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A Study on the Efficacy of selected Anthelmintic Drugs against Cyathostomins in Horses in the Federal State of Brandenburg, Germany

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1. Introduction

As grazing animals, all equines are prone to infections with helminths (worms). The management of domestic horses worldwide faces the constant challenge of controlling these internal parasites. The most common helminths in the horse include redworms (Strongyles), roundworms (*Parascaris (P.) equorum*), tapeworms (*Anoplocephala* spp.), pinworms (*Oxyuris equi*), threadworms (*Strongyloides westeri*), lungworms (*Dictyocaulus arnfieldi*) and the liver fluke, *Fasciola hepatica*. Larvae of bot flies (*Gasterophilus* spp.) also are important internal parasites of the horse (Eckert et al., 2008).

With the advent of highly effective and economical anthelmintic drugs in the 1950s and the subsequent introduction of a new drug class every decade until the 1980s, parasite control has been centred on the use of these chemicals (Kaplan, 2004). Traditionally, a scheme of worming all horses at certain intervals, independently of the status of infestation, is used on most farms to prevent clinical parasitic disease. Currently, there are three broad spectrum drug classes with different modes of action commercially available for horses, the benzimidazoles (BZ), the tetrahydropyrimidines and the macrocyclic lactones (ML).

At present, the Small Strongyles, or cyathostomins (CYA), are considered as the most important parasites in the horse. A representative study on the helminthic burden of horses carried out in 2006 on 126 farms in the Federal State of Brandenburg, Germany, revealed prevalences of 98.5% at farm level and 67% at animal level (Hinney, 2008). Due to the regular use of anthelmintic drugs over the last decades in the developed countries, the formerly significant Large Strongyles (*Strongylus* spp.) are today of less importance in well-managed herds and rarely cause clinical disease. However, the constant use and, often, the considerable over-use of anthelmintic drugs (Kaplan, 2002) has led to the emergence of reduced susceptibility and also resistance to the different classes of anthelmintics in some species of equine helminths.

For the BZ, resistant CYA populations are nowadays widespread and have been described worldwide; the first finding in Germany was in 1983 (Bauer et al., 1983). Resistance for Pyrantel (PYR), the sole member of the tetrahydropyrimidines used in horses, has also been reported from several countries; in Germany, Traversa et al. found resistance on four out of twenty horse yards, and suspected resistance on four others (Traversa et al., 2009).
Due to the appearance of resistance to these two drug classes, the ML have been heavily relied upon during the past two decades, as no suspected or confirmed resistance had been reported until recently. In the German Federal States of Lower Saxony and North Rhine-Westphalia, suspected loss of efficacy for the third drug class, the ML, has been reported

against the potentially highly pathogenic CYA (Fritzen, 2005) and *P. equorum* (Samson-Himmelstjerna et al., 2007). Studies in the Netherlands (Boersema et al. 2002), Brazil (Molento et al., 2008) and the USA (Lyons et al., 2010), have confirmed CYA resistant to Ivermectin (IVM).

So far, very little information on anthelmintic susceptibility in CYA in the Federal State of Brandenburg and the eastern German Federal States is available. The purpose of the present study is the detection of the status quo concerning two commonly used anthelmintic drugs, IVM, a macrocyclic lactone, and PYR, a tetrahydropyrimidine.

2. Review

2.1. Strongyle Infections in Horses

Of the various gastro-intestinal parasites of the domestic horse, the most important helminths are nematodes, or threadworms, of which the family Strongylidae shall be of focal interest in this thesis. They are symmetric, non-segmented worms of cylindrical shape. They are obligatory parasites that undergo the first parts of their life cycle outside the host but mate as sexually mature adults in the intestine of equids. The females then lay eggs which are transported into the external environment with the faeces.

The family Strongylidae belongs to the order Strongylida (subphylum Nematoda, phylum Nematozoa) and is divided into three subfamilies, the Strongylinae, Cyathostominae and Gyalocephalinae (Eckert et al., 2008).
The subfamily Strongylinae includes the genera *Strongylus*, *Bidentostomum*, *Craterostomum*, *Oesophagodontus* and *Triodontophorus* (Lichtenfels et al., 1998). Members of the genus *Strongylus* spp. tend to be slightly longer than the remaining and are considered as Large Strongyles; they also include a stage of somatic migration in their life cycle and are regarded separately from the other genera, which all are counted among the Small Strongyles (Tolliver, 2000; Eckert et al., 2008). Of the subfamily Cyathostominae, 52 Species belonging to the following 13 genera are known to be parasites of the horse and other equids: *Cyathostomum*, *Cylindropharynx*, *Poteriostomum*, *Cylicocyclus*, *Cylicodontophorus, Cylicostephanus*, *Caballonema*, *Petrovinema*, *Tridentoinfundibulum*, *Skrjabinodentus*, *Hsiungia, Coronocyclus* and *Parapoterostomum*. The subfamily Gyalocephalinae has only one member, *Gyalocephalus capitatus* (Lichtenfels et al., 1998 and 2008).

2.1.1. Large Strongyles

The genus *Strongylus* comprises three species that are present in horses in Europe: *S. vulgaris*, *S. equinus* and *S. edentatus*, and one species, *S. asini*, which is found in zebras and donkeys. The members of the genus *Strongylus* are commonly known as “Large Strongyles” and are ca. 1-5cm long nematodes, of a yellowish brown colour. Pre-patent periods (the time between ingestion of the parasite until first appearance of eggs in the faeces) can range from 6 to 11 months, and the life cycle involves extensive somatic migration of the larvae L4 and L5 (Eckert et al., 2008).

2.1.1.1 Prevalence

Large Strongyles have in the past been considered the most important internal parasites of the horse (Herd, 1986a); this was owed to their apparent pathogenicity and frequent findings in post-mortem examinations (Slocombe and McCraw, 1973). Hence, the earliest schemes for parasite control in horses were mainly targeted at these parasites, and prevalences in well managed populations in the developed countries are at present very low (Herd and Coles, 1995; reviewed by Kaplan, 2002). In 2006, Hinney found Large Strongyles in only one of 126 horse yards in the German Federal State of Brandenburg (Hinney, 2008).

2.1.1.2. Development

2.1.1.2.1. External Development

Both Large and Small Strongyles have a direct life cycle, without an intermediate host, and their development outside the equine host is essentially the same. Egg-containing faeces are dropped onto pasture by grazing horses; then, the survival and development of the eggs depends on climatic influences, such as environmental temperature and humidity (Eckert et al., 2008).

Shortly after faeces are passed, first stage larvae (L1) emerge from the eggs. L1 feed on bacteria present in the faeces and then moult to become second stage larvae (L2). The L2 continue the food intake and store lipids in their intestinal cells, they further grow and differentiate. L2 then undergo an incomplete shedding, after which they remain within their old cuticle, and subsequently become the infective larvae (L3) (Eckert et al., 2008). L3 do not feed, as they are enclosed by this double-layered cuticle that impedes any food intake. L3 are quite mobile and can actively migrate out of the faecal patch and onto pasture, where they ultimately are ingested by the host.

The most favourable temperatures for egg hatching are between 25 and 33°C; temperatures below 5-10°C will delay the development (Ogbourne, 1972; Mfitilodze and Hutchinson, 1987), but lower temperatures and even periods of frost do not impede hatching once temperatures rise again. Strongyle eggs and larvae can develop at temperatures between 8 and 38° C provided sufficient humidity. Mfitilodze and Hutchinson found moisture levels of 14% to be the minimum for complete development to the infective stage (Mfitilodze and Hutchinson, 1987).

The rate of development is proportional to the temperature, i. e. development is slow at lower temperatures and it can take as long as 24 days for L3 to develop. At temperatures of 35°C, infective larvae can be observed after as little as 48h (Ogbourne, 1972; Mfitilodze and Hutchinson, 1987).

L1 and L2 are very susceptible to frost and desiccation, but L3 are protected from environmental conditions by their external cuticle and they can survive periods of desiccation or frost (Nielsen et al., 2007). However, if temperatures are as high as 30 to 38°C, and provided moist conditions, L3 tend to be very active. The stored lipids will be consumed faster and the larvae can be depleted of energy, which will compromise their mobility and also infectivity (Mfitilodze and Hutchinson, 1987; Medica and Sukhdeo, 1997).

Nielsen et al. summarised the influences of temperature and humidity on free-living stages of strongyles as shown in Table 1 (Nielsen et al., 2007).

Table 1: Survival of free living equine strongyle stages when exposed to different climatic influences (Nielsen et al., 2007)

Free living stage	Frost	Alternation between frost and thaw	Desiccation	Heat (30-38°C)
unembryonated egg	++	++	[a]	++
embryonated egg	+	-	[a]	++
L1	-	-	-	++
L2	-	-	-	++
L3	+++	+	+++	-

- indicates very susceptible, + weakly resistant, ++ moderately resistant, +++ very resistant

[a] no data available

2.1.1.2.2. Internal Development

The larvae pass the stomach and, once in the intestinal tract, lose their outer sheath (ecdysis) and penetrate the mucosa to undertake different ways of visceral migration. All three species of *Strongylus* spp., and especially *S. vulgaris*, can cause severe damage during their internal development. *S. vulgaris* and *S. equinus* develop to L4 within the intestinal wall before embarking on their passage through the body of their host, whereas *S. edentatus* sets of as L3 and moults to L4 in the liver (McCraw and Slocombe, 1978 and 1985).

S. vulgaris penetrates the mucosa of the ileum, caecum and ventral colon (McCraw and Slocombe, 1976) and moults to L4 within a few days. L4 enter submucosal arterioles and travel against the blood flow through arterioles and arteries into the Arteria (A.) mesenterica cranialis. This is their predilection site, where they grow and develop for about 3-4 months, then moult to become pre-adults and migrate back into the intestine within the arteries. Here, they become encysted into the subserosa, forming numerous nodules of 5-8mm diameter (Duncan and Pirie, 1972). After their emergence into the lumen of the intestine, they become sexually mature adults after another six to eight weeks. Occasionally, aberrant L4 may travel into the wall of the Aorta and can reach the heart or the A. femoralis. Travelling with the bloodstream, these larvae can reach other organs, including the brain. The pre-patent period of *S. vulgaris* is about 6.5 to 7 months (Eckert et al., 2008).

L4 of *S. equinus* pass through the peritoneal cave into the liver, where they migrate within the parenchyma for several weeks. They migrate further on to reach the pancreas and here develop into the adult stage. The adults then migrate retroperitoneally into flanks, perirenal fat and diaphragm. The journey is completed on returning into the intestine at the caecum. The pre-patent period of *S. equinus* is around 8.5 to 9 months (McCraw and Slocombe, 1985).

S. edentatus starts its visceral passage as L3, which penetrate the wall of caecum and right ventral colon and are transported to the liver in the portal vein (McCraw and Slocombe, 1978). In the liver, L3 moult to L4 and later migrate out of the peritoneum via the hepatorenal ligament (Wetzel and Kersten, 1956). In the retroperitoneal space, they reach the adult stage and migrate back into the intestine via the mesentery. The pre-patent period of *S. edentatus* is approximately 10.5 to 11 months (McCraw and Slocombe, 1974).

