraging, however, further research is required validate the categorisation of horses into egg shedding categories and to explore whether leaving low egg shedders (LES) untreated over several grazing seasons leads to an increase in egg shedding overtime, and to ensure that there is no increased risk of clinical disease.

FEC directed treatment strategies incur labour and financial costs, specifically in conducting FEC, and this could act as a disincentive to widespread application. Here, a cost-comparison analysis was performed to assess the financial impact of applying targeted anthelmintic treatments (Section 6.4.2). A saving was achieved on every yard

with the average saving of adopting a targeted programme equal to £294.44. Costs associated with time taken for collecting samples, administering anthelmintic, and postage costs were not included, and should be included in any future analyses. Nevertheless, these results demonstrate that an overall saving can be made by following a FEC directed targeted approach and that the more horses included in a targeted treatment programme, the greater the savings. However, the challenge remains convincing horse owners, yard managers, vets and those that prescribe anthelmintics that utilising FEC can save them money and help to reduce anthelmintic use, which will reduce the selection pressure for resistance and aid sustainable parasite control. One way of promoting the value of FEC directed a targeted approach is to publish these results in scientific and lay publications, and use social media to target appropriate groups of people. Another is to build and promote accessible, robust and validated tools, which use diagnostic FEC data and epidemiological knowledge to support decisions to treat on an evidence basis.

To support FEC directed treatment protocols, it is necessary to have laboratory services to conduct FEC analyses. In the UK, there are several commercial laboratories offering equine FEC analysis. However, at the time of writing this thesis, the majority of these labs currently employ the McMaster method with an egg detection limit of 50 EPG, which as discussed in Chapters 2 and 4 will lead to a greater proportion of horses appearing to require treatment based on a 200 EPG threshold and will lead to more misclassified FECRT results compared to when a more sensitive egg counting method is employed. The challenge now is to convince veterinarians and diagnostic laboratories to adopt more sensitive egg counting methods.

There are several further studies and areas of research to pursue that would complement and clarify the work described in this thesis, which include developing

standardised guidelines for performing FEC analysis and for performing FECRT against all equine helminth species, and to define thresholds for classifying resistance to each anthelmintic so that results can be compared across studies and regions. It is clear from the results obtained in this study that the current use of thresholds are not fit for purpose and currently they are unable to discriminate between efficacy and resistance. To aid the interpretation of FECRT data and confirm resistance, there is an urgent need to develop rapid and sensitive diagnostic tools to detect anthelmintic resistance to complement the FECRT to ensure adequate surveillance of anthelmintic resistance. Molecular-based assays offer future promise, as they will be able to detect genotypic resistance prior to the occurrence of anthelmintic failure (Kaplan, 2002). However, to date, the performance of *in vitro* assays for detecting anthelmintic resistance in equine parasites has been disappointing (Matthews et al., 2012).

As the results from the FECRT conducted here found that IVM and MOX were efficacious on all yards tested, PYR was effective on the 90% of yards tested and FBZ resistance was detected on all yards, it was not possible to determine risk factors for resistance. Further studies need to be conducted in areas where there are documented cases of ML resistance in order to accurately identify risk factors, which will help inform evidence-based control strategies.

A reduction in nematode egg reappearance period (ERP) has been proposed to be an earlier indicator of anthelmintic resistance (Sangster, 2001). ERP was not investigated here as the focus of this study was on measuring anthelmintic efficacy by the FECRT. Further investigation into strongyle ERP after anthelmintic treatment is now warranted, and surveillance of ERP should be encouraged as a part of control programmes. Further studies are also required to examine efficacy of these products against species such as *O. equi* and *P. equorum*.

A major assumption, when interpreting FECRT data and strongyle ERP is that all cyathostomin species respond to an anthelmintic in the same way. There are over 50 recognised cyathostomin species (Lichtenfels et al., 2008) and the role that individual species play in the development of resistance is largely unknown. Several studies looking into the species composition of larvae recovered from horses following treatment with IVM and MOX indicated that cyathostomin species that belong to the genus *Cylicocyclus*, predominate in cases where there is a shortened strongyle ERP (Lyons et al., 2009; van Doorn et al., 2014). This is of particular interest, and requires further investigation as knowledge of which species are contributing to any observed shortened ERP can help inform future research into molecular and *in vitro* tools for the detection of these species, so that species composition can be taken into consideration when performing efficacy testing.

Peregrine et al. (2014) propose that, even in the face of increased reports of resistance in multiple nematode species against all anthelmintic classes, there have been no formal links between anthelmintic resistance and an increase in parasite-associated disease reported in horses. They believe that this is due to a publication bias, where researchers use horses that are in good condition and reside on well-managed establishments. Furthermore, they believe that a lack of an accurate validated tool for the detection of anthelmintic resistance affects the early identification of resistance making associations between clinical cases and resistance difficult. Published data on the incidence of larval cyathostominosis or morbidity associated with *P. equorum* is limited so drawing conclusions as to the interaction of increased resistance with clinical disease is difficult (von Samson-Himmelstjerna, 2012). However, it stands to reason that if anthelmintics fail to control helminths, the resultant accumulation of parasites over time will lead to a greater probability of disease. It could be argued that

at the current time, helminth populations are being adequately controlled, despite widespread cyathostomin resistance to FBZ. Until the levels of ML resistance reaches similar levels, associations between resistance and clinical disease may not be seen. The current situation in sheep illustrates the clinical ramifications of multidrug resistance, and in some countries resistance to all anthelmintic groups and combinations is widespread (with the exception of the new anthelmintic compounds, monepantel and derquantel) (Besier and Love, 2002; Bartley et al., 2004; Waghorn et al., 2006; Kaplan and Vidyashankar, 2012; Guerden et al., 2014b). In the UK, sheep helminth populations resistant to BZ, levamisole, IVM and MOX have been reported (Bartley et al., 2004; Sargison et al., 2005, 2007), and a sheep farm was forced to close due to the failure of all anthelmintic classes to adequately control *Teladorsagia circumcincta* (Sargison et al., 2005). In small ruminant production, multidrug resistance has been reported to lead to increased mortality and morbidity and economic losses such that farm closures or culling of flocks are becoming a reality in many countries across the world (Sargison et al., 2005, 2007; Kaplan and Vidyashankar et al., 2012; Miller et al., 2012; Leathwick and Besier, 2013; Guerden et al., 2014b). Two novel anthelmintics belonging to two new anthelmintic classes have been developed and licensed for use against sheep helminths in many countries including the UK. Monepantel, an amino-acetonitrile derivative was first licensed in New Zealand in 2009 and derquantel, a spiroindole, administered in combination with abamectin (a macrocyclic lactone) was first licensed in New Zealand in 2010. Worryingly, within two years of reaching the market, reduced efficacy following monepantel administration against populations of *T. circumcincta* in New Zealand have been reported (Scott et al., 2013), further demonstrating that even with new compounds, anthelmintic resistance poses a threat to the health and welfare of animals.

Epidemiological studies into the prevalence and incidence of parasite-associated disease in horses to identify the current baseline, which could then be used an indicator for change in incidence rate and to aid surveillance are warranted and multi-site epidemiological studies to determine the impact of anthelmintic resistant helminths on equine health are required. The development and validation of tools that detect prepatent infection are needed as the larval stages of cyathostomins and *S. vulgaris* are responsible for major pathology. Therefore, being able to target these stages would help to reduce the risk of parasite-associated disease. Further, treatments could be targeted at horses with cyathostomin EL so that not all horses receive a dose of MOX in the autumn/winter as per current guidelines, thus reducing the selection pressure for MOX resistance. The cyathostomin EL ELISA under development shows promise as a useful diagnostic tool in the future (McWilliam et al., 2010; Matthews, 2014).

Sustainable equine parasite control depends on the development of recommendations for helminth control programs that are focussed on reducing anthelmintic use to aid the preservation of anthelmintic-sensitive nematode populations while recognising that the primary objective is to avoid clinical disease. The evidence currently available supports the use of FEC directed therapy (Section 5.5.3), and such a strategy is potentially cost effective (Section 6.4.2). The advantage of FEC directed programmes needs to be promoted to ensure further uptake and end users must have easy access to up-to-date knowledge (Matthews, 2014). This can be achieved through publishing in journals that are regularly read by those that prescribe anthelmintics and through educating horse owners via publishing accessible articles in specialist magazines, through social media and webinars. The development of an online decision support tool that advocates the use of appropriate diagnostic tests would provide access to

such knowledge, with the ultimate aim of specifically targeting anthelmintic treatments that are given, and perhaps, increasing levels of refugia whilst minimising the risk of parasite-associated disease.

APPENDICES

Appendix 1. Questionnaire

An Investigation of Wormer Resistance in UK Horses

(Funded by the Elise Pilkington Trust)

Yard Name:	
Yard Address:	

Name of Contact: _____

Telephone No: _____

Section 1: Basic Information

1.1 Basic function of yard:

(Please tick the boxes that apply to your yard)

Private use only	
Livery	
Competitive	
Riding school	
Racing Yard	
Stud	
Other: Please give details	

1.2 Total number of equines **permanently** residing at establishment:

_____

1.3 Of total permanent residents – how many are:

Stallions - aged 4 years or older	
Mares or Geldings - aged 4 years or older	
Youngstock – aged 1-3 years old	
Foals - aged 0-1 year-old	
Donkeys – any age	

1.3.a Of these animals – how many are kept at:

Full livery	
Working or part livery	
D.I.Y or grass livery	
Not applicable – all animals owned by proprietor	

1.4.a On average, how many horses **visit** your establishment per year (e.g. short-term loan): _____

1.4.b Of the visiting population, how frequently do they change: (please tick all that apply)

Weekly	
Monthly	
Every 3 months or seasonally	
Yearly	
Varies depending on individual horses (and/or owners)	

1.5.a Do you quarantine new arrivals?.....**YES / NO**

1.5.b Do you perform faecal egg count before deciding to worm? .....**YES / NO**

1.5.b If **YES,** which wormer do you use?

Ivermectin (e.g. Eqvalan; Eraquell; Vectin; Noromectin)	
Moxidectin (e.g. Equest)	
Benzimidazoles (e.g Panacur; Panacur 5 Day Guard; Telmin)	
Pyrantel (e.g. Strongid-P; Pyratape-P)	
Praziquantel (e.g. Equitape)	
Ivermectin/ Praziquantel (e.g. Eqvalan Duo; Equimax)	
Moxidectin/ Praziquantel (e.g. Equest Pramox)	
Other (please state):	

1.5.c If **YES**, for how long after worming are they quarantined before turnout?:

Turned out immediately	
24 hours	
48 hours	
7 days	
14 days	
Other (please state)	

Section 2: Worm control in PERMANENT Equines (aged 1 year and over)

2.1 Name and role of person in charge of wormer policy/administration:_____

2.2 How frequently are wormers administered at your establishment? (please tick one)

Every 4 weeks or less	
Every 4-6 weeks	
Every 6-8 weeks	
Every 2-6 months	
Every 6-12 months	

Less than every 12 months	

2.3 Which of these wormers have been used at your establishment during the last 12 months?

(please tick all that apply)

Ivermectin (e.g. Eqvalan; Eraquell; Vectin; Noromectin)	
Moxidectin (e.g. Equest)	
Benzimidazoles (e.g Panacur; Panacur 5 Day Guard; Telmin)	
Pyrantel (e.g. Strongid-P; Pyratape-P)	
Praziquantel (e.g. Equitape)	
Ivermectin/ Praziquantel (e.g. Eqvalan Duo; Equimax)	
Moxidectin/ Praziquantel (e.g. Equest Pramox)	
Other (please state):	

2.4 How often do you rotate between wormer **classes** (e.g. between ivermectin and pyrantel)?

(please tick all that apply)

After every application	
Every 2-3 months	
Every 6 months	
Every year	
At random/infrequently	
Never	

2.5 How often do you rotate between wormer **brands** (e.g. between Eqvalan and Noromectin)?

(please tick all that apply)

After every application	
With each change of anthelmintic class	
Every 2-3 months	
Every 6 months	

Every year	
At random/infrequently	
Never	

2.6 Reason(s) for choosing which wormer(s) to use: (please tick all that apply)

Brand name	
Veterinary advice	
Own experience	
Price	
Anthelmintic effectiveness	
The egg reappearance period	
Recommendation from associate	
Rotation between anthelmintics	
Brand loyalty	
Other (please state):	

2.7.a Do you use a weightape to estimate the wormer dose for each horse?
..... **YES / NO**

2.7.b If **NO**, how is wormer dosage calculated at your establishment? (please tick **one**)

Estimation of weight by eye	
One tube/packet of drug per animal	
Average weight of animals in a particular age group	
Average weight of all animals	
Other (please state):	

2.8 In addition to normal worming procedures; which occasions (or times of the year) do you feel are particularly important for administering wormer? (please tick all that apply)

Spring (March; April; May)	
Summer (June; July; August)	
Autumn (September; October; November)	
Winter (December; January; February)	
Prior to grazing turnout	
Prior to introduction of new yard arrivals to existing animals	
Suspicion of parasite-related illness	
Other (please state):	

2.9 Have there been any instances of parasite-related illness at your establishment? (please tick all that apply)

No sign of illness	
Diarrhoea (lasting less than one week)	
Diarrhoea (lasting between 1 and 4 weeks)	
Ill-thrift	
Colic	
Weight loss	
Worms seen in faeces	
Other (please state):	

2.9 Have you heard about wormer resistance?
..... **YES / NO**

2.10 Are you concerned about resistance at your establishment
..... **YES / NO**

2.11 If **YES** have you spoken to your vet or SQP about resistance?
..... **YES / NO**

2.12 If **YES**, how would you rate their advice? (please tick those that apply)

Poor	
Satisfactory	
Excellent	

Other:	

Secti
on 3: Worm control in Foals and Youngstock (please ignore if not applicable)

3.1 At what age are foals **first** treated with wormer:
_____

3.2.a Are foals/youngstock treated differently from practices stated in **Section 2**?.....**YES / NO**

3.2.b If **YES**, how are they treated and with which wormer(s): (please state)

Section 4: Grazing and Pasture Management

4.0 Approximately what area of grazable land do you have at your establishment?

_____

4.2.a Do you poopick?
..... **YES / NO**

4.2.b If **YES,** do you poopick manually or use a machine? (please tick one)

Manually	
Machine	

4.3 At your establishment do you ever: (Please tick those that apply)

	YES	NO
Rotate grazing between groups of equines		
Rest paddocks from grazing		
Graze with animals other than equines (e.g. sheep and cattle)		
Harrow and/or clip		

Section 5: Faecal egg counts

5.1.a Are faecal egg counts carried out on your establishment?.....**YES / NO**

5.1.b If **YES,** where do you get the faecal egg counts performed?

