UNIVERSIDADE DE LISBOA
Faculdade de Medicina Veterinária

THE EFFICACY OF MOXIDECTIN IN EQUINE STRONGYLES AND THE EFFECT OF MANAGEMENT PRACTICES IN STRONGYLE EGG REAPPEARANCE PERIOD AND FAECAL WORM EGG COUNTS IN YARDS ACROSS THE UK

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DISSERTAÇÃO DE MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

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Carvalho

2016

“I might compare a parasitologist to an orchid. He requires long and careful nurturing, he develops slowly, and he is himself a parasite in that he is dependent on many other sciences for material aid. But when he comes to flower he is a rare and beautiful object, scientifically speaking, and is usually slow in going to seed. He may not always smell like and orchid, but we can’t have everything.”

Chandler, 1946 (“The making of a parasitologist”)

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The Horse Trust
Advancing Excellence in Equine Care

Moredun
Foundation

Abstract

The present study was designed to evaluate the efficacy of an oral anthelminic treatment, in particular a combination of moxidectin (MOX) and praziquantel (PRZ) against gastrointestinal strongyles in naturally infected horses. A total of eight yards in the UK were selected to participate in this study: Day 0, the horses were treated with an oral paste containing MOX and PRZ. Faecal samples for faecal egg counts (FECs) analysis were collected at the following time-points: day 0 (treatment day), 14 days post-treatment, 6, 10 and 12 weeks post-treatment (wpt). Faecal egg count reduction tests (FECRTs) were used to determine the product's efficacy. Mean FECR and 95% confidence intervals were calculated using bootstrap analysis. Additional samples were collected at 6, 10 and 12 wpt, to determine the strongyle egg reappearance period (ERP). The ERP was evaluated using two different methods. Faecal cultures were performed to determine the prevalence of the nematode species present in the sample population. The influence of yard management (faecal removal [FR] from the pasture *versus* no faecal removal [NFR]) was analysed using a repeated measures analysis of variance (rANOVA), with the R program. Difference between factors (Group and Time) and their interaction was assessed with a significance level of 0.05. FECRT on day 14 indicated a 100% efficacy in 7 yards and 99.88% in the remaining yard. Using Method 1, the ERP was 6 weeks for all populations examined. Using Method 2, the ERP ranged from 6 weeks to >12 weeks. Only cyathostomin L3 larvae were detected in the faecal cultures throughout the study. Mean FECs of the two groups (FR and NFR) were compared at four time-points: 2 wpt (14 days post-treatment), 6 wpt, 10 wpt and 12 wpt, with significant difference in mean FEC observed between the groups (p = 0.003), since the FR showed lower EPGs. As major conclusions, our results still show an overall excellent efficacy for moxidectin regarding horse strongyle control, although it should not rely only on chemical compounds. Hence, and according to our data, it would appear that this aspect of yard management plays an important role in the levels of strongyle egg excretion after anthelmintic treatment.

Keywords: Horses / strongyles / cyathostomins / moxidectin / Faecal egg count reduction test (FECRT) / Egg reappearance period (ERP) / yard management/ United Kingdom.

Resumo

O presente estudo tem como objectivo avaliar a eficácia de um tratamento anti-helmíntico oral usando uma combinação de moxidectina (MOX) e praziquantel (PRZ) contra estrongilídeos gastrointestinais em cavalos infetados naturalmente. Um total de 8 estabelecimentos equestres no Reino Unido foram selecionados para participar neste estudo. No Dia 0, os cavalos foram tratados com um anti-helmíntico oral contendo MOX e PRZ. As recolhas de amostras fecais para as contagens de ovos fecais foram realizadas nos seguintes tempos: dia 0 (dia do tratamento), 14 dias após tratamento, 6, 10 e 12 semanas após tratamento. Os Testes de Redução de Contagem de Ovos Fecais (TRCOF) foram usados para determinar a eficácia do produto. A média das RCOFs e os intervalos de confiança de 95% foram calculados usando a análise “bootstrap”. As amostras foram recolhidas às 6, 10 e 12 semanas após tratamento para determinar o Período de Reaparecimento de Ovos (PRO). O PRO foi calculado usando dois métodos diferentes. Adicionalmente, foram realizadas culturas fecais para determinar a prevalência das espécies de nematodes presentes nas amostras. A influência do maneio (remoção de fezes [RF] *versus* não remoção de fezes [NRF]) foi analisada usando uma análise de variância com medidas repetidas (rANOVA) através do programa R. A diferença entre dois fatores (grupo e tempo) e a sua interação foram avaliadas com um nível de significância de 0.05. O TRCOF indicou uma eficácia de 100% para 7 quintas equestres e 99.88% na restante. Usando o método 1, o PRO foi de 6 semanas para todas as populações estudadas. Usando o método 2, o PRO variou entre 6 a >12 semanas. Nas culturas fecais foram detetados apenas larvas L3 de ciatostomíneos. As médias das contagens de ovos fecais dos dois grupos (RF e NRF) foram comparadas em 4 pontos no tempo: 2 semanas após tratamento (14 dias após tratamento), 6, 10 e 12 semanas após tratamento, com uma diferença de médias significativa entre os grupos (p=0.003), visto que o grupo RF obteve menores contagens de ovos. Conclui-se que os resultados demonstram que a moxidectina apresenta uma eficácia muito boa no que toca ao controlo de estrongilídeos. Apesar disso, o seu controlo não deveria ser inteiramente químico. Portanto, e de acordo com o nosso estudo, é provável que este aspeto do maneio tem um papel importante nos níveis de excreção de ovos de estrôngilos após tratamento com anti-helmínticos.

Palavras-chave: Cavalos / estrongilídeos / ciatostomíneos / moxidectina / Teste de Redução de Contagem de Ovos Fecais (TRCOF) / Período de Reaparecimento de Ovos (PRO) / maneio / Reino Unido.

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List of abbreviations and symbols

% - percentage

°C – Celsius

µl – Microliter

AAEP – American Association of Equine Practitioners

AR – Anthelmintic Resistance

BZ – Benzimidazoles

cm – centimetre

cm^3 – cubic centimetre

DLP – Dose Limiting Parasites

DNA – deoxyribonucleic acid

e.g. – exempli grati (for example)

ELISA – Enzyme Linked Immunosorbent Assay

EPG – Eggs Per Gram

ERP – Egg Reappearance Period

FEC – Faecal Egg Count

FECRT – Faecal Egg Count Reduction Test

i.e. – id est (that is)

IVM – Ivermectin

L – Litre

LCL – Lower Confidence Limits

L1 – first stage larvae

L2- second stage larvae

L3 – third stage larvae

LL3 – luminal third stage larvae

L4 – fourth stage larvae

LL4 – Luminal forth stage larvae

L5 – fifth stage larvae

mL – millilitre

ML – Macrocyclic Lactones

MOX - Moxidectin

MRI – Moredun Research Institute

NaCl – sodium chloride

PCR – Polymerase Chain Reaction

PRZ – Praziquantel

rANOVA – Repeated Measures Analysis of Variance

rpm – Rotations Per Minute

SAR – Suspected Anthelmintic Resistance

THP – Tetrahydropyrimidines

TST – Targeted Selective Treatment

WAAVP – World Association for the Advancement of Veterinary Parasitology

wpt – Weeks Post Treatment

UK – United Kingdom

USA – United States of America

Chapter 1 - Introduction

1.1 Cyathostomins: are they a threat?

The large strongyle parasite *Strongylus vulgaris* was considered the principal parasitic threat to equine health due to its high pathogenic potential (Duncan & Pirie, 1975), but after the implementation of regular anthelmintic treatment in the 1960s, the prevalence of *S. vulgaris* dropped considerably (Drudge & Lyons, 1966). Nowadays, *S. vulgaris* is only found in very few samples (von Samson-Himmelstjerna, 2012). Decades of interval-based anthelmintic treatment programmes lead to a selection of high levels of anthelmintic resistance (AR) within nematode populations, especially in the cyathostomin populations (Kaplan & Nielsen, 2010). Currently, the great majority of strongyle eggs detected in faecal egg counts (FECs) performed on managed horses originate from cyathostomin species (AAEP, 2013). This drastic change in species prevalence has caused an important shift in the relative importance of cyathostomins and it is now considered a major threat to equine health (Kaplan & Nielsen, 2010). Adult cyathostomins are considered fairly benign. Disease is caused by the synchronous emergence of larvae encysted within the mucosa of the caecum and colon, known as larval cyathostominosis. Larval cyathostominosis is a protein-losing enteropathy characterized by generalized typhlocolitis, profuse, watery diarrhea, sudden weight loss and colic, and in death in 50% of the cases (Love, Murphy, & Mellor, 1999).

Furthermore, cyathostomins have a relatively high propensity to develop resistance to the three anthelmintic drugs classes licensed for their control (Matthews, 2014b). For the last 50 years anthelmintic control was based on the regular use of anthelmintic compounds (von Samson-Himmelstjerna, 2012), which lead to resistant cyathostomin populations being reported against all three available classes of anthelmintic drugs in Brazil (Molento, Antunes, Bentes, & Coles, 2008). Cyathostomin resistance to benzimidazole and pyrantel classes are now a relatively common finding in managed horse populations (AAEP, 2013), with reports of resistance to both classes in single populations. Hence, only Macrocyclic Lactones remains fairly effective against adult cyathostomin infections (Kaplan et al., 2004).

Only two anthelmintic drugs have approved label claims against encysted cyathostomin larvae: moxidectin administered once orally, and a daily oral administration for five consecutive days of fenbendazole (Xiao, Herd, & Majewski, 1994; Duncan, Bairden, & Abbott, 1998). However, some studies reported failure to achieve acceptable egg count reduction after treatment with fenbendazole (Rossano, Smith, & Lyons, 2010; Mason, Voris, Ortis, Geeding, & Kaplan, 2014). Hence, in several regions, MOX is the only effective larvicidal anthelmintic for horses, which makes the preservation of its effectiveness highly important (Mason et al., 2014). To date, there are no other published reports of cyathostomin resistance against MOX apart from that one isolated case in Brazil (Molento et al., 2008). Unfortunately, reports of shorter Egg Reappearance Period (ERP) after treatment with ivermectin and MOX have been observed in several regions (Rossano et al., 2010; Relf, Lester, Morgan, Hodgkinson, & Matthews, 2014). A reduction in the ERP is thought to be an early indicator of resistance (Sangster, 1999b). Thus, the eggs that reappear earlier than expected might be from the cyathostomin populations that are shifting towards resistance. It is essential that non-chemical practices be implemented at all levels in a control programme, such as improved pasture hygiene. The removal of faeces from pastures can break the parasite life cycle, reducing the re-infection level and delaying progression towards resistance (Corbett et al., 2014).

1.2 The Moredun Research Institute

The Moredun Foundation (2010) was established in 1920 as the “Animal Disease Research Association”. Over the years the Animal Disease Research Association grew into the internationally recognised Moredun Research Institute (MRI). The MRI, based at Pentlands Science Park, conducts world class scientific research to improve farm animal health and welfare through the prevention and control of infectious diseases of livestock (Moredun Foundation, 2010).

The MRI is divided into several teams, made up of scientists, veterinarians and scientific support staff. The institute has particular scientific expertise in veterinary bacteriology, virology, parasitology, immunology, molecular biology, pathology, surveillance and bioservices (Moredun Foundation, 2010).

I had the pleasure of joining *The Horse Trust* project, under the supervision of Professor Jacqui Matthews and Doctor Thomas Tzelos, a project included in the Disease Control division of the institute. During 3 months, for a total of 600 hours, I actively participated in faecal egg counts (FECs) and larvae cultures, in order to collect data for this thesis. For the FECs, we used a fairly new technique called "centrifugal floatation faecal egg counting method" (described further on). This method has a sensitivity of 1 egg per gram (EPG), a much higher sensitivity when compared to the classic McMaster technique, which has a sensitivity of 15 to 100 EPG (Lester & Matthews, 2014).

Additionally, I followed and participated in other projects that occurred simultaneously, including egg extraction from the same samples used for the FECs which were stored for future research. Further on, I visited the molecular laboratory, where I followed DNA extraction (from the previous egg extraction) and subsequent PCR reactions for the detection of *Strongylus vulgaris* DNA. However the protocols and results are not included in this thesis due to some problems with the optimisation of the PCR protocol. As there were still no results at the end of my traineeship, the only protocol for the prevalence of *Strongylus vulgaris* will be the larvae culture (also described further on).

On the 9th of March, I attended the WormBase ParaSite Workshop. WormBase Parasite (http://parasite.wormbase.org/) is a new online resource for the display, analysis and comparison of helminth sequence data. During the workshop, the participants had to opportunity to be guided through the website and shown how search and analyse genome sequences, protein and nucleotide sequences, etc.

Finally, I also had the privilege to follow the validation of a blood-based ELISA diagnostic test that informs of the presence of encysted cyathostomins. In this test, cyathostomin recombinant antigens were coated on the wells of 96-well plates and used to detect antibody levels in the blood serum of the patient horse. Various antigen-cocktails were tested. The different cocktails were coated on different plates and tested with the blood serum of infected horses. If successful, this ELISA test will prove to be an innovation of great use to the improvement of the diagnosis of cyathostomin infections, but unfortunately, I was unable to follow the entire progress, as my traineeship ended before the final steps.

Chapter 2 – Important Equine Helminths

The word parasite comes from the ancient Greek *parasitos*, which means a person who eats at the table of another. Parasites are small organisms that live on or inside of a larger organism called the host. The host can tolerate the presence of parasites without any evident signs of illness. However, depending on the host's immunity, the degree of injury the parasites inflict and the number of parasites present, the host may show clinical signs of a large spectrum, ranging from minor discomfort and even to death (Bowman, 2014). The word helminth also comes from the Greek *hélmins*, which can be simply translated to worm. According to Satyu Yamaguti's studies regarding taxonomical classification (Yamaguti, 1959, 1961), the most important equine helminths belong to the Platyhelminthes and Nemathelminthes phylum (Taylor & Coop, 2007).

The phylum Platyhelminthes is subdivided into various classes, one of them being the class Cestoda. This class is characterized by having a flat, segmented body, each segment containing one and sometimes two sets of male and female reproductive organs (Taylor & Coop, 2007). Members of this class are known as *tapeworms* due to their body shape. Unlike nematodes, equine cestodes do not release individual eggs on a regular basis. Rather, terminal gravid proglottids detach from the main body and are expelled to the external environment, resulting in an irregular distribution of cestode eggs (Reinemeyer & Nielsen, 2012).

The Nemathelminthes, commonly known as *roundworms*, is subdivided into six classes, but only one of those, the class Nematoda, contains worms of veterinary importance. Nematodes are one of the most important groups of endoparasites that affect domestic animals. These parasites are relatively small, wormlike organisms covered by a tough skin called the cuticle. It has been estimated that about 16,000 – 17,000 nematode species have been described and that as least 40,000 species exist (Anderson, 2000).

2.1 Strongylidae

The Class Nematoda is subdivided into various Superfamilies, including the superfamily Strongyloidea, which contains the most common parasites infecting mammals. This superfamily is further divided into various families. In this study, only the members of the family Strongylidae will be described. The great majority of the nematodes of the family Strongylidae feed and reproduce on the mucosal surfaces of the gastrointestinal and respiratory tracts of the host (Taylor & Coop, 2007), and can be found in the gut of a diverse group of hosts, being richly represented in equines (Lichtenfels, Kharchenko, & Dvojnos, 2008). In horses, there are two important subfamilies, the Cyathostominae and the Strongylinae. Together, they represent a group known as the equine strongyles, all having a direct life cycle, which means that intermediate or paratenic hosts are never found (Reinemeyer & Nielsen, 2012).

Strongyle eggs are commonly found during routine FECs. However, there a number of factors that influence the degree of strongyle egg-shedding, these include the level of exposure to the parasites, the specific immune response of the host, stress levels of the host (Reinemeyer & Nielsen, 2012) and the age of the host (Boersema, Eysker, Maas, & van der Aar, 1996; Boersema, Eysker, & van der Aar, 1998).

a) Not all horses acquire protective immunity against strongyle infections, therefore individual susceptibility can persist throughout an animal's life; horses with large parasite burdens as foals may maintain high burdens as adults (Nielsen, Monrad, & Olsen, 2006). As a result of this diverse susceptibility among the herd's individuals, only a relatively small number of horses contribute to the majority of pasture contamination with parasite eggs, the generally accepted rule is that approximately 20% of horses excrete approximately 80% of the eggs (Gomez & Georgi, 1991; Matthews, 2008).

b) Horses with large grazing areas instinctively divide the pasture into two distinct zones: "roughs", where horses go to defecate; and "lawns", where they normally graze (Reinemeyer, 2009a). Horses naturally avoid grazing in the "roughs" areas, and they also do not defecate on the "lawns". Consequently, the "rough" pasture can have a much higher (sometimes 15 times more) strongylid larval number (Herd & Willardson, 1985).

c) When horses are introduced to a new herd, it is highly probable that the new horses will develop higher strongyle egg counts than the permanent residents of the farm.

Adaptation and adjustment to a new diet (including interacting with a different microbiota environment) and to a new social hierarchy cause stress to the animals, which leads to higher counts (Reinemeyer & Nielsen, 2012).

d) Young horses are more likely to have higher levels of strongyle egg shedding (and consequently higher FECs) than adult mature individuals (Boersema et al., 1996; Boersema et al., 1998).

Presently, cyathostomins constitute 99% of all strongyle eggs detected in FEC analysis performed on managed horses in developed regions (AAEP, 2013). The prevalence of *Strongylus vulgaris* dropped considerably since implementation of regular anthelmintic treatment with broad spectrum anthelmintics (Drudge & Lyons, 1966). Studies have shown that either they are not found at all or they are found only in a few individuals (von Samson-Himmelstjerna, 2012).

2.1.1 Cyathostomins

The Cyathostominae subfamily (also known as cyathostomins or small strongyles) is now considered the most important group of all nematodes that infect horses because of their high prevalence, pathogenicity and ability to develop anthelmintic resistance when under selection pressure (Love et al., 1999; Matthews, 2014b). This group of nematodes comprises approximately 50 different species. However, the majority of horses are infected by 5 – 12 common species (Ogbourne, 1976; Reinemeyer, Smith, Gabel, & Herd, 1984). Various authors suggest that 99% of the total cyathostomin burdens are composed of only 12 species, particularly: *Cyathostomum catinatum, Cyathostomum pateratum, Coronocyclus coronatus, Coronocyclus labiatus, Coronocyclus labratus, Cycocyclus nassatu, Cycocyclus leptostomus*, *Cycocyclus insigne*, *Cylicostephanus longibursatus, Cylicostephanus goldi, Cylicostephanus calicatus* and *Cylicostephanus minutus* (Bucknell, Gasser, & Beveridge, 1995; Lyons et al., 1996). Another study confirmed that similar burden patterns regarding species proportions have been documented in diverse geographic regions (Kaplan, 2002). Moreover, three of the above species – *Cylicostephanus longibursatus*, *Cyathostomum catinatum* and *Cycocyclus nassatus* – represented 70 – 80% of the total cyathostomin burden (Kaplan, 2002).