2.1.1.3. Pathogenicity

The migratory larval stages of the Large Strongyles can cause severe damage; particularly *S. vulgaris'* extensive travels through the artery system lead to fibrotic thickening of arterial walls, thromboarteritis and aneurysms, especially in the cranial mesenteric artery and caecal and colic arteries. Detached thrombi can cause embolisms, local ischemia and infarction of the intestine, leading to severe colics (Duncan and Pirie, 1975; Eckert et al., 2008; McCraw and Slocombe, 1976). This syndrome of thrombo-embolic colic has long been associated with *S. vulgaris* (Enigk, 1951) and in severe cases, the prognosis is poor. L4 that enter the wall of the Aorta and travel caudally into the A. femoralis or associated arteries can cause intermittent lameness. Other aberrant larvae can produce considerable destruction of tissue in the organs they end up in, for example leading to ataxia if the brain or spinal cord are compromised. Depending on the number of ingested larvae and the general physical and immunological

condition of the animal, further symptoms of an infestation with *S. vulgaris* include mild abdominal pain, lethargy, and fever (Duncan and Pirie, 1975).
Larvae of *S. equinus* and *S. edentatus* both mainly cause damage in the liver, omentum and the intraperitoneal space, where they are responsible for inflammation, fibrosis, adhesions and peritonitis (Eckert et al., 2008; McCraw and Slocombe, 1974). Larval migration leaves clearly visible tortuous tracks widely distributed throughout the liver (McCraw and Slocombe, 1978 and 1985). *S. equinus* can furthermore cause large areas of fibrosis and loss of secretory tissue in the pancreas (McCraw and Slocombe, 1985).

The adult stages of the Large Strongyles in turn cause erosions on the intestinal wall of the caecum and ventral colon by sucking a small piece of mucosa into their buccal cavity. This tissue is digested, and small amounts of blood are withdrawn. When large numbers of worms are present, low levels of blood and albumin will be lost through these erosions, leading to anaemia. The intestinal motility can be compromised (Eckert et al., 2008).

2.1.2. Small Strongyles

The members of the family Strongylidae bar *Strongylus* spp. are considered as Small Strongyles (Tolliver, 2000). This includes all species that do not undergo migration outside the intestinal tract, and entails some members of the subfamily Strongylinae, all Cyathostominae and the only genus in the subfamily Gyalocephalinae. Following the recommendation of the Third WAAVP International Workshop on the Systematics of the Cyathostominea of Horses, the subfamily Cyathostominae should be referred to as cyathostomins (CYA) (Lichtenfels et al., 2002).
They are of a yellowish white or red colour (hence the term “redworms”) and measure ca. 0.5–3cm. The pre-patent period differs between species and ranges from 5.5 to 14 weeks (Eckert et al., 2008).

2.1.2.1. Prevalence

The major focus since the introduction of anthelmintic drugs has been on the eradication of large strongyles (Herd, 1986a), whereas the pathogenicity of CYA has long been underestimated. This was partly due to the overshadowing effect of the lesions caused by large strongyles as opposed to CYA, and partly to the fact that large amounts of CYA might be present in the large intestine of clinically healthy horses (Reinemeyer, 1986). Nowadays, CYA

are considered the predominant internal parasite of horses worldwide, and they often provide the major part of worm egg output of adult grazing horses (Herd, 1993).

The distribution of infestation rates within a horse herd differs largely, where most of the worm burden is usually carried by only a few animals. The number of parasites within individual horses can vary greatly and ranges from a few thousand to up to 3 million worms (Eckert et al., 2008). Largest numbers, however, are generally found in young animals, as repeated and prolonged exposure to the parasites induces age-dependent immunity (Anders et al., 2008).
The number of species most commonly found within an individual or a herd is variable, with around 12 different species being among the most frequent. The composition of CYA populations worldwide is comparable, as the most frequently found species are generally almost the same (Mfitilodze and Hutchinson, 1990; Matthews et al., 2004; Chapman et al., 2002a). These most common species are: *Cyathostomum catinatum, Cyathostomum pateratum, Coronocyclus coronatus, Coronocyclus labiatus, Coronocyclus labratus, Cylicocyclus nassatus, Cylicocyclus leptostomus, Cylicocyclus insigne, Cylicostephanus longibursatus, Cylicostephanus goldi, Cylicostephanus calicatus* and *Cylicostephanus minutus* (reviewed by Kaplan, 2002).
Steinbach found a total of fourteen species in her study conducted in the German Federal State of Hessen (Steinbach, 2003), with three being the most common: *Cylicostephanus longibursatus*, *Cylicostephanus goldi* and *Cyathostostomum catinatum*.

Whilst infestation of healthy horses and with small numbers of CYA may pass unnoticed in many cases, they carry a high pathogenic potential.

2.1.2.2. Development

The external development is principally the same as for the Large Strongyles (see 2.1.1.2.1.). The internal development lacks the migratory stages, and can include a period of arrested larval development of variable length.

Infective third stage larvae are ingested and travel to the intestine along with the chyme. In the small intestine, the larvae lose their sheath and reach the large intestine to undergo further development in the intestinal wall: These early third stage larvae (EL3) enter the crypts of caecum and colon and proceed to mucosa and submucosa where a granuloma and later a capsule of connective tissue are formed around them (Eysker and Mirck, 1986, Steinbach, 2003). The majority of the encysted larvae are located in the caecum and proximal 75% of the ventral colon (Reinemeyer and Herd, 1986; Steinbach, 2003). Here, they mature into late L3

(LL3), which are more developed and bigger in size. Still within the capsule, the LL3 moult and become developing L4 which rest for a varying time of usually 30 to 60 days. They then leave the capsule and enter the lumen. Back in the intestine, they moult yet again to the pre-adult 5th stage, which eventually reaches sexual maturity as adult male and female worms (Eckert et al., 2008).

2.1.2.2.1. Hypobiosis

The pre-patent period is usually 5.5-14 weeks, but early stage 3 larvae (EL3) can enter a dormant stage called hypobiosis, in which they remain within the mucosa of the large intestine for several months or even years (Duncan et al., 1998; Gibson, 1953) and may accumulate in large numbers.

It is thought that negative feedback from adult luminal worms (Gibson, 1953), trickle infections with larvae as well as the host's immune response favour hypobiosis (Chapman et al., 2002b). Eysker et al. assumed seasonal conditioning of larvae on pasture in late summer and early autumn to be responsible for the induction of hypobiosis (Eysker et al., 1990).

The hypobiosis allows CYA to survive within the host during the winter, when environmental conditions are not favourable for the external development, and to continue their development to sexually mature stages once chances for completion of the life cycle outside the host have improved. As a result, stabled horses can show an increased egg output in springtime, even though they were not exposed to new infection on pasture (Herd, 1986a), as formerly arrested larvae will have completed the development to adults.
A simultaneous eruption of the encysted larvae can lead to a clinical syndrome called "larval cyathostominosis" (see 2.1.2.4.). There is currently no reliable diagnostic tool for the detection of encysted larvae intra vitam (Matthews et al., 2004). But recent studies have shown promising results for the establishment of a diagnostic method based on an immunodiagnostic marker specific to CYA developing larvae in the future (Dowdall et al., 2002; McWilliam et al., 2010).

2.1.2.3. Pathogenicity

Adult CYA are mostly localized in the dorsal and ventral colon, only 10% in the caecum (Steinbach, 2003). They adhere onto the mucosa and can cause erosions and ulcerations, as well as the rupture of small capillaries. If large numbers are present, the surface of the mucosa

may be disrupted in large areas (Uhlinger, 1991). Clinical signs of an infestation with adult CYA are not specific and vary from a reduction in athletic performance, coarse coats and failure to shed winter coat, to mild colics, chronic diarrhoea and weight loss. Of greater clinical importance are the encysted larvae. L3 can be very abundant and can constitute the great majority of the population of CYA present in the host (Eysker et al., 1984), and granulomas and hyperplasia are found around the encysted larvae (Steinbach, 2003). An inflammatory enteropathy is the response to penetration of larvae into and, later on, their emergence from the mucosa (Love et al., 1999).

2.1.2.4. Larval Cyathostominosis

Larval cyathostominosis is a syndrome caused by the simultaneous reactivation of encysted larvae and subsequent emergence of L4 into the intestinal lumen. This eruption leads to a massive inflammatory response triggering acute enteropathy. The barrier function of the damaged mucosa is impeded, causing enterotoxemia. Symptoms of larval cyathostominosis include: rapid weight loss, slight to profuse watery diarrhoea irresponsive to treatment, unspecific signs of colic and ventral oedema. Fever can be present or absent. Even prior to death, no loss of appetite may occur (Kelly, et al., 1993). In many cases, this condition is fatal despite intensive medical care, with mortality reaching up to 50% (Kelly et al., 1993, Love et al., 1999).

Larval cyathostominosis most commonly affects young stock, possibly because the lack of acquired immunity may predispose to the accumulation of large worm burdens. It most oftenly occurs in the late winter or early spring period, when the mucosal stages start to continue their development and emerge from the mucosa. The sudden re-activation of large numbers of encysted larvae has also been associated with the recent administration of anthelmintics (Reid et al., 1995; Wobeser and Tataryn, 2009). It is thought that the removal of adult stages from the intestinal lumen can trigger the simultaneous release of the encysted larvae (Gibson, 1953).

As the clinical signs are not pathognomonic, definite diagnosis of larval cyathostominosis can be difficult. In affected horses, egg shedding often is low or even absent, therefore the FEC is not a reliable tool for diagnosis, but the history and the age of the horse must be considered. In addition to the clinical presentation, laboratory parameters are indicative for the diagnosis such as neutrophilia, hypoalbuminemia and, less often, hyper-β-globulinemia and increased alkaline phosphatase (Giles et al., 1985; Love et al., 1999). Sometimes, L4 are present in the Ampulla recti and can be found during rectal examination, indicating larval cyathostominosis.

2.1.3. Immune Response

Natural infection with strongyles induces weak immunity; it is slow to develop and incomplete, as horses of all ages can carry high worm burdens. However, highest worm counts are frequently found in young horses (Klei and Chapman, 1999), which also are more likely to show signs of clinical disease caused by CYA. Advanced age seems to weaken the immune status, as geriatric animals frequently have higher FEC. Encysted larvae trigger T helper 2 type responses linked with Interleukin-10 (IL-10) and IL-4 (Davidson et al., 2002). The reactivation of the encysted larvae and subsequent inflammatory response is associated with Tumour Necrosis Factor α (TNFα) (Davidson et al., 2002).
Low-level exposure to strongyles is necessary to induce natural immunity, and it is not considered desirable to create a worm-free environment on a stud farm. Otherwise, the horses reared worm-free would be likely to show clinical symptoms when they eventually encounter strongyles in a different environment (Herd and Coles, 1995; Uhlinger, 1993).