Perform it yourself	
Veterinary Surgery	
Online service	

5.1.c If **YES**, how frequently are they carried out? (please tick all that apply)

Monthly OR every 2-3 months	
Every six months	
Yearly	
Under suspicion of parasite-related illness	
During Quarantine	
Infrequently OR at random	

5.1.d If you perform FECs do you: (Please tick the box that applies)

Treat all horses regardless of FEC	
Treat all with a positive FEC	
Treat horses with a FEC of 200 epg or greater	
Other:	

5.2.a Do you know what a faecal egg count reduction test is?.....**YES / NO**

5.2.b If **YES**, have you had one carried out in the last 5 years?.....**YES / NO**

5.2.c If **YES**, which wormer(s) were investigated?:

Wormer	**Date**
Ivermectin (e.g. Eqvalan; Eraquell; Vectin; Noromectin)	
Moxidectin (e.g. Equest)	
Benzimidazoles (e.g Panacur; Panacur 5 Day Guard; Telmin)	
Pyrantel (e.g. Strongid-P; Pyratape-P)	
Praziquantel (e.g. Equitape)	
Ivermectin/ Praziquantel (e.g. Eqvalan Duo; Equimax)	
Moxidectin/ Praziquantel (e.g. Equest Pramox	
Other (please state):	

5.2.d If **YES**, what did the results of the FECRT show? (Please tick the boxes that apply)

Wormer	**Susceptible**	**Resistant**
Ivermectin		
Moxidectin		
Benzimidazoles		
Pyrantel		
Other (please state):		

5.3 Which wormer did you last use and on what date did you administer it?

Wormer	**Date**
Ivermectin (e.g. Eqvalan; Eraquell; Vectin; Noromectin)	
Moxidectin (e.g. Equest)	
Benzimidazoles (e.g Panacur; Panacur 5 Day Guard; Telmin)	
Pyrantel (e.g. Strongid-P; Pyratape-P)	
Praziquantel (e.g. Equitape)	
Ivermectin/ Praziquantel (e.g. Eqvalan Duo; Equimax)	

Moxidectin/ Praziquantel (e.g. Equest Pramox	
Other (please state):	

Completed by: _____(please print name)
Date:_____

If you have any specific questions regarding this questionnaire, feel free to email me at: hannah.lester@moredun.ac.uk, or telephone: (0131) 44 55 111 (extension 7479/7465)

Thank you for taking the time to complete this questionnaire

Please return to: Hannah Lester, Division of Parasitology, Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik, near Edinburgh, EH26 0PZ

or

hannah.lester@moredun.ac.uk

Appendix 2 – Population distribution data from Chapter 5

Appendix 2a. The distribution of age according to the last anthelmintic administered, the year, month and season in which the sample was collected, the country and region where the horse resided, whether or not quarantine measures were implemented on the yard, anthelmintic treatment frequency, the grazing area for each horse and whether or not the pasture was poopicked.

		No. Yards	Adults	Foals	Geriatric	Youngster	Total
Last anthelmintic	IVM	6	142	18	25	25	210
	MOX	20	366	12	97	44	519
	PYR	1	9	1	0	5	15
Year	2011	11	274	21	64	40	399
	2012	16	243	10	58	34	345
Month	MAR	3	52	5	17	4	78
	APR	7	86	6	37	22	151
	MAY	6	126	0	15	12	153
	JUN	3	51	0	12	10	73
	JUL	3	113	13	31	19	176
	AUG	4	77	7	3	6	93
	SEP	1	12	0	7	1	20
Season	Spring	16	264	11	69	38	382
	Summer	10	241	20	46	35	342
	Autumn	1	12	0	7	1	20
Country	England	16	243	10	58	34	345
	Scotland	11	274	21	64	40	399
Region	SE England	13	199	5	49	19	272
	SW England	3	44	5	9	15	73
	NE Scotland	1	41	12	4	12	69
	SE Scotland	9	223	9	56	24	312
	SW Scotland	1	10	0	4	4	18
Quarantine	Y	16	321	29	70	50	470
	N	11	196	2	52	24	274
Treatment frequency	2 times/year	18	342	31	64	57	494
	4 times/year	6	103	0	40	10	153
	FEC	3	72	0	18	77	167
Grazing area	<1 acre/horse	4	41	6	5	13	65
	1 - 1.9 acres/horse	14	289	19	75	32	415
	>2 acres/horse	3	36	5	8	13	62
	NA	5	151	1	34	15	201
Poopick	Y	3	398	29	96	57	580
	N	24	119	2	26	17	164

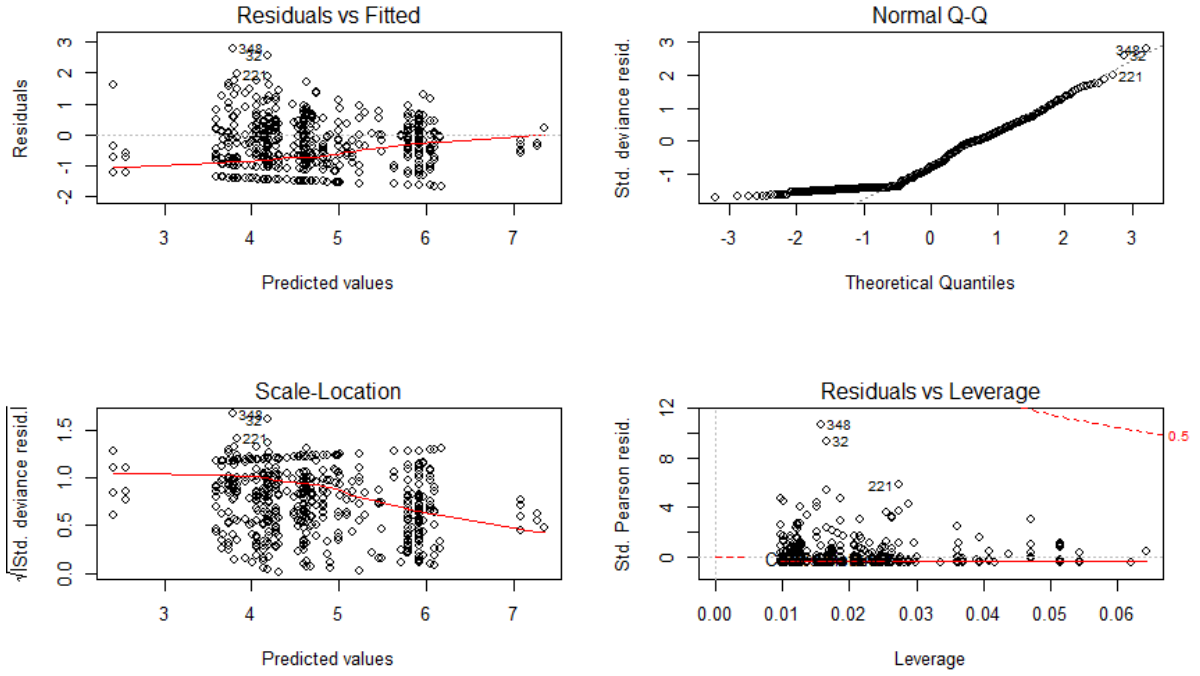
Appendix 2b. Distribution of horses according to yard type by age, last anthelmintic, the year, month and season that the samples were taken, country and region, whether quarantine measures and poopicking were employed, anthelmintic treatment frequency and grazing area

		Yard type				
		CTN	DLR	LVY	STD	STY
	No. Yards	3	1	17	2	4
	No. Horses	49	14	468	36	177
Age	Adults	44	11	338	21	103
	Foals	0	0	8	5	18
	Geriatric	0	1	91	4	26
	Youngster	5	2	31	6	30
Last anthelmintic	5FBZ	0	0	24	0	0
	IVM	14	0	28	36	132
	MOX	35	14	401	0	45
	PYR	0	0	15	0	0
Year	2011	0	0	249	18	132
	2012	49	14	219	18	45
Month	March	0	0	60	18	0
	April	0	0	120	0	31
	May	9	0	129	0	14
	June	25	0	30	18	0
	July	0	0	44	0	132
	August	14	14	65	0	0
	September	0	0	20	0	0
Season	Spring	10	0	309	18	45
	Summer	39	14	139	18	132
	Autumn	0	0	20	0	0
Country	England	49	14	219	18	45
	Scotland	0	0	249	18	132
Region	SE England	39	14	187	18	14
	SW England	10	0	32	0	31
	NE Scotland	0	0	0	0	69
	SE Scotland	0	0	249	0	63
	SW Scotland	0	0	0	18	0
Quarantine	Y	39	0	299	18	114
	N	10	14	169	18	63
Treatment Frequency	2 times/year	24	14	261	18	177
	4 times/year	0	0	135	18	0
	FEC	25	0	72	0	0
Grazing area	<1 acre/horse	0	14	0	18	0
	1 to 1.9 acres/horse	22	0	288	0	69
	>2 acres/horse	0	0	0	0	31
	NA	27	0	180	18	77

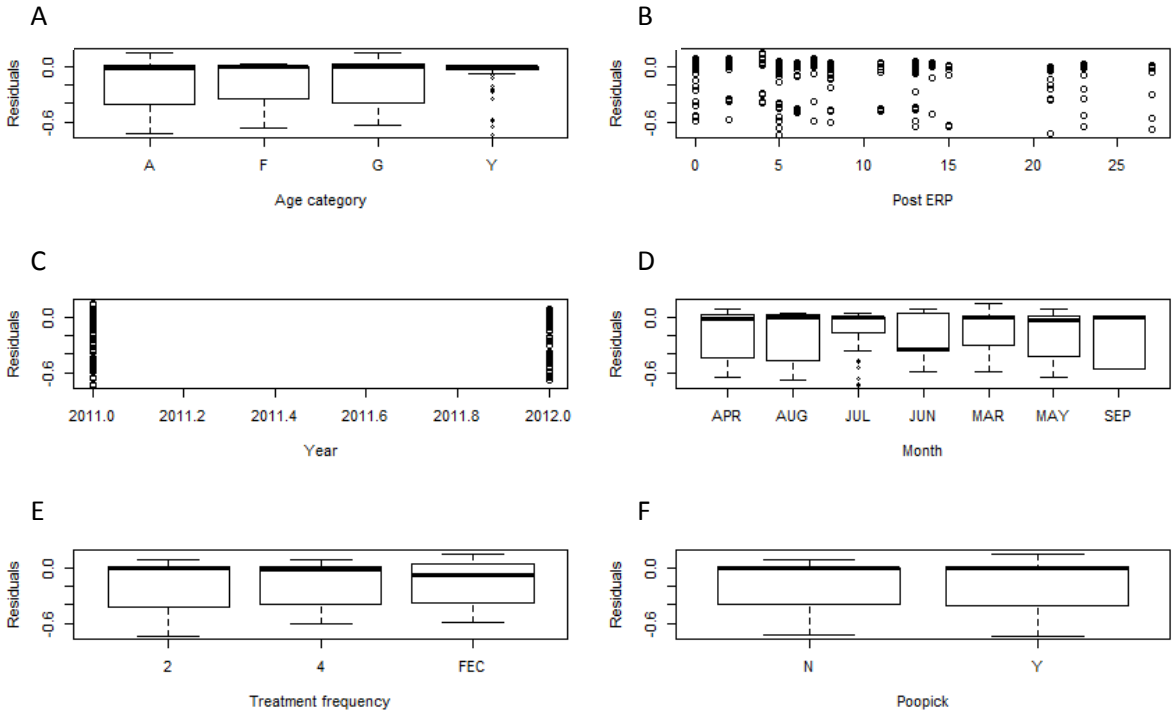
Poopick	Y	49	14	367	36	114
	N	0	0	101	0	63

Appendix 3 - Residuals from negative binomial generalised linear mixed (NBGLMM) model

Appendix 3a. Residuals and model checking from the final generalise linear mixed model (GLMM). A. Plot of model residuals plotted against the fitted values to evaluate the constancy of variance. B. Normal quantile-quantile plot to check that the errors are normally distributed. C. Plot of the square root of the standardised residuals. D. Plot of the standardised residuals as a function of leverage, along with Cook's distance to highlight any *y* values that have the biggest effect on parameter estimates.



Appendix 3b. Model residuals for each significant term retained in the final GLMM model. A. Residuals for age category. B. Residuals for ERP. C. Residuals for month. D. Residuals for year. E. Residuals for treatment frequency. F. Residuals for poopicking.



References

Abbott, J.B., Barrett, E.J., 2008. The problem of diagnosing tapeworm infections in horses. Equine Veterinary Journal 40, 5-6.

Allison, K., Taylor, N.M., Wilsmore, A.J., Garforth, C., 2011. Equine anthelmintics: survey of the patterns of use, beliefs and attitudes among horse owners in the UK. Veterinary Record.168, 483.

American Association of Equine Practitioners (AAEP). 2013. Parasite control guidelines. Available at: http://www.aaep.org/custdocs/ParasiteControlGuidelinesFinal.pdf

Andersen, U.V., Howe, D.K., Olsen, S.N., Nielsen, M.K., 2013. Recent advances in diagnosing pathogenic equine gastrointestinal helminths: The challenge of prepatent detection. Veterinary Parasitology 192, 1-9.

Anderson, R.M., May, R.M., 1978. Regulation and Stability of Host-Parasite Population Interactions: I. Regulatory Processes. Journal of Animal Ecology 47, 219-247.

Anderson, N . 1990. The challenge of endoparasitism: developments in the control of nematode infections of sheep. Proceedings of the New Zealand Society of Animal Production, 50: 215–227.

Arundel J.H. 1985. Parasitic diseases of the horse: Veterinary Review University of Sydney Postgraduate Foundation in Veterinary Science. 1985, 28-150.

Bain, S. K., Kelly, J. D. 1977. Prevalence and pathogenicity of *Anoplocephala perfoliata* in a horse population in South Auckland. New Zealand Veterinary Journal, 25, 27-28.

Bairden, K., Brown, S.R., McGoldrick, J., Parker, L.D., Talty, P.J., 2001. Efficac of moxidectin 2 per cent gel against naturally acquired strongyle infections in horses, with particular reference to larval cyathostomes. Veterinary Record 148, 138-141.

Bairden, K., Davies, H.S., Gibson, N.R., Hood, A.J., Parker, L.D., 2006a. Efficacy of moxidectin 2 per cent oral gel against cyathostomins, particularly third-stage inhibited larvae, in horses. Veterinary Record 158, 766-767.

Bairden, K., Davies, H.S., Gibson, N.R., Hood, A.J.O., Parker, L.D., 2006b. Efficacy of moxidectin 2 per cent oral gel against cyathostomins, particularly third-stage inhibited larvae, in horses. Veterinary Record 158, 766-768.