2.1.1.1 Life Cycle

Small strongyles have a direct and non-migratory life cycle. Eggs are shed in the faeces, hatch and develop to third stage larvae (L3) in faeces before migration onto pasture. Eggs hatch, when exposed to temperatures around 6-29ºC, to release the first stage larvae (L1), which subsequently develop to second stage larvae (L2), and finally to infective L3. These infective L3 migrate from the faeces onto the surrounding herbage. Time range between hatching and development to infective L3 greatly depends upon temperature and moisture (Ogbourne, 1972). Development is complete within 2 weeks during the summer in temperate areas (Ramsey et al., 2004). No successful larval development occurs at temperatures below 5ºC or above 37ºC (Ogbourne, 1972). The duration of larval survival is inversely proportional to environmental temperatures: larvae die quickly at temperatures above 32ºC, but can tolerate freezing conditions particularly well (Lucker, 1941). Horses are infected when they ingest infective L3s with forage. After ingestion, L3 invade the wall of the caecum and colon and remain encysted – process known as hypobiosis – in the mucosa and submucosa layers. The larvae may reside in the cysts for just a few weeks or for as long as 2.5 years (Smith, 1976). Henceforth, the larvae develop to late L3 (LL3) and then to forth stage larvae (L4). The latter emerges into the gut lumen and moults to become young adult worms – the fifth stage larvae (L5) – which then finally matures to adult male and female worms. After mating, the females lay eggs that are passed to the pasture with the faeces, completing the cycle.

2.1.1.2 Pathogenicity and Clinical Signs

Clinical signs are not always related to the cyathostomin burden. In moderate burdens the adults are relatively non-pathogenic, as they feed mostly of organic material within the intestinal lumen. Horses may harbour thousands of cyathostomins without developing detectable illness (Love et al., 1999). Clinical signs are most commonly caused by the presence of large numbers of mucosal larval stages, and burdens to the order of several million larvae have been reported (Proudman & Matthews, 2000). Some horses can develop a sub-clinical alteration in gastrointestinal function characterized by a mild inflammatory enteropathy, which leads to alterations of intestinal microcirculation and motility and subsequently to a protein losing enteropathy (Love et al., 1999). In temperate areas of the northern hemisphere, inhibition in development of cyathostomin larvae in the mucosa occurs primarily during autumn and winter, when these stages can comprise up to 90% of the total burden (Dowdall et al., 2002). If these larvae synchronously re-emerge in large numbers, especially over a short period of time, inflammation of the caecum and ventral colon, haemorrhages and excess mucus production occurs, which can lead to severe clinical signs (Love & Duncan, 1992). This can result in a fatal typhlocolitis, known as larval cyathostominosis (Giles, Urquhart, & Longstaffe, 1985). Larval cyathostominosis often occurs in late winter or early spring (Love & Duncan, 1992) and is more common in animals 1 to 3 years old, even when they are treated regularly with anthelmintics (Lyons, Drudge, & Tolliver, 2000). Usual clinical signs include sudden weight loss, subcutaneous oedema, colic and diarrhoea and in 50% of cases death (Love et al., 1999).

2.1.2 *Strongylus vulgaris*

Members of the subfamily Strongylinae, often referred to as "large strongyles", are parasites of the large intestine of equines and are distributed in five genera: *Strongylus*, *Triodontophorus*, *Craterostomum*, *Bidentostomum* and *Oesophahodont* (Lichtenfels et al., 2008). There are three major species acknowledged to infect horses: *Strongylus vulgaris*, *Strongylus edentatus* and *Strongylus equinus*, the most important clinically being *Strongylus vulgaris*.

Strongylus vulgaris is a ubiquitous, cosmopolitan parasite of the caecum and right ventral colon of wild and domestic horses (Anderson, 2000). It was considered for many years the major parasitic threat to equine health (Duncan & Pirie, 1975) because of its high pathogenic potential (Enigk, 1951).

2.1.2.1 Life Cycle

The life cycle was fully established in 1984 (Ogbourne & Duncan, 1984), although it is possible that its presence in the cranial mesenteric artery was already known in ancient Rome (Enigk & Grittner, 1921). Eggs are shed in the host faeces, thus reaching the environment. Hatching and larval development to infective L3 takes place at temperatures that range between 8 – 39ºC. At 30ºC, development to L3 stage occurs rapidly, requiring 3 – 4 days. On the other hand, at 12ºC development to L3 stage is slower, requiring 16 – 20 days (Anderson, 2000). Infective larvae can remain alive under dry conditions or months and even years (Anderson, 2000). Infection occurs when horses ingest the L3 on the herbage. Once ingested, the L3s exsheath in less than 2 hours in the small intestine (Bowman, 2014). After 1 – 3 days post-infection, the exsheathed larvae penetrate the intestinal mucosa and moult to L4 in the submucosa. The L4 then migrate and enter the lumen of submucosal arterioles. They migrate along the endothelium, into the caecal and colic arteries, which they reach in 14 days post-infection, and then to the cranial mesenteric artery and its main branches, normally 21 days after infection (Duncan & Pirie, 1975). There, the larvae undertake their final moulting into immature adults (L5), a process which can take up to 3 to 4 months (Wetzel, 1942). Afterwards, the larvae return to the intestinal wall, carried by the bloodstream to the small arteries in the subserosa of the intestinal wall. On arrival, the larvae can occlude these small arteries, whose walls then become inflamed and in due course are destroyed. Subsequently, the larvae enter the surrounding tissue and then form pea-sized nodules (Bowman, 2014). Finally, these nodules rupture and release the young larvae adults into the lumen of the intestine, where they sexually mature into adults and commence reproduction.

2.1.2.2 Pathogenicity and Clinical Signs

Similar to cyathostomins, *S. vulgaris* pathogenicity is caused by the presence of larval stages. Infection is not detectable until the parasites complete a full life cycle and by that time serious arterial damage might have occured. The prognosis is often poor (Drudge, 1979). The migration of L4 in the cranial mesenteric artery normally causes verminous endarteritis characterized by roughened *intima*, thickening of the arterial wall, and thrombus formation (Duncan, 1974). Sometimes, emboli may break away and lodge in smaller vessels of the intestinal track, causing a thrombo-embolic colic syndrome (Enigk, 1951). This leads to local ischemia and infarction of the corresponding intestinal portion (Enigk, 1951; Duncan & Pirie, 1975). Clinical signs include a very painful colic with elevated heart rates, profuse sweating and cardiovascular shock (Drudge, Lyons, & Szanto, 1966; Duncan & Pirie, 1975). When nodules rupture, and larvae are released into the intestinal track, considerable haemorrhage might occur. In very heavy burdens, bleeding can be fatal (Radostits, Gay, Arundel, & Blood, 2000).

2.1.3 Diagnosis of strongyle infections

The only *ante-mortem* method available to distinguish between large *versus* small strongyle infections is by performing larval coprocultures. Larvae are then identified and differentiated between cyathostomins, *S. vulgaris*, *S. equi*, *S. edentatus* and other strongyle species. Identification of larvae is based on the number of intestinal cells (Thienpont, Rochette, & Vanparijs, 1986; Madeira de Carvalho et al., 2008) and will be described further in this thesis. Coproculture is time consuming (a minimum of two weeks are required until final diagnosis), it requires a trained technician, and the logistics for large numbers of samples may be difficult (Nielsen, Olsen, Lyons, Monrad, & Thamsborg, 2012a). Moreover, the superior prevalence of cyathostomins nowadays – when compared with the relative low frequency of *Strongylus* species – makes the sensitivity of this method likely to be poor (Nielsen et al., 2008). The advantages are that, apart from the microscope, it does not require further expensive equipment, and the cost of laboratory supplies is very low (Cernea et al., 2008; Nielsen et al., 2012a). Nielsen et al., (2008) developed and validated a real-time PCR assay capable of detection and semi-quantification of *S. vulgaris* DNA, extracted from eggs. This procedure is easily manageable as quantitative (q)PCR machines are increasingly common in diagnostic laboratories, the test can be completed within one working day and it is more sensitive than traditional larval culture (Nielsen et al., 2008). However, a study in 2012 by the same author (Nielsen et al., 2012a) stated that the qPCR assay had the same sensitivity as the larval culture, hence more studies are needed to optimise this technique. When compared to larval culture, qPCR has obvious advantages: the culture period is skipped and samples can be analysed immediately. In addition, eggs can be kept in 70% ethanol for a considerable time until further analysis. On the other hand, PCR reagents and DNA extraction kits are quite expensive (Nielsen et al., 2012a). However, the two methods share a common disadvantage: both are limited to measuring egg output in faecal samples, and as mentioned before, strongyle egg shedding is variable, which can challenge the accuracy of the methods (Nielsen et al., 2012a). Infection is not detectable until the life cycle has been completed, by which time serious arterial damage may have already occurred. Approximately 25 years ago, a diagnostic ELISA assay to detect IgG(T) antibodies was attempted, but without success (Klei, Chapman, Torbert, & McClure, 1983).

As encysted cyathostomin larvae are in a state of dormancy, they do not produce eggs, resulting in low (i.e. <100 eggs per gram) or negative FEC results (Dowdall et al., 2002). Unfortunately, there are still no validated diagnostic tests that give information about mucosal or total cyathostomin burden (Proudman & Matthews, 2000; Dowdall et al., 2002; Cernea et al., 2008) which can constitute up to 90% of the total worm burden during certain times of year (Murphy & Love, 1997). Efforts have been made to develop a diagnostic assay for the detection of larval cyathostomin (McWilliam, Nisbet, Dowdall, Hodgkinson, & Matthews, 2010). This paper reports the identification of a likely protein component of a protein, designated cyathostomin gut-associated larval antigen-1 (Cy-GALA-1). This protein shows to be a target of serum IgG(T) responses in experimentally and naturally infected horse populations. The recombinant Cy-GALA-1 exhibited no reactivity to serum from horses mono-specifically infected with other helminth species, and antisera raised to the recombinant protein did not bind to adult stage extracts of heterologous species. However, to be truly useful, this diagnostic tool must be semi-quantitative and the test is still being patented and developed for commercialization. More recently, segments of the Cy-GALA gene were isolated from 13 further common species and the associated proteins expressed in the recombinant form. Four of these proteins have been tested in the ELISA with the objective of informing on the presence of encysted larval infection and the level of burden (Mitchell et al., 2016).

2.2 Ascarididae

The family Ascarididae also belongs to the class Nematoda. These larger parasites occur in most domestic animals and have a great veterinary importance due to their migratory behaviour (Taylor & Coop, 2007).

2.2.1 *Parascaris equorum*

Parascaris equorum, also known as the equine roundworm, is the largest nematode that infects horses, donkeys and zebras. This common and ubiquitous nematode usually affects animals younger than 2 years-old (Clayton, 1986). Prevalence and intensity of infection decrease markedly as horses mature. It is thought that horses develop a strong acquired immunity against ascarid infections during the first year of their lives, which means that infections are limited to foals and yearlings, and rarely diagnosed in horses over two years of age (Clayton, 1986; Reinemeyer, 2012).

2.2.1.1 Life Cycle

P. equorum has a direct life cycle, in which eggs are passed into the environment with the faeces. Eggs are spherical with roughened shells and have a sticky surface, which causes them to adhere firmly to each other and to objects in the environment (Anderson, 2000). Development to infective larvae takes place inside the egg, and occurs within 9-10 days under suitable environmental conditions (25ºC to 35ºC). It is thought that embryonated eggs can remain viable for several years due to their thick, three-layered protective shell. Horses, mainly foals, become infected by ingesting eggs containing the L3 (Bowman, 2014). After ingestion, the eggs hatch in the small intestine, releasing the larvae. These larvae then undergo a complex migration before returning to the gastrointestinal tract. Approximately 24 hours after ingestion, the larvae penetrate the liver, via the portal vein, and remain there for approximately a week. In 7 – 14 days, the larvae move to the hepatic vein and are carried to the caudal vena cava, heart and pulmonary artery to the lungs, where they enter the alveoli. Larvae can be found in the pulmonary parenchyma and the airways during the first four weeks after infection. After completing a moult in the lungs, the larvae ascend in the expectorant mucus and reach the tracheoesophageal area, where they are swallowed and return to the intestinal tract. The adults start laying eggs approximately 75 – 80 days after infection (Clayton, 1986) and usually remain in the duodenum and the proximal jejunum. In heavy infections, adult *P. equorum* can be found throughout the length of the small intestine (Clayton & Duncan, 1979). Foals are normally infected soon after birth and maintain infections for 6 – 12 months, after that period the animals develop immunity against infection.

2.2.1.2 Pathogenicity and Clinical Signs

When high burdens occur, the animals will show severe clinical signs such as coughing, nasal discharge, lethargy, diarrhoea, illthrift, rough hair coat, gastrointestinal impaction and colic (Clayton & Duncan, 1978; Cribb, Cote, Boure, & Peregrine, 2006). In addition, substantive burdens may be associated with spontaneous non-strangulating obstruction, and occasionally perforation of the small intestine (Cribb et al., 2006; Nielsen, 2016). Clinical signs of infection are mainly seen in foals (Lyons, Tolliver, & Collins, 2006). A recent study (Nielsen, 2016) suggests that deworming heavily parasitized foals with an anthelmintic that has a paralytic mode of action may cause acute small intestinal impaction. Furthermore, larvae migration has been associated with clinical nervous syndromes, including signs of vertigo and epilepsy (Soulsby, 1965). Moreover, migrations often trigger an eosinophilic response, with a subsequent eosinophilia. The rise in blood eosinophils is proportional to the magnitude of the infection. However, eosinophilia is a typical feature of many parasitic migrations and should not be considered specific for parascariasis (Clayton, 1986).

2.2.1.3 Diagnosis of *Parascaris equorum* infection

Diagnosis is based on the clinical signs and the presence of *P.equorum* eggs in faeces. In heavy burdens, the number of EPG may be high. However, clinical sings may be due to large number of immature worms and in this case few or no eggs will be found by the parasitological analysis (Soulsby, 1965).

2.3 Oxyuridae

The family Oxyuridae belong to the class Nematoda. The members of this family are found in the large intestine or caecum of vertebrates. It consists of eight genera, which includes *Oxyuris* spp., a nematode of horses.

2.3.1 *Oxyuris equi*

Oxyuris equi, also known as pinworm, due to the sharp, tapering tails of female parasites, is a cosmopolitan parasite of horses. These nematodes inhabit the distal gastrointestinal tract, and female worms deposit eggs outside the host. *Oxyuris equi* has global distribution. Typical infections involve low numbers of mature worms but, on the other hand, the number of L4 stages may exceed 10,000 in a single horse (Lyons, Tolliver, & Collins, 2007).

2.3.1.1 Life Cycle

O. equi has a direct life cycle, in which horses get infected by ingesting the eggs. The ingested eggs hatch in the small intestine, and the infective L3 are released in the small intestine. They, subsequently, invade the crypts of Lieberkühn in the anterior large bowel. After 3 to 11 days, the larvae moult to L4, which possess a marked buccal capsule, and emerge into the large intestine, where they attach to the mucosa of the ventral colon (Wetzel, 1930). After approximately 50 days, the final moult to L5 occurs, and full maturation to reproducing adults is accomplished about 139-156 days later (Enigk, 1949). Adults usually inhabit the right dorsal colon, but may be also found in more proximal sections when worm burdens are high (Enigk, 1949). After fertilization, the adult gravid females migrate into the rectum and then to the perineum, where they lay clusters of eggs (8000 – 60.000 eggs/female) in a yellowish-grey gelatinous material. The surface temperature of the skin and the high relative humidity of the perineum offer the optimal microclimate for development of eggs. After this process, females are expelled and die (Enigk, 1949). Development of the egg is rapid within 24 to 36 hours the eggs containing L1 are found, and after 3 to 5 days of being laid the eggs contain the infective larvae (L3).

2.3.1.2 Pathogenicity and Clinical Signs

Little harm is caused by the adult parasites in the large intestine and only in severe infections the L4s produce any clinical manifestations (Soulsby, 1965). This fact is easily explained, since adults have a less well developed buccal cavity than that of L4 stages, which prevents them from attaching and feeding on the mucosa (Enigk, 1949). The presence of L4s and their feeding habits causes superficial erosion and irritation of the colonic mucosa, which subsequently leads to oedema and erythema of the colon and caecum walls (Enigk, 1949). Clinical signs include lymphocytosis, rough hair coats and poor body condition. In high burdens, the presence of larvae produces an inflammation of the mucosa, resulting in mild colic symptoms (Urguhart, Armour, Duncan, Dunn, & Jennings, 1996). The main pathogenic effect is caused by the ovigerous females when they lay eggs on the perineum area. The gelatinous material, which contain the eggs, is markedly irritant and causes an intense pruritus of the anal region and a subsequent marked inflammation of the area, leading to tail-rubbing behaviour, loss of hair and later thickening and scaling of the skin. The latter symptoms results in a distinct appearance, known as “rat-tailed” condition. The excessive rubbing may injure the skin and lead to secondary bacterial infection and more deep-seated damage (Soulsby, 1965).

2.3.1.3 Diagnosis of *Oxyuris equi* infection

Diagnosis is based on the clinical signs. Adult female worms may be found crawling on the perineum area and scrapings from that region will reveal large number of eggs (Soulsby, 1965). Egg collection can be done by the Scotch Tape Method. A small piece of transparent scotch tape, approximately 4 cm in length, is pressed onto the skin of the anus and then placed on a microscopic slide (Kaufmann, 1996). Eggs are not normally passed in the faeces and consequently a faecal examination may fail to confirm diagnosis (Soulsby, 1965). However, if the faeces are collected directly from the rectum with an obstetric glove, eggs might be found in the FECs (Reinemeyer & Nielsen, 2012).

2.4 Anoplocephalidae

The family Anoplocephalidae, unlike the previous described parasites, belongs to the class Cestoda. In this family, there are three species capable of infecting horses: *Anoplocephala perfoliata*, *Anoplocephala magna* and *Anoplocephaloides mamillana* (formely known as *Paranoplocephala mamillana*). The latter two species are rarely encountered (Borgsteede & van Beek, 1996; Meana et al., 2005) and relatively harmless, so focus will be on the more prevalent *A. perfoliata*.

2.4.1 *Anoplocephala perfoliata*

Anoplocephala perfoliata is the most common tapeworm of equids. This specie dwells in the posterior part of the small intestine, the anterior part of the caecum and less frequently in the colon of horses. The majority of horses have low burdens, which subsequently do not cause any obvious ill-thrift (Soulsby, 1965).

2.4.1.1 Life cycle

A. perfoliata has an indirect life cycle, which means it needs an intermediate host to complete its life cycle. Each worm has male and female segments. Reproduction occurs via hermaphroditism, in which the male segments fertilise the female segments. Afterwards, the reproductive organs deteriorate, leaving only a uterus full of eggs. The fertilised segment – mature proglotid – detaches from the rest of the worm's body and migrates to the large intestine. Eggs are passed into the pasture with the faeces, individually or, more usually, in the gravid segment. The latter having the appearance of a grain of rice (Soulsby, 1965). The eggs are infective to the intermediate host: an oribatid mite. These mites live on the ground, feeding of plant debris. They are found especially in moist environmental conditions. In arid areas low numbers of oribatid mites are present, which consequently leads to a lower incidence of infection. After ingestion, an oncosphere is digested from the egg within the alimentary tract of the mite. The oncosphere migrates into the hemocoel (body cavity) of the mite, and develops into an infective stage known as a cysticercoid (Reinemeyer & Nielsen, 2012). Cysticercoids are produced in the mites 2 to 4 months after infection and horses are infected by ingestion of these mites along with the herbage. The cysticercoids released from the mite's tissue in the gastrointestinal tract, and primitive scolices attach to the lining of the preferred region of gut (Reinemeyer & Nielsen, 2012). Development to adult form is accomplished within 6 to 10 weeks (Soulsby, 1965).