2.1.4. Detection of Infestation

The most commonly used method for the diagnosis of patent infestation with strongyles is the Faecal Egg Count (FEC), for which a sample of faecal material collected freshly from the host is diluted in a saline solution, sieved to clear it of debris, and examined for the presence or absence of helminth eggs.
To quantify the egg output, the World Association for the Advancement in Veterinary Parasitology (WAAVP) recommends the McMaster technique (Coles et al., 1992), where a defined amount of faeces is suspended in a defined volume of saline solution. This sample is microscopically examined, and the eggs floating in the solution are counted. The McMaster slide contains a grid with a defined volume and allows for the quantification per gram of faeces of the eggs found (for a description of the modified McMaster method used in this study, see 3.4.).

The FEC is an indicator for the presence of sexually mature, female worms in the host. It does, however, not give exact information about the magnitude of the worm burden. The use of a faecal sample bears the problem of high variation in egg count, as the faecal egg output of one single host can differ largely from day to day. Egg shedding can be intermittent, and a high fecundity of a low number of worms at a given time will yield high FECs, whereas a high number of larval or sexually immature stages, or indeed a high proportion of male helminths present in the host can lead to low egg counts (Osterman Lind et al., 2003; Uhlinger, 1993). This could be partly made up for by using a pooled sample from several days, although repeated sampling is generally not considered under field conditions.

Furthermore, the eggs are distributed unevenly within the faecal material, and for one identical faecal sample different FEC can be obtained (Kraemer, 2005; Uhlinger, 1993). Because a standardized weight is used (4g of faeces for the McMaster method), a sample with a high water content will appear to have a lower egg count than a drier sample, because it contains less faecal material per gram, and vice versa (Uhlinger, 1993). The age (Herlich, 1960) and immune status (Michel, 1968; Roberts et al., 1951) of the host can also influence the faecal egg output.

Another factor influencing the consistency of FECs is that techniques based on dilution inherently are subjected to large variance (Peters and Leiper, 1940), as they rely on the even suspension of the faecal mass. Repeatability is further negatively influenced by the necessity to select an aliquot of the mixture (Uhlinger, 1993).
Nonetheless, Steinbach found that worm burden and egg count can be related, as in her study the horse with the highest FEC had the highest worm burden in the post mortem examination, and the horse with the lowest FEC showed the smallest worm burden (Steinbach, 2003).

2.1.5. Species Differentiation

Until recently, species differentiation of CYA was only possible by microscopically examining the anatomical features of adult specimen. This requires vast skills and experience of the researcher or technician (Tolliver, 2000) and can be very time-consuming. The differentiation of larvae CYA from large strongyles is based on differences in the number and shape of the intestinal cells of the L3. In a FECRT, one does not avail of L3 or adult worms, but can only use the obtained eggs for larval cultures, which are time-consuming and do not allow prompt results.

Polymerase Chain Reaction (PCR) assays have been developed for DNA-based approaches for the identification of species of CYA (Gasser et al., 1996; Hung et al., 1999; Hodgkinson et al., 2003). These allow the species-specific amplification of DNA derived from faecal samples and do not require L3 or adult specimen, as any larval stages and eggs can be utilized.

Traversa et al. developed a new technique which allows fast and specific identification of all developmental stages of CYA. This Reverse Line Blot hybridization (RLB) method can unequivocally identify the PCR-amplified DNA of 13 common species of CYA, and at the same time discriminate them from *Strongylus* spp. (Traversa et al., 2007).
This method holds many advantages, as it allows the accurate identification of the most common species of CYA, comparison of their occurrence in different equine populations and recognition of potentially resistant species by using larval stages.

2.2. Endoparasite Control

The control of endoparasites has traditionally been based on drug treatment alone for the past decades, although some horse owners resort to alternative medicine (e. g. homoeopathy, herbal medicines). The second aspect involved, and of growing importance, is the stable management.

2.2.1. Stable Management

2.2.1.1. Hygiene of Fields and Paddocks

In a population of parasitic helminths, the developmental stages outside the host greatly outnumber the parasitic stages within the host. Therefore, the major epidemiological variable influencing worm burdens of grazing animals is the number of infective larvae ingested from pasture each day (Barger, 1999).
The higher the amount of infective larvae on the pasture, the higher the intake consequently will be. The availability of infective larvae may be relatively constant, or may vary throughout the year, depending on the climate, and is determined by environmental factors such as temperature and humidity. The infectivity of a pasture is determined by the input of developmental stages to it such as the eggs in faeces, exposure to environmental factors and the return of the hosts to the pasture.
Several management techniques are used with horses to reduce this infectivity, such as the collection of faeces, grazing with alternate hosts (usually sheep or cattle) or by cropping, for example for silage or hay (Herd, 1986b; Soulsby, 2007). Roughage should be offered not directly from the ground, but on racks, during the periods of supplementary feeding, or if feeding on sand paddocks.

Horses that are infestated with worms can contaminate pastures with millions of worm eggs every day. During evolution, horses have developed a natural aversion to faeces as a part of their instincts. They tend not to graze in the close proximity of horse droppings. A typical, unmanaged field where horses are kept will be separated in areas of grazed grass (short “lawns”) and areas of ungrazed grass around faeces (long “roughs”) (Herd, 1986b). Up to 50% of a field can be transformed into roughs, which are usually avoided by the horses unless overcrowding forces them to forage on them (Herd, 1986b). This can be of importance if limited pasture is available.
Under favourable conditions, i.e. humidity, larvae migrate out of the faecal patches onto the grass. Nonetheless, faeces are the main reservoir of larvae in the field (Kuzmina et al., 2006),

and larvae are more likely to survive the winter in disintegrated faecal patches than on the grass.

Twice weekly removal of faeces from pasture significantly reduces parasitic burden, as this pattern does not leave enough time for strongyle eggs to develop to infective larvae and migrate to pasture, or the dispersal of ascarid eggs (Herd, 1986b). The collection of faeces also reduces areas of rough grazing, increasing the area available for grazing on the pasture; an advantage especially on farms with a high concentration of horses on limited pasture (Herd, 1986b and 1986c, Kelly, et al., 1993). The collection of faeces from pastures can be performed manually or mechanically with a vacuum sweeper.

Herd conducted a study in which he compared six groups of ponies that were kept on grass. In one group, faeces were collected twice weekly and no anthelmintic treatment was given, four groups of ponies did receive different anthelmintic treatments and faeces were not collected, and one control group was left untreated and faeces were not collected. The twice-weekly collection of faeces provided a superior parasite control than the anthelmintic treatments (Herd, 1986b). Corbett et al. (2014) performed a large-scale study on grazing donkeys: FEC was significantly reduced in those herds where removal of faecal patches was performed twice weekly, compared to those where faeces remained in the field. No difference in FEC could be found between herds in fields with manual and mechanical removal (Corbett et al., 2014).

The cost and time involved in field hygiene can be outweighed by reduced spending on anthelmintic drugs, and, more importantly, a reduced risk for developing resistance. Cleaning of pasture is a procedure implemented by some horse owners (Lloyd et al., 2000; Meier and Hertzberg, 2005b; Hinney, 2008), even though it is not yet a widely used practice.

Another method to decrease parasitic contamination is to allow the fields to rest for long periods. This allows larvae to die out over time and is an effective measure, if the resting period is long enough. It might be necessary to remove horses for up to a year (Herd, 1986b), and it could prove difficult for small farms to do so.

Mixed or alternated grazing is traditionally implemented, mostly with sheep but also with cattle, and allows for the continuous use of the pasture. A large proportion of the equine parasites will then be eaten by the ruminants, and vice versa. With the exception of *Trichostrongylus axei*, helminths of horses and ruminants do not reach patency in the other host, and the infection chain is interrupted (Eckert et al., 2008). Another approach is to use the same pasture for different age groups of the same species, thus reducing the relative amount of young stock which shed larger numbers of larvae, and therefore reducing the overall contamination of the grass.

2.2.1.2. Hygiene of Stables

The horse's stable provides a suitable environment for larval development. If the stable is not regularly cleaned, temperatures within the bedding, together with the humidity from urine and faeces will allow larvae to develop into infective L3.
By removing faeces from the stables on a regular basis, the dispersion of helminth eggs into the bedding can be minimized to a great extent (Vercruysse and Eysker, 1989). Ideally, droppings and wet material should be removed twice daily, with an additional removal of droppings in the evening time in order to keep contamination at a minimum. If removed less often, the horse's movements around the stable will disperse the droppings, disintegrating them into small pieces impossible to be collected. Washing the empty stable, including walls, windows and doors, with a high pressure hot water system before disinfecting, at least once yearly, will further increase hygiene. Hinney found that daily mucking out was correlated with a lower risk of CYA infection than less regular stable hygiene (Hinney, 2008).

2.2.2. Anthelmintic Drugs

There are four different drug classes currently available on the market for equine helminths: benzimidazoles (BZ), tetrahydropyrimidines, macrocyclic lactones (ML) and Praziquantel (PZQ). Each drug class has a different mode of action and a characteristic efficacy profile against susceptible parasites. The aforementioned are broad-spectrum drugs and act against strongyle nematodes, amongst others; PZQ is used exclusively against cestodes (tapeworms). Ivermectin (IVM), a ML, and Pyrantel (PYR), a tetrahydropyrimidine, were used to perform FECRTs in the present study.

2.2.2.1. Macrocyclic Lactones

There are two groups of macrocyclic lactones (ML), the avermectins and the milbemycins, which are fermentation products derived from the soil fungi *Streptomyces avermilites* and *Streptomyces cyanogriseus*, respectively. They are chemically similar, and rely on the same mechanism of action. The mode of action is two-fold, as they firstly bind on glutamate-gated ion channels in neurons and muscle cells of nematodes, causing hyperpolarization which leads to non-spasmic paralysis. Secondly, they increase the effect of γ-aminobutyric acid (GABA), which acts as an inhibitory neurotransmitter (Ungemach, 2010).

The blood-brain barrier of vertebrates efficiently keeps concentrations of ML in the brain very low at therapeutic doses, and they exert no effect on the host animal. Horses show adverse reactions at concentrations of more than tenfold of the therapeutic dosing, when neurotoxic effects lead to depression of the central nervous system with somnolence, tremor, salivation, ataxia and hyper-excitability (Ungemach, 2010).

Two members of this class are commercially available for horses, Ivermectin (IVM), an avermectin, and Moxidectin (MOX), a milbemycin.

Since its introduction for the use against endoparasites in horses in 1981, IVM has become one of the most used broad spectrum anthelmintics in equine practice (Daugschies et al., 1995; Klei et al., 2001; Osterman Lind et al., 2005). It is quickly absorbed, and maximum blood concentration of 20-30ng are reached after 3-8 hours (Ungemach, 2010), the half-life is 4 days. It is very effective against luminal L4, pre-adults and adults, but not against mucosal larvae (Eysker et al., 1992). Since the patent period for IVM ended in 2002, generic products have been brought onto the market, leading to even more frequent use of this drug (Samson-Himmelstjerna et al., 2007).