Barclay, W. P., Phillips, T. N., Foemer, J.J.1982. Intussusceptions associated with *Anoplocephala perfoliata* infection in five horses. Journal American Veterinary Medicine Association 180, 752-753.

Barger, I.A. 1999. The role of epidemiological knowledge and grazing management for helminth control in small ruminants. International Journal for Parasitology 29, 41–47.

Bartley, D.J., Jackson, F., Jackson, E., Sargison, N. 2004. Characterisation of two triple resistant field isolates of Teladorsagia from Scottish lowland sheep farms. Veterinary Parasitology 123, 189-199.

Bartley, D., Elsheika H.M. 2011. Essentials of Veterinary Parasitology. Horizon Scientific Press. 143-148.

Bashkirova, E.J. 1941. Study of biology of *Anoplocephala perfoliata* (Goeze,1782)—cestode helminth of the horse. Proc. Academic Science USSR 30 (6), 570–572 (in Russian).

Bates, D., Maechler, M., Bolker, B. 2015. lme4: Linear-mixed Effects Models Using'Eigen' and S4. Retrieved from: https://cran.r-project.org/web/packages/lme4/lme4.pdf

Beaver, P.C. 1950. The standardization of faecal smears for estimating egg producion and worm burden. Journal of Parasitology 36, 451-456.
Becher, A.M., Mahling, M., Nielsen, M.K., Pfister, K., 2010. Selective anthelmintic therapy of horses in the Federal states of Bavaria (Germany) and Salzburg (Austria): An investigation into strongyle egg shedding consistency. Veterinary Parasitology 171, 116-122.
Beech, J. 1979. Diseases of the lung. Veterinary Clinic North America (Large Animal Practice), 1 pp. 149–169.
Bernard, F., van Doorne, D. 2010. Natural dewormer does not do the trick in donkeys. Proceedings; European Veterinary Conference. p342.
Beroza, G. A., Barclay, W. P., Phillips, T. N., Foemer, J. J. and Donawick, W. J. 1983. Caecal perforation and peritonitis associated with *Anoplocephala perfoliata* infections in three horses. Journal American Veterinary Medicine Association 183, 804-806.
Besier, R.B., Love, S.C.J . 2003. Anthelmintic resistance in sheep nematodes in Australia: the need for new approaches. Australian Journal of Experimental Agriculture, 43, 1383–1391
Bewick, V., Cheek, L., Ball, J., 2005. Statistics review 14: Logistic regression. Critical care (London, England) 9, 112-118.
Blanek, M., Brady, H.A., Nichols, W.T., Hutcheson, D.P., Jackson, S.P., Pond, K.R., Wilson, K.R., 2006. Investigation of anthelmintic resistance and deworming regimens in horses The Professional Animal Scientist 22, 346-352.
Bliss, C.I., Fisher, R.A., 1953. Fitting the Negative Binomial Distribution to Biological Data. Biometrics 9, 176-200.
Bodecek, S., Jahn, P., Dobesova, O., Vavrouchova, E. 2010. Equine cyathostomosis: case reports. Veterinarni Medicina 55, 187-193.
Boersema, J.H., Borgsteede, F.H.M., Eysker, M., Saedt, I. 1995. The reappearance of strongyle eggs in feces of horses treated with pyrantel embonate. Veterinary Quarterly 17, 18 – 20.
Boersema, J.H., Eysker, M., Maas, J., van der Aar, W.M. 1996. Comparison of the reappearance of strongyle eggs in foals, yearlings, and adult horses after treatment with ivermectin or pyrantel. Veterinary Quarterly 18, 7 – 9.
Boersema, J.H., Eysker, M., Nas, J.W. 2002. Apparent resistance of *Parascaris equorum* to macrocylic lactones. Veterinary Record 150, 279-281.
Bolker, B.M. 2008. Ecological Models and Data in R. Princeton University Press.
Bollinger, O. 1870. Die kolik der Pferde und das Wurmaneurysma der Eingeweidearterien. Akad. Wiss. Munchen Sitzber. 1, 539–544.
Borji, H., Azizzadeh, M., Kameli, M. 2012. A retrospective study of abattoir condemnation due to parasitic infections: economic importance in Ahwaz, Southeastern Iran. Journal of Parasitology 98, 954-957.
Borgsteede, F.H., Boersma, J.H., Gaasenbeek, C.P., van der Burg, W.P. 1993. The reappearance of eggs in faeces of horses after treatment with ivermectin. Veterinary Quarterly 15, 24-26.
Boxell, A.C., Gibson, K.T., Hobbs, R.P., Thompson, R.C.A. 2004. Occurrence of gastrointestinal parasites in horses in metropolitan Perth, Western Australia. Australian Veterinary Journal 82, 91–95.
Boyle, A.G., Houston, R. 2006. Parasitic Pneumonitis and Treatment in Horses. Clinical Techniques in Equine Practice 5, 225-232.
Brady, H.A., Nichols, W.T. 2009. Drug Resistance in Equine Parasites: An Emerging Global Problem. Journal of Equine Veterinary Science 29, 285-295.

Brazik, E.L., Luquire, J.T., Little, D. 2006. Pyrantel pamoate resistance in horses receiving daily administration of pyrantel tartrate. Journal of American Veterinary Medicine Association 228, 101-103.
Brunsdon , R.V. 1980. Principles of helminth control. Veterinary Parasitology 6, 185–215.
Burks, B.S. 1998. Parasitic pneumonitis in horses. Continuing Education in Practice Veterinary 20, 378–383.
Burns, G.N., Karcher, L.F., Cummings, J.F. 1990. Equine myenteric ganglionitis: a case of chronic intestinal pseudo-obstruction. Cornell Veterinary 80, 53–63.
Cabaret, J., Berrag, B. 2004. Faecal egg count reduction test for assessing anthelmintic efficacy: average versus individually based estimations. Veterinary Parasitology 121, 105-113.
Calabrese J.M., Brunner J.L., Ostfeld R.S. 2011. Partitioning the Aggregation of Parasites on Hosts into Intrinsic and Extrinsic Components via an Extended Poisson-Gamma Mixture Model. *PLoS ONE* 6(12): e29215. doi:10.1371/journal.pone.0029215.
Calvete, C., Uriarte, J. 2013. Improving the detection of anthelmintic resistance: Evaluation of faecal egg count reduction test procedures suitable for farm routines. Veterinary Parasitology 196, 438-452.
Canever, R.J., Braga, P.R.C., Boeckh, A., Grycajuck, M., Bier, D., Molento, M.B. 2013. Lack of Cyathostomin sp. reduction after anthelmintic treatment in horses in Brazil. Veterinary Parasitology 194, 35-39.
Cawthome, R.J.G., Whitehead, J.D. 1983. Isolation of benzimidazole resistant strains of *Ostertagia circumcincta* from British sheep. Veterinary Record 112, 274–277.
Chandler, K.J., Collins, M.C., Love, S. 2000. Efficacy of a five-day course of fenbendazole in benzimidazole-resistant cyathostomes. Veterinary Record 147, 661-662.
Chandler, K.J., Love, S. 2002. Patterns of equine faecal egg counts following spring dosing with either fenbendazole or moxidectin. Veterinary Record 151, 269-270.
Chowaniec, W., Darski, J. 1970. Investigations on excretion time of liver fluke eggs after killing the parasite Bulletin of the Veterinary Institute of Pulawy 14, 108–110.
Christie, M., Jackson, F. 1982. Specific identification of strongyle eggs in small samples of sheep faeces. Research in Veterinary Science 32, 113-117.
Clayton, H. 1986. Ascarids: recent advances. Veterinary Clinics of North America: Equine Practice 2, 313 - 328.
Clayton, H.M., Duncan, J.L. 1977. Experimental Parascaris equorum infection of foals. Research in Veterinary Science 23, 109-114.
Clayton, H.M., Duncan, J.L. 1979a. The development of immunity to Parascaris equorum infection in the foal. Research in Veterinary Science 26, 383-384.
Clayton, H.M., Duncan, J.L. 1979b. The migration and development of Parascaris equorum in the horse. International Journal of Parasitology 9, 285-292.
Clayton, H.M., Duncan, J.L. 1981. Natural infection with *Dictyocaulus arnfieldi* in pony and donkey foals. Research in Veterinary Science 31, 278-280.
Clements, R.J.M., McNeilly, T.M., Pickles, K.J., Keen, J.A., Matthews, J.B. 2012. Evaluation of rectal mast cell responses as a novel method to estimate equine cyathostomin burdens. Journal of Equine Veterinary Science 32, S32.
Cobb, R., Boeckh, A. 2009. Moxidectin: a review of chemistry, pharmacokinetics and use in horses. Parasites and Vectors 2 Suppl 2, S5.
Coles, G., Bauer, C., Borgsteede, F., Geerts, S., Klei, T., Taylor, M., Waller, P. 1992. World Association for Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. Veterinary Parasitology 44, 35 - 44.

Coles, G.C., Roush, R.T. 1992. Slowing the spread of anthelmintic resistant nematodes of sheep and goats in the United Kingdom. Veterinary Record 130, 505–510.

Coles, G.C., Hillyer, M.H., Taylor, F.G., Villard, I. 2003, Efficacy of an ivermectin-praziquantel combination in equids against bots and tapeworms. Veterinary Record 152, 178-179.

Coles, G.C., Jackson, F., Pomroy, W.E., Prichard, R.K., von Samson-Himmelstjerna, G., Silvestre, A., Taylor, M.A., Vercruysse, J. 2006. The detection of anthelmintic resistance in nematodes of veterinary importance. Veterinary Parasitology 136, 167-185.

Colglazier, M.L., Enzie, F.D., Kates, K.C. 1977. Critical anthelmintic trials in ponies with four benzimidazoles: mebendazole, cambendazole, fenbendazole, and albendazole. Journal of Parasitology 63, 724-727.

Collobert-Laugier, C., Hoste, H., Sevin, C., Chartier, C., Dorchies, P. 2002. Mast cell and eosinophil mucosal responses in the large intestine of horses naturally infected with cyathostomes. Veterinary Parasitology 107, 251-264.

Comer, K., Hillyer, M., Coles, G. 2006. Anthelmintic use and resistance on thoroughbred training yards in the UK. Veterinary Record 158, 596 - 598.

Corbett, C., Love, S., Moore, A., Burden, F., Matthews, J., Denwood, M. 2014. The effectiveness of faecal removal methods of pasture management to control the cyathostomin burden of donkeys. Parasites and Vectors 7, 48.

Cornwell, R.L., Jones, R.M. 1968. Critical tests in horses with anthelmintic pyrantel tartrate. Veterinary Record 82, 483-484.

Cosgrove J.S., Sheeran J.J., Sainty T.J. 1986. Intussusception associated with infection with *Anoplocephala perfoliata* in a two year old Thoroughbred. Irish Veterinary Journal 40, 35–36.

Craig, T.M., Bowen, J.M., Ludwig, K.G. 1983. Transmission of equine cyathostomes (Strongylidae) in central Texas. American Journal of Veterinary Research 44, 1867–1869.

Craig, T., Diamond, P., Ferwerda, N., Thompson, J. 2007. Evidence if ivermectin resistance by *Parascaris equorum* on a Texas horse farm. Journal of Equine Veterinary Science 27, 67 - 71.

Craven, J., Bjorn, H., Henriksen, S., Nansen, P., Larsen, M., Lendal, S. 1998. Survey of anthelmintic resistance on Danish horse farms, using 5 different methods of calculating faecal egg count reduction. Equine Vet Journal 30, 289 - 293.

Crawley, M.J. 2012. Generalized Linear Models, In: The R Book. John Wiley & Sons, Ltd, pp. 557-578.

Cringoli, G., Rinaldi, L., Veneziano, V., Capelli, G., Scala, A. 2004. The influence of flotation solution, sample dilution and the choice of McMaster slide area (volume) on the reliability of the McMaster technique in estimating the faecal egg counts of gastrointestinal strongyles and *Dicrocoelium dendriticum* in sheep. Veternary Parasitolology 123, 121-131.

Crofton, H.D. 1971. A model of host–parasite relationships. Parasitology 63, 343-364.

Cully, D.F., Vassilatis, D.K., Liu, K.K., Paress, P.S., van der Ploeg, L.H., Schaeffer, J.M., Arena, J.P. 1994. Cloning of an avermectin-sensitive glutamate-gated chloride channel from *Caenorhabditis elegans.* Nature 371, 707–711.

Davidson, A.J., Hodgkinson, J.E., Proudman, C.J., Matthews, J.B. 2005. Cytokine responses to Cyathostominae larvae in the equine large intestinal wall. Research in Veterinary Science 78, 169-176.

Demeulenaere, D., Vercruysse, J., Dorny, P., Claerebout, E. 1997. Comparative studies of ivermectin and moxidectin in the control of naturally acquired cyathostome infections in horses. Veterinary Record 15, 383–386.

Denwood, M., Reid, S., Love, S., Nielsen, M., Matthews, L., McKendrick, I., Innocent, G. 2010. Comparison of three alternative methods for analysis of equine faecal Egg count reduction test data. Preventative Veterinary Medicine 93, 316 - 323.

Denwood, M.J., Love, S., Innocent, G.T., Matthews, L., McKendrick, I.J., Hillary, N., Smith, A., Reid, S.W.J. 2012. Quantifying the sources of variability in equine faecal egg counts: Implications for improving the utility of the method. Veterinary Parasitology 188, 120-126.

Deprez, P., Vercruysse, J. 2003. Treatment and follow-up of clinical cyathostominosis in horses. Journal of Veterinary Medicine and Physiology Patholology Clinical Medicine 50, 527-529.

DiPietro, J.A., Lock, T.F., Todd, K.S. *and* Davis, J.L. 1989. Efficacy of ivermectin in the treatment of induced *Parascaris equorum* infection in pony foals. Journal of American Veterinary Medicine Association 195, 1712–1714.

DiPietro, J.A., Hutchens, D.E., Lock, T.F., Walker, K., Pau, A.J., Shipley, C., Rulli, D. 1997. Clinical trial of moxidectin oral gel in horses. Veterinary Parasitology 72, 167–177.

Dobson, R.J., Hosking, B.C., Jacobson, C.L., Cotter, J.L., Besier, R.B., Stein, P.A., Reid, S.A. 2012. Preserving new anthelmintics: A simple method for estimating faecal egg count reduction test (FECRT) confidence limits when efficacy and/or nematode aggregation is high. Veterinary Parasitology 186, 79-92.

Dobson, R.J., Sangster, N.C., Besier, R.B., Woodgate, R.G. 2009. Geometric means provide a biased efficacy result when conducting a faecal egg count reduction test (FECRT). Veterinary Parasitology 161, 162-167.