2.4.1.2 Pathogenicity and Clinical Signs

A. perfoliata, differs greatly from the nematodes. Not only does the life cycle involve an intermediate host, but also, adult stages appear to be more pathogenic than larval stages (Nielsen, 2012). *A. perfoliata* tends to cluster in the area near to the ileocaecal valve. There, several dozen and even hundreds of parasites can be found, with their scolices attached to the mucosa and the bodies tightly packed together. The mucus membrane shows oedema and inflammatory changes and where the parasites are attached an ulcerous depression occurs. The base of the ulcer is quite haemorrhagic, and surrounded by an elevated ring of granulation tissue, leading to a decrease in valve elasticity (Soulsby, 1965). The severity is usually proportional to parasite infection intensity (Pearson, Davies, White, & O'Brien, 1993). The presence of this cestode in the ileo-caecal junction has been associated with two specific types of colic: spasmodic colic and ileal impaction colic (Proudman, French, & Trees, 1993; Nielsen, 2016). In a case-controlled study, horses with tapeworms were 26 times more likely to have a spasmodic colic than non-infected animals (Proudman et al., 1993).

2.4.1.3 Diagnosis of tapeworm infection

Diagnosis is based on clinical signs and the presence of tapeworm eggs in the faeces. However, faeces usually contain small numbers of tapeworms eggs, resulting in poor sensitivity which makes conventional flotation methods inadequate for diagnosing cestodosis. A modification of a centrifugation-based egg counting method, in which 40 grams of faeces are analysed, has been validated to have a diagnostic sensitivity and specificity of 0,61 and 0,98, respectively (Proudman & Edwards, 1992). In 1996, the first validated and commercially available diagnostic test was released (Proudman & Trees, 1996), which measures specific antibody levels in blood, but antibody levels tend to reflect exposure rather than actual infection, and horses can remain seropositive for months after treatment (Abbott, Mellor, Barrett, Proudman, & Love, 2008). Most recently, a saliva-based ELISA has been validated for tapeworm diagnostic and made commercially available in the United Kingdom (Lightbody, Davis, & Austin, 2016).

Chapter 3 – Anthelmintic Drug Classes

Helminth control in horses has been accomplished through the administration of broad-spectrum anthelmintics. The various anthelmintics are grouped into classes, according to their chemical structure as well as their site of action (Sangster, 2001). Currently there are three drug classes of anthelmintics licensed for use in horses: benzimidazoles (BZ; fenbendazole, oxfendazole), tetrahydropyrimidines (THP; pyrantel salts) and macrocyclic lactones (ML; ivermectin and moxidectin). All details can be seen in tables 1 and 2. Those three classes have good efficacy (>90%) against four groups of target parasites: large strongyles, cyathostomins, ascarids and pinworms. However, they do not act uniformly against all parasitic targets; usually one parasite requires a higher dosage than the others – also known as dose-limiting parasites (DLPs) – and *P. equorum* is the DLP for most equine anthelmintics (Reinemeyer, 2009b). How this may influence development towards resistance will be discussed further on. Currently, none of the new anthelmintic drugs classes used in dogs and cats (e.g. emodepside) and sheep (e.g. derquantel and monepantel) are commercially available for horses. Some of these new drugs have shown to be affective against a range of nematodes, including cyathostomins, however there is no indication of registration intent for horses (Epe & Kaminsky, 2013).

Table 1 - The most common anthelmintics used against equine helminths. Adapted from Proudman & Matthews (2000) and AAEP Parasite Control Guidelines, 2013

Drug class	Mechanism of Action	Active Ingredient	Spectrum of Activity
Benzimidazoles (BZs)	Binds to beta tubulin and prevent its polymerization into microtubules, which interfere with worm's energy metabolism, leading to paralysis and death.	Fenbendazole	Efficacy against adult cyathostomins and other roundworms (*O. equi*, *P. equorum*, etc). Five-day course effective against inhibited mucosal stages of cyathostomins. Ovicidal effect on nematode eggs.
		Oxibendazole	Efficacy against adult cyathostomins and other roundworms (*O. equi*, *P. equorum*, etc). Ovicidal effect on nematode eggs.
Tetrahydropyrimidines (THPs)	Causes an irreversible rigid paralysis at the neuromuscular junction	Pyrantel embonate or pyrantel pamoate	Efficacy against roundworms, such as: large and small adult strongyles, *Oxyuris equi*; mainly effective against ascarids such as *P. equorum*. Efficacy against tapeworms.
Macrocyclic Lactones (MLs)	Act as agonist of the GABA neurotransmitter in nerve cells and on glutamate-gated chloride channels in nematode nerve and muscle cells, disturbing the normal transmission of nervous stimuli to muscles, which results in flaccid paralysis.	Ivermectin	High efficacy against roundworms, such as: adult cyathostomins, large strongyles, pinworm larval forms and *P. equorum*.
		Moxidectin	High efficacy against roundworms, such as: adult cyathostomins, large strongyles, pinworm larval forms and *P. equorum*. Also effective against developing encysted cyathostomins.

Table 2 - The most common association of anthelmintics used against equine helminths. Adapted from Proudman & Matthews (2000) and AAEP Parasite Control Guidelines, 2013

Association of Drug Classes	**Mechanism of Action**	**Active Ingredient**	**Spectrum of Activity**
Macrocyclic Lactones (MLs) **+** **Isoquinoline Pyrazines**	Act as agonist of the GABA neurotransmitter in nerve cells and on glutamate-gated chloride channels in nematode nerve and muscle cells, disturbing the normal transmission of nervous stimuli to muscles, which results in flaccid paralysis. + Affect the permeability of calcium ions in the muscular membrane, leading to paralysis and death.	Moxidectin + Praziquantel	High efficacy against roundworms, such as: adult cyathostomins, large strongyles, pinworm larval forms and *P. equorum*. Also effective against developing encysted cyathostomins. + High efficacy against tapeworms.
		Ivermectin + Praziquantel	High efficacy against roundworms, such as: adult cyathostomins, large strongyles, pinworm larval forms and *P. equorum*. + High efficacy against tapeworms.

3.1 Anthelmintic Resistance

Anthelmintic resistance (AR) occurs when a larger proportion of individuals in a parasite population, usually affected by an anthelmintic treatment, ceases to be affected, or a greater concentration of drug is needed to reach a certain level of efficacy (Prichard, Hall, Kelly, Martin, & Donald, 1980). AR in horses has been reported for small strongyles (Sangster, 1999a), *P. equorum* (Sangster, 1999a; Kaplan, 2004) and, more recently, for *O. equi* (Scháňková et al., 2013; Wolf, Hermosilla, & Taubert, 2014). Anthelmintic safety and effectiveness, combined with the fact that they are rather low-cost, led to several years of overuse, i.e. frequent "blanket" treatments of all animals based on the egg reappearance period (ERP) of the drug at the time of licencing (Kaplan, 2002). Moreover, very few horse owners/managers calculate the exact weight of the animals, and instead choose to estimate the animals' weight and then administer an approximate dose. This method can easily lead to administration of sub-therapeutic doses, which will be ineffective in terms of killing parasites, and will also promote the development of AR (Proudman & Matthews, 2000). In addition, parasitic nematodes have great genetic diversity and rapid generation rates, which added to their considerable population sizes, makes resistance development a rapid process (Gilleard & Beech, 2007). Therefore, host, parasite and environmental factors can deeply influence the development of AR. Of the factors influencing the development of AR, those affecting the parasite itself are the most complex. Examples of these are: genetic mechanisms and mutations among species, the number of genes involved, and anthelmintic pharmacokinetics. Host factors include, immunity and anthelmintic pharmacokinetics; whereas drenching frequency and climatic variables must be included in environmental factors (Nielsen et al., 2014). Unfortunately, AR is a permanent genetic feature, which cannot revert to susceptibility (Leathwick, Pomroy, & Heath, 2001). Among the factors related to the parasites, the population of worms *in refugia* is now considered one of the most important features that mitigate the development of anthelmintic resistance (van Wyk, 2001; Kaplan, 2004).

3.2 Refugia

Refugia is defined as "the proportion of the parasite population that is not exposed to a particular given control measure, thus escaping selection for resistance" (van Wyk, 2001). Parasites residing in untreated animals, free-living stages in the environment at the time of the treatment, and those that are not affected by the drug (i.e. encysted parasites during a treatment with a non-larvicidal anthelmintic) are considered *in refugia* (van Wyk, 2001). Parasites *in refugia* are not exposed to selection pressure for resistance, and thereby provide a reservoir of non-resistant alleles to dilute resistant genes in the remaining population (Sangster, 1999b; van Wyk, 2001). Thus, the size of the population *in refugia* has a very direct influence on the degree of selection pressure for resistance (Martin, Le Jambre, & Claxton, 1981; van Wyk, 2001). It has been proposed that the more parasites within a population that are *in refugia*, the slower resistance will develop (Kaplan & Nielsen, 2010; Nielsen et al., 2014). When a drug is excessively used and eliminates the great majority of the anthelmintic susceptible parasite burden (leaving only the resistant individuals), and the number of worms *in refugia* is low, resistance is likely to be established faster. Thus, it is most likely that a combination of the proportion of a given population *in refugia* and drenching frequency are the major factors that determine the degree of selection pressure for resistance (van Wyk, 2001). Climate is also likely to have an indirect impact on the development of AR. Climate conditions regulate the development and survival of free-living stages. As a result, the size of the refugia varies during different seasons of the year (Nielsen, Kaplan, Thamsborg, Monrad, & Olsen, 2007). Extra care should be taken when the proportion of parasites *in refugia* is known to be small such as cold winters, hot summers and droughts (Nielsen et al., 2007; AAEP, 2013).

3.3 Indicators of Anthelmintic Resistance

In horses, the only *in vivo* test suitable for detecting AR in all available drug classes is the Faecal Egg Count Reduction Test (FECRT), which follows the guidelines of the World Association for the Advancement of Veterinary Parasitology (WAAVP) published by Coles et al. (1992, 2006).

3.3.1 Faecal Egg Count Reduction Test

The Faecal Egg Count Reduction Test (FECRT) is the current “gold standard” *in vivo* method to determine the efficacy of an anthelmintic treatment. It is a simple test and can be used for several species – horses included – and for all three classes of anthelmintics. The test was originally developed for use in small ruminants (Coles et al., 1992; Coles et al., 2006). Later on, it was adapted for use in horses (Vidyashankar, Hanlon, & Kaplan, 2012). Anthelmintic efficacy is estimated based on the arithmetic group mean reduction in FECs observed between Day 0 (treatment day) and Day 14 post-treatment.

$$\text{Group mean FECRT (\%)} = \frac{(\text{Pre treatment mean FEC} - \text{14 days post treatment mean FEC}) \times 100}{\text{Pre treatment mean FEC}}$$

There are no equine-specific guidelines at the moment. Until they are established, it is highly recommended to follow the 2013 Guidelines for Parasite Control developed by the American Association of Equine Practitioners (AAEP, 2013). For equines, a minimum of 5 – 10 animals should be assessed for more accurate results. Also, horses should not have received anthelmintic treatment at least 8 weeks prior to the FECRT. If moxidectin was used, the FECRT should be performed after 12 weeks (Kaplan & Nielsen, 2010; AAEP, 2013). Several FEC methods can be used, however it is recommended to employ the most sensitive technique possible. Ideally, an egg detection limit of 10 EPG or less is preferred (Matthews & Lester, 2015), but the detection limit could be extended up to 25 EPG (AAEP, 2013). The same methodology has to be followed for the FECs on Day 0 and Day 14 post-treatment for the results to be valid (Kaplan & Nielsen, 2010).

The term “detection limit” is used to describe the sensitivity of a FEC method. If the detection limit is high, the sensitivity will not be as high, as the method may not detect changes in egg abundance below or around the detection limit. Therefore, a lower detection limit means that the method is more sensitive to slight changes. Moreover, the protocols with high detection limit inflate the high FECs and resistance might be reported when there is not (Matthews & Lester, 2015). The presence of anthelmintic resistant parasites is confirmed when the tested drug fails to achieve high levels of egg reduction post-treatment. There are currently no agreed formal cut-off values, therefore different values have been used in various studies. However, the most accepted cut-off values for each anthelmintic class are shown in table 3 (AAEP, 2013).

Table 3 - Suggested cut-off values regarding the FECRT. Adapted from AAEP Parasite Control Guidelines, 2013 (Revised February 2016).

Anthelmintic	Sensitive	Suspected Resistance	Resistant
Benzimidazoles (Fenbendazole and Oxibendazole)	>95%	90 – 95%	<90%
Tetrahydropyrimidines (Pyrantel salts)	>90%	85 – 90%	<85%
Macrocyclic Lactones (Ivermectin and Moxidectin)	>98%	95 – 98%	<95%

Before making any assumptions, it is important to take under consideration other causes of decreased efficacy, such as misdosing, inadequate sample storage, inadequate FEC protocol, etc. (Kaplan & Nielsen, 2010).

Unfortunately, the FECRT has certain limitations. First of all, it cannot quantify the level of resistance, it can only lead to a strong suspicion (Kaplan, 2002). In a previous study with sheep it was suggested to consider an insensitivity for detecting resistance when the proportion of genetically resistant worms is below 25% (Martin, Anderson, & Jarrett, 1989). Thus, low levels of resistance may be impossible to detect. Another study with sheep showed that FECRT provide poor correlations with the actual nematode numbers (Miller, Waghorn, Leathwick, & Gilmour, 2006).

In addition, inherent variability in FEC data can affect the outcome and interpretation of the FECRT results in horses (Lester et al., 2013b). These factors include:

- Differences in egg shedding; As parasites tend to reduce egg shedding during seasons when parasite transmission is less likely to occur (Poynter, 1954), FEC performed during these periods might be less reliable.

- Over-dispersion of strongyle eggs in faeces, as FECs performed in the same horse may vary at different times (Denwood et al., 2012).
- Non-uniform distribution of eggs in faecal solutions (Vidyashankar et al., 2012).
- Type of FEC methodology used, as different methods have different sensitivities (Lester & Matthews, 2014).
- Sampling and storage of the faecal samples (Nielsen et al., 2010).

Nevertheless, the FECRT is a very important tool, especially considering that detecting resistance at an early stage may allow the maintenance of anthelmintic efficacy (von Samson-Himmelstjerna, 2012). This is achieved by combining several appropriate measures, such as reducing treatment frequency and promoting anthelmintic non-resistant parasites *in refugia* (van Wyk, 2001; von Samson-Himmelstjerna, 2012).

3.3.2 Egg Reappearance Period

The Egg Reappearance Period (ERP) is the post-treatment period in which egg shedding remains negligible (Duncan, 1985). A shortening of this period can be considered an early indicator that the parasite populations are shifting towards resistance, hence, the ERP may be a more sensitive indicator of resistance than the standard FECRT (Sangster, 1999b). One must not forget to consider that the ERP is irrelevant if AR on a farm is already established, as there is no egg disappearance (Kaplan & Nielsen, 2010). In the same manner as for the FECRT, the ERP cut-off values vary with the different anthelmintics. Reinemeyer & Nielsen (2012) stated that, in susceptible populations, the ERP should be more than:

- Pyrantel and benzimidazoles – 4 - 5 weeks
- Ivermectin – 6 - 8 weeks
- Moxidectin – 10 - 12 weeks

Those values have been decreasing over the time, which is expected due to their common use and the overall spread of AR. When released for horse use, moxidectin had an ERP of 16 – 22 weeks (Jacobs, Hutchinson, Parker, & Gibbons, 1995), whereas nowadays some studies report an ERP of 4 – 5 weeks (Rossano et al., 2010; Relf et al., 2014).

There is no standard method to determine the ERP, and various methods have been used over the last years:

1) ERP defined as the week of first positive strongyle FEC after anthelmintic administration (Lyons, Tolliver, Ionita, & Collins, 2008a; Molento et al., 2008).

2) ERP defined as the week in which the mean egg count reaches a fixed threshold, such as 100 or 200 EPG (Jacobs et al., 1995; Boersema et al., 1996).

3) ERP defined as the week in which the arithmetic mean of strongyle FEC exceeds the arithmetic mean of FEC obtained at Day 0 by 10% (von Samson-Himmelstjerna et al., 2007; Larsen, Ritz, Petersen, & Nielsen, 2011).

4) ERP defined as the week in which the arithmetic mean of strongyle FEC exceeds the arithmetic mean of FEC obtained at Day 0 by 20% (Tarigo-Martinie, Wyatt, & Kaplan, 2001; Reinemeyer, 2009a).

Those differences may have led to inconsistencies in the reported ERP in some of these studies. Therefore, it is of maximum importance to establish a standard method of ERP measurement, so that comparative analysis can be made successfully among studies (Relf et al., 2014) and to create baseline ERPs for different regions, and monitoring that value regularly, especially in foals (Sangster, 1999b).

3.4 Anthelmintic Resistance in Cyathostomin Populations

Resistant cyathostomin populations have now been reported against all three available anthelmintic classes (Molento et al., 2008). Reversion to susceptibility does not seem to occur, meaning that once drug resistance is established, it becomes a permanent trait (Kaplan, 2004). In fact, a known BZD-resistant cyathostomin population remained resistant even after being left untreated for approximately 20 years (Lyons et al., 2007). In another study (Lyons, Tolliver, Drudge, Collins, & Swerczek, 2001), a BZD-resistant cyathostomin population was exposed to bimonthly treatments with pyrantel for eight years, which resulted in pyrantel resistance but did not change the BZD resistance status.

3.4.1 Benzimidazole Resistance

Benzimidazole resistance was the first AR to be reported among the three anthelmintic classes (Wescott, Jen, Hellier, Stenslie, & Torbeck, 1982). Nowadays, BZD resistance is the most common type of AR and can be found in various countries worldwide (Kaplan, 2004; Wirtherle, Schnieder, & von Samson-Himmelstjerna, 2004; Traversa et al., 2007; Kuzmina & Kharchenko, 2008). A daily oral administration for five consecutive days of fenbendazole was considered to be effective against equine encysted cyathostomins (Duncan et al., 1998). However, several more recent studies reported a failure to achieve acceptable egg count reduction after fenbendazole administration (Chandler, Collins, & Love, 2000; Rossano et al., 2010; Mason et al., 2014; Relf et al., 2014).

3.4.2 Pyrantel resistance

The first cases of resistance to pyrantel were reported in the USA (Chapman, French, Monahan, & Klei, 1996). It was thought that this resistance was associated with the use of a pyrantel tartrate formulation administrated at a low dosage, on a daily basis, in feed, throughout the year (Kaplan, 2002). Recently, however, reports about pyrantel resistance across Europe have become more common (Madeira de Carvalho, Farrim, Afonso-Roque, & Fazendeiro, 2003; Kaplan, 2004; Traversa et al., 2009), where the daily administration product is not available. Worryingly, one study stated that pyrantel resistance was found in approximately one third of the 102 horse farms examined in Germany, Italy and the UK (Traversa et al., 2009).