MOX was introduced in the late 1990s and is also widely used (Nielsen et al., 2006b; Osterman Lind et al., 2007b). It is readily absorbed and distributed into all tissues; however, it is about 100 times more lipophilic than IVM and therefore has a higher affinity to fat tissue (Sangster, 1999). It is slowly released and has a long half-life of up to 28 days and good residual activity (Ungemach, 2010; reviewed by Cobb and Boeckh, 2009). Its efficacy on adult stages is backed up by the removal of mucosal L4 (Xiao et al., 1994; Bairden et al., 2001), which is probably due to its lipophilic character, as this leads to high concentrations of this drug in the mucosa (DiPietro et al., 1997).

Excretion of ML is through the faeces, and ecotoxicological effects have to be considered when administering these drugs, as insects and soil nematodes can be affected when they come in contact with dung containing the drug (Lumaret et al., 2012). Active compounds can be found in the faeces for up to eight days after the administration, but peak levels are reached after 24h (Gokbulut et al., 2001). By keeping horses stabled for a minimum of 24h after treatment, effects on the fauna can be minimized.

2.2.2.2. Tetrahydropyrimidines

Pyrantel (PYR) is the only member of the tetrahydropyrimidines commercially available for horses. Low solubility of the PYR embonate salt leads to low resorbtion, and consequently the toxicity after oral administration is very low, and it has a wide therapeutic window (Ungemach, 2010).

PYR exerts its action on luminal stages of most nematode parasites of the horse, with the exception of Strongyloides westeri, and is effective against cestodes only at elevated doses. Its effect on strongyles is limited to adult luminal stages, as it has no effect on their encysted or migratory larvae (Ungemach, 2010). Its mode of action is that of a nicotinic agonist at acetylcholine receptors for neuromuscular transmission, causing spastic paralysis of the nematodes (Martin, 1997). Subsequently, they lose their ability to attach to the intestinal wall, and are expulsed from the host.
The host escapes the cholinergic effect of PYR as bioavailability is very low, and adverse reactions in horses are usually only seen in the case of severely damaged intestinal mucosa, as in the case of intense helminthosis. Symptoms include tachypnoea, salivation, tremor and diarrhoea (Ungemach, 2010).

PYR was introduced to the market in the early 1970ies and is still widely used (Lloyd et al., 2000; Nielsen et al., 2006b; Osterman Lind et al., 2007b). In Canada and the USA, PYR is also used as a daily feed additive for low-dose preventive medication since the early 1990ies (Uhlinger, 1991; Lyons et al., 1999).

2.2.2.3. Treatment Schemes

Anthelmintic drugs are often applied by a traditional scheme of regular treatments, usually by horse owners or stable managers with minimal reference to veterinary advice. These treatments generally ignore the need for treatment or the character of the parasitic burden, should there be one (Soulsby, 2007). Generally, there are two main programmes followed by horse owners in Germany to be distinguished, either interval treatments approximately every 8 weeks, or calendar based treatments in spring and autumn, the later often in combination with a third treatment during the winter for *Gasterophilus* spp. and sometimes a fourth treatment in the summer if the horses appear “wormy”.

In Germany, horse owners can obtain anthelmintic drugs through the veterinarian only. However, in many countries such as the United Kingdom, Ireland and the USA, anthelmintics are readily available in pet shops and tack stores as well. Clearly, veterinary practitioners are

not often enough involved in the anthelmintic programme used on horse farms (Kaplan et al., 2004). They might at times also be willing to pass anthelmintics on to the horses owners without assessing the situation on the farm, as the mark-up on these drugs is considerable.

The frequent use of anthelmintic drugs has been identified as a risk factor associated with the development of resistance (Coles, 1999), as it increases the selection pressure. It is therefore desirable to reduce the frequency of the applications of these drugs and only use them when worm burdens are present.

In Denmark, a change in legislation made anthelmintics prescription-only drugs in 1999 (Nielsen et al., 2006b), prohibiting the routine prophylactic use. Nielsen et al. found a reduction in the frequency of treatments since the introduction of this new legislation. In this study, 97% of veterinary practices participating in a questionnaire stated that they use routine FEC, 11% perform FECRT (Nielsen et al., 2006b).

2.2.3. Biological Control

2.2.3.1. Nematophagous Fungi

The most successful research into the biological control of parasite nematodes has been with nematophagous fungi. The predacious micro fungus *Duddingtonia flagrans* can colonize faecal mounds, and sticky networks on its hyphae trap nematode larvae present in the faeces. The larvae then are destroyed. The spores survive the passage through the intestinal tract and subsequently germinate in the faeces. Larsen et al. have shown that effective reduction of the contamination of pasture is possible when resting spores of *D. flagrans* are fed to the horses on a daily basis (Larsen et al., 1996). The long-term viability of this method, however, has not yet been studied comprehensively with regards to ways of administration of the spores to animals kept on pasture, the manufacturing of the fungal material and the potential environmental impact (Larsen, 2000).

2.3. Resistance against Anthelmintic Drugs

2.3.1. Definition

Resistance per definition is present “when there is a greater frequency of individuals within a population able to tolerate doses of a compound than in a normal population of the same species and is heritable” (Prichard et al., 1980).
There is a natural genetic variation in a worm population, and some individuals of the population naturally have alleles of genes (R alleles) coding for low susceptibility to anthelmintic drugs (Dargatz et al., 2000). If the survivors of a treatment reproduce, their genes are passed on to the following generation and, subsequently, the frequency of these R alleles increases. If the use of the same drug or drug class is repeated, further selection favouring R alleles will occur, and eventually, enough worms in the population will survive the treatment to cause disease (Dargatz et al., 2000).

A reversion to susceptibility is in theory possible if the drug in question is discontinued and the resistant worms are less fit than the remaining part of the population. Fitness in this context includes all attributes positively contributing to the completion of the life cycle, such as survival in the environment, infectivity and persistence in the host (Coles, 2005). However, if the drug to which resistance existed is reintroduced, individuals carrying R alleles will have a distinct advantage and therefore, the proportion of resistant worms will quickly increase (Kaplan, 2004).

Resistance can begin on a farm due to selection, or resistant worms can be imported in horses that join the herd from outside. To avoid the introduction of resistant worms, the treatment of all new animals with a drug or a drug combination that is known to be effective is recommended (Coles, 2003; Eysker et al., 2006). They should be let out onto pasture only after the efficacy of treatment has been determined (Eysker et al., 2006). However, the movement of horses could also help to spread susceptible CYA. It therefore can be very important to know the resistance status of the farms in question if moving animals (Coles, 2005).

2.3.2. Diagnostic Methods

2.3.2.1. Field Studies: Faecal Egg Count Reduction Test

2.3.2.1.1. Method

The faecal egg count reduction test (FECRT) is currently the most common method for the detection of anthelmintic resistance in CYA populations in the field (Coles et al., 2006; Kaplan, 2002). It is the method recommended by the WAAVP guidelines (Coles et al., 1992) and consists in counting the worm eggs present in faecal samples of naturally infected animals shortly before and 7-14 days after treatment. The quantitative McMaster technique is used to count the eggs present in the faecal samples (see 3.4. for the description of a modified McMaster technique as used in this study).

The FECRT provides an estimation of the efficacy of the anthelmintic drug used in the test. It is cost-effective, apart from the labour involved, as it does not entail complex technology. In fact, the parasitological procedures required can be performed in any veterinary consultancy with a small laboratory. As there is no need to take animals out of their environment, the FECRT is very little invasive to the animals and does not interfere with the population in question.
However, there are several limitations to this technique, as outlined in 2.1.4., that are innate to quantitative FECs. Also, it can be difficult to find sufficient numbers of horses on the farms in question (Pook et al., 2002), as a minimum of 6 horses each for treatment and a control group are required (Coles et al., 2006; Herd and Coles, 1995). To perform a FECRT in the field, a prolonged period of time is required (at least 7- 14 days), and it therefore does not allow instant results as might be wished for e. g. in the case of an outbreak of acute disease, that might suggest insufficient efficacy of the drugs previously used, such as larval cyathostominosis or colic symptoms.
Another problem of the FECRT is its low sensitivity. According to Martin et al., at least 25% of the population of worms need to be resistant for resistance to be detected (Martin et al., 1989).

Egg Reappearance Period

The time between treatment with an anthelmintic drug and the first time for eggs to be detected in the faeces is known as egg reappearance period (ERP). Some authors do not count the appearance of the first eggs, but rather give a certain threshold, either a set EPG (e.g., 200) or when the FEC reaches 20% of the initial value (Tarigo-Martinie et al., 2001). It has been

suggested that a shortening of the ERP can be an indicator for a loss in efficacy (Sangster, 1999), even before FECR would be compromised. Eysker et al. highlight the importance of performing a faecal egg count reduction test (FECRT; see 2.3.3.1.) once a shortened ERP has been detected (Eysker et al., 2006). In a study conducted by Slocombe and Cote (1984) it took six weeks after the administration of IVM until eggs were detected in the faeces.

2.3.2.1.2. Calculation

In the literature, there is no common denominator for the calculation of FECR. However, it has been demonstrated that the outcome of FECRTs can depend on the mathematical technique used for analysing the data (Craven et al., 1998; Togerson et al., 2005). Several approaches to encounter this problem exist, nonetheless, with or without the inclusion of control groups. In addition to the calculation, there is further discussion about the interpretation of the outcome of FECRTs, and different approaches exist to when the line should be drawn to regard a reduction in drug efficacy as "resistance" (see below).

One of the difficulties met when performing FECRTs is that the size of the herd not always allows for the inclusion of a control group. Hence, some investigators calculate their FECRTs without control groups, while the WAAVP include control groups into their recommendation (Coles et al., 1992).

The second basic question is if to use arithmetic means (AM) or geometric means (GM). The GM approximates the median in the case of an asymmetric distribution and hence is an estimate of the FEC of the "average" animal in the herd. The AM on the contrary is proportional to the total egg count of the group of animals studied.
Therefore, Dash et al. suggest the use of AM in FECRTs, as in these tests it is important to observe the FECR of the group of animals rather than that of the "average" animal. According to Dash et al., the outcome of the GM is generally lower than the AM and consequently underestimates the total egg output (Dash et al., 1988). These authors also show that this difference can be of little importance when anthelmintic efficacy is either very low or very high, but gains importance with FECR of less extreme values (Dash et al., 1988).

Kochapakdee also found that the egg count reduction calculated with GM is generally higher than when AM of the same data are used (Kochapakdee et al., 1995). This might hinder the early detection of AR.

The WAAVP recommends comparing post-treatment AM of the egg counts with the AM of a control group; the AM to calculate FECR is preferred for its ease of calculation, and its more

accurate estimation of egg output (Coles et al., 1992). These authors also consider the AM as the more conservative measure of anthelmintic efficacy. Presidente on the other hand suggested a method of calculating FECR including a control group and taking post- and pre-treatment GM of both groups into account; the cut-off point for resistance is set at 90% (Presidente, 1985).