Dopfer, D., Kerssens, C., Meijer, Y., Boersema, J., Eysker, M., 2004. Shedding consistency of strongyle-type eggs in Dutch boarding horses. Veterinary Parasitology 124, 249 - 258.

Dowdall, S.M., Matthews, J.B., Mair, T., Murphy, D., Love, S., Proudman, C.J. 2002. Antigen-specific IgG(T) responses in natural and experimental cyathostominae infection in horses. Veterinary Parasitology 106, 225-242.

Dowdall, S.M., Proudman, C.J., Klei, T.R., Mair, T., Matthews, J.B. 2004. Characterisation of IgG(T) serum antibody responses to two larval antigen complexes in horses naturally- or experimentally-infected with cyathostomins. International Journal for Parasitology 34, 101-108.

Dowdall, S.M.J., Proudman, C.J., Love, S., Klei, T.R., Matthews, J.B. 2003. Purification and analyses of the specificity of two putative diagnostic antigens for larval cyathostomin infection in horses. Research in Veterinary Science 75, 223-229.

Drogemuller, M., Beelitz, P., Pfister, K., Schnieder, T., Samson-Himmelstjerna, G.v. 2004. Amplification of ribosomal DNA of Anoplocephalidae: *Anoplocephala perfoliata* diagnosis by PCR as a possible alternative to coprological methods. Veterinary Parasitology 124, 205-215.

Drudge, J.H., Lyons, E.T. 1966. Control of internal parasites of the horse. Journal of the American Veterinary Medicine Association 148, 378-383.

Drudge, J.H., Szanto, J., Wyant, Z.N., Elam, G. 1963. Critical tests of thiabendazole as an anthelmintic in the horse. American Journal of Veterinary Research 24, 1217–1222.

Drudge, J.H., Elam, G.W. 1961. Preliminary observations on the resistance of horse strongyles to phenothiazine, Journal of Parasitology 47 (suppl.), 38–39.

Drudge, J., Lyons, E., Tolliver, S., Kubis, J. 1979. Critical tests and clinical trials on oxibendazole in horses with special reference to removal of *Parascaris equorum*. American Journal of Veterinary Research 40, 758 - 761.

Drudge, J.H., Lyons, E.T., Tolliver, S.C. 1979, Benzimidazole resistance of equine stronygles--critical tests of six compounds against population B. American Journal of Veterinary Research 40, 590-594.
Drudge, J.H., Lyons, E.T., Tolliver, S.C., Kubis, J.E. 1982. Pyrantel in horses– clinical trials with emphasis on a paste formulation and activity onbenzimidazole resistant small strongyles. Veterinary Medicine Small Animal Clinician 77, 956–967.
du Toit, N., McGorum, B.C., Pemberton, A.D., Brown, J., Dacre, K.J. 2007. The involvement of mast cells and mast cell proteinases in the intestinal response to equine cyathostomin infection. Veterinary Immunology and Immunopathology 115, 35-42.
Duncan, J. L., Pirie, H. M. 1972. The life cycle of *Strongylus vulgaris* in horses. Research in Veterinary Science 13(4), 374-379.
Duncan, J.L. 1974a. Field studies on the epidemiology of mixed strongyle infection in the horse. Veterinary Record 94, 337-345.
Duncan, J.L. 1974b. S*trongylus vulgaris* infection in the horse. Veterinary Record 95, 34-37.
Duncan, J.L., McBeath, D.G., Preston, N.K. 1980. Studies on the efficacy of fenbendazole used in a divided dosage regime against strongyle infections in ponies. Equine Veterinary Journal 12, 78-80.
Duncan, J.L. 1985. Internal parasites of the horse and their control. Equine Veterinary Journal 17, 79-82.
Duncan, J.L., Bairden, K., Abbott, E.M. 1998. Elimination of mucosal cyathostome larvae by five daily treatments with fenbendazole. Veterinary Record 142, 268-271.
Duncan, J.L., Love, S. 1991. Preliminary observations on an alternative strategy for the control of horse strongyles. Equine Veterinary Journal 23, 226-228.
Duncan, J.L., Abbott, E.M., Arundel, J.H., Eysker, M., Klei, T.R., Krecek, R.C., Lyons, E.T., Reinemeyer, C., Slocombe, J.O.D., 2002. World association for the advancement of veterinary parasitology (WAAVP): second edition of guidelines for evaluating the efficacy of equine anthelmintics. Veterinary Parasitology 103, 1-18.
Dunn, A.M. 1978. Veterinary Helminthology, 2nd edn. Butler and Tanner, London.
Dunn, A., Keymer, A., 1986. Factors affecting the reliability of the McMaster technique. Journal of Helminthology 60, 260-262
Durham, A., Coles, G. 2010. Possible resistance in equine pinworms. Veterinary Record 16, 913–914.
Earle, C., Kington, H., Coles, G. 2002. Helminth control used by trainers of thoroughbreds in England. Veterinary Record 150, 405 - 408.
Efron, B. 1979. Bootstrap methods: another look at the jackknife. The Annals of Statistics 7, 1-26.
Efron, B. Tibshirani, R., 1986. Bootstrap Methods for Standard Errors, Confidence Intervals, and Other Measures of Statistical Accuracy. 54-75.
El-Abdellati, A., Charlier, J., Geldhof, P., Levecke, B., Demeler, J., von Samson-Himmelstjerna, G., Claerebout, E., Vercruysse, J. 2010. The use of a simplified faecal egg count reduction test for assessing anthelmintic efficacy on Belgian and German cattle farms. Veterinary Parasitology 169, 352-357.
Elsheikha, H., Khan, N.A. 2011. Essentials of Veterinary Parasitology. Caister Academic Press.
Elston, D.A., Moss, R., Boulinier, T., Arrowsmith, C., Lambin, X. 2001. Analysis of aggregation, a worked example: numbers of ticks on red grouse chicks. Parasitology 122, 563-569.

English, A. 1979. The epidemiology of equine strongylosis in Southern Queensland 1. The Bionomics of the free-living stages in faeces and on pasture. Australian Veterinary Journal 55, 299 - 305.
Enna, S.J., Gallagher, J.P. 1983. Biochemical and electrophysiological characteristics of mammalian GABA receptors. International Review of Neurobiology 24, 181-212.
Enigk, K. 1934. Die Wiederstandsfa¨higkeit der Entwicklungsstadien der Strongyliden ausserhalb des Wirtstieres. Archiv fu¨ r Tierheilkunde 67, 363–376.
Enigk, K. 1949. Zur Biologie und Bekämpfung von *Oxyuris equi*. Z. Tropenmed.
Enigk, K. 1950. Zur Entwicklung von *Strongylus vulgaris* (nematodes) im Wirtsier. Zeitschrift fur Tropmenmedizin un Parasitologie, 2; 287-306 [English translation in Cornell Vet. (1973) 63, 223-246.
Eysker, M., Boersema, J.H., Kooyman, F.N.J. 1990. Seasonally inhibited development of Cyathostomine nematodes in Shetland ponies in the Netherlands. Veterinary Parasitology 36, 264–269.
Eysker, M., Jansen, J., Mirck, M.H. 1984. Inhibited development of cyathostominae in the horse in the early third stage. Research in Veterinary Science 37, 355-356.
Fairweather, I., McShane, D.D., Shaw, L., Ellison, S.E., O'Hagan, N.T., York, E.A., Trudgett, A., Brennan, G.P. 2012. Development of an egg hatch assay for the diagnosis of triclabendazole resistance in *Fasciola hepatica*: Proof of concept. Veterinary Parasitology 183, 249-259.
Fallon, P.G., Sturrock, R.F., Niang, A.C., Doenhoff, M.J. 1995. Short report: diminished susceptibility to praziquantel in a Senegal isolate of *Schistosoma mansoni.* American Journal of Tropical Medicine and Hygiene 53, 61-62.
FDA. 1997. U.S Food and Drug Administration. NADA 141-087 Quest™ moxidectin 2% Equine Oral Gel – original approval. July 11, 1997. Retrieved November 11, 2012 from www.fda.gov/AnimalVeterinaryProducts/ApprovedAnimalDrugProducts/FOIADrugSummaries/ucm117065.htm.
FDA. 2003. U.S Food and Drug Administration. Supplement to NADA 141-087 Freedom of Information Summary. Quest™ (moxidectin) Gel. May 29, 2003. Retrieved November 11, 2012 from…….. http://www.fda.gov/downloads/AnimalVeterinary/Products/ApprovedAnimalDrugProducts/FOIADrugSummaries/ucm117074.pdf
Fisher, M., Jacobs, D., Grimshaw, W., Gibbons, L. 1992. Prevalence of benzimidazole-resistance in equine cyathostome populations in south east England. Veterinary Record 130, 315-318.
Flanagan, A.M., Edgar, H.W.J., Forster, F., Gordon, A., Hanna, R.E.B., McCoy, M., Brennan, G.P., Fairweather, I. 2011. Standardisation of a coproantigen reduction test (CRT) protocol for the diagnosis of resistance to triclabendazole in *Fasciola hepatica*. Veterinary Parasitology 176, 34-42.
Fritzen, B., Rohn, K., Schnieder, T., Von Samson-Himmelstjerna, G. 2010. Endoparasite control management on horse farms – lessons from worm prevalence and questionnaire data. Equine Veterinary Journal 42, 79-83.
Gaba, S., Ginot, V., Caberet, J. 2005. Modelling macroparasite aggregation using a nematode-sheep system: the Weibull distribution as an alternative to the Negative Binomial distribution? Parasitology 131, 393-401.
Gasbarre, L.C., Leighton, E.A., Bryant, D. 1996. Reliability of a single fecal egg per gram determination as a measure of individual and herd values for trichostrongyle nematodes of cattle. American Journal of Veterinary Research 57, 168-171.
Gates, M.C., Nolan, T.J. 2009. Endoparasite prevalence and recurrence across different age groups of dogs and cats. Veterinary Parasitology 166, 153-158.

Gawor, J.J. 1996. Occurrence of *Parascaris equorum* in foals and adult horses under different breeding conditions. Wiadomosci parazytologiczne 42, 213-219.
Geenen, P.L., van der Gaag, L.C. 2005. Developing Bayesian neteoks for clinical diagnosis in veterinary medicine: from individual to the herd. Computing Science. Accessed via: http://www.computingscience.nl/groups/DSS/publications/construction/pigs-modelling.pdf
Geurden, T., Hoste, H., Jacquiet, P., Traversa, D., Sotiraki, S., Frangipane di Regalbono, A., Tzanidakis, N., Kostopoulou, D., Gaillac, C., Privat, S., Giangaspero, A., Zanardello, C., Noé, L., Vanimisetti, B., Bartram, D. 2014a. Anthelmintic resistance and multidrug resistance in sheep gastro-intestinal nematodes in France, Greece and Italy. Veterinary Parasitology 201, 59-66.
Geurden, T., van Doorn, D., Claerebout, E., Kooyman, F., De Keersmaecker, S., Vercruysse, J., Besognet, B., Vanimisetti, B., di Regalbono, A.F., Beraldo, P., Di Cesare, A., Traversa, D. 2014b. Decreased strongyle egg re-appearance period after treatment with ivermectin and moxidectin in horses in Belgium, Italy and The Netherlands. Veterinary Parasitology 204, 291-296.
Gibson, T. E. 1953. The effect of repeated anthelmintic treatment with phenothiazine on the faecal egg counts of housed horses, with some observations on the life cycle of *Trichonema* spp. in the horse. Journal of Helminthology 27, 29–40.
Gibson, T. 1960. Some experiences with small daily doses of phenothiazine as a means of control of strongylid worms in the horse. Veterinary Record 72, 37 - 41.
Giles, C.J., Urquhart, K.A., Longstaffe, J.A. 1985. Larval cyathostomiasis (immature trichonema-induced enteropathy): A report of 15 clinical cases. Equine Veterinary Journal 17, 196-201.
Gilleard, J.S., Beech, R.N. 2007, Population genetics of anthelmintic resistance in parasitic nematodes. Parasitology 134, 1133-1147.
Gomez, H.H., Georgi, J.R. 1991. Equine helminth infections: control by selective chemotherapy. Equine Veterinary Journal 23, 198-200.
Gordon, H.M., Whitlock, H.V. 1939. A new technique for counting nematode eggs on sheep faeces. Journal of Scientific and Industrial Research 12, 50-52.
Gordon, D.K., Zadoks, R.N., Stevenson, H., Sargison, N.D., Skuce, P.J., 2012. On farm evaluation of the coproantigen ELISA and coproantigen reduction test in Scottish sheep naturally infected with *Fasciola hepatica*. Veterinary Parasitology 187, 436-444.
Gordon, D.K., Roberts, L.C.P., Lean, N., Zadoks, R.N., Sargison, N.D., Skuce, P.J. 2013. Identification of the rumen fluke, *Calicoporon daubneyi*, in GB livestock: possible implications for liver fluke diagnosis. Veterinary Parasitology 195, 65-71.
Greer, G.J., Bello, T.R., Ambroski, G.F. 1974. Experimental infection of *Strongyloides westeri* in parasite-free ponies. Journal of Parasitology 60, 466-472.
Grenfell, B.T., Wilson, K., Isham, V.S., Boyd, H.E.G., Dietz, K. 1995. Modelling patterns of parasite aggregation in natural populations: trichostrongylid nematode–ruminant interactions as a case study. Parasitology 111, S135-S151.
Gronvold, J. 1991. Laboratory diagnoses of helminths common routine methods used in Denmark. In: Seminars on Parasitic Problems in Farm Animals Related to Fodder Production and Management, Eds: P. Nansen, J. Gronvold and H. Bjorn, The Estonian Academy of Sciences, Tartu, Estonia. pp 47-48.
Habermann, R.T., Harwood, P.D., Hunt, W.H. 1941. Critical tests with phenothiazine as an anthelmintic in horses North American Veterinary Journal 22, 85–92.
Hall, M.C. 1917. Notes in regard to bots, *Gastrophilus* spp. Journal American Veterinary Medicine Association 52, 177–184.

Hampshire, V.A., Doddy, F.M., Post, L.O., Koogler, T.L., Burgess, T.M., Batten, P.O., Hudson, R., McAdams, D.R., Brown, M.A. 2004. Adverse drug event reports at the United States Food and Drug Administration Center for Veterinary Medicine. Journal American Veterinary Medicine Association 225, 533-536.

Hasslinger, M.A. 1990. Oxyuriasis. Sonderdruck HandlexikonTierärztlichen Praxis 186, 639.

Hayes, P.W. 1994. Moxidectin: understanding the unique persistant anthelmintic activity of this second generation macrocyclic lactone. In: Australian Veterinarians in Industry, Australian Veterinary Association, 1994 Annual Conference, Canberra, Australia.