3.4.3 Macrocyclic Lactones Resistance

Until 1999, macrocyclic lactones (MLs) were considered highly effective against cyathostomins, with no reports of resistance (Sangster, 1999b). Unfortunately, evidences of resistance after treatment with ivermectin have been reported in Brazil (Molento et al., 2008), Italy and the UK (Traversa et al., 2009). Also, reports of shortened ERP have been observed in several regions (Lyons, Tolliver, Ionita, Lewellen, & Collins, 2008b; Molento et al., 2008; Relf et al., 2014). Regarding moxidectin, reports of resistance have been confined to Brazil (Molento et al., 2008). However, reports of shortened ERP following treatment were submitted in Brazil (Molento et al., 2008), the UK (Relf et al., 2014) and the USA (Rossano et al., 2010; Lyons et al., 2011a). In 2009, a study performed in the USA (Lyons, Tolliver, & Collins, 2009), showed that a shortened ERP observed after moxidectin and ivermectin treatment may be due to development of ML resistance on the luminal fourth larval stage (LL4). This would lead to incomplete elimination of cyathostomins or to a reduced time of maturation of the parasite, thus leading to a shorter ERP (Lyons & Tolliver, 2013). More importantly, the ML moxidectin is the only remaining product effective against all stages of cyathostomins, including the encysted form EL3; therefore, preservation of its efficacy is crucial (Matthews, 2011; Stratford, McGorum, Pickles, & Matthews, 2011).

3.5 Anthelmintic Resistance in *Parascaris equorum* populations

As mentioned before, *P. equorum* is a dose-limiting parasite (DLP) for most equine anthelmintic (Reinemeyer, 2009b). DLP have a lower threshold for the development of resistance, as they need a higher dosage of anthelmintic to accomplish a successful treatment (Reinemeyer, 2009b). A fact that may explain why there are reports of resistance in this particular species. Reports of resistance to MLs in *P. equorum* were first documented in the Netherlands (Boersema, Eysker, & Nas, 2002). Thereafter, more studies in other countries, including Canada (Hearn & Peregrine, 2003), Denmark (Schougaard & Nielsen, 2007), Germany (von Samson-Himmelstjerna et al., 2007), Brazil (Molento et al., 2008) and Italy (Veronesi, Fioretti, & Genchi, 2010) confirmed the presence of ML resistance in this parasite. Resistance to pyrantel seems to be restricted to the USA and may be associated with the practice of daily treatment with low dosage of this anthelmintic drug mixed into the feed (Lyons et al., 2008a; Lyons, Tolliver, Kuzmina, & Collins, 2011b). However, new reports of reduced efficacy in Australia (Armstrong et al., 2014) show that resistance is spreading outside the USA.

3.6 Anthelmintic Resistance in *Oxyuris equi* populations

All three drug classes have demonstrated efficacy against *O. equi* infections (Reinemeyer, 2012). However, some reports delivered in the last few years stated a lack of efficacy using MLs (Reinemeyer, 2012; Schánková et al., 2013; Wolf et al., 2014) even with a higher dosage. It is still unclear if this is due to the development of resistance or to an incomplete efficacy. In addition, there is no FECRT for pinworm, which translates in a poor documentation of clinical efficacy of oxyuricidal treatments (Reinemeyer, 2012).

Chapter 4 – Chemical and Non-chemical helminth control practises

4.1 Interval Treatment

The Interval Treatment was the first to attempt strategic parasite control for horses, and was designed to eliminate parasite-associated disease related to *S. vulgaris* infections. All horses were treated bimonthly with rotation of anthelmintic classes between successive treatments, to prevent parasite maturation and pasture contamination (Drudge & Lyons, 1966). This approach became widely adopted and proved to be extremely effective in reducing morbidity and mortality due to *S. vulgaris* infection (Lyons, Tolliver, & Drudge, 1999). As a result, this parasite is no longer considered a threat to horse health and is rarely diagnosed in developed regions (Kaplan, 2002). Unfortunately, another consequence of this practice was the development of AR, particularly in cyathostomin species (Kaplan, 2004), which until then were considered a lesser threat compared to the highly pathogenic *S. vulgaris* (Drudge & Lyons, 1966). The old concept of keeping FEC near zero by frequent use of anthelmintics is not anymore considered a sustainable approach and may actually increase the risk of parasitic disease from cyathostomin infections (Herd, 1990). However, this method is still highly followed by Thoroughbred studs farms all over the UK, which, in addition, usually use the same anthelmintic class in every treatment (Relf, Morgan, Hodgkinson, & Matthews, 2012). A questionnaire study conducted in Ireland (O'Meara & Mulcahy, 2002), reported that a large majority of respondents indicated that owner/managers were responsible for helminth control strategies instead of veterinarians. Furthermore, 38% of respondents stated that the primary anthelmintic method used was interval treatment at intervals of 4-6 weeks, followed by deworming at intervals of 6-8 weeks (34%) and 2-6 months (28%).

4.2 Strategic Treatment

Strategic Treatment involves using anthelmintic drugs at particular times of the year based on parasite life cycles (Proudman & Matthews, 2000) but without taking into account variation in parasite burden and nematode egg excretion, which is known to differ markedly among individuals (Gomez & Georgi, 1991). Regarding horse strongyles, this method can be easily established due to their seasonality. Thus, all horses are given an anthelmintic drug in the middle of the grazing season and again in autumn. However, as weather patterns can vary markedly over different years, drenching might not be coincident with the peak of larvae burden in pasture, leading to an inefficient anthelmintic treatment. Moreover, this method is also susceptible to breakdown when heavily infected horses are added to a herd (Proudman & Matthews, 2000).

4.3 Targeted Selective Treatment

Targeted Selective Treatment (TST) is now considered the best approach for equine gastrointestinal helminth control (Matthews, 2008). Before an anthelmintic administration, FECs for all horses must be performed to identify the individuals that need to be treated. As mentioned previously, individuals of the same herd differ in susceptibility to strongyle infection, the generally accepted proportions being that 20% of horses excrete 80% of the eggs contaminating the pasture (Gomez & Georgi, 1991; Matthews, 2008). Only horses with moderate to high FEC (>200 EPG) – "high shedders" – should receive anthelmintic treatment, whilst those with low or negative results – "low shedders" – are left untreated (Uhlinger, 1993; Relf, Morgan, Hodgkinson, & Matthews, 2013). Thus, a TST is able to reduce high levels of pasture contamination and simultaneously leave a number of parasites with anthelmintic-sensitivity *in refugia* (van Wyk, 2001). A study demonstrated that treating all adult horses exceeding a strongyle FEC of 200 EPG only leads to treating approximately 50% of the herd, but still provides about 95% reduction of egg shedding (Kaplan & Nielsen, 2010). For these reasons, targeted control programmes are being promoted to slow down the spread of AR (Stratford et al., 2014). Matthews (2008) goes a step further and suggests a combination of strategic and targeted treatment, in which FECs should be performed during the grazing season, but still use an annual dosing against cyathostomins encysted larvae and tapeworms.

One disadvantage of this method is the possible re-emergence of *S. vulgaris* (Nielsen, Vidyashankar, Olsen, Monrad, & Thamsborg, 2012b). As individuals, horses tend to maintain a consistent value of FEC throughout their lives (Matthews, 2008), some of them may always be below the 200 EPG threshold for anthelmintic treatment. Without regular treatment, *Strongylus* species are able to complete their life cycles, which may increase prevalence of these parasites in the lower egg count horses (Nielsen et al., 2012b).

4.4 Anthelmintic (slow) Rotation

Theoretically, anthelmintic rotation should be one of the best ways to prevent/delay anthelmintic resistance. If a parasite population is resistant to drug “A”, then treatment with drug “B” (with a different mode of action) would remove adults resistant to drug “A”, so the latter would not gain a sustained reproductive advantage (Reinemeyer & Nielsen, 2012). However in practical terms this does not happen, and may actually increase the development of resistance by selecting for resistance to more than one drug simultaneously (Uhlinger & Kristula, 1992; Kaplan, 2002). However, a slow annual rotation seems to be able to slow the rate of resistance (Coles & Roush, 1992). This method consists of using one anthelmintic drug during a whole year, and a second one during the next. Unfortunately, it is difficult to implement this approach on a practical level. Of the three classes of anthelmintics, widespread resistance is reported in the benzimidazoles class (Lyons et al., 1999), which leaves only two relatively effective classes. Of the two, pyrantel is the only one effective against tapeworms. On the other hand, moxidectin is the only effective methods against developing mucosal cyathostomin larvae. Thus, a complete parasite control method that eliminates strongyles and tapeworms, must contain pyrantel and moxidectin, which makes annual rotation extremely difficult (Proudman & Matthews, 2000). Of course, a drug rotation strategy does not substitute a routine testing of AR using a FECRT annually (Kaplan & Nielsen, 2010).

4.5 Farm Management

Good pasture hygiene is now considered one of the pillars of parasite control. Even though concerns about farm management are being greatly discussed nowadays, the original concept goes back to 1969, when the method known as “Drench and Move” was created. It consisted in moving all animals to a clean pasture (left ungrazed for an established period of time) immediately after anthelmintic treatment (Michel, 1969). Later on, this practice was considered to favour the development of resistance, as moving newly dewormed animals to new pastures will drastically reduce the population *in refugia* (van Wyk, 2001; Waghorn, Miller, Oliver, & Leathwick, 2009) and also contaminate the new pasture with resistant parasite eggs, if any. Nevertheless, it is recommended to reduce major contact between horses and pastures contaminated with faeces. Therefore, faeces from stabled horses must be removed several times a day; they must not eat on a faecally contaminated pasture; and they must not contact with other horses' faeces (Proudman & Matthews, 2000). It is important to do this at intervals shorter than the time it takes for eggs to develop into infective stage larvae, thus stopping the parasite of completing its life cycle (Reinemeyer & Nielsen, 2012). Herd (1986) demonstrated that good pasture management, in which faeces were removed twice a week, was more effective than anthelmintic therapy in reducing pasture contamination. More recently, a study with donkeys demonstrated that removing faeces from the pasture two times per week will reduce the FEC shedding significantly (Corbett et al., 2014). The latter had a larger sample size and rigorous statistical analysis to enhance results. Thus, farm management can be considered an effective way to control strongyle burdens (Corbett et al., 2014).

Larval cyathostominosis appears to be primarily associated with the ingestion of heavily contaminated pasture, which makes it even more important to implement preventive measures to minimize pasture contamination. Removal of faeces from pasture at least twice per week, and the reduction of stocking density to 1 horse per 2 acres can be a very effective way to decrease cyathostomin burdens (Peregrine, McEwen, Bienzle, Koch, & Weese, 2006). Known factors that increase the possibility of parasite infection include: high stocking density; over-grazed pasture; the presence of horses with high FECs as well as foals and yearlings (which tend to be more heavily infected); and warm climatic conditions (Proudman & Matthews, 2000). A recent publication in the UK confirms these previous statements by claiming that an inadequate rotation in grazing practises was associated with higher prevalence of cyathostomin egg excretion on Thoroughbred stud farms (Relf et al., 2013). An important fact to bear in mind is that a combination of pasture management and strategic deworming may result in an excessive reduction of the cyathostomin population *in refugia*, which might increase the likelihood of developing anthelmintic resistance (Corbett et al., 2014).

4.6 Co-grazing

Co-grazing consists on alternating hosts of different species on the same pasture in order to reduce parasite transmission (Reinemeyer & Nielsen, 2012). Grazing infected pastures with ruminants can assist in worm control, as the majority of equine strongyle larvae are quite host-specific and do not infect cattle, sheep or goats (Eysker, Jansen, & Mirck, 1986). However, it is necessary to carefully monitor the presence of parasites that can be transmitted from the aforementioned species to horses, especially *Fascicola hepatica* in areas where fluke infection is endemic in ruminants (Matthews, 2014a). One study investigated the effects of co-grazing Shetland ponies and sheep; the results showed an increase in the prevalence of *Trichostrongylus axei*, although overall strongylid infection was reduced (Eysker et al., 1986).

Chapter 5 – Material and Methods

5.1 Research Aims

1. To determine the efficacy of moxidectin against cyathostomins by using the faecal egg count reduction test (FECRT).

2. To estimate the strongyle egg reappearance period (ERP) after moxidectin treatment. The ERP was estimated in two distinct ways so that results could be analyzed and compared.

3. To study the prevalence of small and large strongyles.

4. To examine the influence of farm management (faecal removal *vs* no faecal removal) on the strongyle ERP.

5.2 Study Design

For this study, 8 yards were recruited based on the responses of an online questionnaire. The questionnaire was designed by Dr Thomas Tzelos and Professor Jaqueline B. Matthews and distributed over the UK from April to July 2015. The questionnaire was promoted via social media websites, and also sent directly by e-mail to several UK equine practices, hospitals and equid premises, including riding schools and livery yards (Tzelos et al., unpublished data). At the end of the questionnaire the respondents were asked whether they would be willing to participate in parasitological studies in the near future. Equid premises with a minimum of 20 resident equines were contacted, and 8 of those agreed to participate in the Egg Reappearance Period (ERP) study. All yards were located in the United Kingdom; 6 across England, 1 in Wales and 1 in Scotland (figure 1).

Figure 1 - Geographic location of the 8 yards. They were located in the following three regions: England (1,2,3,4,6,8), Wales (5), Scotland (7).

There were five livery farms, a multipurpose stable, a stud farm and a private yard. A total of 246 horses were recruited. The number of horses per yard ranged from 17 to 59 animals (mean 31). All farms started with more than 20 horses per yard, but due to unpredictable circumstances (one death and animals sold), two yards finished the study with only 17 and 18 horses. More details are shown in table 4.

Table 4 - Data of the 8 yards used in the present study. Information includes the type of yard, number of horses and age median, and all the dates of samples collection. Yard number 5 also collected samples in 8 weeks post-treatment (wpt) due to high faecal egg counts (FECs) on 6 wpt.

Yards	**Type of Yard**	**Number of horses (age median)**	**Samples Collection**					
			Day 0 (treatment day)	Day 14 pt	6 wpt	8 wpt	10 wpt	12 wpt
1	Multipurpose stable	31 (11)	22-11-2015	06-12-2015	03-01-2016	----	31-01-2016	14-01-2016
2	Livery	21 (12)	29-11-2015	13-12-2015	10-01-2016	----	07-02-2016	21-02-2016
3	Stud farm	59 (10)	29-11-2105	13-12-2015	10-01-2016	----	07-02-2016	21-02-2016
4	Livery	17 (15)	29-11-2105	13-12-2015	10-01-2016	----	07-02-2016	21-02-2016
5	Livery	33 (11)	22-11-2015	06-12-2015	03-01-2016	17-01-2016	31-01-2016	14-02-2016
6	Private	23 (16)	10-01-2016	24-01-2016	07-02-2016	----	06-03-2016	20-03-2016
7	Livery	44 (13)	22-11-2015	06-12-2015	03-01-2016	----	31-01-2016	14-02-2016
8	Livery	18 (14)	22-11-2015	06-12-2015	03-01-2016	----	31-01-2016	14-02-2016

The study took place from November 2015 to March 2016. Fresh faecal samples were collected from each horse on Day 0, from identified individual horses and placed into zip-lock plastic bags. Instructions regarding the sampling protocol were based on a previous study (Lester et al., 2013b). Briefly, the horses' owners and/or those who manage the horses were asked to collect small samples from several different areas – minimum 3 to 5 areas – from a freshly voided faecal pile, to maximize the likelihood of detecting eggs, because strongyle eggs are not evenly distributed among balls of horse dung (Lester, Bartley, Morgan, Hodgkinson, & Matthews, 2012). After collecting the samples, the air had to be expelled from the bags, creating an anaerobic environment, to delay egg hatching. After collecting the samples, the horses were treated with a combination of moxidectin and praziquantel (Equest Pramox, Zoetis UK, Ltd., UK).

The horse owners/managers were asked to store the samples at 4ºC until posted to Moredun Research Institute (MRI) under the regulations of the Royal Mail, UK. Once received, all samples were processed on the same day or within two days. Prior to processing, information of every sample was recorded, including the date of arrival, the date of processing and each horse's name, age and information regarding the last anthelmintic treatment (product and date). Apart from Day 0, samples from each yard were collected 14 days post-treatment, 6 weeks post-treatment (wpt), 10 wpt and 12 wpt. Due to high FECs at 6 wpt, "yard 5" additionally collected samples at 8 wpt. The precise dates of collection periods can be found in table 4.

Additionally, yards were grouped based on their farm management. One group (yard 1, 2, 3, 4 and 6) comprised premises that had stated in the questionnaires that they removed faeces twice a week, whilst the other group (yards 5, 7 and 8) stated that they did not remove faeces at all.

5.3 Centrifugal Floatation Faecal Egg Counting Method

All FECs were performed using a modification of a salt-flotation method (Christie & Jackson, 1982) with a detection limit of 1 egg per gram (EPG) of faeces (Bartley & Elsheikha, 2011). The protocol was as follows:

Each faecal sample was manually homogenised in the sealed bag, and a sub-sample of 10 grams of faeces transferred into a polythene bag. The air was expelled from each sub-sample and left lying flat while the others sub-samples were being prepared. Next, 100 mL of tap water were added to each sub-sample (10 mL per gram of faeces). The subsamples were mixed thoroughly with a stomacher for 5-10 seconds until they reached an even consistency. Then, the bags were re-agitated and 10 mL were transferred to beakers through sieves using a pipette. The sieves were used to withhold large faecal debris, whilst the eggs would pass into the beaker with the water (figure 2). Extra 5 mL of tap water were added to the beakers to rinse any eggs left within the pipette-tip. The faecal debris caught in the sieves were manually pressed until as much liquid as possible had been removed from the debris. The beakers were agitated and the contents were poured into a labelled thin wall polypropylene 17 mL centrifuge tubes (Beckman Coulter). Once all samples were in the tubes, they were centrifuged at 1,000 rotations per minute (rpm) for 2 minutes. In this step, the faecal debris and any parasite eggs would pellet. The supernatant was aspirated with a vacuum line, leaving approximately 1 mL of the supernatant to ensure that the egg containing pellet would not be disturbed. Subsequently, approximately 10 mL of saturated sodium chloride (NaCl) solution were added to the tubes. The saturated NaCl solution made the parasite eggs float to the surface. The samples were inverted at least 3 times to re-suspend the egg containing pellet. The inversion was done gently to prevent formation of air bubbles, which would interfere with posterior egg enumeration.

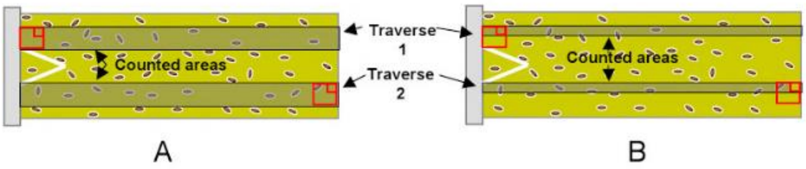
Figure 2 – On the left, beakers with sieves on top. On the right, debris is retained in the sieves, whilst eggs are retained in the beaker with the water.