Lyndal-Murphy et al. state that it is important to include the 95% upper confidence limit (UCL) when classifying FECRT results, and that the presence of resistance can only be confirmed if the UCL is also <95% (Lyndal-Murphy et al., 2014).

2.3.2.1.3. Interpretation

The third challenge for the calculation of FECRTs is that cut-off points for establishing resistance have not yet been standardized (Kaplan, 2002), and hence results obtained in different studies cannot always be compared directly.
The current recommendations of the WAAVP advise to assume the presence of resistance if firstly, the FECR is <95% and secondly, the 95% lower confidence level (LCL) is <90%. This accounts for BZMs and IVM in sheep and goats; the cut-off point is set at FECR <90% for BZMs in horses, and no LCL value is given (Coles et al., 1992).

According to Pook et al., a number of disadvantages are involved in these recommendations, as the efficacy limit suggested by the WAAVP is extrapolated from trials in sheep, whilst ovine and equine populations are not comparable; and they consider the 90% efficacy limit in horses as an arbitrary value that does not appear to have a statistical explanation. Consequently, a different approach is suggested, where for PYR resistance is considered when the FECR is <90% and the LCL <80% (Pook et al., 2002), a value that has been followed by other authors (Osterman Lind et al., 2007a).

Tarigo-Martinie et al. interpreted treatment with fenbendazole, PYR and IVM as effective if FECR was >90%, as equivocal if it was between 80 and 90%, and as ineffective if it was <80% (Tarigo-Martinie et al., 2001). Kaplan used the same cut-offs in FECRTs performed with fenbendazole, oxibendazole, PYR and IVM; this author opted for the more conservative cut-off of 80% in order to minimize the chance of overestimating the prevalence of resistance (Kaplan, 2004).

Craven et al. compared 5 different methods for calculating FECR, and found the method recommended by the WAAVP for the detection of resistance in sheep as being the most

sensitive method (Craven et al., 1998). A FECR of < 95% after treatment with PYR was seen as indicative for resistance against this drug (Craven et al., 1999).

Dargatz et al. suggested cut-off values of 90% FECR for PYR and 95% for ML and BZ. They also recommend transforming the individual FEC data by angular transformation before calculating group means in order to approximate a normal distribution (Dargatz et al., 2000).

The efficacy initially determined for PYR by Lyons et al. was 92% (Lyons et al., 1974); efficacy determined by Bauer et al. in 1986 was 99% (Bauer et al., 1986).

The efficacy originally determined for a drug class when used against a susceptible population of CYA should be considered when establishing cut off levels for resistance, so that resistance is suspected when the efficacy is actually considerably less than when the drug was first introduced to the market (Kaplan, 2002, Pook et al., 2002). The threshold at which resistance is declared has direct consequences on the prevalence at which AR is reported (Kaplan, 2002), and it is therefore important to identify levels for the declaration of resistance for the different drug classes (Coles et al., 2006).

A further complication to the discussion is that FECRT data usually do not follow a Gaussian distribution and confidence intervals cannot be easily calculated. The numbers of horses available on the farms are generally low, and the variability in the data can be large. This variability of FECRT data, including frequent zero observations, composes a challenge for statistical application, and some authors have applied different statistical methods, such as bootstrap analysis (Vidyashankar et al., 2007; Traversa et al., 2012). This method is based on frequent re-sampling and can be applied when the distribution of the underlying data is unknown (Denwood et al., 2010).

Denwood et al. compared three different methods for calculating FECRT data, firstly the method as recommended by the WAAVP, secondly, a bootstrapping method, and thirdly a Markov Chain Monte Carlo method (MCMC) (Denwood et al., 2009). The authors favoured the MCMC, because it consistently generated more accurate, albeit larger 95% confidence intervals.

In this model, pre-treatment data are assumed to follow a Gamma-Poisson distribution, and post-treatment data are distributed as a different Gamma-Poisson distribution (mean value scaled relative to the pre-treatment mean, and variability scaled relatively to the pre-treatment variability) (Denwood et al., 2010). That way, inference can be made on the true change in mean egg-shedding and the variability between egg counts. The different causes of variability can be considered, thus leading to a higher accuracy in predicting parameter estimates (Denwood et al., 2009). In the study performed by Denwood et al., confidence intervals

provided by the MCMC method were slightly larger, but more accurate when compared to two other methods that require less computational effort, one being the method recommended by the WAAVP (Denwood et al., 2009).

The MCMC method facilitates the calculation of more accurate and not merely larger confidence intervals. By using a different method, which generates smaller confidence intervals, data might be subjected to too much inference, because the high numbers of zero values complicate the correct analysis. A disadvantage of this method is the high computational effort involved.

2.3.2.2. Experimental Studies

2.3.2.2.1. In-vivo-Techniques

There are two tests for the evaluation of anthelmintic efficacy, the critical and the controlled test. The critical test is primary method for the evaluation of the efficacy of anthelmintic drugs (Drudge and Lyons, 1977). Naturally or experimentally infected horses are stabled individually and treated with an anthelmintic drug. Faeces are then collected and examined for the presence of parasites until the day of necropsy, when the number of remaining parasites is established. Each horse serves as its own control, as the number of expelled parasites is compared with the total number.
For controlled tests, also called dose-and-slaughter trials, naturally or experimentally infected animals are allocated into treatment and control groups and then killed at a given time after anthelmintic treatment in order to establish the number and identity of the remaining parasites in the gastro-intestinal tract through necropsy.
These studies are rarely used in horses, and only in experimental environments, as they are cost intensive and cannot be implemented in equine populations in the field.

2.3.2.2.2. In-vitro-Techniques

There are several in vitro techniques being used to demonstrate anthelmintic resistance in animals, such as larval motility assays, egg hatch assays (EHA) and larval development assays (LDA).
The latter is frequently used for sheep parasites, and efforts have been made to establish the LDA in horses, however with varying outcomes. In this assay, larvae are incubated with

increasing concentrations of anthelmintics, and the LC50 is established. LC50 is the concentration at which 50% of larvae are inhibited in their development. Meier and Hertzberg found results to be satisfactorily consistent between a LDA and FECRT performed on 33 premises (Meier and Hertzberg, 2005b). However, Craven et al. compared the results of FECRT, EHA and LDA and found correlations to be poor (Craven et al, 1999). Pook et al. suggest that, once refined, the LDA has potential to detect resistance in CYA of horses (Pook et al., 2002); whereas other authors deduce that the LDA is not sufficiently reliable to be used as a general diagnostic means (Tandon and Kaplan, 2004; Osterman Lind et al., 2005; Osterman Lind et al., 2007a).

The EHA is widely used for the in vitro testing of BZ resistance in nematodes of horses (Kelly et al., 1981; Ihler, 1995). It consists in incubating nematode eggs at serial concentrations of the anthelmintic in order to establish the LD50 concentration, at which 50% of the eggs are killed.
An advantage of both LDA and EHA over the FECRT is that they do not interfere with the de-worming scheme on the horse farms involved. However, they require more laboratory effort and are more cost-intensive than the FECRT.

2.3.2.3. Biomolecular Tests

BZ resistance in ruminants is associated with a mutation at codon 200 of the β-tubulin gene (Elard et al., 1999). A PCR has been developed for the detection of resistance to BZ. However, it is suspected that more than one factor contributes to the development of resistance (Coles, 2005; Pape, 2001), and Matthews et al. suggested that the mechanisms of drug resistance in CYA may be more complex (Matthews et al., 2004). Therefore, molecular tests based on the use of a single mutation potentially hold the risk of false results if resistance is induced by more than one mutation (Coles, 2005).

As the molecular mechanisms of resistance to both tetrahydropyrimidines and the ML are not known, no PCR based tests can be expected to be developed in the near future. Nevertheless, it is desirable to avail of such tests in the future, as they would be able to identify genotypic changes before therapeutic failure can detect phenotypic resistance (Kaplan, 2002).

2.3.3. Occurrence

For more than four decades, anthelmintic resistance of CYA has been acknowledged. The first drug commercially used against CYA was phenotiazine, introduced in the 1940s and broadly used for about 20 years. Resistance arouse in Great Britain and the USA by the 1960s (reviewed by Meier and Hertzberg, 2005a).
Thiabendazole was the first drug of the group of BZs and was widely used due to its low toxicity and good efficacy as a broad-spectrum anthelmintic. The first reports for thiabendazole resistance date from 1965, only a few years after its introduction to the market in 1962 (Drudge and Lyons, 1965; reviewed by Kaplan, 2004).
For the group of BZs, resistance has now been reported throughout Europe (Germany, Bauer et al., 1986; Norway, Ihler, 1995; Denmark, Craven et al., 1998; Switzerland, Meier and Hertzberg, 2005a; Sweden, Osterman Lind et al., 2007a; Italy, Traversa et al., 2007b) and is wide spread throughout the world. There is side resistance between the different pro-BZs and BZs, with the only exception of oxibendazole, which can still be effective against otherwise BZ-resistant helminths (Lyons et al., 1994). However, if oxibendazole is used on a farm with BZ-resistant CYA, resistance to oxibendazole develops quickly (Uhlinger and Kristula, 1992).

PYR has been used against internal parasites in horses since 1974; the first finding of resistance was in 1996 in the USA (Chapman et al., 1996), and it has been found in many countries worldwide since, including Brazil (Molento et al., 2008). In Europe, resistance against PYR has been reported from various countries: Norway (Ihler, 1995), Denmark (Craven et al., 1998), Italy (Traversa et al., 2007b), Finland (Näreaho et al., 2011), France (Traversa et al., 2012).
On some farms in Canada and the USA since the 1990ies, PYR is fed as a preventive measure at low doses (2.64mg/kg body weight) on a daily basis. This might have led to the early establishment of resistance against this drug (Kaplan et al., 2004; Tarigo-Martinie et al., 2001).

The sole drug class that until recently had maintained full efficacy against small strongyles is the ML group. Klei et al. (Klei et al. 2001) assessed IVM efficacy in two trials performed in Louisiana, USA, and Bavaria, Germany, in 2001 and confirmed high efficacy (>99% reduction) of IVM against CYA. Wirtherle found 100% efficacy of IVM on all ten farms (77 horses) in the German Federal State of Lower Saxony (Wirtherle, 2003).

Nevertheless, even this group may be under threat, as studies from the German Federal State of North Rhine Westphalia (Samson-Himmelstjerna et al., 2007) and Kentucky (USA) (Lyons et al., 2008) show a reduced egg-reappearance period following treatment with IVM. Indication of developing resistance of strongyles to IVM has been seen in Finland (Näreaho et al., 2011). Molento et al. found inadequate efficacy of IVM and MOX on CYA in horses in

Brazil (Molento et al., 2008), and one case of IVM resistance in a single horse in Australia has been reported (Edward and Hoffmann, 2008). IVM and MOX, respectively, showed reduced efficacy in two different individual horses on two different farms in France (Traversa et al., 2012).
Lyons et al. also reported a reduced egg-reappearance period after treatment with MOX in the same herd in Kentucky (Lyons et al., 2008) and suspected a decreased activity on encysted larvae (Lyons et al., 2010). Suspected resistance of CYA to MOX was also found in donkeys in the United Kingdom (Trawford et al., 2005).