Henriksen, S.A., Aagaard, K.A. 1976. A simple flotation and McMaster method. Nordic Veterinary Medicine 28, 392-397.

Hearn, F.P., Peregrine, A.S., 2003. Identification of foals infected with *Parascaris equorum* apparently resistant to ivermectin. Journal American Veteterinary Medicine Assocociation 223, 482-485.

Herd, R.P., Miller, T.B., Gabel, A.A. 1981. A field evaluation of pro-benzimidazole, benzimidazole, and non-benzimidazole anthelmintics in horses. Journal of American Veterinary Medicine Associaton 179, 686-691.

Herd, R.P., Willardson, K.L. 1985. Seasonal distribution of infective strongyle larvae on horse pastures. Equine Veterinary Journal 17, 235-237.

Herd, R.P. 1986. Epidemiology and control of equine strongylosis at Newmarket. Equine Veterinary Journal 18, 447-452.

Herd, R., Gabel, A. 1990. Reduced efficacy of anthelmintics in young compared with adult horses. Equine Veterinary Journal 22, 164 - 169.

Herd, R.P. 1990a. Equine parasite control - Problems associated with intensive anthelmintic therapy. Equine Veterinary Education 2, 41-47.

Herd, R.P. 1990b. Equine parasite control - solutions to anthelmintic associated problems. Equine Veterinary Education 2, 86-91.

Herd, R. 1993. Control strategies for ruminant and equine parasites to counter resistance, encystment, and ecotoxicity in the USA. Veterinary Parasitology 48, 327 - 336.

Herd, R., Coles, G. 1995. Slowing the spread of anthelmintic resistant nematodes of horses in the United Kingdom. Veterinary Record 136, 481 - 485.

Hertzberg, H., Schwarzwald, C.C., Grimm, F., Frey, C.F., Gottstein, B., Gerber, V. 2014. Helminth control in the adult horse: the need for a re-orientation. Schweizer Archiv für Tierheilkunde., 156, 61–70.

Hilborn, R., Mangel, M. 1997. The ecological detective: confronting models with data. Princeton University Press.

Hillyer, M.H., Mair, T.S. 1997. Recurrent colic in the mature horse: A retrospective review of 58 cases. Equine Veterinary Journal 29, 421-424.

Hinney, B., Wirtherle, N., Kyule, M., Miethe, N., Zessin, K.-H., Clausen, P.H. 2011. Prevalence of helminths in horses in the state of Brandenburg, Germany. Parasitology Research 108, 1083-1091.

Hoglund, J., Ljungstrom, B.L., Nilsson, O., Lundquist, H., Osterman, E., Uggla, A. 1997. Occurrence of *Gasterophilus intestinalis* and some parasitic nematodes of horses in Sweden. Acta Veterinaria Scandinavica 38, 157-165.

Holden-Dye, L., Walker, R.J. 1990. Avermectin and avermectin derivatives are antagonists at the 4-aminobutyric acid (GABA) receptor on the somatic cells of *Ascaris*; is this the site of anthelmintic action? Parasitology 101, 265–272.

Hood GM. 2010. PopTools version 3.2.5. Available at http://www.cse.csiro.au/poptools.

Hosmer, D.W., Lemeshow, S., 2005. Assessing the Fit of the Model, In: Applied Logistic Regression. John Wiley & Sons, Inc., pp. 143-202.
Houe, H., Ersbøll, A.K., Toft, N., landbohøjskole, K.V.-o. 2004. Introduction to Veterinary Epidemiology. Biofolia.
Hughes, P.L., Dowling, A.F., Callinan, A.P.L. 2007. Resistance to macrocyclic lactone anthelmintics and associated risk factors on sheep farms in the lower North Island of New Zealand. New Zealand Veterinary Journal, 55, 177–183.
Hummelinck, P.W. 1946. Investigation of the eggs of horse strongyles. Tijdschrift voor Diergeneeskunde 71, 411–427.
Ihler, C.F., Rootwelt, V., Heyeraas, A., Dolvik, N.I. 1995. The prevalence and epidemiology of *Anoplocephala perfoliata* infection in Norway. Veterinary Research Communications 19, 487-494.
Ismail, M., Metwally, A., Farghaly, A., Bruce, J., Tao, L.F., Bennett, J.L. 1996. Characterization of isolates of *Schistosoma mansoni* from Egyptian villagers that tolerate high doses of praziquantel. American Journal of Tropical Medicine and Hygiene 55, 214-218.
Jackson, F., Coop, R.L. 2000.The development of anthelmintic resistance in sheep nematodes. Parasitology 120 Suppl, S95-107.
Jacobs, D. E. 1986. Tapeworm infection - *Anoplocephala* spp. A colour atlas of equine parasites. Gower Publishing Ltd, London. pp. 5-16.
Jell, K., Barnes, T., Seddon, J. M., Coleman, G. T., Morton, J., Kotze, A. 2008. The Use of ZeroInflated Modelling to Identify High and Low Gastrointestinal Nematode Egg Shedders in Groups of Horses. In Proc. Australian Equine Science Symposium volume 2, p. 51.
Johnson, P.J., Mrad, D.R., Schwartz, A.J., Kellam, L. 1999. Presumed moxidectin toxicosis in three foals. Journal of American Veterinary Medicine Associaton 214, 678-680.
Kania, S.A., Reinemeyer, C.R., 2005. *Anoplocephala perfoliata* coproantigen detection: a preliminary study. Veterinary Parasitology 127, 115-119.
Kaplan, R. 2002. Anthelmintic resistance in nematodes of horses. Veterinary Research 33, 491 - 507.
Kaplan, R. 2004a. Drug resistance in nematodes of veterinary importance: a status report. Trends in Parasitology 20, 477 - 481.
Kaplan, R., Klei, T., Lyons, E., Lester, G., Courtney, C., French, D., Tolliver, S., Vidyashankar, A., Zhao, Y. 2004b. Prevalence of anthelmintic resistant cyathostomes on horse farms. Journal American Veterinary Medicine Assocociation 225, 903-910.
Kaplan, R., Reinemeyer, C., Slocombe, J., Murray, M. 2006. Confirmation of ivermectin resistance in a purportedly resistant Canadian isolate of *Parascaris equorum* in foals. Proceedings of the American Association of Veterinary Parasitologists, 51st Annual Meeting, 15-18 July, 2006, 69 - 70.
Kaplan, R.M., Nielsen, M.K. 2010. An evidence-based approach to equine parasite control: It ain't the 60s anymore. Equine Veterinary Education 22, 306-316.
Kaplan, R.M., Vidyashankar, A.N. 2012. An inconvenient truth: Global worming and anthelmintic resistance. Veterinary Parasitology 186, 70-78.
Kassai, T. 1999. Veterinary Helminthology, Butterworth-Heinemann, Oxford.
Kearney, A. 1974. *Fasciola hepatica* in equines as a reservoir host on hill and marginal land. Proc. 3rd Int. Congr. Parasit. Munich. 1, 511-512.
Kenyon, F., Sargison, N.D., Skuce, P.J., Jackson, F. 2009. Sheep helminth parasitic disease in south-eastern Scotland arising as a possible consequence of climate change. Veterinary Parasitology 163, 293-297.

King, A., Love, S., Duncan, J. 1990. Field investigation of anthelmintic resistance of small strongyles in horses. Veterinary Record 127, 232-233.
Kjaer, L.N., Lungholt, M.M., Nielsen, M.K., Olsen, S.N., Maddox-Hyttel, C. 2007. Interpretation of serum antibody response to *Anoplocephala perfoliata* in relation to parasite burden and faecal egg count. Equine Veterinary Journal 39, 529-533.
Klei, T., Chapman, M. 1993. Evaluation of ivermectin at an elevated dose against encysted equine cyathostome larvae. Veterinary Parasitology 47, 99 - 106.
Klei, T.R., Chapman, M.R. 1999. Immunity in equine cyathostome infections. Veterinary Parasitology 85, 123-136.
Kornas, S., Skalska, M., Nowosad, B. 2006. Occurrence of roundworm (*Parascaris equorum*) in horses from small farms based on necropsy. Wiadomosci parazytologiczne 52, 323-326.
Krauth, S.J., Coulibaly, J.T., Knopp, S., Traoré, M., N'Goran, E.K., Utzinger, J. 2012. An In-Depth Analysis of a Piece of Shit: Distribution of Schistosoma mansoni and Hookworm Eggs in Human Stool. PLoS Neglected Tropical Diseases 6, e1969.
Krecek, R.N., Guthrie, A.J., Nieuwenhuizen, L.V., Booth, L.M. 1994. A comparison between the effects of conventional and selective antiparasitic treatments on nematode parasites of horses from two management schemes. Journal of South African Veterinary Association 65, 97–100.
Kuhnert-Paul, Y., Schmäschke, R., Daugschies, A. 2012. Einfluss der Verteilung von Strongyliden- und Ascarideneiern in Pferdekotproben auf den Nachweis mit einem kombinierten Sedimentations-Flotations-Verfahren. Tierarztliche Praxis Groaytiere 40, 21-26.
Kuzmina, T., Dzeverin, I., Karchenko, V. 2012. Fecundity of various species of strongylids (Nematoda: Strongylidae) parasites of domestic horses. Parasitology Research 111, 2265-2271
Lacey, E. 1990. Mode of action of benzimidazoles. Parasitol Today 6, 112-115.
Larsen, M.L., Ritz, C., Petersen, S.L., Nielsen, M.K. 2011. Determination of ivermectin efficacy against cyathostomins and *Parascaris equorum* on horse farms using selective therapy. The Veterinary Journal 188, 44-47.
Laugier, C., Sevin, C., Ménard, S., Maillard, K. 2012. Prevalence of *Parascaris equorum* infection in foals on French stud farms and first report of ivermectin-resistant *P. equorum* populations in France. Veterinary Parasitology 188, 185-189.
Lawrence , K.E., Rhodes , A.P., Jackson, R., Leathwick, D.M., Heuer, C., Pomroy, W.E., West, D.M., Waghorn, T.S., Moffat, J.R. 2006. Farm management practices associated with macrocyclic lactone resistance on sheep farms in New Zealand. *New* Zealand Veterinary Journal 54, 283–288.
Le Jambre, L.E. 1978. The Epidemiology and Control of Gastrointestinal Parasites of Sheep in Australia, Edited by: Donald, A.D., Southcott, W.H., Dineen, JK. 109–120. Melbourne, Australia: CSIRO.
Le Jambre, L.F., Dominik, S., Eady, S.J., Henshall, J.M., Colditz, I.G., 2007. Adjusting worm egg counts for faecal moisture in sheep. Veterinary Parasitology 145, 108-115.
Leathwick, D.M., Vlassoff, A., Barlow, N.D. 1995. A model for nematodiasis in New Zealand lambs: The influence of drenching and grazing management on the development of anthelmintic resistance. International Journal for Parasitology 25, 1479–1491.
Leathwick, D.M., Miller, C.M., Atkinson, D.S., Haack, N.A., Alexander, R.A., Oliver, A.M., Waghorn, T.S., Potter, J.F., Sutherland, I.A. 2006a. Drenching adult ewes: implications of anthelmintic treatments pre- and post-lambing on the

development of anthelmintic resistance. New Zealand Veterinary Journal 54, 297-304.
Leathwick, D.M., Waghorn, T.S., Miller, C.M., Atkinson, D.S., Haack, N.A., Oliver, A.M. 2006b. Selective and on-demand drenching of lambs: impact on parasite populations and performance of lambs. New Zealand Veterinary Journal 54, 305-312.
Lee, D.L., Tatchell, R.J. 1964. Studies on the tapeworm *Anoplocephala perfoliata* (GOEZE, 1782). Parasitology 54, 467-479.
Leland, S.E., Drudge, J.H., Wyant, Z.N., Elam, G.W. 1961. Studies on *Trichostrongylus axei* (Cobbold, 1879). VII. Some quantitative and pathologic aspects of natural and experimental infections in the horse. American Journal Veterinary Research 22, 128-138.
Lele, S.R. 2009. A new method for estimation of resource selection probability function. Journal for Wildlife Management 73, 122-127.
Lester, H., Bartley, D., Morgan, E., Hodgkinson, J., Stratford, C., Matthews, J. 2012. A comparison of three methods for calculating anthelmintic efficacy in equids. Journal of Equine Veterinary Science 32, S34-S35.
Lester, H.E., Matthews, J.B. 2014. Faecal worm egg count analysis for targeting anthelmintic treatment in horses: Points to consider. Equine Veterinary Journal 46, 139-145.
Levecke, B., Dobson, R.J., Speybroeck, N., Vercruysse, J., Charlier, J. 2012a. Novel insights in the faecal egg count reduction test for monitoring drug efficacy against gastrointestinal nematodes of veterinary importance. Veterinary Parasitology 188, 391-396.
Levecke, B., Rinaldi, L., Charlier, J., Maurelli, M.P., Bosco, A., Vercruysse, J., Cringoli, G. 2012b. The bias, accuracy and precision of faecal egg count reduction test results in cattle using McMaster, Cornell-Wisconsin and FLOTAC egg counting methods. Veterinary Parasitology 188, 194-199.
Lichtenfels, J.R., Kharchenko, V.A., Dvojnos, G.M. 2008. Illustrated identification keys to strongylid parasites (strongylidae: Nematoda) of horses, zebras and asses (Equidae). Veterinary Parasitology 156, 4-161.
Lind, E.O., HÖglund, J., LjungstrÖm, B.L., Nilsson, O., Uggla, A. 1999. A field survey on the distribution of strongyle infections of horses in Sweden and factors affecting faecal egg counts. Equine Veterinary Journal 31, 68-72.
Little, D., Flowers, J., Hammerberg, B., Gardner, S. 2003. Management of drug-resistant cyathostominosis on a breeding farm in central North Carolina. Equine Veterinary Journal 35, 246 - 251.
Lloyd-Smith, J.O. 2007. Maximum Likelihood Estimation of the Negative Binomial Dispersion Parameter for Highly Overdispersed Data, with Applications to Infectious Diseases. PloS one 2, e180.
Lloyd, S., 2009. Effects of previous control programmes on the proportion of horses shedding small numbers of strongyle-type eggs. Veterinary Record 164, 108-111.
Love, S., McKeand, J. 1997. Cyathostominosis: practical issue of treatment and control. Equine Veterinary Education 9, 253 - 256.
Love, S., Murphy, D., Mellor, D. 1999. Pathogenicity of cyathostome infection. Veterinary Parasitology 85, 113-121.
Lucker, J.T. 1941a. Survival and development at low temperatures of eggs and preinfective larvae of horse strongyles. Journal of Agricultural Research 63, 193–218.
Lucker, J.T. 1941b. Notes on the survival of infective horse strongyle larvae. In: Proceedings of the Helminthological Society of Washington, vol. 8, pp. 11–13.