The samples were centrifuged again at 1,000 rpm for 2 minutes. After this step, the faecal debris would pellet whilst the parasite eggs would float to the surface. The centrifuge tubes were clamped using arterial forceps to isolate the top 1 mL of liquid containing the eggs, and poured into spectrophotometer cuvettes (figure 3). Approximately 0.5 mL of saturated NaCl solution was added to rinse the upper chamber of the tube to rinse potential eggs left in the tube and poured again into the same cuvette. The cuvette was then gently inverted at least 3 times to evenly distribute the eggs. The cuvette was topped up with saturated NaCl until a positive meniscus was formed and a lid used to close the cuvette. To reduce the possibility of introducing an air bubbles into the cuvettes, saturated NaCl solution was also added to the lid before closing it. Once the cuvette was closed, it was turned horizontally with the reading side (i.e. clear pane) facing upwards. The cuvettes were allowed to settle for at least 1 min to allow the eggs present to float to the reading side. The reading side of the cuvette was examined under a microscope at 4x magnification (figure 3) and eggs were identified following published guidelines (Thienpont et al., 1986; Cernea et al., 2008) .

Figure 3 – The content of a centrifugal tube being poured into a cuvette (on the left), and the microscope with the reading side of the cuvette facing upwards (on the right).

A Millar graticule was used and there were 3 options to calculate the number of parasite eggs, depending on an initial evaluation of the density of eggs present. These included: i) count all eggs in the reading field; ii) use the large reading square of the Millar graticule to count eggs within the large square in two traverses (figure 4); or, iii) use the small reading square of the Millar graticule to count eggs within the small square in two traverses (figure 4). If the large or small square was used to count eggs, the total number of eggs counted in the 2 traverses was multiplied by 3 or 9, respectively. There was no multiplication factor when the whole reading field was examined. The results represented the total number of eggs per gram (EPG) of faeces, since 10 grams of faeces were in 100 ml of water and 10 ml, i.e. 1 gram of faeces, was subsequently processed.

Figure 4 - Diagrammatical representation of areas examined (traverse 1 and 2) in a FEC when using the large (A) and small (B) squares. Original from Bartley & Elsheikha (2011).



If necessary, the protocol could be paused after the first centrifugation when the eggs are pelleted with the faecal debris. At this stage, the samples could be stored at 4ºC to be analysed during the following day because the cold environment and the anaerobic conditions in the pellet delay any parasite eggs present from hatching. It is not possible to effectively distinguish a large strongyle from a small strongyle egg. Therefore, eggs were differentiated as strongyle eggs, *P. equorum* eggs and tapeworm eggs as shown in table 5.

Table 5 - Egg differentiation regarding the egg size, shape, shell and content. Adapted from Thienpont et al. (1986).

	Egg size	**Egg shape**	**Egg shell**	**Egg content**
***Anoplocephala* spp.**	Medium-sized	D shape	Thin with various layers	Hexacanth embryo
Strongyles	Medium-sized	Ovoid	Thin with smooth surface	Morula* Gastrula* or larva*
Parascaris equorum	Medium-sized	Nearly spherical	Thick with various layers	One or two cells

* Strongyle eggs change from an un-embryonated egg (morula and gastrula) to an embryonated egg (containing a visible larva).

5.4 Larval Culture Method

In order to identify the type of strongyle species present (large or small strongyles), larval cultures were performed. After performing the initial FECs, samples with positive counts were selected to do a larval culture for the eggs to develop to the infective L3 stage.

A 500 mL container was placed within a polythene bag, so that faeces would not contact with the container. A portion of every positive sample (fist-sized balls) was disposed homogeneously on the container. The container with the samples was covered by another polythene bag, which was twisted at the end and closed using a stapler. The polythene bag was pierced several times, allowing air to circulate, thus creating an aerobic environment (figure 5). The containers were labeled with the name of the yard, the date the sample was cultured and the date sample was due to be extracted. The containers were then incubated at approximately 22ºC for at least 14 days, as the optimum temperature for development of eggs and larvae is in the range 20-30ºC (Ogbourne, 1972). After 14 days, the containers were removed from the incubator, and flooded with tepid tap water (~ 2L) and allowed to rest for 4 hours at room temperature (figure 5).

Figure 5 - Containers with the faeces before incubation (on the left). On the right, the containers (after incubation) were flooded with tepid tap water and left to rest for 4 hours.

Afterwards, the contents were poured through a laboratory test sieve with 1 mm diameter, which retained debris and allowed the water (containing the larvae) to be collected in a 2L jug. The contents of the jug were then poured through a Baermann apparatus made from two layers of nappy-liners, retaining the larvae. The filter was placed on top of a labeled jar (same information placed on the containers), previously filled with tepid tap water, so that the filter paper was flush with water, and left overnight at room temperature. Any larvae present on the filter would migrate through the filter paper into the jar. During the following day, the filter was removed and the jar was left to rest for another 30 minutes to ensure all larvae have settled at the bottom. Afterwards, the water was carefully vacuumed using a vacuum line until approximately 2 cm of liquid remained. Finally, the solution was transferred into a small (25-35 cm^3) tissue culture flask, with a vented lid so it can reproduce an aerobic environment, and labeled with the same information placed on the containers of the cultures The culture flasks were then stored in the fridge at 4ºC lying flat until larval identification.

Identification and classification of the first 100 larvae encountered was based on previously established guidelines (Thienpont et al., 1986; Madeira de Carvalho et al., 2008). Small and large strongyle larvae can be easily confused due to their similar size and long tail. However, discernment between species can be accomplished by comparing the body/tail ratio and the number of intestinal cells, as seen in table 6. Cyathostomin L3 larvae are easily identified due to their characteristic 8 intestinal cells (figure 6).

Table 6 - Comparasion of the body/ratio and the number of intestinal cells of Small and Large Strongyles. Adapted from Thienpont et al. (1986) and Madeira de Carvalho et al. (2008).

	Body/Tail Ratio	**Intestinal cells**
Cyathostomins	1.5/1	8
S. vulgaris	2.5/1	28 – 32
S. edentatus	2/1	20
S. equinus	2.8/1	16

Figure 6 - Third stage larva of small strongyle at 4x magnification (original). Note the 8 intestinal cells that characterizes these species (Madeira de Carvalho, Fazendeiro, & Afonso-Roque, 2008).

5.5 Data Analysis

Microsoft® Office Excel® 2013 was used for recording data and subsequent analysis. Summary data for each yard included the number of horses recruited, age range, last anthelmintic treatment (including the product used and date), mean and range of FECs.

5.5.1 Faecal Egg Count Reduction Test

Group FECRTs were performed for each yard using the recommendations of the World Association of the Advancement of Veterinary Parasitology (WAAVP) (Coles et al., 1992, 2006). Percentage reductions were calculated for each yard, to make it possible to estimate the anthelmintic efficacy. FECR was calculated using the ulterior formula (Coles et al., 1992; Coles et al., 2006):

$$\text{Group mean FECRT (\%)} = \frac{(\text{Pre treatment mean FEC} - \text{14 days post treatment mean FEC}) \times 100}{\text{Pre treatment mean FEC}}$$

As there are no clearly defined guidelines regarding appropriate cut-off values, this thesis follows recently published recommendations of the American Association of Equine Practitioners (AAEP, 2013); i.e. appropriate anthelmintic efficacy was declared when arithmetic mean FECR was >95%. In addition, 95% lower confidence limits (LCLs) were calculated to give an indication of the range of the data (Vidyashankar, Kaplan, & Chan, 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% confident limits (Efron, 1979; Lester et al., 2013b). PopTools software (CSIRO, Australia) was used for bootstrapping (http://www.poptools.org/). LCLs of 90% were selected to classify resistance. To declare anthelmintic resistance (AR), both mean of FECRT and LCLs need to fall below the previously established cut-offs. If just one value falls below the cut-offs, suspected anthelmintic resistance (SAR) was declared.

5.5.2 Egg Reappearance Period

To calculate the ERP, the most common methods used in other studies were chosen:

1. ERP was defined as the week of first positive strongyle FEC after anthelmintic administration (Lyons et al., 2008a; Molento et al., 2008).

2. ERP corresponds to the week in which the strongyle FEC exceeds the values of FEC obtained at Day 0 by 10% (von Samson-Himmelstjerna et al., 2007; Larsen et al., 2011).

5.5.3 Farm management

The influence of good farm management (faecal removal) was analysed using a Repeated Measures Analysis of Variance Method (rANOVA), with the R program (R Core Team, 2013). This particular test is used in studies that examine changes in the mean scores over different points in time. The yards that participated in this study were grouped into two different groups according to their faecal removal habits, i.e. faecal removal (yards 1, 2, 3, 4, 6) and no faecal removal (yards 5, 7, 8). Thereafter, the measure variable (i.e. mean FECs for each yard) was analysed at 4 time points (i.e. 2 weeks post-treatment, 6 wpt, 10 wpt and 12 wpt). Difference between factors (Group and Time) and their interaction were assessed with a significance level of 0.05. However, the test does not specify at which chronologic point this difference occurs.

Chapter 6 – Results

6.1 Faecal Egg Counts

A total of 1262 FECs were performed during this study. All FECs for each yard are summarized in Appendix A. The exact numbers of strongyle FEC-positive horses are shown in table 7. Two yards (yard 1 and 2) had fairly low FECs throughout the studied weeks and the threshold of 200 EPG was never reached. On yard 3, the number of FEC-positive horses was fairly low at 6 wpt. However, the number of positive counts increased, with some of them reaching a value >200 EPG. Those animals received anthelmintic treatment and their FECs returned to 0 EPG at 12 wpt. At 12 wpt, two other animals had FECs >200 EPG. On yard 4, FECs were fairly low, except for one animal that reached a value of >200 EPG at 10 wpt. However, for medical reasons, the animal had to be euthanized. On yard 5, approximately one third of the animals had positive FECs at 6 wpt. Thus, FECs were performed additionally at 8 wpt. At 8, 10 and 12 wpt, a relatively large number of positive FECs was documented again, with some animals reaching a FEC >200 EPG. On yard 6, FECs remained low or negative through the studied time, except for one animal. That particular horse showed a fairly high FEC at day 0, and it was the only one (of the five FEC-positive animals) to have a high positive FEC at 6 wpt, reaching a value >200 EPG at 10 wpt. On yard 7, approximately one fourth of the studied animals had positive FECs. At 10 and 12 wpt the number of positive-FEC horses was fairly high with some horses reaching a FEC >200 EPG. On yard 8, positive FECs at 6 wpt were relatively few. However, more animals with positive FECs appeared at 10 and 12 wpt. At 12 wpt, one horse had a FEC >200 EPG.

Table 7 - Number of strongyle FEC-positive (EPG<0) horses through the studied time for each yard.

Yard	Number of strongyle FEC-positive horses				
	Day 0	Day 14 post-treatment	6 wpt	10 wpt	12 wpt
1	17/31	0/31	4/31	10/31	10/31
2	13/21	0/21	4/21	6/21	5/21
3	56/59	0/59	10/59	28/59	23/59
4	9/17	0/17	1/17	3/17	2/17
5	24/33	0/33	11/33	21/33	19/33
6	7/23	0/23	5/23	7/23	3/23
7	38/44	1/44	14/44	34/44	34/44
8	14/18	0/18	5/18	15/18	15/18

6.2 Moxidectin efficacy – FECRTs and ERP

Moxidectin efficacy was tested by performing a FECRT for each yard. On 7 yards, the mean FEC at Day 14 was zero, which translates into a result of 100% efficacy for moxidectin. On yard number 7, four eggs were found in one horse, resulting in a mean FECR of 99.88%, with a LCL of 99.55%. Both values are above the designated efficacy cut-off values. Hence, moxidectin was effective on all yards. The FECRTs as well as the arithmetic mean FECs of Day 0 and 14 days post-treatment are displayed in table 8.

Table 8 - The moxidectin faecal egg count reduction test (FECRT) result for each yard. Mean strongyle FECs are shown for Day 0 and Day 14 post-treatment. Arithmetic mean faecal egg count reduction (FECR %) and the respective 95% lower confidence limit (L.C.L. %) are displayed for each yard.

Yards	**Mean Day 0 FEC (EPG) (range)**	**Mean Day 14 FEC (EPG) (range)**	**FECRT (%)**	**L.C.L. (%)**
1	97.90 (0 - 1044)	0 (0 - 0)	100	100
2	18.27 (0 - 108)	0 (0 - 0)	100	100
3	146.58 (0 - 999)	0 (0 - 0)	100	100
4	88.35 (0 - 693)	0 (0 - 0)	100	100
5	101.65 (0 - 477)	0 (0 - 0)	100	100
6	46.87 (0 - 810)	0 (0 - 0)	100	100
7	82.13 (0 - 774)	0.09 (0 - 4)	99.88	99.55
8	202.47 (0 - 882)	0 (0 - 0)	100	100

Strongyle ERP data was collected from all yards and two different methods were applied. Both results are summarized in table 9.

Table 9 - Strongyle egg reappearance period calculated using the two different methods as described in Materials and Methods section.

Yards	**Method 1**	**Method 2**
1	6 weeks	>12 weeks
2	6 weeks	10 weeks
3	6 weeks	10 weeks
4	6 weeks	6 weeks
5	6 weeks	6 weeks
6	6 weeks	6 weeks
7	6 weeks	10 weeks
8	6 weeks	10 weeks

Using Method 1 (i.e. the first positive FEC post-treatment), the strongyle ERP was 6 weeks for all yards examined. Using Method 2 (i.e. the week in which the arithmetic mean FEC exceeded the values of the arithmetic mean FEC obtained at Day 0 by 10%, meaning a FECRT reduction falling below 90%), strongyle ERP ranged from 6 weeks to >12 weeks. For Method 2, a FECR was performed at 6 wpt, 10 wpt and 12 wpt. Values below 90% were considered the ERP. All FECRs are shown in table 10.

Table 10 - Faecal egg count reductions (FECRs) performed at 3 different time points.

Yard	6 wpt	10 wpt	12 wpt
1	99,85	96,29	94,54
2	95,16	42,85[a]	76,28
3	99,20	85,84[a]	80,97
4	57,49[a]	54,83	97,98
5	85,47[a]	59,46	72,20
6	89,39	68,73[a]	95,58
7	97,54	72,55[a]	39,24
8	96,14	79,22[a]	81,37

[a] Values under 90%, translating into the ERP when using Method number 2 (details in the material and methods section).

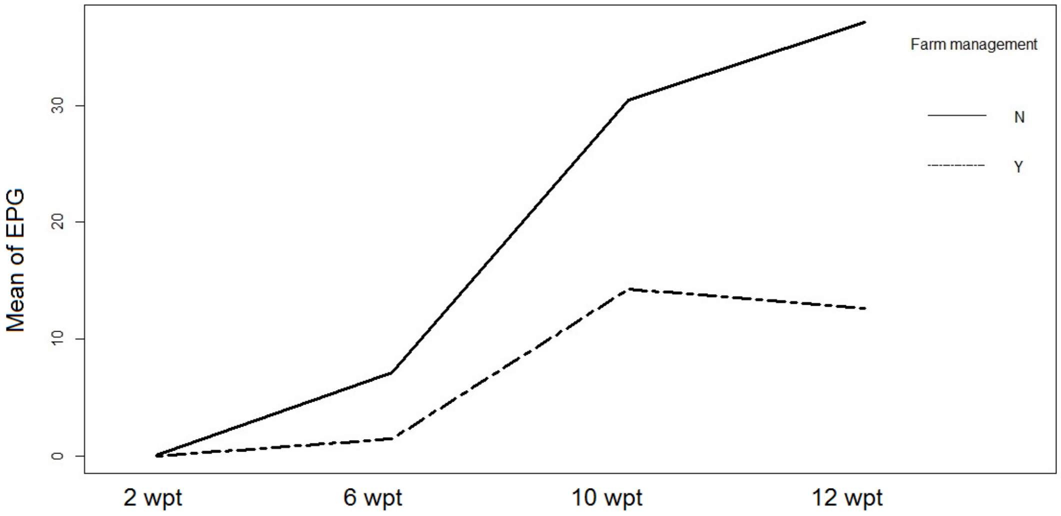
6.3 Larval Cultures

In all yards, faecal samples that were positive for strongyle eggs were pooled and cultured to allow strongyles to develop to the infective L3 stage, using the Baerman migration method, and the larvae examined under a microscope. This procedure was made at Day 0, 14 days post-treatment, 6 wpt, 10 wtp and 12 wpt for every yard. No large strongyles were identified in any samples. All larvae identified belonged to small strongyles species.

6.4 Yard Management

The influence of a faecal removal from pasture *versus* no faecal removal was analysed using a repeated measures ANOVA, in which two groups were compared. The mean FECs of the two groups were compared over 4 times points: 2 wpt (14 days post-treatment), 6 wpt, 10 wpt and 12 wpt, as shown in graph 1. The p-value was of 0.003, meaning that there was a significant statistical difference between the mean FECs of the two groups.

Graph 1 - Repeated measures ANOVA graphic comparing the two groups mean faecal egg counts (FECs). The mean FECs of the group with no faecal removal (N) is higher than the mean FECs of the group with faecal removal (Y).



Chapter 7 – Discussion

The present study examined the effect of moxidectin (MOX) against equine strongyles in eight different horse populations in the UK. This study is not representative of the country as a larger number of yards would be needed to achieve that. Hence, results regard only the yards involved. MOX proved to be highly effective, achieving a reduction in strongyle FECs at day 14 post-treatment of 100% in seven yards, and by 99.88% in one yard. This result was not surprising, as recent studies also demonstrated high efficacy of MOX against strongyle infections (Traversa et al., 2012; Stratford, Lester, Pickles, McGorum, & Matthews, 2013). However, it is important to bear in mind that FEC methods are incapable of diagnosing pre-patent infections of helminths and, accordingly, the FECRT is only capable of estimating anthelmintic efficacy against adult nematodes. Both facts can lead to false conclusions, particularly within cyathostomin species, as encysted larvae can constitute up to 90% of the total burden during autumn/winter in the UK (Matthews, 2014b).

The FECRT is considered the “gold standard” for determining anthelmintic efficacy in domestic animals (Coles et al., 1992; Coles et al., 2006). However, there is no standardised method for its use in horses, which makes direct comparison between studies difficult. For the time being, the WAAVP method remains the most widely implemented. Additionally, many factors can influence the test’s results negatively; one of them being the type of FEC methodology used (Lester & Matthews, 2014), because different methods have different sensitivities. The common McMaster has an egg detection limit (EDL) of 15 to 100 EPG (Lester & Matthews, 2014), depending on the amount of area of the slide that is examined. Particularly, counting all eggs present in one grid of the McMaster slide gives an EDL of 100 EPG, and counting eggs in two grids gives an EDL of 50 EPG, whilst enumerating all eggs in one chamber gives and EDL of 30 EPG, whilst enumerating all eggs in both chambers gives and EDL of 15 EPG. The American Association of Equine Practitioners (AAEP) suggests that only egg counting techniques with a limit for detection of less than 25 EPG should be used for estimating the efficacy of an anthelmintic. Therefore, for this study, a centrifugal flotation method with an EDL of 1 EPG was used (Bartley & Elsheikha, 2011). However, this technique requires specific equipment, such as a Miller eye piece and cuvettes, and is considered more complex than the McMaster techique (Lester & Matthews, 2014). As a result, this technique is less used than the McMaster. Nevertheless, by analysing different variations of sensitivity between methods (and sometimes within the same technique), it is easy to understand why it is important to define an official FEC method to do the FECRTs, so that results among studies can be compared.