Strains of *P. equorum* resistant to IVM have first been found in the Netherlands (Boersema et al., 2002), and since then in several other countries, including Kentucky, USA (Lyons et al., 2006), Germany (Samson-Himmelstjerna, 2007), Brazil (Molento et al., 2008) and Finland (Näreaho et al., 2011).

2.4. Sustainable Approach to Endoparasite Control

2.4.1. Refugia

Establishing and maintaining refugia can be a means of counter-acting the development of resistance (van Wyk, 2001). Helminths in refugia are not exposed to anthelmintic drugs and therefore no selection on susceptibility occurs in this part of the population, which is primarily composed of those helminths living on pasture and not within the host at the time of treatment. Larvae encysted in the intestinal wall are also considered as being in refugia, as they are not subjected to the effects of the drugs administered. This, however, does not apply to anthelmintics with effect on these larvae, as is the case for MOX and multiple doses of fenbendazole on consequent days.

The size of the population in refugia influences the degree of selection for resistance (van Wyk, 2001). With the exception of MOX, single-dose anthelmintics are not active against encysted L3, these are consequently in refugia. According to Coles, this could explain why resistance is (still) quite low (Coles, 2005).

It has in the past been suggested to move animals onto “safe”, worm-free pasture after treatment with anthelmintics (the so-called drench-and-move system) in order to keep re-infection at a minimum (van Wyk, 1990). However, the weakness of this approach which does not “give susceptible worms the chance to reproduce” (van Wyk, 2001), as only resistant specimen will be shed onto the new pasture and hence be ingested by the host, is now widely recognized (van Wyk, 2001; Coles, 2003); it is in contrast suggested to either not treat the entire herd before moving them to a “safe” pasture, or indeed to leave them on the contaminated pasture for some time after the treatment. This way, re-infection of the treated animals with unselected worms can be obtained. Molento et al. propose to treat the animals after moving them onto pasture that contains little or no worms in refugia (Molento et al., 2004), as these animals will then shed unselected larvae onto the grass before being treated, thus allowing for re-infection with presumably susceptible worms.
The implementation of these approaches, however, may be difficult, as farmers and animal owners wish for as much a worm-free environment as possible and might not understand the need of re-infection with susceptible worms.

2.4.2. Alternation of Anthelmintic Drugs

It is common practice among horse owners and stable managers to rotate anthelmintic drugs in order to gain a broader spectrum of effectiveness, and to avoid AR. A distinction has been made between “slow” (change of drug class every year or even less often) and “fast” (change of drug class every treatment) rotation.

In a study performed by Uhlinger and Kristula, the most commonly practised fast rotation did not slow down the development of AR against BZ (Uhlinger and Kristula, 1992).
Van Wyk even suggests not to alternate anthelmintics at all, unless FECRT are done on a regular basis in order to monitor the development of resistance, as the rotation with effective drugs could disguise the impact of resistance (van Wyk, 2001). Most recommendations however aim at “slow” rotation, i.e. to use a drug for an entire year before a different drug class is used again for one year and so on (Coles and Roush, 1992; Herd, 1993; Kelly et al., 1981; Prichard et al., 1980). A problem with this method is the treatment of *Gasterophilus* spp. (Meier and Hertzberg, 2005a) which may require the use of a different drug, as only ML have an effect on larvae of *Gasterophilus* spp. Kaplan et al. recommend to choose the drugs individually, considering the efficacy against different parasites and the time of year (Kaplan et al., 2004).

2.4.3. Selective Treatment

A modern tactic to control parasitic infestation is to consider the worm burden in the herd and/or the individual instead of the indiscriminate application of anthelmintic drugs. This approach combines the detection of FEC with the targeted use of anthelmintic drugs.

In a herd of horses, worm burdens are distributed unevenly, and the majority of worms is carried by the minority of hosts (Kaplan et al., 2004; Relf et al., 2013). This implies that the majority of hosts are able to control their burdens and are not in need of anthelmintic medication (Soulsby, 2007). Egg shedding rates in fact can differ largely between individuals within an equine population, depending on age, genetic disposition and access to pasture (Döpfer et al., 2004; Gruner et al., 2002; Krecek et al., 1994). Thus, “low egg shedders“ can be identified and can be excluded from drug treatment when a selective anthelmintic treatment system is applied (Döpfer et al., 2004). In addition, the frequency of routine faecal egg counts in mature horses that prove to have consistently low egg counts can be reduced long-term (Eysker et al., 2006).

The concept that all members of a population should be treated with anthelmintics probably weakens the immune status of the host population to its parasites (Soulsby, 2007). By not treating all animals in the herd, refugia are created, thus decreasing the selection pressure for the parasites (Duncan and Love, 1991; Kaplan et al., 2004). Leading parasitologists suggested that the limit for anthelmintic treatment should be at 100-300 EPG for the herd mean in adult horses (Uhlinger, 1993).

Krecek et al. found a financial advantage in a programme that included worm counts and only implemented anthelmintic drugs when individual egg counts were ≥300 EPG, compared to a system that de-wormed four times a year regardless of the egg counts (Krecek et al., 1994). Little et al. implemented a selective treatment programme on a farm with CYA resistant to both BZ and PYR. Although frequent FECs and FECRTs were initially necessary on this farm, the authors still found a 25% decline in costs in mature horses, and a 10% overall cost reduction (Little et al., 2003).

One of the problems of targeted treatment is that CYA are more pathogenic before they become patent. Another aspect is the fact that horses with a very high worm burden might still have very low egg counts, as the amount of eggs shed is not necessarily representative of the real worm burden, and furthermore, FECs are merely indicative of the actual number of eggs present in the faeces (see 2.1.4. for details on FEC). Samson-Himmelstjerna et al. suggest that a selective treatment programme should be tailored towards each individual horse farm, respecting local factors such as climate, animal density on the pasture, pasture management, age and group composition of the herds etc., and should include yearly FECRTs for the drugs used (Samson-Himmelstjerna et al., 2011). The authors also recommend, for practical means and to put an economically viable system in place, to routinely perform FECs with pooled samples and worm the entire herd if any egg shedding is detected in the FEC (Samson-Himmelstjerna et al., 2011). This approach includes the use of FECs and relatively low numbers of treatments of the entire herd throughout the year, combined with FECRT if deemed necessary, i.e. if FEC continue to be high. Nonetheless, the skewed distribution of egg shedding within a population demands the identification of the high egg-shedders in order to minimize pasture contamination.

Therefore, the regular monitoring of anthelmintic efficacy through FECRTs should be incorporated into the management programme of all horse farms (Kelly et al., 1981; Tarigo-Martinie et al., 2001). These programmes need veterinary expertise and supervision to be implemented in the field. Stratford et al. emphasise the importance of the veterinarian as the key holder to preserve anthelmintic efficacy (Stratford et al., 2011).

3. Materials and Methods

3.1. Study Design – An Overview

A prevalence study performed on 126 equine farms in the Federal State of Brandenburg in 2006 yielded 75 premises presenting a higher FEC for CYA than the average (Hinney et al., 2011). The objective of the present study was the further investigation of a part of these premises regarding AR. Following the hypothesis that frequent use of anthelmintic drugs enhances the development of resistance, farms that frequently de-wormed were to be compared with farms that rarely de-wormed.

The pre-requisites for participation in the present study included:

- The size of the horse population: A minimum of 20 horses was required in total, of which at least eight horses showing an EPG of 150 or higher had to be available for the study
- Horses with access to grassland, either continuously or at least for two hours every day
- Confirmation that the horses had not been treated with anthelmintics for at least eight weeks prior to testing
- Consent from the owners for the repeated equine rectal sampling and the administration of the anthelmintic drug by the author

Based on these criteria, 24 farms were selected (Fig. 1), out of which seven farms de-wormed 4 times per year or more often, and the remaining 7 farms de-wormed two times per year or less often (Hinney, 2011).

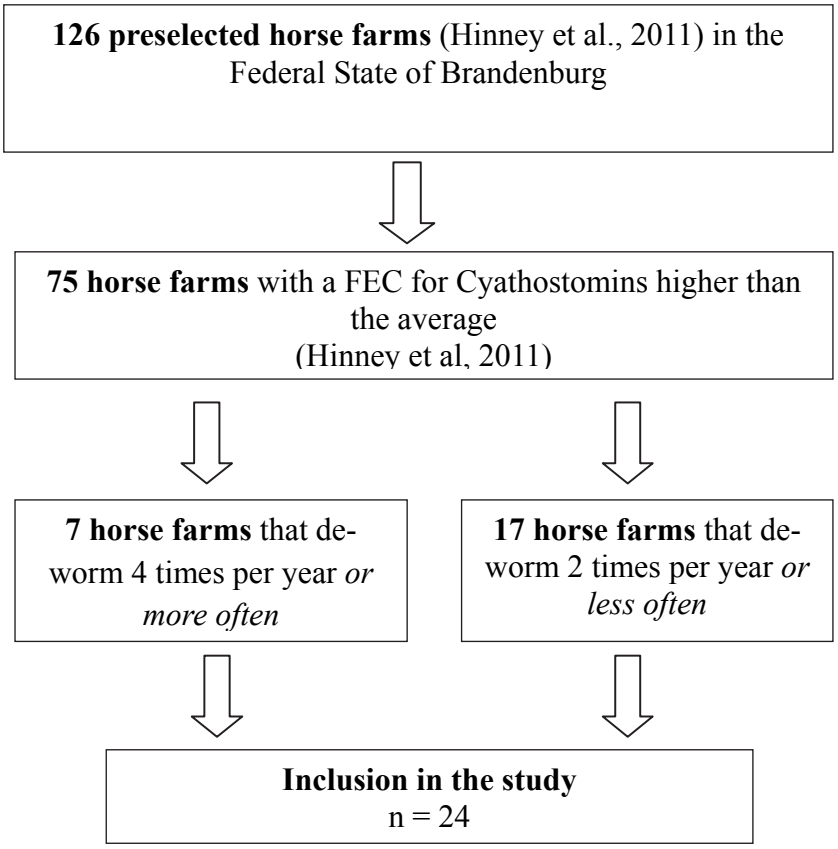

Figure 1: Selection of horse farms for the study on anthelmintic resistance in the Federal State of Brandenburg, Germany, 2007/2008

In 2007, a FECRT was performed on 23 of these 24 equine premises. On the remaining farm, anthelmintic drugs had been administered within eight weeks prior to sampling, and it could not be included in the study. The active drug substance, Ivermectin, was administered using the product Eqvalan Duo® (Merial, Hallbergmoos, Germany). Eqvalan Duo® contains both IVM and Praziquantel. As Praziquantel only acts on cestodes, the presence of this compound had no influence on the CYA.