Lucas, P.J., van der Gaag, L.C, Abu-Hanna, A. 2004. Bayesian networks in biomedicine and health-care. Artificial Intelligence in Medicine 30, 201-214.

Ludwig, K.G. 1982. Dynamics of intestinal parasite transmission from mare to foal. Master's thesis. College Station. Texas A & M University.

Lyons, E.T., Drudge, J.H., Tolliver, S.C. 1973. On the life cycle of Strongyloides westeri in the equine. Journal of Parasitology 59, 780-787.

Lyons, E.T., Drudge, J.H., Tolliver, S.C., Breukink, H.J. 1974. Critical test of three salts of pyrantel against internal parasites of the horse. American Journal of Veterinary Research 35, 1515-1522.

Lyons, E.T., Drudge, J.H., Tolliver, S.C. 1975. Field tests of three salts ofpyrantel [hydrochloride, tartrate, pamoate] against internal parasitesof the horse [*Strongylus vulgaris*, *Parascaris equorum*, *Strongylus edentatus*]. American Journal Veterinary Research 36, 161–166.

Lyons, E., Tolliver, S., Drudge, J. 1999. Historical perspective of cyathostomes: prevalence, treatment and control programs. Veterinary Parasitology 85, 97 - 111.

Lyons, E.T., Drudge, J.H., Tolliver, S.C. 2000. Larval cyathostomiasis. The Veterinary clinics of North America. Equine practice 16, 501-513.

Lyons, E., Tolliver, S., Ionita, M., Lewellen, A., Collins, S. 2008. Field studies indicating reduced activity of ivermectin on small strongyles in horses on a farm in Central Kentucky. Parasitology Research 103, 209 - 215

Lyons, E.T., Tolliver, S.C., Collins, S.S. 2009. Probable reason why small strongyle EPG counts are returning "early" after ivermectin treatment of horses on a farm in Central Kentucky. Parasitology Research 104, 569-574.

MacKay, R.J., Urquhart, K.A. 1979. An outbreak of eosinophil bronchitis in horses possibly associated with *Dictyocaulus arnfieldi* infection. Equine Veterinary Journal 11, 110–112.

MAFF. 1986. Manual of Veterinary Parasitological Laboratory Techniques. Her Majesty's Stationery Office, London, UK.

Mair, T.S. 1993. Recurrent diarrhoea in aged ponies associated with larval cyathostomiasis. Equine Veterinary Journal 25, 161-163.

Mair, T., Divers, T., Ducharme, N. 2002. Manual of Equine Gastroenterology. W.B Saunders.

Malan, F.S., Reinecke, R.K., Scialdo, R.C. 1981. Anthelmintic efficacy fenbendazole paste in equines. Journal South African Veterinary Association 52, 127-130.

Martin , P.J., Anderson, N., Jarrett, R.G. 1985. Resistance to benzimidazole anthelmintics in field strains of *Ostertagia* and *Nematodirus* in sheep. Australian Veterinary Journal 62, 38–43.

Martin, P.J., Le Jambre, L.F., Claxton, J.H. 1981. The impact of refugia on the development of thiabendazole resistance in *Haemonchus contortus*. International Journal for Parasitology 11, 35-41.

Martin, P.J. 1989. Selection for thiabendazole resistance in *Ostertagia* spp. by low efficiency anthelmintic treatment. International Journal for Parasitology, 19, 317–325

Martin, R.J., Robertson, A.P., Wolstenholme, A.J. 2002. Mode of action of the macrocyclic lactones. *In:* Macrocyclic Lactones in Antiparasitic Therapy, *Eds:* J. Vercruysse *and* R.S.Rew, *CABI Publishing, New York. pp* 125–140.

Martin, T.G., Wintle, B.A., Rhodes, J.R., Kuhnert, P.M., Field, S.A., Low-Choy, S.J., Tyre, A.J., Possingham, H.P. 2005. Zero tolerance ecology: improving ecological inference by modelling the source of zero observations. Ecology letters 8, 1235-1246.

Matthee, S. 2003. Anthelmintic treatment in horses: the extra-label use of products and the danger of under-dosing. Journal of South African Veterinary Association 74, 53-56.
Matthee, S., McGeoch, M.A. 2004. Helminths in horses: use of selective treatment for the control of strongyles. Journal of South African Veterinary Association. 75, 129–136.
Matthews, J. 2008. An update on cyathostomins: Anthelmintic resistance and worm control. Equine Veterinary Education 20, 552-560.
Matthews, J.B. 2011. Facing the threat of equine parasitic disease. Equine Veterinary Journal 43, 126-132.
Matthews, J.B., McArthur, C., Robinson, A., Jackson, F. 2012. The in vitro diagnosis of anthelmintic resistance in cyathostomins. Veterinary Parasitology 185, 25-31.
Matthews, J., Burden, F. 2013. Common helminth infections of donkeys and their control in temperate regions. Equine Veterinary Education 25, 461 - 467.
Matthews, J.B. 2014. The future of helminth control in horses. Equine Veterinary Journal 46, 10-11.
Mathieson, A. O. 1964 A study into the distribution of, and tissue responses associated with, some internal parasites of the horse. Thesis, University of Edinburgh.
McCaughey, W.J., Hatch, C. 1964. Routine faecal examination for the detection of fluke (*Fasciola hepatica*) eggs. Irish Veterinary Journal 18, 181–187.
McCraw, B.M., Slocombe, J.O. 1974. Early development of and pathology associated with *Strongylus edentatus*. Canadian journal of comparative medicine. Revue canadienne de medecine comparee 38, 124-138.
McKenna, P.B. 1990. The detection of anthelmintic resistance by the faecal egg count reduction test: an examination of some of the factors affecting performance and interpretation. New Zealand Veterinary Journal 38, 142-147.
McWilliam, H.E., Nisbet, A.J., Dowdall, S.M., Hodgkinson, J.E., Matthews, J.B. 2010. Identification and characterisation of an immunodiagnostic marker for cyathostomin developing stage larvae. International Journal for Parasitology 40, 265-275.
Meana, A., Luzon, M., Corchero, J., Gómez-Bautista, M. 1998. Reliability of coprological diagnosis of *Anoplocephala perfoliata* infection. Veterinary Parasitology 74, 79-83.
Medica, D.L., Sukhdeo, M.V.K.,1997. Role of lipids in the transmission of the infective stage (L3) of *Strongylus vulgaris* (Nematoda: Strongylida). Journal of Parasitology 83, 775–779.
Menzel, M., Becher, A.M., Reist, M., Pfister, K. 2012. Implementation of selective anthelmintic treatment in an equine practice in Upper Bavaria (Germany): 1st experiences. Journal of Equine Veterinary Science 32, S35-S36.
Mercier, P., Chick, B., Alves-Branco, F., White, C.R. 2001. Comparative efficacy, persistent effect, and treatment intervals of anthelmintic pastes in naturally infected horses. Veterinary Parasitology 99, 29-39.
Mes, T.H.M. 2003. Technical variability and required sample size of helminth egg isolation procedures. Veterinary Parasitology 115, 311-320.
Mezo, M., Gonzalez-Warleta, M., Carro, C., Uberia, F.M. 2004. An ultrasensitive capture ELISA for detection of *Fasciola hepatica* coproantigens in sheep and cattle using a new monoclonal antibody (MM3). Journal of Parasitology 90, 845-852.
Mfitilodze, M.W., Hutchinson, G.W. 1987. Development and survival of free-living stages of equine strongyles under laboratory conditions. Veterinary Parasitology 23, 121-133.

Mfitilodze, M.W., Hutchinson, G.W. 1988. Development of free-living stages of equine strongyles in faeces on pasture in a tropical environment. Veterinary Parasitology 26, 285-296.
Michel , J.F . 1985. Strategies for the use of anthelmintics in livestock and their implications for the development of drug resistance. Parasitology 90, 621–628.
Miller, Wm. C. 1953. The general problem of parasitic infestation in horses. Veterinary Record 65, 213-217.
Miller, C.M., Waghorn, T.S., Leathwick, D.M., Gilmour, M.L. 2006. How repeatable is a faecal egg count reduction test? New Zealand Veterinary Journal 54, 323-328.
Mitchell M. J. 1979. Energetics of oribatid mites (Atari: Cryptostigmata) in an aspen woodland soil. Pedobiologia 19, 89-98.
Mitchell, G. 2002. Update on fasciolosis in cattle and sheep. In Practice 24, 378-385.
Molento, M., Antunes, J., Bentes, R. 2008. Anthelmintic resistance in Brazilian horses. Veterinary Record 162, 384 - 385.
Monahan, C., Chapman, M., French, D., Taylor, H., Klei, T. 1995. Dose titration of moxidectin oral gel against gastrointestinal parasites of ponies. Veterinary Parasitology 59, 241 - 248.
Monahan, C., Chapman, M., Taylor, H., French, D., Klei, T. 1996. Comparison of moxidectin oral gel and ivermectin oral paste against a spectrum of internal parasites of ponies with special attention to encysted cyathostome larvae. Veterinary Parasitology 63, 225 - 235.
Monahan, C.M., Klei, T.R. 2002. Macrocyclic Lactones in Antiparasitic Therapy. CABI Publishing, New York.
Morgan, E.R., Cavill, L., Curry, G.E., Wood, R.M., Mitchell, E.S.E. 2005. Effects of aggregation and sample size on composite faecal egg counts in sheep. Veterinary Parasitology 131, 79-87.
Morrill, A., Forbes, M.R., 2012. Random parasite encounters coupled with condition-linked immunity of hosts generate parasite aggregation. International Journal for Parasitology 42, 701-706.
Morrison, D.A. 2004. Technical variability and required sample size of helminth egg isolation procedures: revisited. Parasitology Research 94, 361-366.
Muiño, L., Perteguer, M.J., Gárate, T., Martínez-Sernández, V., Beltrán, A., Romarís, F., Mezo, M., González-Warleta, M., Ubeira, F.M. 2011. Molecular and immunological characterization of Fasciola antigens recognized by the MM3 monoclonal antibody. Molecular and Biochemical Parasitology 179, 80-90.
Muller, J.M., Feige, K., Kastner, S.B., Naegeli, H. 2005. The use of sarmazenil in the treatment of a moxidectin intoxication in a foal. Journal of Veterinary Internal Medicine 19, 348-349.
Murphy, D., Love, S. 1997. The pathogenic effects of experimental cyathostome infections in ponies. Veterinary Parasitology 70, 99-110.
Murphy, T.M., Fahy, K.N., McAuliffe, A., Forbes, A.B., Clegg, T.A., O'Brien, D.J. 2006. A study of helminth parasites in culled cows from Ireland. Preventative Veterinary Medicine 76, 1-10.
Nansen, P., Andersen, S., Hesselholt, M. 1975. Experimental infection of the horse with *Fasciola hepatica*. Experimental Parasitology 37, 15-19.
Neal, M.J. 2002. Medical Pharmacology at a Glance, 4th edn. Blackwell Publishing, Malden. p 89.
Nielsen, M., Haaning, N., Olsen, S. 2006. Strongyle egg shedding consistency in horses on farms using selective therapy in Denmark. Veterinary Parasitology 135, 333 - 335.

Nielsen, M., Kaplan, R., Thamsborg, S., Monrad, J., Olsen, S. 2007. Climatic influences on development and survival of free-living stages of equine strongyles: implications for worm control strategies and managing anthelmintic resistance. Veterinary Journal 174, 23-32.

Nielsen, M., Vidyashankar, A., Olsen, S., Monrad, J., Thamsborg, S. 2012a. *Strongylus vulgaris* associated with usage of selective therapy on Danish horse farms - Is it reemerging? Veterinary Parasitology 189, 260-266.

Nielsen, M.K. 2012b. Sustainable equine parasite control: Perspectives and research needs. Veterinary Parasitology 185, 32-44.

Nielsen, M.K., Baptiste, K.E., Tolliver, S.C., Collins, S.S., Lyons, E.T. 2010a. Analysis of multiyear studies in horses in Kentucky to ascertain whether counts of eggs and larvae per gram of feces are reliable indicators of numbers of strongyles and ascarids present. Veterinary Parasitology 174, 77-84.

Nielsen, M.K., Vidyashankar, A.N., Andersen, U.V., DeLisi, K., Pilegaard, K., Kaplan, R.M. 2010b. Effects of fecal collection and storage factors on strongylid egg counts in horses. Veterinary Parasitology 167, 55-61.

Nielsen, M.K., Vidyashankar, A.N., Hanlon, B.M., Diao, G., Petersen, S.L., Kaplan, R.M. 2013. Hierarchical model for evaluating pyrantel efficacy against strongyle parasites in horses. Veterinary Parasitology 197, 614-622.

Nielsen, M.K., Pfister, K., von Samson-Himmelstjerna, G. 2014a. Selective therapy in equine parasite control—Application and limitations. Veterinary Parasitology 202, 95-103.

Nielsen, M.K., Reinemeyer, C.R., Donecker, J.M., Leathwick, D.M., Marchiondo, A.A., Kaplan, R.M. 2014b. Anthelmintic resistance in equine parasites—Current evidence and knowledge gaps. Veterinary Parasitology 204, 55-63.

Nilsson, O., Ljungstrom, B.L., Hoglund, J., Lundquist, H., Uggla, A. 1995. *Anoplocephala perfoliata* in horses in Sweden: prevalence, infection levels and intestinal lesions. Acta Vet Scand 36, 319-328.

O'Meara, B., Mulcahy, G. 2002. A survey of helminth control practices in equine establishments in Ireland. Veterinary Parasitology 109, 101-110.

Ober-Blo¨baum, W. von. 1932. Untersuchungen u¨ber die Einwirkungenphysikalischer Einflu¨ sse auf die Larven von Pferdestrongyliden.Tiera¨rtzliche Rundschau 47, 812–815.

Ogbourne, C.P. 1972. Observations on the free-living stages of strongylid nematodes of the horse. Parasitology 64, 461-477.

Ogbourne, C., 1973. Survival on herbage plots of infective larvae of strongylid nematodes of the horse. Journal of Helminthology 47, 9 - 16.

Ogbourne, C.P. 1975. Epidemiological studies on horses infected with nematodes of the family trichonematidae (Witenberg, 1925). International Journal for Parasitology 5, 667–672.

Ogbourne, C.P. 1976. The prevalence, relative abundance and site distribution of nematodes of the subfamily Cyathostominae in horses killed in Britain. Journal of Helminthololology 50, 203-214.

Oju, J.P.E., Mpoame, M. 2006. Periodic Release of Gastrointestinal Helminth Eggs in Native Chickens from Dschang in the Western Highlands of Cameroon. Veterinary Research Communications 30, 39-43.