Other known factors that influence the FECRT result are the sampling protocol and the storing conditions of the samples until processing (Nielsen et al., 2010).We could confirm this statement in this study as the results of one yard were possibly altered by improper sampling. On yard 2, FECs at 12 wpt were slightly lower than at 10 wpt, especially regarding one horse that dropped from 150 to 27 EPG (graphs are shown in Appendix A). This was due to the small size of the samples sent to the MRI laboratory for the 12 wpt FECs. The majority of the samples weighed less than 10 grams, and some weighed less than 5 grams. In order to have confidence regarding the results of FECs at least 10 grams are needed (Bartley & Elsheikha, 2011). Moreover, yards were asked to collect from 3 to 5 different areas of the faecal pile (which in total is always more than 10 grams), to maximize the likelihood of detecting a representative amount of strongyle eggs, since they are not evenly distributed in the faecal balls (Lester et al., 2012). Therefore, the sudden drop of FECs on this particular yard could be due to human error during the sampling process.

Until now, there is only one case of reported anthelmintic resistance to MOX (Molento et al., 2008). Apart from that study, reduced efficacy of MLs (ivermectin and moxidectin) is essentially measured by shortened ERPs. The ERP corresponds to the time period a parasite populations needs to restore its reproducing adults. Hence, a reduced ERP is believed to provide an early indicator of the parasite populations shifting into being less susceptible to anthelmintic treatment (Sangster, 2001). In this study, the ERP ranged from 6 to >12 weeks depending on the methodology used to determine the date that the eggs “re-appeared”. A shortened ERP was observed in all yards using method 1 (ERP = 6 weeks), and in 7 yards using method 2 (ERP = 6 and 10 weeks). Other studies that used the same two methods to examine strongyle ERP after MOX administration reached similar results. A recent study in the UK (Relf et al., 2014), reported an ERP of 4 wpt using method 1, and an ERP of 6, 8 and 9 wpt using method 2. However, it is noteworthy that all animals with shortened ERP were yearlings and foals. Similarly, a study in the USA reported a shortened strongyle ERP in yearlings after MOX treatment (Rossano et al., 2010). The strongyle ERPs was of 5 and 6 weeks using methods 1 and 2, respectively (Rossano et al., 2010). Van Doorn et al. (2014) reported a shortened strongyle ERP after

MOX administration in adult horses, with an ERP of 6-8 weeks using method 2. Thus far, this is the only published study that reported a shortened strongyle ERP in adult horses after MOX treatment (van Doorn et al., 2014). Hence, the results of the present study are of concern, since the majority of horses were adults, which means adult horses showed a shortened strongyle ERP.

The shortened strongyle ERP observed in this study may be caused by the frequent and continuous use of MOX. A questionnaire performed in Scotland stated that 92% of the participating yards had used MOX in their premises in the preceding 12 months. Additionally, 22% of the respondents reported that anthelmintics were administered every 13-15 weeks, and 20% every 4-6 months without performing FECs (Stratford et al., 2014). In another study, 84% horses of a racing yard survey in the UK, received anthelmintic treatment even when their FEC were less than 50 EPG (Comer, Hillyer, & Coles, 2006). Interval-based treatment protocols will select resistance alleles within parasite populations (Kaplan & Nielsen, 2010). Regarding small strongyles, shorter ERPs after treatment with MLs appear to be associated with survival of luminal L4 (Lyons et al., 2009; Lyons & Tolliver, 2013). After a MOX treatment, those surviving L4 (with the resistant allele) are recruited to resume maturation and emerge into the gut lumen to replace the (susceptible) adults killed with the anthelmintic treatment. In a recent study (Reinemeyer, Prado, & Nielsen, 2015), necropsies were performed 18-20 days after MOX administration and luminal L4s were recovered from five of six horses at only two weeks after treatment with MOX. Furthermore, cyathostomins worms were enumerated during the necropsies, and moxidectin exhibited an efficacy of 85.2% against LL3/L4 mucosal larvae. A previous study, performed in 2006, in which horses were necropsied at 56 days after MOX treatment showed efficacies of 99.9% against LL3/L4 stages (Bairden, Davies, Gibson, Hood, & Parker, 2006). The difference of efficacies may reinforce the hypothesis that MOX efficacy is declining.

Furthermore, interpretation of the ERP values is complicated because there is a lack of consensus in the definition of the term itself. “Yard 1” had the first positive FEC at 6 wpt (ERP = 6 wpt using method 1), but using method 2 the ERP was of >12 wpt. These two methods are often used in studies of anthelmintic efficacy, being the reason why they were chosen for this thesis. However, as the two methods show different results, it should be an urgent objective to establish a validated and recommended method, so that comparative analysis can be made among studies. In the authors’ opinion, the official method should be a cross between the two methods used in this thesis. The first method can be considered too vague, as an observation of a few eggs can scarcely count as a shortened ERP. Thus, using a method with a defined cut-off value (e.g. 50 or 100 EPG) probably would be a more sensitive and sensible way to establish the ERP. Additionally, the threshold should be defined through a multi-dimensional study so that different areas and different types of premises have different ERP. Nematode eggs tend to reappear more quickly in younger animals (Matthews, 2010). For example, stud farms are an establishment for selective breeding, which means they have a greater percentage of young animals. Here, there might be expected a shorter ERP than in a normal premise (with more adult animals). The cut-off values for the ERP should have all these factors in account.

In order to delay the development of anthelmintic resistance, all premises should use a targeted selective treatment (TST) approach for gastrointestinal helminth control (Matthews, 2008). Before deworming, FECs for all horses should be performed to identify those individuals that require anthelmintic treatment and those who do not. Only horses above a stipulated threshold should receive anthelmintic treatment, which would insure a safe number of worms *in refugia* in a given population (Matthews, 2014b). Matthews (2014b) advised that anthelmintic treatment for adult horses should be based on an annual anthelmintic rotation, where there is sensitivity to >1 class of anthelmintic. Rotation is performed for adulticidal treatments (with ivermectin or other effective anthelmintic drug classes) and based on previous FECs analysis in spring through autumn, and MOX reserved for larvicidal treatment in late autumn/early winter for all horses.

The cut-off EPG value that defines when the horses should be treated varies greatly among parasitologists (Uhlinger, 1991). Efforts should be made to define official cut-off values, depending on the region where the horses are, which can only be accomplished by large-scale multi-year epidemiological studies, including variables such as the climate, anthelmintic resistance history, number of horses/foals, etc. (Nielsen, 2012). During the present study, horses with FECs of 200 EPG or higher were treated with an anthelmintic, dropping back to 0 EPG 14 days after the new treatment (Appendix A). Horses with high counts on Day 0 tended to be the first ones to have positive FECs and higher counts, which was expected, since individual susceptibility persist throughout the animal’s life (Nielsen et al., 2006).

One disadvantage of performing selective therapy is the great number of faecal samples that need to be analysed, which makes horse owners/yard managers regard targeted treatment as more expensive than traditional interval treatments. However, data from a recent study (Lester et al., 2013a) indicated that might not be the case. In that study, FECs were performed in three different times per year, and only horses with high FECs were treated with anthelmintic. By using a targeted method, anthelmintic use was reduced by 82%, and an average saving of £294.44 (approximately 372€) per yard per year was achieved. Strategic anthelmintic control can have another negative outcome: the reemergence of the highly pathogenic *Strongylus vulgaris*, a threat considered rare due to its long cycle and its (still) high susceptibility to commonly used anthelmintic drug classes (Nielsen et al., 2007). Only cyathostomin larvae were identified in the larvae culture method. This is an expected result due to the low prevalence of large strongyle in managed horse populations (von Samson-Himmelstjerna, 2012; AAEP, 2013). *S. vulgaris* is still highly susceptible to any of the currently available anthelmintic drugs, which is the main explanation as to why this parasite has a low prevalence in managed equine premises (Nielsen et al., 2014). In the questionnaire, all yards stated that all resident animals are dewormed, at least once a year, with one of the three available anthelmintic drug classes. Knowing this, the negative result for large strongyle larvae was not a surprise.

Since eggs are reappearing sooner after anthelmintic treatment, efforts should be made to implement a TST combined with improved pasture hygiene. It is clear that good hygiene practices can prevent reinfection and the development of resistance by breaking the worm cycle (Corbett et al., 2014). Removing faeces twice a week greatly reduced pasture contamination and lower the levels of infection (Corbett et al., 2014). In this study, 3 yards (yards 5, 7, 8) did not remove the faeces from the paddocks. Those yards showed a faster progress towards higher positive FECs and more infected animals compared to the yards (yards 1, 2, 3, 4, 6) that removed the faeces from the paddocks (Appendix A). To compare the mean FECs of the yards that removed faeces and to the ones that did not, a Repeated Measures ANOVA was used, reaching a p-value of 0.003. Hence, it would appear that faecal removal from pasture influenced the mean FECs and the levels of re-infection post-treatment; the mean FECs in the yards that did not remove faeces were higher than the yards that did. Moreover, the difference between the mean FECs of the two groups (faeces removal *versus* no faeces removal) increased over time, i.e. the difference between mean FECs is greater at 12 wpt than at 10 wpt and 6 wpt. This could be explained by the fact that removing faeces from pastures reduces the level of strongyle re-infection. Additionally, it is highly probable that the eggs are originated from the luminal L4 cyathostomin that survived treatment, since there are signs that shorter ERPs after treatment with MOX appear to be associated with survival of luminal L4 (Lyons & Tolliver, 2013). Thus, a good pasture hygiene is essential so that those eggs coming from less susceptible parasites are removed from pasture, and will not infect other horses and reproduce. A recent study in the UK stated that 395 out of 492 (~80%) of respondents removed faeces from pasture on their premises (Easton et al., 2016). In addition to this, a study in Germany and Austria compared two groups of horses; the first group consisted of horses who lived in premises where faeces were removed from pasture at least once weekly. This group had a monthly mean FEC consistently lower that the monthly mean FEC of animals which grazed pastured that were cleaned less frequently or not cleaned at all (Becher, Mahling, Nielsen, & Pfister, 2010). Another study associated removal of faeces from pasture and reduction of stocking density as a very effective approach to reduce cyathostomin burdens (Peregrine et al., 2006). Nevertheless, the mean FECs depend on several factors (e.g. farm management, deworming protocol, animals' mean age, etc.). More studies are needed in this particular area, for veterinarians to advise owners on how best to combine chemical and non-chemical parasite control strategies in an integrated approach (Nielsen, 2012). With the alarmingly increase of reports of reduced strongyle ERP after treatment with MOX, it is a major concern to maintain its efficacy for as long as possible. This chemical approach should be combined with good pasture hygiene in order to reduce pasture contamination and strongyle reinfection.

Chapter 8 - Conclusion

Our results showed that there is no sign of anthelmintic resistance in the farms included in the study, as the faecal egg count reduction test (FECRT) was above the established cut-off value. However, the shortened egg reappearance period (ERP) in some farms shows that positive faecal egg counts (FECs) are occurring sooner than expected. This might indicate that there is a slight evolution of the parasite populations towards resistance.

The validation of an official protocol to perform FECs should be a major concern, because it is the only way of comparing results of different studies. Different studies follow different FEC protocols with different sensitivity levels. The same should be done for estimating the ERP method because as seen in this study, the method used can greatly influence the final results and, consequently, comparison of results between studies can be a challenge.

During spring until late autumn, only horses with high FECs (e.g. above an established threshold) should receive an adulticidal treatment. Only a minority of the horses used in this study did reach the cut-off value for treatment, and therefore only those received an anthelmintic treatment. This strategy is important to delay the development of resistance.

Good farm management can positively influence the FECs, as the yards that removed faeces twice a week had lower mean FECs than the yards that did not. Nevertheless, more research is needed to understand how farm management truly influences the FECs. Many variables have to be strictly controlled (i.e. use groups of healthy horses of the same age, same deworming protocol, etc.) so that farm management is the only analysed variable.

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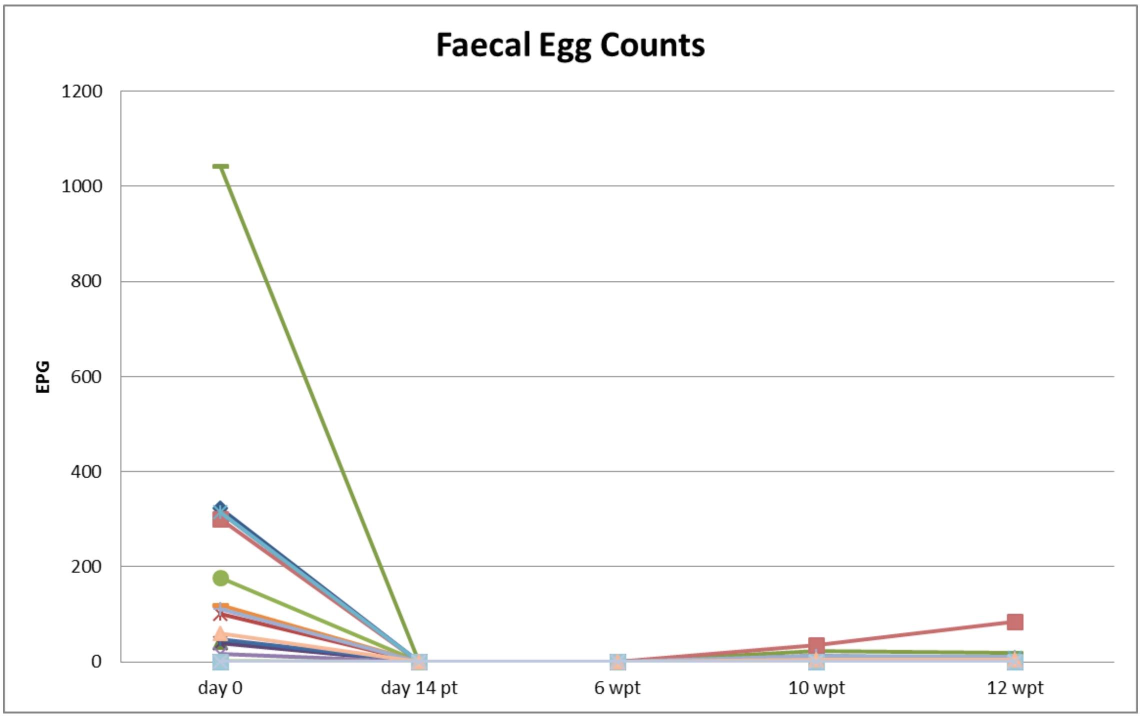
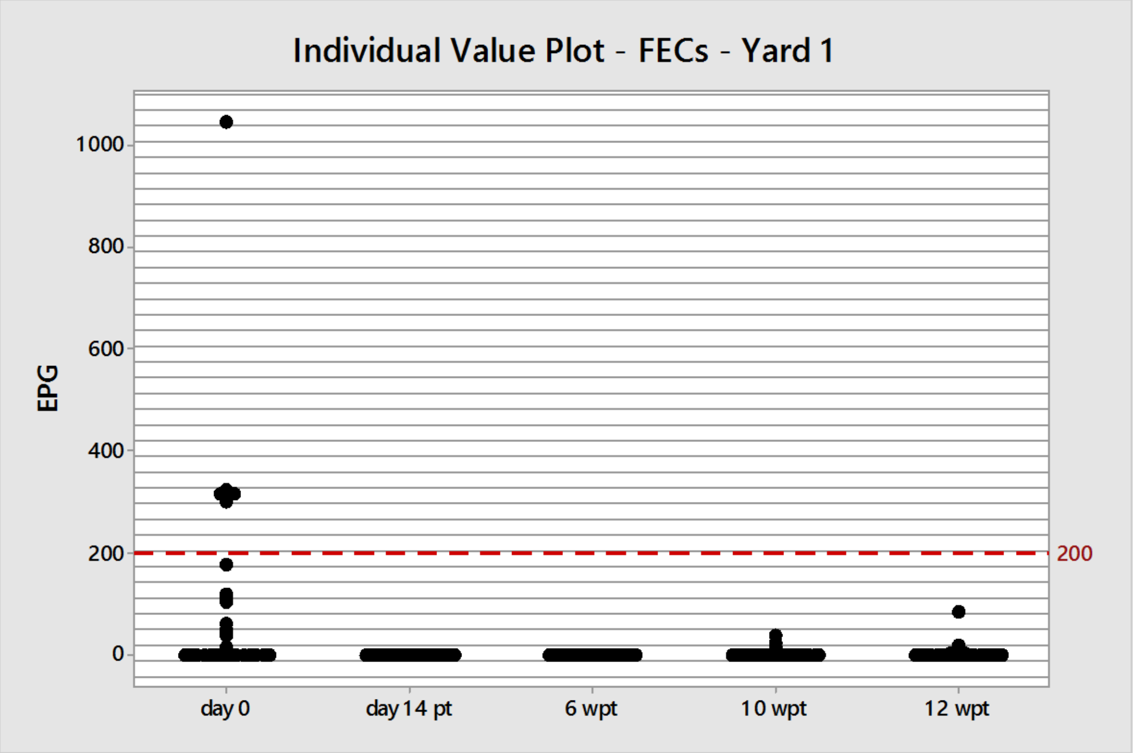
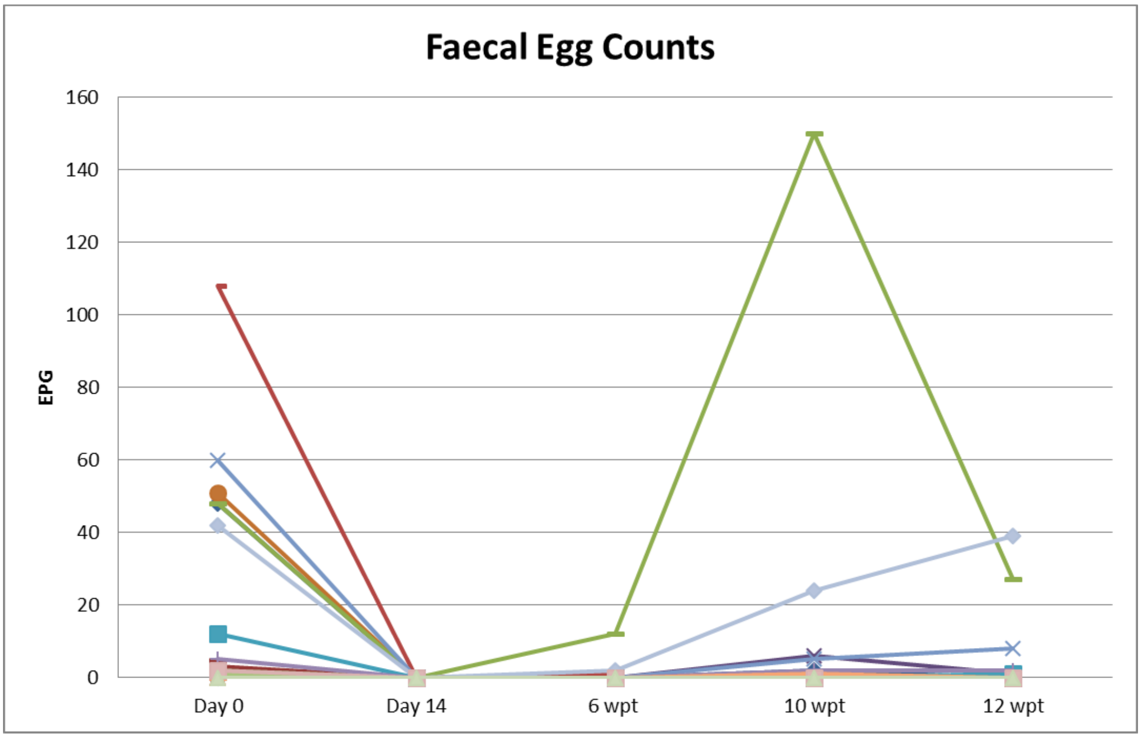
Appendix A – Individual faecal egg counts (FECs) and individual value box plots for every yard.