In 2008, a FECRT was performed on 21 of these 24 equine premises. This included the farm that had not participated in 2007. Two of the premises included in the previous year could not participate due to recent administration of anthelmintic drugs to the animals. In one yard, the owner did not wish to participate again without giving a specific reason. The active drug substance, Pyrantel embonate, was administered using the product Banminth® (Pfizer, Berlin, Germany). On one farm (N° 2), the FECRT was repeated ten weeks after the first treatment in order to validate the results previously obtained.

Size and purpose of the equine premises varied broadly, including large scale stud farms, riding schools and livery yards, but mostly a blend of all three types. Also, the time that the individual horses spent on grassland differed largely. A few horses spent two hours daily in the field and were stabled the rest of the time, some were turned out during the day and stabled in the night, and others lived in the field day and night.

The FECRT was initiated with the first sampling on day 0. Horses allocated to the treatment group were de-wormed with the respective anthelmintic drug on the following day (day 1). Faecal samples of the horses in the treatment group and the control group were again taken on day 14 to determine the FECR. In 2007, the yards were again visited on day 42 to establish the ERP for IVM. On these occasions, only the horses included in the treatment group were sampled.

3.2. Selection of the Animals on the respective Premises

On the first visit, all available horses or up to 60 horses were sampled to determine the FEC. In the rare case of an individual owner not consenting to the rectal sampling and/or worming of their horse, that particular horse was not considered. Horses that showed a FEC of 150 EPG or more were included in the study. The minimum number of horses included per farm was 8 and the maximum 40 horses. If there were more than 40 horses that fulfilled the criteria, 40 were selected with the aid of random numbers. Half of the thus obtained animals were allocated into the treatment group, and the other half into the control group. This allocation was again done with the aid of random numbers, with the exception of pregnant mares, which were allocated into the control group. In the case of an uneven number, the treatment group would receive one more animal than the control group. Horses of all ages and sexes were included equally, except young foals (younger than 6 months), which were not included in the study.

3.3. Diagnosis of Egg Shedding

The faecal samples were taken rectally, using arm length plastic gloves and lubricant. When fresh, warm droppings were available in a horse's stable and could be designated to the specific animal, a sample was taken in lieu of a rectal sample. The sample was then removed from the top of the pile to avoid contamination. The samples were protected in a plastic glove which was labelled and stored in a refrigerator box. Great care was taken not to freeze or overheat the samples on the way back to the laboratory. Once in the laboratory, they were

stored at 4°C and processed as soon as possible, mostly immediately, and latest within 24 hours.
Each sample was manually crumbled, and 4g were weighed out on digital scales.
These 4g were transferred into a labelled plastic, single use Petri dish where they were dispersed in a saturated saline solution. This solution had previously been obtained by dissolving 360g of Sodium Chloride per litre of water, density being verified with a densimeter. The mixture was then sifted through a tea strainer. Saline was added to make 60ml of the suspension.
This suspension was transferred into capped bottles and, with a pipette, a part of it was extracted four times, in order to fill two McMaster slides (McMaster, Chalex, Wallowa, USA). Each time, the bottle was previously shaken five times immediately before the pipette was inserted. Thus, the contents were not allowed to settle prior to extraction. Once filled, the McMaster slides were allowed to settle for 10min, and accordingly the Strongyle eggs in all four chambers were counted, including the eggs resting on the outside lines of the grids. The eggs found in all four chambers were summed up and multiplied by the factor 25 to yield the EPG value for each sample.
The EPG sensitivity is 25 EPG, as the volume of each chamber is 0.15ml and four chambers are counted (4×0.15ml=0.6ml). The dilution factor (60/4=15) is divided by this volume (15/0.6=25). The lowest detection level for egg shedding was thus an FEC of 25 EPG.

3.4. Treatment

Each of the horse farms was visited again on the day after the first sampling. Horses allocated into the treatment groups were then treated with the respective drug according to the manufacturers' recommendations. IVM in 2007 was administered per os with 0.2 mg per kg bodyweight and PYR in 2008 with 6.6 mg per kg bodyweight.
The body weights of the horses were independently estimated by the author and the owner or stable manager, and the mean was calculated. Also, a commercially available girth measuring tape ("weighband") was used. The tape was put around each horse's body right behind the withers, and an estimation of the horse's weight was read off. When large discrepancies occurred, the highest estimated weight was employed for dosing the animal. In addition, a body condition scoring system was applied to every horse, ranging from 1 (cachectic) to 9 (severely obese) (Kienzle, 2006).

3.5. Larval Cultures and Morphologic Differentiation

In 2008, pooled larval cultures for each horse farm were obtained from the faecal material taken on the day before the administration of PYR for two different reasons: On one hand, the overall prevalence of large strongyles was to be assessed by differentiating the third stage larvae.

The second purpose was, where applicable, to determine any changes in the composition of the sample pre and post treatment: From samples showing presence of strongyle eggs on day 14, larval cultures were obtained, and the species of CYA present on day 14 were compared with the original pooled sample taken on day 0.

At least ten grams per horse were taken to prepare a pooled sample of the corresponding farm. In the case of two particular horses which showed an unusually high FEC on day 14 post treatment, individual larval cultures were obtained using the faecal material taken on that day.

The sample was mixed with vermiculite (Rajapack, Birkenfeld, Germany) to make a crumbly mixture and filled into 1l glass jars. Water was added to provide moisture, and the jars were covered, but not sealed, and incubated at 26°C for 7 days. Following the incubation period, the jars were topped up with water to the rim and turned over onto large Petri dishes which then also were filled with water. 10-14 hour later, the liquid from the margin of each Petri dish was pipetted off and filled into a centrifuge tube. Centrifugation was performed at 2000 rpm for 20 min; the sediment was pipetted off and transferred onto microscopic slides.
For the morphologic differentiation, the larvae were immobilised with direct heat, and the number of intestinal cells of 100 larvae per sample was counted to differentiate the third stage larvae of CYA from those of large strongyles.
Where further species differentiation was desired, the obtained larvae were kept refrigerated and then stored in alcohol for dispatching. The genetic differentiation was performed via Reverse Line Blot hybridization (RLB) by and courtesy of Dr. Donato Traversa, University of Teramo, Italy. Traversa et al. developed a RLB hybridization assay with which the most common large and small strongyles can be identified simultaneously (Traversa et al., 2007).

3.6. Statistical Methods

For the FECRT with IVM, the method recommended by the WAAVP (Coles et al., 1992) for FECRT in ruminants, pigs and horses was used for calculating the results. Only this method was employed, as the outcomes of all FECRTs were unequivocally 100% FECR or very close to 100% FECR.

The outcome of the FECRT with PYR was calculated with three different methods, as the results varied between 65.3% and 100% FECR. The aim of using more than one method was to evaluate the influence of the statistical method on the results. The first method was again the procedure recommended by the WAAVP. Additionally, a Bootstrapping method was applied to the data. This approach was chosen as Bootstrapping is a method based on frequent re-sampling in order to evaluate confidence intervals on non Gaussian distributions, like FECRT data. The "BootStreat" computer programme calculates the mean efficacy of treatments with 95% confidence intervals (Cabaret and Antoine, BootStreat, 2008).

Denwood et al. compared three different methods of calculating FECR, demonstrating the differences in the outcome of FECRTs depending on the statistical method applied to the data. The authors favoured the MCMC method (Denwood et al., 2009). Considering these results, the data were also subjected to the MCMC method as used by Denwood et al.

3.6.1. Method 1 – as recommended by the WAAVP

This method is recommended by the World Association for the Advancement of Veterinary Parasitology (WAAVP, Coles et al., 1992). The following equation is used to calculate FECR:

$$FECR\% = 100 \times \frac{AM\ post-treatment\ EPG}{AM\ post-treatment\ control\ EPG}$$

Arithmetic means (AM) are used, and the EPG values of a control group are considered. The AM is directly proportional to the total egg count of the group of animals, as opposed to the geometric mean (GM), which provides a better estimate of the FEC of the "average" animal in the herd. By considering the FEC of a control group, changes in the EPG caused by factors other than the administration of the anthelmintic drug are being accounted for. Pre-treatment EPG are not considered in this equation.

The 95% confidence intervals are calculated as follows:
Upper confidence limit:

$$100 \times \left(1 - \left(\frac{AM\ post - treatment}{AM\ pre - treatment}\right) \times \exp\left(-2.048 \times \sqrt{\sigma^2 reduction}\right)\right)$$

Lower confidence limit:

$$100 \times \left(1 - \left(\frac{AM\ post - treatment}{AM\ pre - treatment}\right) \times exp\left(+2.048 \times \sqrt{\sigma^2 reduction}\right)\right)$$

Where $\sigma^2 reduction$ is the variance of reduction:

$$\sigma^2 reduction = \frac{\sigma_2^2}{N \times (AM\ post - treatment\ FEC)^2} + \frac{\sigma_1^2}{N \times (AM\ pre - treatment\ FEC)^2}$$

Where σ_1^2 is the variance in FEC pre-treatment, σ_2^2 is the variance in FEC post-treatment and *N* is the number of animals (according to Coles et al., 1992, modified according to Denwood et al., 2009).

3.6.2. Method 2 - Bootstrapping

In addition to the calculation as described above, a Bootstrapping method was employed. Bootstrap is a method to evaluate confidence intervals on non Gaussian distributions, like FECRT data. The "BootStreat" computer programme developed by Antoine and Cabaret calculates the mean efficiency of treatments; with 95% confidence intervals based on 2000 times re-sampling bootstraps (Cabaret and Antoine, BootStreat, 2008).

Four different equations were employed with the "BootStreat" programme:

Equation 1

In order to compare the outcome of two different calculations based on the same equation, the equation described as Method 1 was used again for the BootStreat programme:

$$FECR\% = 100 \times \frac{AM\ post - treatment\ EPG}{AM\ post - treatment\ control\ EPG}$$

Equation 2

Equation 2 employs arithmetic means and no control groups (Kochapakdee, 1995):

$$FECR\% = 100 \times \frac{AM\ pre\ treatment\ -\ AM\ post-treatment\ EPG}{AM\ pre-treatment\ EPG}$$

Thus, the post-treatment FEC values are compared with the pre-treatment values of the same horses (but taken 14d earlier), and environmental factors that may contribute to a reduction in EPG counts are not considered.

Equation 3

This equation, described by Dash et al. (Dash et al., 1988) takes into consideration potential changes in the control group egg counts pre and post treatment. It also employs the arithmetic means:

$$FECR\% = 100 \times \left[1 - \frac{AM\ post-treatment\ EPG}{AM\ pre-treatment\ EPG} \times \frac{AM\ pre-treatment\ control\ EPG}{AM\ post-treatment\ control\ EPG}\right]$$

Dash et al. suggest that the AM should be used when calculating FECR, as the egg count reduction of the herd is more important than that of the "average" animal (Dash et al., 1988).