Olaechea, F., Lovera, V., Larroza, M., Raffo, F., Cabrera, R. 2011. Resistance of *Fasciola hepatica* against triclabendazole in cattle in Patagonia (Argentina). Veterinary Parasitology 178, 364-366.

Osterman Lind, E., Kuzmina, T., Uggla, A., Waller, P., Hoglund, J. 2007. A field study on the effect of some anthelmintics on cyathostomins of horses in Sweden. Veterinary Research Communication 31, 53 - 65.
Owen, J.M. 1977. Liver fluke infection in horses and ponies. Equine Veterinary Journal 9, 29-31.
Owen, R., Jagger, D. W. and Quan-Taylor, R. 1989. Caecal intussusceptions in horses and the significance of *Anoplocephala perfoliata*. Veterinary Record 124, 34-37.
Pankhurst, J.W. 1963. Liver fluke in donkeys. Veterinary Record 75, 434.
Pavone, S., Veronesi, F., Genchi, C., Fioretti, D.P., Brianti, E., Mandara, M.T. 2011. Pathological changes caused by *Anoplocephala perfoliata* in the mucosa/submucosa and in the enteric nervous system of equine ileocecal junction. Veterinary Parasitology 176, 43-52.
Pereckienė, A., Kaziūnaitė, V., Vyšniauskas, A., Petkevičius, S., Malakauskas, A., Šarkūnas, M., Taylor, M.A. 2007. A comparison of modifications of the McMaster method for the enumeration of *Ascaris suum* eggs in pig faecal samples. Veterinary Parasitology 149, 111-116.
Peregrine, A.S., McEwen, B., Bienzle, D., Koch, T.G., Weese, J.S. 2006. Larval cyathostominosis in horses in Ontario: an emerging disease? The Canadian veterinary journal. La revue veterinaire canadienne 47, 80-82.
Peregrine, A.S., Molento, M.B., Kaplan, R.M., Nielsen, M.K. 2014. Anthelmintic resistance in important parasites of horses: Does it really matter? Veterinary Parasitology 201, 1-8.
Perez, 1999. Comparison of the pharmacokinetics of moxidectin (EQUEST) and ivermectin (EQVALAN) in horses. JVPT 22, 174 - 180.
Phillips, T.N., Kolviet, A. 1958. A case of equine diarrhoea associated with heavy parenteral *Strongylus edentatus* infection. Illinois Veterinary Journal 1, 37-39.
Pickles, K.J., Mair, J.A., Lopez-Villalobos, N., Shaw, D.J., Scott, I., Pomroy, W. 2010. Large intestinal mast cell count and proteinase expression is associated with larval burden in cyathostomin-infected horses. Equine Veterinary Journal 42, 652-657.
Pinheiro, J.C., Bates, D. 2009. Mixed-Effects Models in S and S-PLUS. Springer.
Picquet, M., Vercruysse, J., Shaw, D.J., Diop, M., Ly, A., 1998. Efficacy of praziquantel against Schistosoma mansoni in northern Senegal. Transactions of the Royal Society of Tropical Medicine and Hygiene 92, 90-93.
Pook, J.F., Power, M.L., Sangster, N.C., Hodgson, J.L., Hodgson, D.R. 2002. Evaluation of tests for anthelmintic resistance in cyathostomes. Veterinary Parasitology 106, 331-343.
Poulin, R. 2013. Explaining variability in parasite aggregation levels among host samples. Parasitology 140, 541-546.
Poynter, D. 1954. Seasonal fluctuation in the number of strongyle eggs passed by horses. Veterinary Record 66, 74–78
Poynter, D., Hughes, D. 1958. Phenothiazine and piperazine, an efficient anthelmintic mixture for horses. Veterinary Record 70, 1183 - 1188.
Poynter, D. 1970. Some observations on the nematode parasites of horses. Proceedings of the Second International Conference on Equine Infectious Diseases. New York, 1970. pp. 269-289.
Presidente, P.J.A. 1985. Methods for the detection of resistance to anthelmintics. In: Anderson, N., Waller, P.J. (Eds.), Resistance in Nematodes to Anthelmintic Drugs. Division of Animal Health, CSIRO, Australia, pp. 13–27.
Presland, S.L., Morgan, E.R., Coles, G.C. 2005. Counting nematode eggs in equine faecal samples. Veterinary Record 156, 208-210.

Prichard, R.K., Hall, C.A., Kelly, J.D., Martin, I.C. and Donald, A.D. 1980. The problem of anthelmintic resistance in nematodes. Australian Veterinary Journal 56, 239-251.
Prichard, R.K. 1990. Anthelmintic resistance in nematodes: Extent, recent understanding and future directions for control and research. International Journal for Parasitology 20, 515-523.
Proudman, C., Edwards, G. 1992. Validation of a centrifugation/flotation technique for the diagnosis of equine cestodiasis. Veterinary Record 131, 71-72.
Proudman, C.J., Edwards, G.B. 1993. Are tapeworms associated with equine colic? A case control study. Equine Veterinary Journal 25, 224-226.
Proudman, C.J., French, N.P., Trees, A.J. 1998. Tapeworm infection is a significant risk factor for spasmodic colic and ileal impaction colic in the horse. Equine Veterinary Journal 30, 194-199.
Proudman, C.J., Matthews, J.B. 2000. Control of intestinal parasites in horses. In Practice 22, 90-97.
Proudman, C.J., Trees, A.J. 1996. Use of excretory/secretory antigens for the serodiagnosis of *Anoplocephala perfoliata* cestodosis. Veterinary Parasitology 61, 239-247.
Proudman, C.J., Trees, A.J. 1999. Tapeworms as a cause of intestinal disease in horses. Parasitology Today 15, 156-159.
Quenouille, M.H. 1949. A Relation between the Logarithmic, Poisson, and Negative Binomial Series. Biometrics 5, 162-164.
Rehbein, S., Lutz, W., Visser, M., Winter, R. 2002. Investigation of the parasitic fauna of game animals of North Rhine-Westfalia. 3. Endoparasites of red deer. Zeitschrift furJadgwissenschaft 48, 69-93.
Rehbein, S., Visser, M., Winter, R. 2013. Prevalence, intensity and seasonality of gastrointestinal parasites in abattoir horses in Germany. Parasitology Research 112, 407-413.
Reid, S.W., Mair, T.S., Hillyer, M.H., Love, S. 1995. Epidemiological risk factors associated with a diagnosis of clinical cyathostomiasis in the horse. Equine Veterinary Journal 27, 127-130.
Rieder N., Beelitz P., Gothe R. 1995. Incidence of *Parascaris equorum* in foals and their mares after strategic use of wide-spectrum anthelmintics for several years. Tierarztl Prax. 23, 53-58.
Reinemeyer, C.R. 1986. Small strongyles. Recent advances. The Veterinary clinics of North America. Equine practice 2, 281-312.
Reinemeyer, C., Farley, A., Clymer, B. 2003. Comparisons of Cyathostome Control and Selection for Benzimidazole Resistance Using Larvicidal Regimens of Moxidectin Gel or Fenbendazole Paste. The International Journal of Applied Research 1, 1.
Reinemeyer, C. 2009. Diagnosis and control of anthelmintic-resistant *Parascaris equorum*. Parasites and Vectors 2, S8.
Reinemeyer, C.R., Nielsen, M.K. 2009. Parasitism and Colic. Veterinary Clinics of North America: Equine Practice 25, 233-245.
Reinemeyer, C.R. 2012. Anthelmintic resistance in non-strongylid parasites of horses. Veterinary Parasitology 185, 9-15.
Reinemeyer, C.R., Nielsen, M.K. 2013. Handbook of Equine Parasite Control. Wiley-Blackwell.
Relf, V.E., Morgan, E.R., Hodgkinson, J.E., Matthews, J.B. 2012. A questionnaire study on parasite control practices on UK breeding Thoroughbred studs. Equine Veterinary Journal 44, 466-471.

Relf, V.E., Morgan, E.R., Hodgkinson, J.E., Matthews, J.B. 2013. Helminth egg excretion with regard to age, gender and management practices on UK Thoroughbred studs. Parasitology 140, 641-652.
Relf, V.E., Lester, H.E., Morgan, E.R., Hodgkinson, J.E., Matthews, J.B. 2014. Anthelmintic efficacy on UK Thoroughbred stud farms. International Journal for Parasitology 44, 507-514.
Rinaldi, L., Levecke, B., Bosco, A., Ianniello, D., Pepe, P., Charlier, J., Cringoli, G., Vercruysse, J. 2014. Comparison of individual and pooled faecal samples in sheep for the assessment of gastrointestinal strongyle infection intensity and anthelmintic drug efficacy using McMaster and Mini-FLOTAC. Veterinary Parasitology 205, 216-223.
Rinaldi, L., Veneziano, V., Morgoglione, M.E., Pennacchio, S., Santaniello, M., Schioppi, M., Musella, V., Fedele, V., Cringoli, G. 2009. Is gastrointestinal strongyle faecal egg count influenced by hour of sample collection and worm burden in goats? Veterinary Parasitology 163, 81-86.
Ripley, B. D. 1987. Stochastic simulation. Wiley, New York.
Robertson, D. 1939. Intestinal parasites of Shetland Ponies in the North of Scotland. Veterinary Record 51, 779–781.
Rock, C., Pomroy, W., Gee, E., Scott, I. 2013.Macrocyclic lactone resistant *Oxyuris equi* in New Zealand. Proc. 24th. Int. Conf. of the WAAVP, 25–29 August, p. 520.
Rojo-Vazquez, F.A., Meana, A., Valcarcel, F., Martinez-Valladares, M. 2012. Update on trematode infections in sheep. Veterinary Parasitology 189, 15-38.
Ross, J.G. 1966. An abattoir survey of cattle liver infections with *Fasciola hepatica*. British Veterinary Journal 122, 489-494.
Rossano, M.G., Smith, A.R., Lyons, E.T. 2010. Shortened strongyle-type egg reappearance periods in naturally infected horses treated with moxidectin and failure of a larvicidal dose of fenbendazole to reduce fecal egg counts. Veterinary Parasitology 173, 349-352.
Round, M.C. 1976. Lungworm infection (*Dictyocaulus arnfieldi*) of horses and donkeys. Veterinary Record 99, 393-395.
Rupashinge, D., Ogbourne, C.P. 1978. Laboratory studies on the effect of temperature on the development of the free-living stages of some strongylid nematodes of the horse. Zeitschrift fu¨r Parasitenkunde 55, 249–253.
Sangster, N.C. 2001. Managing parasiticide resistance. Veterinary Parasitology 98, 89-109.
Sargent, R.G. 2013. Verification and validation of simulation models. Journal of Simulation 7, 12-24.
Sargison, N.D., Jackson, F., Bartley, D.J., Moir, A. 2005. Failure of moxidectin to control benzimidazole, levamisole and ivermectin resistant *Teladorsagia circumcincta* in a sheep flock. Veterinary Record 156, 106–109.
Sargison, N.D., Jackson, F., Bartley, D.J., Wilson, D.J., Stenhouse, L.J., Penny, C.D. 2007. Observations on the emergence of multiple anthelmintic resistance in sheep flocks in the south-east of Scotland. Veterinary Parasitology 145, 65-76.
Sargison, N.D., Scott, P.R. 2011. Diagnosis and economic consequences of triclabendazole resistance in *Fasciola hepatica* in a sheep flock in south-east Scotland. Veterinary Record 168, 159.
Schnyder, M., Maurelli, M., Morgoglione, M., Kohler, L., Deplazes, P., Torgerson, P., Cringoli, G., Rinaldi, L. 2011. Comparison of faecal techniques including FLOTAC for copromicroscopic detection of first stage larvae of *Angiostrongylus vasorum.* Parasitology Research 109, 63-69.

Schumacher, J., Taintor, J. 2008. A review of the use of moxidectin in horses. Equine Veterinary Education 20, 546-551.
Scott, I., Pomroy, W.E., Kenyon, P.R., Smith, G., Adlington, B., Moss, A. 2013. Lack of efficacy of monepantel against *Teladorsagia circumcincta* and *Trichostrongylus colubriformis*. Veterinary Parasitology 198, 166-171.
Shaw, D.J., Dobson, A.P. 1995. Patterns of macroparasite abundance and aggregation in wildlife populations: a quantitative review. Parasitology 111, S111-S133.
Shaw, D.J., Grenfell, B.T., Dobson, A.P. 1998. Patterns of macroparasite aggregation in wildlife host populations. Parasitology 117, 597-610.
Sinniah, B. 1982. Daily egg production of *Ascaris lumbricoides*: the distribution of eggs in the faeces and the variability of egg counts. Parasitology 84, 167-175.
Skotarek, S.L., Colwell, D.D., Goater, C.P. 2010. Evaluation of diagnostic techniques for *Anoplocephala perfoliata* in horses from Alberta, Canada. Veterinary Parasitology 172, 249-255.
Slocombe, J.O., McCraw, B.M. 1975. Suppression of the pathogenic effects of *Strongylus edentatus* larvae with thiabendazole. Canadian journal of comparative medicine. Revue canadienne de medecine comparee 39, 256-260.
Slocombe, J.O. 1979. Prevalence and treatment of tapeworms in horses. The Canadian veterinary journal. La revue veterinaire canadienne 20, 136-140.
Slocombe, J. O. D. 1985. Pathogenesis of helminths in equines. Veterinary Parasitology 18,139-153.
Slocombe, J.O., de Gannes, R.V. 2006. Cyathostomes in horses in Canada resistant to pyrantel salts and effectively removed by moxidectin. Veterinary Parasitology 140, 181-184.
Slocombe, J., de Gannes, R., Lake, M. 2007. Macrocyclic lactone-resistant *Parascaris equorum* on stud farms in Canada and effectiveness of fenbendazole and pyrantel pamoate. Veterinary Parasitology 145, 371 - 376.
Smith, G., Guerrero, J. 1993. Mathematical models for the population biology of *Ostertagia ostertagi* and the significance of aggregated parasite distributions. Veterinary Parasitology 46, 243-257.
Smith, H.J. 1976. Strongyle infections in ponies. I. Response to intermittent thiabendazole treatments. Canadian journal of comparative medicine. Revue canadienne de medecine comparee 40, 327-333.
Soulsby, E. J. L. 1968. Helminths, arthropods and protozoa of domesticated animals. Bailliere, Tindall and Cassell. London, UK. pp. 96.
Squires, S., Fisher, M., Gladstone, O., Rogerson, S., Martin, P., Martin, S., Lester, H., Sygall, R., Underwood, N. 2012. Comparative efficacy of flubendazole and a commercially available herbal wormer against natural infections of *Ascaridia galli*, *Heterakis gallinarum* and intestinal *Capillaria* spp. in chickens. Veterinary Parasitology 185, 352-354.
Stear, M., Bishop, S., Doligalska, M., Duncan, J., Holmes, P., Irvine, J., McCririe, L., McKellar, Q., Sinski, E. and Murray, M. 1995. Regulation of egg production, worm burden, worm length and worm fecundity by host responses in sheep infected with *Ostertagia circumcincta*. Parasite Immunology17, 643–652.
Stear, M. J. and Bishop, S. C. 1999. The curvi-linear relationship between worm length and fecundity of *Teladorsagia circumcincta*. International Journal for Parasitology29, 777–780.
Stear, M.J., Abuagob, O., Benothman, M., Bishop, S.C., Innocent, G., Kerr, A., Mitchell, S. 2006. Variation among faecal egg counts following natural nematode infection in Scottish Blackface lambs. Parasitology 132, 275-280.