Yard 1

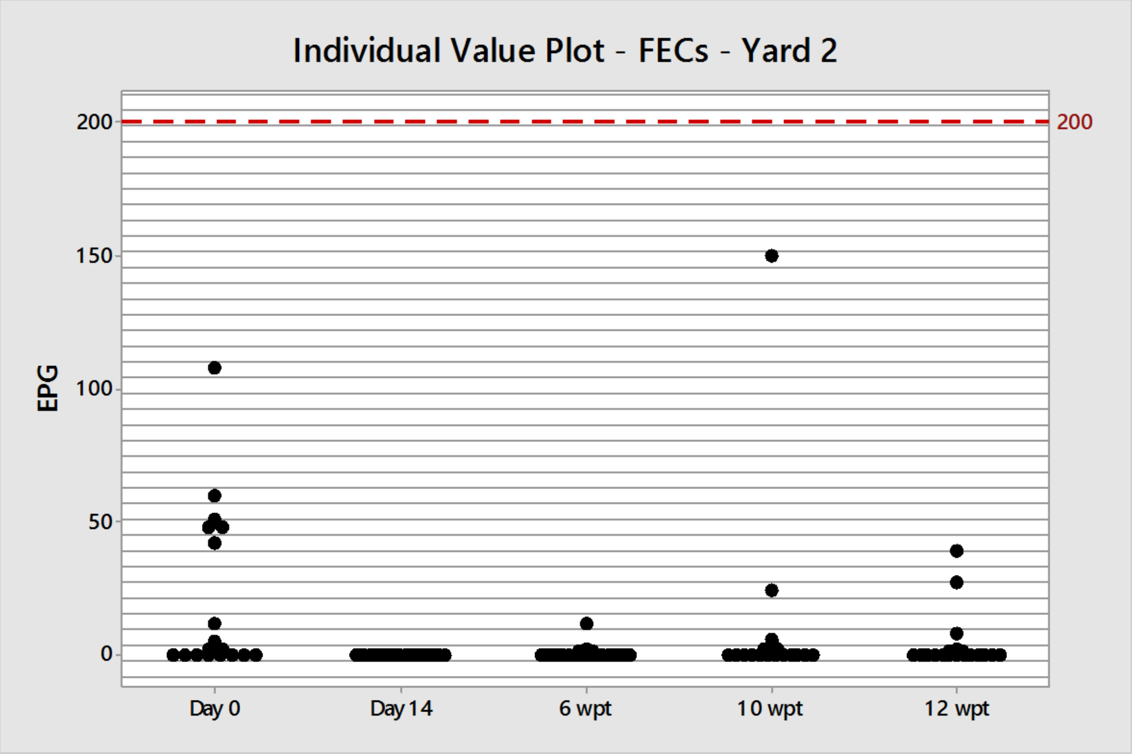




Yard 2

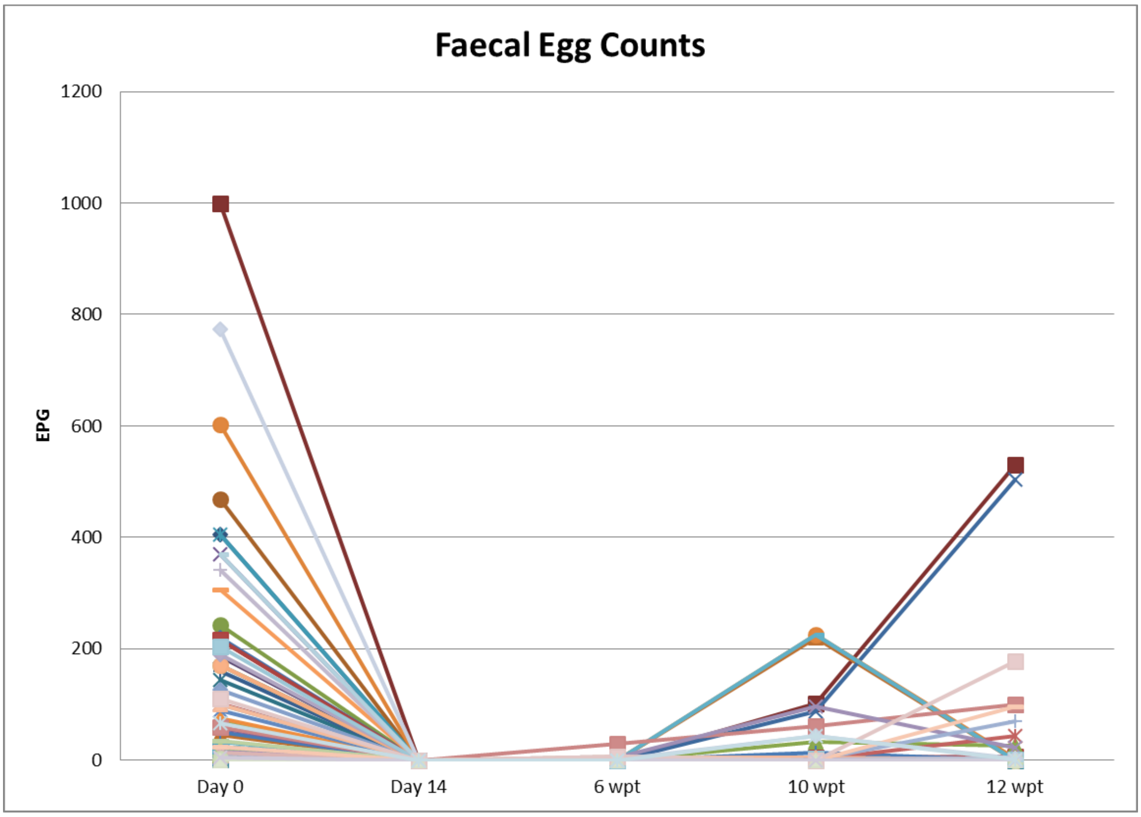


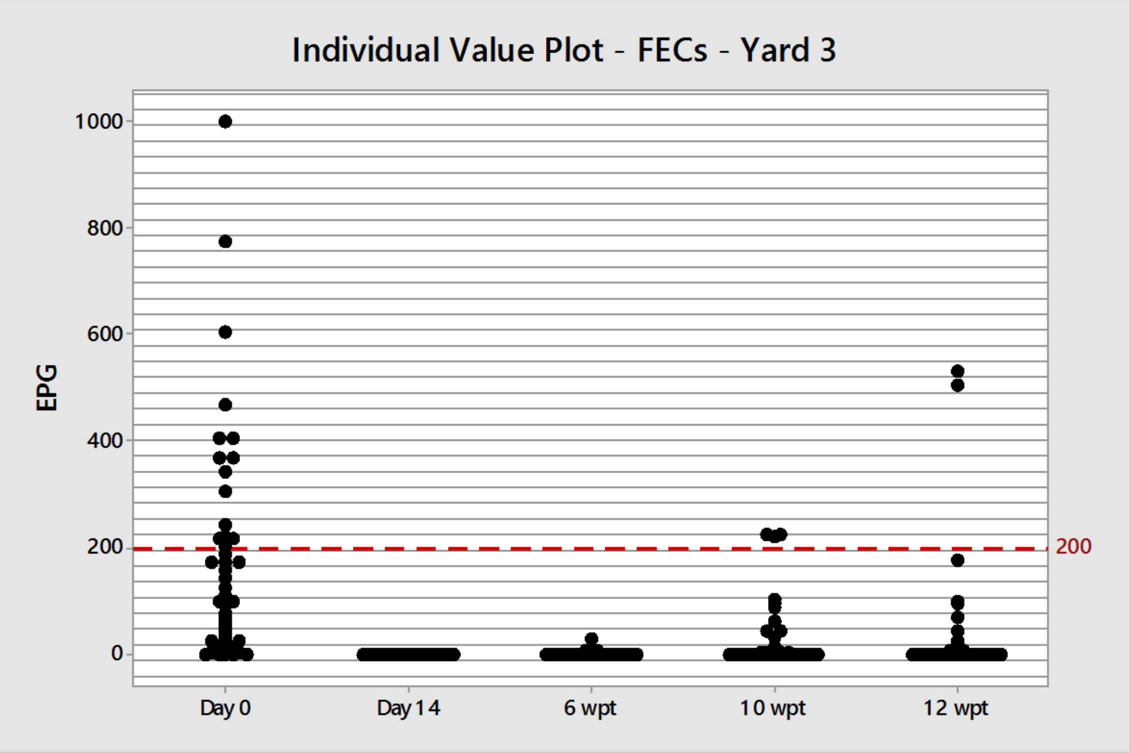
Faecal Egg Counts
EPG
160
140
120
100
80
60
40
20
0
Day 0
Day 14
6 wpt
10 wpt
12 wpt



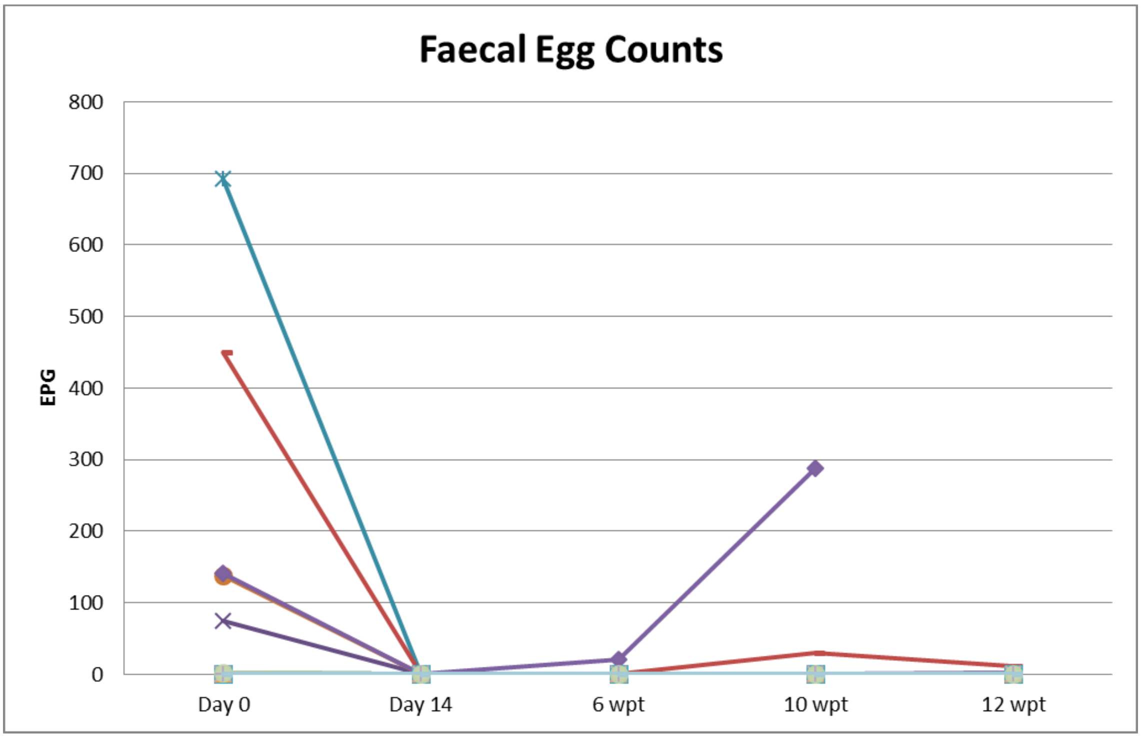
Individual Value Plot - FECs - Yard 2
EPG
200
150
100
50
0
200
Day 0
Day 14
6 wpt
10 wpt
12 wpt

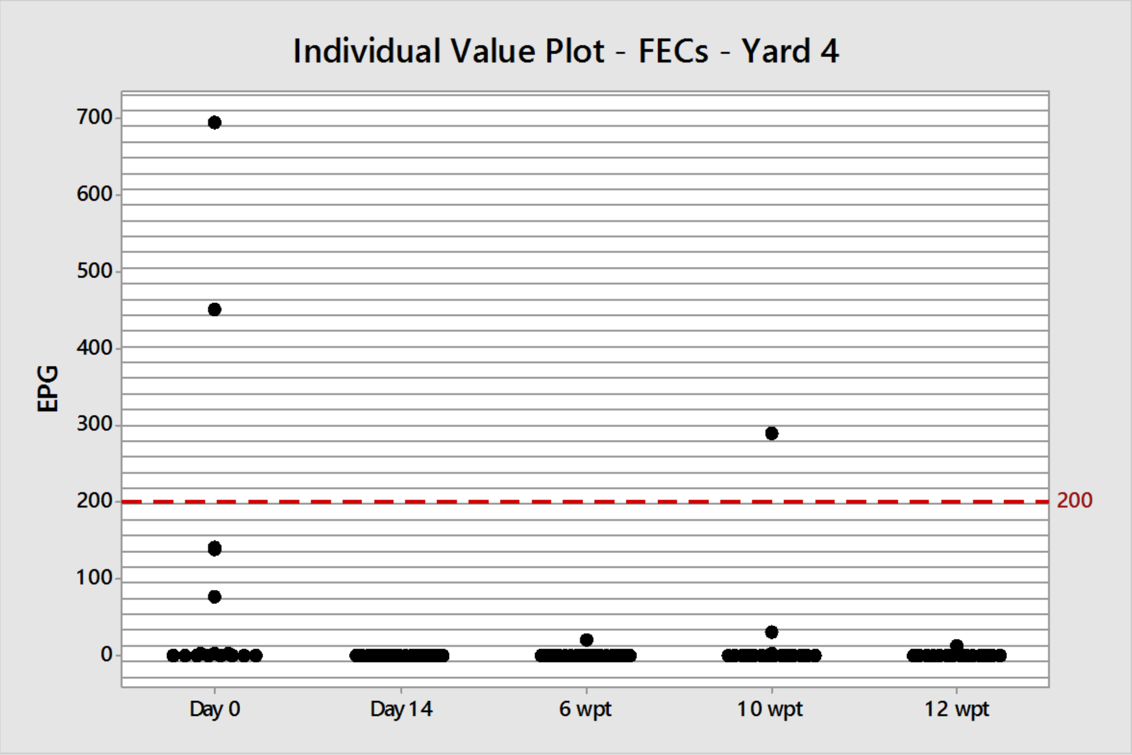
Yard 3


Faecal Egg Counts
EPG
1200
1000
800
600
400
200
0
Day 0
Day 14
6 wpt
10 wpt
12 wpt

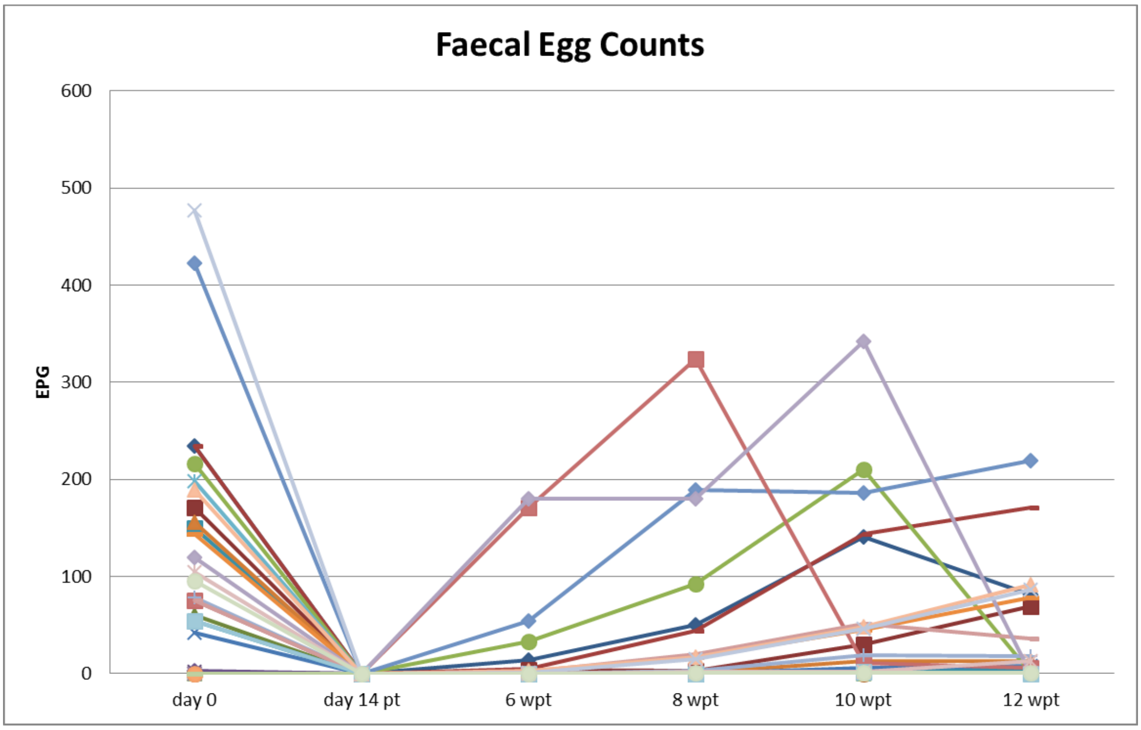

Individual Value Plot - FECs - Yard 3
EPG
1000
800
600
400
200
0
200
Day 0
Day 14
6 wpt
10 wpt
12 wpt

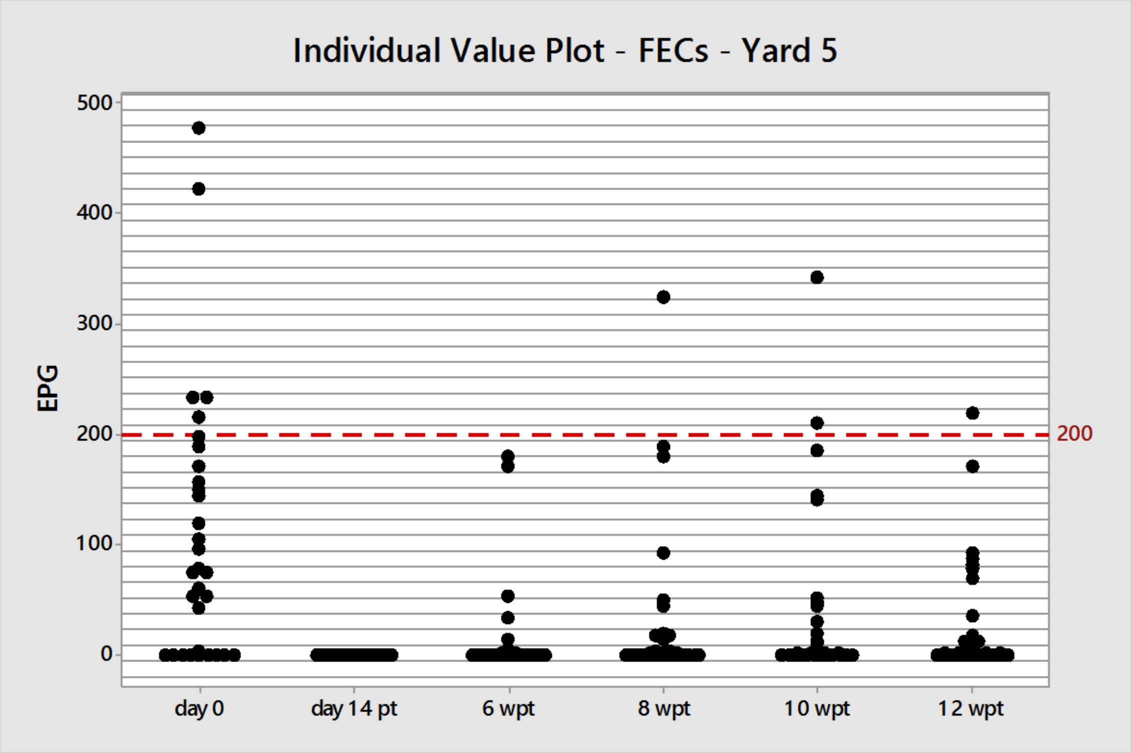
Yard 4


Faecal Egg Counts
EPG
800
700
600
500
400
300
200
100
0
Day 0
Day 14
6 wpt
10 wpt
12 wpt


Individual Value Plot - FECs - Yard 4
EPG
700
600
500
400
300
200
100
0
200
Day 0
Day 14
6 wpt
10 wpt
12 wpt

Yard 5


Faecal Egg Counts
EPG
600
500
400
300
200
100
0
day 0
day 14 pt
6 wpt
8 wpt
10 wpt
12 wpt


Individual Value Plot - FECs - Yard 5
EPG
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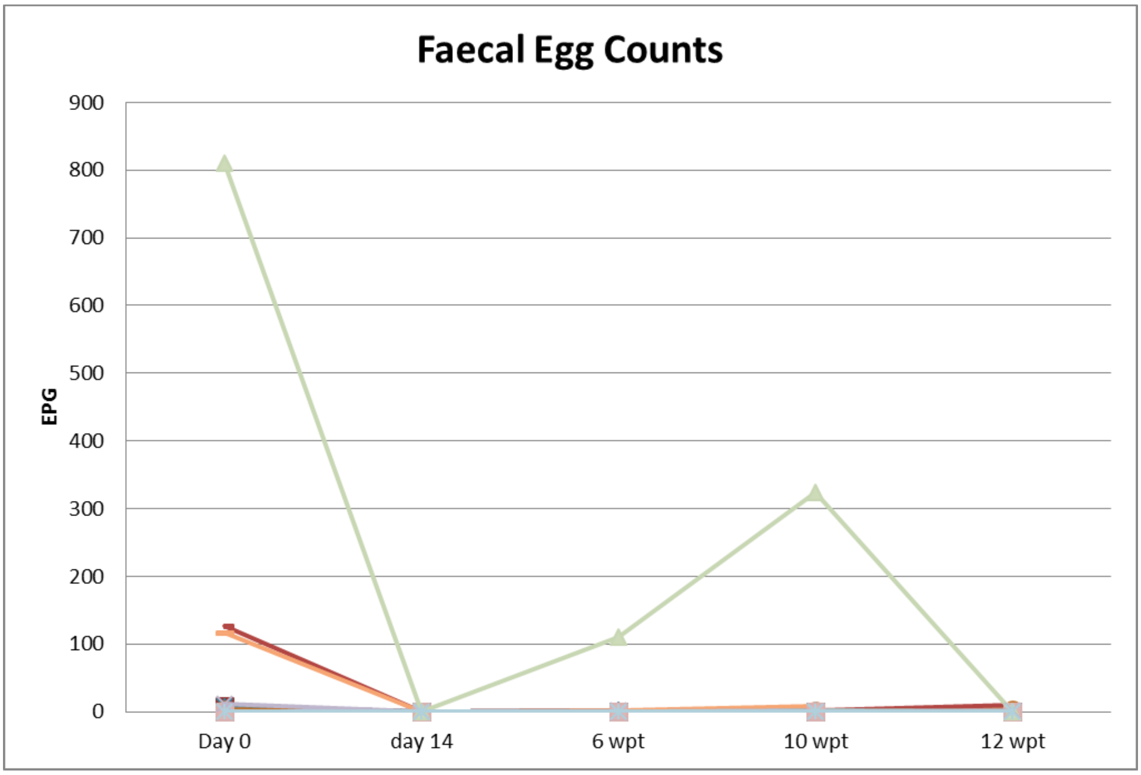
400
300
200
100
0
200
day 0
day 14 pt
6 wpt
8 wpt
10 wpt
12 wpt

Yard 6


Faecal Egg Counts
EPG
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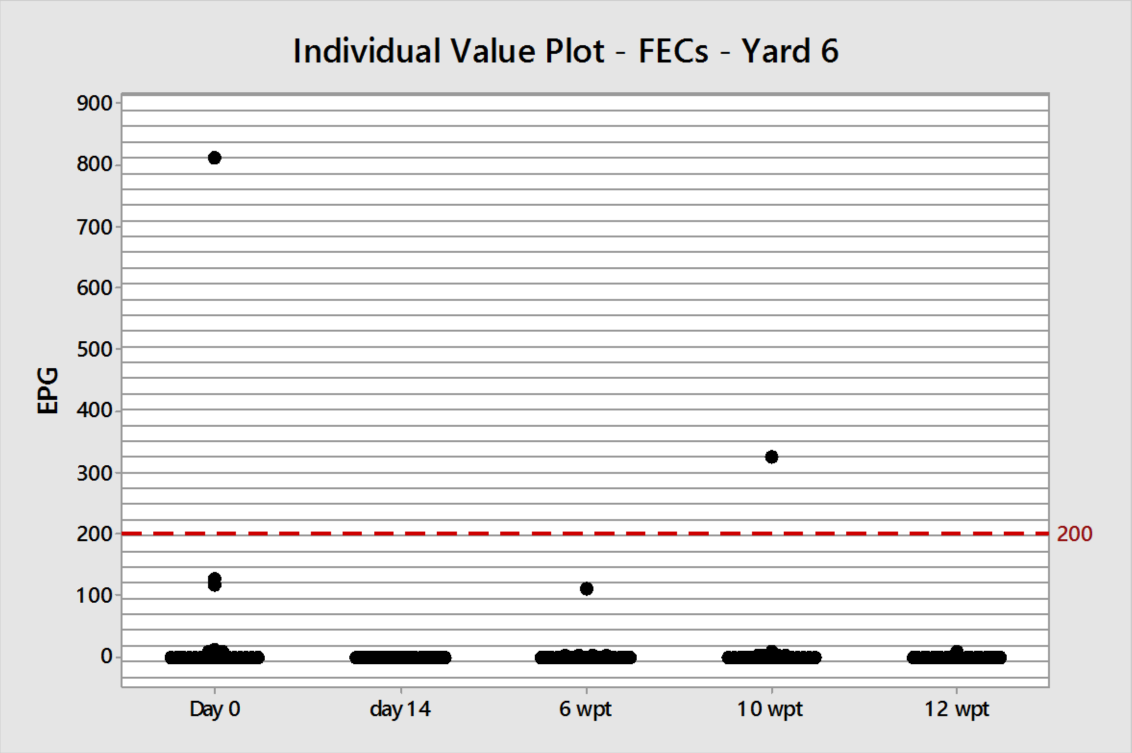
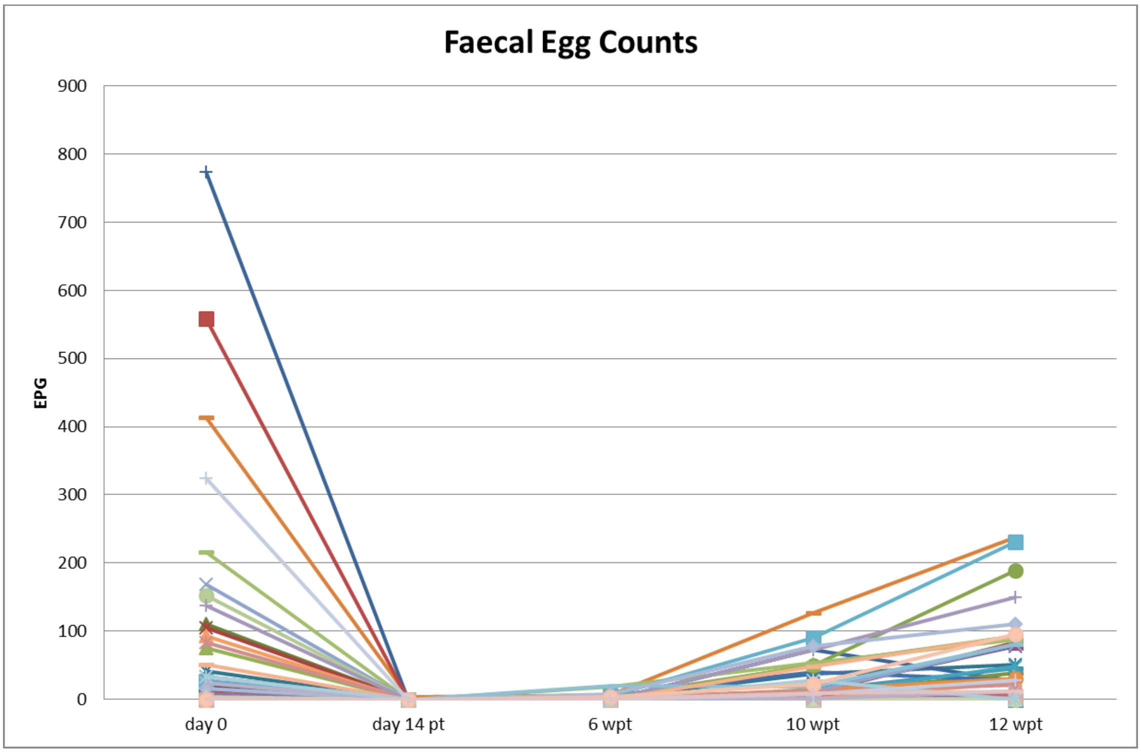
800
700
600
500
400
300
200
100
0
Day 0
day 14
6 wpt
10 wpt
12 wpt


Individual Value Plot - FECs - Yard 6
EPG
900
800
700
600
500
400
300
200
100
0
200
Day 0
day 14
6 wpt
10 wpt
12 wpt

Yard 7


Faecal Egg Counts
EPG
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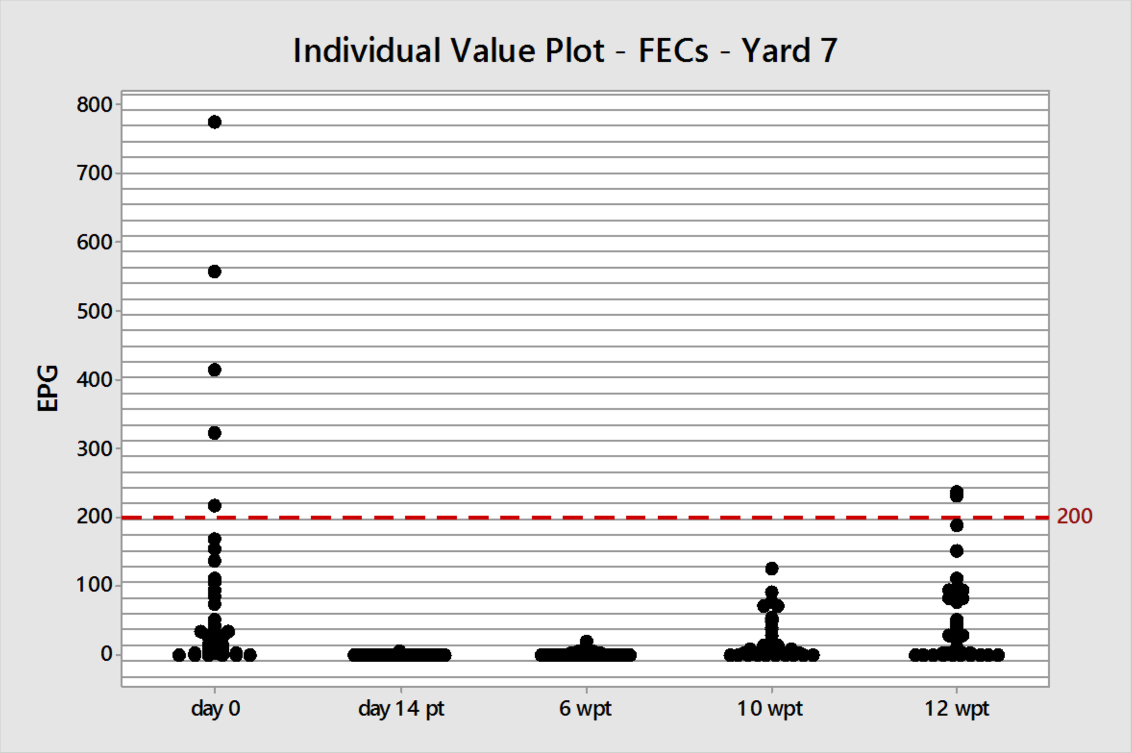
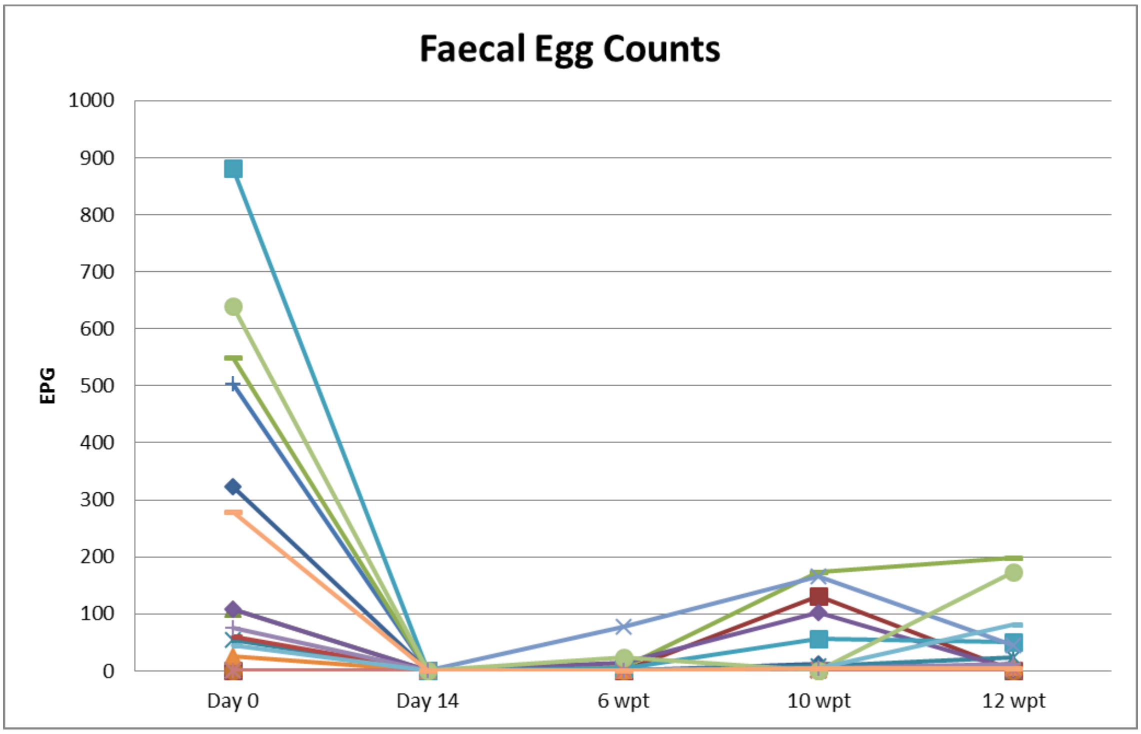
800
700
600
500
400
300
200
100
0
day 0
day 14 pt
6 wpt
10 wpt
12 wpt


Individual Value Plot - FECs - Yard 7
EPG
800
700
600
500
400
300
200
100
0
200
day 0
day 14 pt
6 wpt
10 wpt
12 wpt

Yard 8


Faecal Egg Counts
EPG
1000
900
800
700
600
500
400
300
200
100
0
Day 0
Day 14
6 wpt
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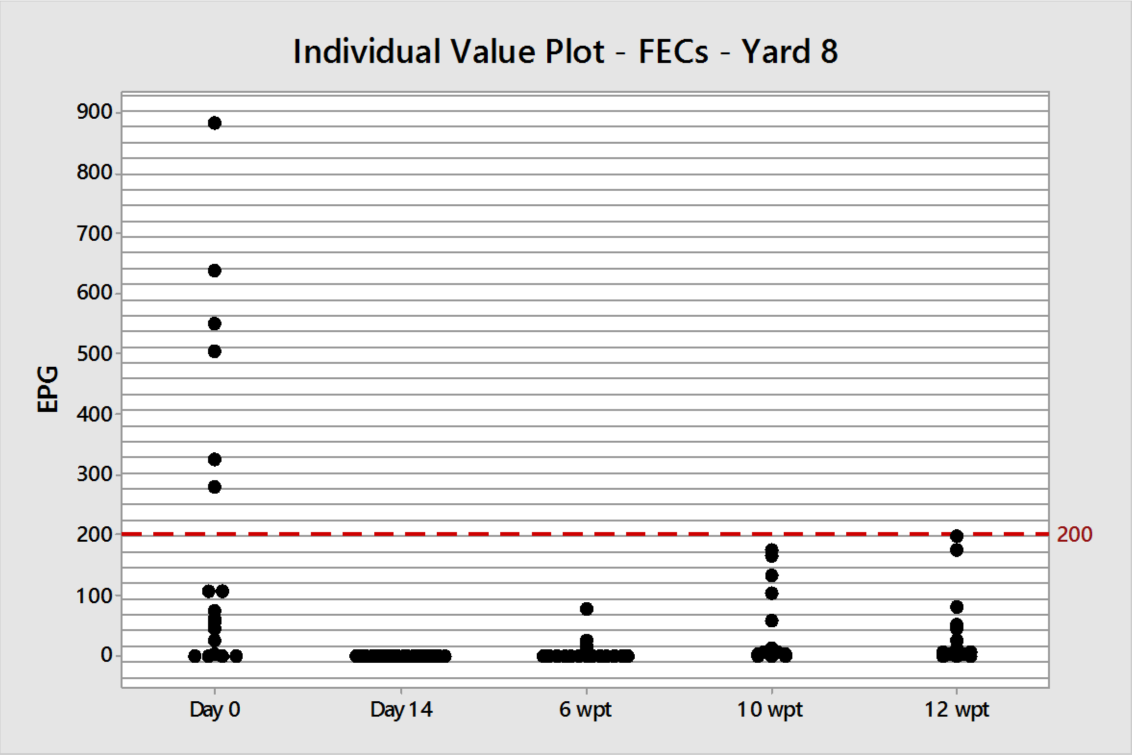
12 wpt


Individual Value Plot - FECs - Yard 8
EPG
900
800
700
600
500
400
300
200
100
0
200
Day 0
Day 14
6 wpt
10 wpt
12 wpt