Steinbach, T., Bauer, C., Sasse, H., Baumgartner, W., Rey-Moreno, C., Hermosilla, C., Damriyasa, I.M., Zahner, H. 2006. Small strongyle infection: consequences of larvicidal treatment of horses with fenbendazole and moxidectin. Veterinary Parasitology 139, 115-131.

Stratford, C.H., McGorum, B.C., Pickles, K.J., Matthews, J.B. 2011. An update on cyathostomins: Anthelmintic resistance and diagnostic tools. Equine Veterinary Journal 43, 133-139.

Stratford, C.H., Lester, H.E., Morgan, E.R., Pickles, K.J., Relf, V., McGorum, B.C., Matthews, J.B. 2014a. A questionnaire study of equine gastrointestinal parasite control in Scotland. Equine Veterinary Journal 46, 25-31.

Stratford, C.H., Lester, H.E., Pickles, K.J., McGorum, B.C., Matthews, J.B. 2014b. An investigation of anthelmintic efficacy against strongyles on equine yards in Scotland. Equine Veterinary Journal 46, 17-24.

Summers, B.A., Cummings J.F., Lahunta, A. 1995. Diseases of the peripheral nervous system (Eds.), Veterinary Neuropathology, Mosby, St. Louis, pp. 402–501.

Suter, R.J., Besier, R.B., Perkins, N.R., Robertson, I.D., Chapman, H.M. 2004. Sheep-farm risk factors for ivermectin resistance in *Ostertagia circumcincta* in Western Australia. Preventive Veterinary Medicine, 63, 257–269.

Taylor, M.A., Hunt, K.R. 1988. Field observations on the control of ovine parasitic gastroenteritis in south-east England. Veterinary Record, 123, 241–245.

Taylor, M.A., Hunt, K.R. 1989. Anthelmintic drug resistance in the UK. Veterinary Record, 125: 143–147.

Taylor, M.A. 2012. Emerging parasitic diseases of sheep. Veterinary Parasitology 189, 2-7.

Tarigo-Martinie, J., Wyatt, A., Kaplan, R., 2001b. Prevalence and clinical implications of anthelmintic resistance in cyathostomins of horses. Journal of American Veterinary Medicine Associaton 218, 1957 - 1960.

Thiele, J., Markussen, B. 2012. Potential of GLMM in modelling invasive spread. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources, vol 7, no. 16., 10.1079/PAVSNNR20127016.

Thienpont, D., Rochette, F., Vanparijs, O.F.J. 1986. Diagnosing Helminthiasis by Coprological Examination, 2nd edn., Janssen Research Foundation, Beerse, Belgium.

Thrusfield, M. 2007. Veterinary Epidemiology. Wiley.

Tiunov, V.I. 1951. Nodular trichonematosis of the horse large intestine. Trudy Gel'mintologicheskoi Laboratorii, Akademiya Nauk USSR, 5, 318–1237.

Tiunov, V.I. 1953. The development of the nematode *Trichonema longibursatum* as it occurs in the horse Trudy Vsesoyunz Instituta Gelmintologie, 5, 65–67.

Tolliver S. C., Lyons, E. T. and Drudge, J. H. 1987. Prevalence of internal parasites in horses in critical tests of activity of parasiticides over 28 years period (1956-1983) in Kentucky. Veterinary Parasitology 23,273-284.

Torgerson, P.R., Schnyder, M., Hertzberg, H. 2005. Detection of anthelmintic resistance: a comparison of mathematical techniques. Veterinary Parasitology 128, 291-298.

Torgerson, P.R., Paul, M., Lewis, F.I. 2012. The contribution of simple random sampling to observed variations in faecal egg counts. Veterinary Parasitology 188, 397-401.

Torgerson, P.R., Paul, M., Furrer, R. 2014. Evaluating faecal egg count reduction using a specifically designed package "eggCounts" in R and a user friendly web interface. International Journal for Parasitology 44, 299-303Traversa, D., Castagna, G., von Samson-Himmelstjerna, G., Meloni, S., Bartolini, R., Geurden, T., Pearce, M.C., Woringer, E., Besognet, B., Milillo, P., D'Espois, M., 2012. Efficacy of major

anthelmintics against horse cyathostomins in France. Veterinary Parasitology 188, 294-300.
Traversa, D., Fichi, G., Campigli, M., Rondolotti, A., Iorio, R., Proudman, C.J., Pellegrini, D., Perrucci, S. 2008. A comparison of coprological, serological and molecular methods for the diagnosis of horse infection with Anoplocephala perfoliata (Cestoda, Cyclophyllidea). Veterinary Parasitology 152, 271-277.
Traversa, D., Klei, T., Iorio, R., Paoletti, B., Lia, R., Otranto, D., Sparagano, O., Giangaspero, A. 2007. Occurrence of anthelmintic resistant equine cyathostome populations in central and southern Italy. Preventative Veterinary Medicine 82, 314 - 320.
Traversa, D., von Samson-Himmelstjerna, G., Demeler, J., Milillo, P., Schurmann, S., Barnes, H., Otranto, D., Perrucci, S., di Regalbono, A., Beraldo, P., Boeckh, A., Cobb, R. 2009. Anthelmintic resistance in cyathostomin populations from horse yards in Italy, United Kingdom and Germany. Parasites and Vectors 2, S2.
Trawford, A.F., Burden, F.A., Hodgkinson, J. 2005, Suspected moxidectin resistance in cyathostomes in two donkey herds at The Donkey Sanctuary, UK. In: Proceedings of the 20th International Conference World Association for Advanced Veterinary Parasitology, New Zealand, p. 196.
Trawford, A., Burden, F. 2012. Drug resistant cyathostomins in donkey herds; lessons in management for All equids. Journal of Equine Veterinary Science 32, 43.
Turner, W.C., Cizauskas, C.A., Getz, W.M. 2010. Variation in faecal water content may confound estimates of gastro-intestinal parasite intensity in wild African herbivores. Journal of Helminthology 84, 99-105.
Uhlinger, C.A. 1993. Uses of faecal egg count data in equine practice. Continuing Education in Practice Veterinary. 15, 742-749.
Vadlejch, J., Petrtýl, M., Zaichenko, I., Čadková, Z., Jankovská, I., Langrová, I., Moravec, M. 2011. Which McMaster egg counting technique is the most reliable? Parasitology Research 109, 1387-1394.
Valero, M.A., Ubeira, F.M., Khoubbane, M., Artigas, P., Muiño, L., Mezo, M., Pérez-Crespo, I., Periago, M.V., Mas-Coma, S. 2009. MM3-ELISA evaluation of coproantigen release and serum antibody production in sheep experimentally infected with *Fasciola hepatica* and *F. gigantic*a. Veterinary Parasitology 159, 77-81.
van Dijk, J., Sargison, N.D., Kenyon, F., Skuce, P.J. 2010. Climate change and infectious disease: helminthological challenges to farmed ruminants in temperate regions. animal. Animal 4, 377-392.
van Doorn, D.C.K., Ploeger, H.W., Eysker, M., Geurden, T., Wagenaar, J.A., Kooyman, F.N.J. 2014. Cylicocyclus species predominate during shortened egg reappearance period in horses after treatment with ivermectin and moxidectin. Veterinary Parasitology 206, 246-252.
van Wyk, J.A. 2001. Refugia--overlooked as perhaps the most potent factor concerning the development of anthelmintic resistance. The Onderstepoort Journal of Veterinary Research 68, 55-67.
Venables, W.N., Ripley, B.D. 2002. Modern Applied Statistics with S. Springer.
Veneziano, V., Di Loria, A., Masucci, R., Di Palo, R., Brianti, E., Gokbulut, C. 2011. Efficacy of eprinomectin pour-on against *Dictyocaulus arnfieldi* infection in donkeys (*Equus asinus*). The Veterinary Journal 190, 414-415.
Vercruysse, J., Eysker, M., Demeulenaere, D., Smets, K., Dorny, P. 1998. Persistence of the efficacy of moxidectin gel on the establishment of cyathostominae in horses. Veterinary Record 143, 307 - 309.

Veronesi, F., Fioretti, D.P., Genchi, C. 2010. Are macrocyclic lactones useful drugs for the treatment of *Parascaris equorum* infections in foals? Veterinary Parasitology 172, 164-167.

Veronesi, F., Moretta, I., Moretti, A. 2009. Field effectiveness of pyrantel and failure of *Parascaris equorum* egg count reduction following ivermectin treatment in Italian horse farms. Veterinary Parasitology 161, 138 - 141.

Vidyashankar, A.N., Kaplan, R.M., Chan, S., 2007. Statistical approach to measure the efficacy of anthelmintic treatment on horse farms. Parasitology 134, 2027-2039.

Vidyashankar, A.N., Hanlon, B.M., Kaplan, R.M. 2012. Statistical and biological considerations in evaluating drug efficacy in equine strongyle parasites using fecal egg count data. Veterinary Parasitology 185, 45-56.

Villanua, D., Perez-Rodriguez, L., Gortazar, C., Hofle, U., Vinuela, J. 2006. Avoiding bias in parasite excretion estimates: the effect of sampling time and type of faeces. Parasitology 133, 251-259.

Vlassoff, A., Kettle, P.R . 1980. Benzimidazole resistance in *Haemonchus contortus*. New Zealand Veterinary Journal 28, 23–24.

von Samson-Himmelstjerna, G., Fritzen, B., Demeler, J., Schurmann, S., Rohn, K., Schneider, T., Epe, C. 2007. Cases of reduced cyathostomin egg-reappearance period and failure of Parascaris equorum egg count reduction following ivermectin treatment as well as survey on pyrantel efficacy on German horse farms. Veterinary Record 144, 74 - 80.

Waghorn, T.S., Leathwick, D.M., Miller, C.M., Atkinson, D.S . 2008. Brave or gullible: Testing the concept that leaving susceptible parasites in refugia will slow the development of anthelmintic resistance. New Zealand Veterinary Journal, 56, 158–163.

Wakelin, D., Blackwell, J. 1988. Genetics of Resistance to Bacterial and Parasitic Infection. Taylor and Francis, London.

Waller, P. 1987. Anthelmintic resistance and the future for roundworm control. Veterinary Parasitology 25, 177 - 191.

Walker, M., Hall, A., Anderson, R. M., Basanez, M.G. 2009. Density-dependent effects onthe weight of female *Ascaris lumbricoides* infections of humans and its impact on patterns of eggproduction. Parasitology and Vectors, 2:11.

Waltner-Towes, D. 1983. Questionnaire design and administration. In Proceedings of 3rd International Symposium of Veterinary Epidemiology and Economics, Arlington, VA, USA, pp.31-37.

Wang, W., Dai, J.R., Li, H.J., Shen, X.H., Liang, Y.S. 2010. Is there reduced susceptibility to praziquantel in *Schistosoma japonicum*? Evidence from China. Parasitology 137, 1905-1912.

Wetzel, R. 1940. Zur Entwicklund de grossen palisadenwurmes (*Strongylus equinus*) im pferd. Arch. Wis. Prakt. Tier., 76, 81-118.

Wetzel, R. 1952. Die Entwicklungsdauer (praepatente-periode) von *Strongylus edentatus* im pferd. Deutche tierarztliche Wochenchrift 59, 129-130.

Wetzel, R., Kertzen, W. 1956. Die leberphase der Entwicklund von *Strongylus edentatus*. Wiener tierarcztliche Monatsschrift 43; 664-673.

Wickham, H. 2015. An implementation of the grammar of graphics. Retrieved from: https://cran.r-project.org/web/packages/ggplot2/ggplot2.pdf

Williams, B.G., Dye, C. 1994. Maximum likelihood for parasitologists. Parasitology Today 10, 489-493.

Winkelhagen, A.J., Mank, T., de Vries, P.J., Soetekouw, R. 2012. Apparent triclabendazole-resistant human *Fasciola hepatica* infection in The Netherlands. Emerging Infectious Diseases 18, 1028–1029.

Wilson, K., Grenfell, B. 1997. Generalized linear modelling for parasitologists. Parasitology Today 13, 33 - 38.

Wirtherle, N., Schnieder, T., von Samson-Himmelstjerna, G. 2004. Prevalence of benzimidazole resistance on horse farms in Germany. Veterinary Record 154, 39-41.

Wolf, D., Hermosilla, C., Taubert, A. 2014. *Oxyuris equi*: Lack of efficacy in treatment with macrocyclic lactones. Veterinary Parasitology 201, 163-168.

Wood, E.L.D., Matthews, J.B., Stephenson, S., Slote, M., Nussey, D.H. 2013. Variation in fecal egg counts in horses managed for conservation purposes: individual egg shedding consistency, age effects and seasonal variation. Parasitology 140, 115-128.

Xiao, L., Herd, R.P., Majewski, G.A. 1994. Comparative efficacy of moxidectin and ivermectin against hypobiotic and encysted cyathostomes and other equine parasites. Veterinary Parasitology 53, 83-90.

Yet, B., Bastani, K., Raharjo, H., Lifvergren, S., Marsh, W., Bergman, B., 2013. Decision support system for Warfarin therapy management using Bayesian networks. Decision Support Systems 55, 488-498.

Yu, J.M., de Vlas, S.J., Yuan, H.C., Gryseels, B. 1998. Variations in fecal *Schistosoma japonicum* egg counts. The American Journal of Tropical Medicine and Hygiene 59, 370-375.

Zulalian, J., Stout, S.J., DaCunha, A.R., Garcés, T. *and* Miller, P. 1994. Absorption, tissue distribution, metabolism and excretion of moxidectin in cattle. Journal of Agriculture and Food Chemistry 42, 381–387.

Zuur, A.F., Ieno, E.N., Elphick, C.S. 2010. A protocol for data exploration to avoid common statistical problems. Methods in Ecology and Evolution 1, 3-14.

Zuur, A., Ieno, E.N., Walker, N., Saveliev, A.A., Smith, G.M. 2009. Mixed Effects Models and Extensions in Ecology with R. Springer.