Equation 4

Equation 4 (Presidente, 1985), is identical with equation 3, except the fact that it uses the geometric mean (GM) to reduce the impact of extreme values of individuals. The GM approximates the median and is an estimate of the FEC of the "average" animal in the herd. Egg count reduction calculated with GM is generally higher than when calculated with AM.

$$FECR\% = 100\ \times \left[1\ - \frac{GM\ post-treatment\ EPG}{GM\ pre-treatment\ EPG}\ \times \frac{GM\ pre-treatment\ control\ EPG}{GM\ post-treatment\ control\ EPG}\right]$$

3.6.3. Method 3 - Markov Chain Monte Carlo

A Bayesian Markov Chain Monte Carlo method (MCMC) suggested by Denwood et al. to calculate FECRT data was employed. In this model, pre-treatment data are assumed to follow a Gamma-Poisson distribution, and post-treatment data are distributed as a different Gamma-Poisson distribution (mean value scaled relative to the pre-treatment mean, and variability

scaled relatively to the pre-treatment variability) (Denwood et al., 2009). This method is based on frequent re-sampling and can be applied when the distribution of the underlying data is unknown (see 2.3.2.1.3. for a description of the MCMC method).

The procedures for this method were performed by and courtesy of Dr. Matthew Denwood, Boyd Orr Centre for Population and Ecosystem Health, Institute of Comparative Medicine, Faculty of Veterinary Medicine, University of Glasgow, UK.

3.6.4. Definition of Anthelmintic Resistance

Using each of these sets of results as calculated above, the FECR was classified using two different sets of criteria.
For criteria 1, the reductions were classified as 'Susceptible', 'Suspect resistant' and 'Confirmed resistant' using the observed FECR according to the WAAVP recommendations for BZ in horses (Coles et al., 1992). These recommendations propose a value of <90% as indicative of resistance in horses but give no value for the 95% LCL. Hence, in line with other recent equine AR-studies (Lester et al. 2013; Relf et al. 2014), the cut off was set at 90% FECR and the 95% LCL was set at 80%:

Resistant:	LCL below 80% and observed FECR below 90%
Susceptible:	LCL at/above 80% and observed FECR at/above 90%
Inconclusive:	only one of the above conditions met

For criteria 2, a method similar to that advocated by Lyndal-Murphy et al. (2014) was used to classify the FECR as "Resistant", "Inconclusive" or "Susceptible" based on the following conditions, which include the 95%UCL:

Resistant:	LCL below 80%, observed FECR below 90%, and UCL below 95%
Susceptible:	LCL at/above 80% and observed FECR at/above 90%
Inconclusive:	neither of the above conditions met

4. Results

4.1. Treatment with Ivermectin in 2007

In 2007, 755 horses on 23 horse farms were first sampled, of which 428 fulfilled the criteria for the study and therefore were incorporated. 224 (52.3%) were allocated into treatment groups, and 204 (47.7%) into control groups.

Treatment with IVM was done in the autumn, during a period in which most horse owners would be using this drug. Horses were still kept at grass, for at least two hours daily, but much longer on most farms. The first sampling (day 0) occurred between October 30th and December 5th. Horses allocated into the treatment groups were treated on the day after the first faecal sample had been taken (day 1). All participating horses were re-sampled on day 14, and the horses that had been treated were again sampled on day 42 to assess the Egg Reappearance Period.

4.1.1. Egg Count Reduction two Weeks after Treatment with Ivermectin

4.1.1.1. Calculations

FECR was calculated according to the recommendations of the WAAVP (Coles et al., 1992).
On 21 out of 23 farms (91.3%) the FECR was 100%, and all horses in the treatment group had a zero egg count on the second day of sampling. Only two farms had one horse each that showed a positive egg count of 25 EPG on day 14. The FECR on these farms was 99.7% (Farm Nº1) and 98.3% (Farm Nº21), respectively.

In other words, 222 out of 224 horses (99.1%) had a zero egg count 14 days after treatment with IVM, as shown in Fig. 2.

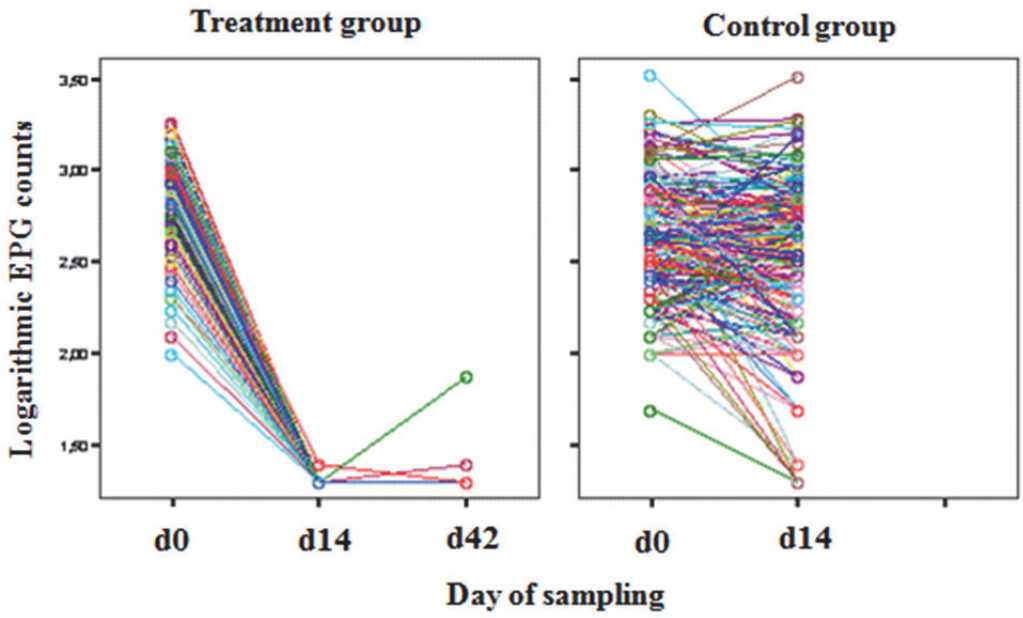

Figure 2: Logarithmic visualization of FECR after treatment with IVM in the individual animals, showing FEC on days 0, 14, and 42 (day 42 for the treatment group only).

Table 2 shows the number of horses in the treatment and the control groups, the arithmetic mean egg count pre and post treatment, and the FECR for each farm.

Table 2: Egg count reduction on equine premises in the Federal State of Brandenburg following treatment with Ivermectin (Eqvalan Duo®) in 2007. Calculated according to Coles et al. (Coles et al., 1992)

Farm	Nº of horses		EPG: arithmetic mean (range)		Egg count
Nº	control group	treatment group	control group	treatment group	Reduction in %
1	**6**	**7**	**1325** (200-3250)	**3** (0-25)	**99.7**
2	**13**	**14**	**721** (125-1900)	**0**	**100**
3	**5**	**5**	**375** (125-675)	**0**	**100**
4	**8**	**11**	**334** (125-800)	**0**	**100**
5	**13**	**14**	**625** (50-1550)	**0**	**100**
6	**5**	**6**	**385** (250-775)	**0**	**100**
7	**4**	**5**	**291** (100-450)	**0**	**100**
8	**13**	**14**	**251** (0-600)	**0**	**100**
9	**4**	**5**	**425** (200-600)	**0**	**100**
10	**8**	**9**	**428** (0-1050)	**0**	**100**
11	**10**	**11**	**417** (100-675)	**0**	**100**
12	**4**	**5**	**558** (100-825)	**0**	**100**
13	**5**	**6**	**205** (75-500)	**0**	**100**
14	**4**	**6**	**733** (475-1200)	**0**	**100**
15	**15**	**15**	**558** (100-1025)	**0**	**100**
17	**15**	**15**	**568** (25-1425)	**0**	**100**
18	**4**	**5**	**191** (50-275)	**0**	**100**
19	**18**	**19**	**348** (150-800)	**0**	**100**
20	**12**	**13**	**368** (25-1050)	**0**	**100**
21	**5**	**5**	**300** (100-675)	**5** (0-25)	**98.3**
22	**9**	**9**	**652** (275-975)	**0**	**100**
23	**15**	**15**	**417** (125-1025)	**0**	**100**
24	**9**	**10**	**497** (125-175)	**0**	**100**

The confidence intervals were very narrow on 21 farms, ranging from 99.99 to 100% in all cases. The two farms with a FECR lower than 100% had a LCL of 97.6% (FECR 99.7) and 84.5 (FECR 98.3), respectively.

Table 3: Upper and Lower 95% confidence intervals for the two farms with FECR <100%, calculated according to the recommendations of the WAAVP (Coles et al., 1992).

Farm Nº	FECRT%	LCL (95%)	UPL (95%)
1	**99.7**	**97.6**	**99.9**
21	**98.3**	**84.5**	**99.8**

Those premises with a high frequency of de-worming were not among the farms with an ECR <100%.

In the case of the two individual horses with an EPG of 25 on day 14, original egg count on day had been 975 and 200 EPG. The individual FECR was 97.4% and 87.5%, respectively.

4.1.1.2. Interpretation

Overall efficacy of IVM was adequate on all farms, with FECR of 100% or close to 100%. Only two out of 220 horses (0.9%) showed an EPG of 25 (lowest detection level) on day 14.

4.1.2. Egg Reappearance Period (ERP)

Faecal samples on day 42 were available from 203 out of 224 horses in the control groups. One farm with eleven treated horses was not accessible on or around day 42 due to organisational problems in the management; and ten horses on three different farms had been sold or moved in the meantime.

4.1.2.1. Results ERP

Faecal egg counts of the samples taken on day 42 yielded the following results: 198 samples (97.5%) showed a negative EPG count, and five samples (2.5%) showed a positive, while low EPG. These samples stemmed from five different farms and from horses that had shown a negative EPG on day 14. The two horses with an EPG of 25 on day 14 had a negative egg count on day 42.

Table 4: Egg count on day 0, 14 and 42 for five horses that had a positive egg count on day 42 following treatment with IVM (Eqvalan Duo®)

Farm Nº	Horse Nº	EPG Day 0	EPG Day 14	EPG Day 42
13	**11**	**250**	**0**	**25**
17	**40**	**1050**	**0**	**75**
18	**13**	**125**	**0**	**25**
22	**34**	**1075**	**0**	**25**
23	**29**	**525**	**0**	**25**

4.1.2.2. Interpretation ERP

In 97.5% of the individuals and 78.3% of the farms, the ERP was longer than 42d and therefore satisfactory. Five individuals on five different farms had a positive, albeit very low egg count, ranging from 25epg (four horses) to 75epg (one horse). No shortening of the ERP can be concluded from the data of 203 horses. 21 animals (10.3%) were unavailable on day 42 and therefore are not included in the calculation.

4.2. Treatment with Pyrantel Embonate in 2008

In 2008, 685 horses on 21 horse farms were sampled, of which 414 fulfilled the criteria for the study and therefore were incorporated in the study. 218 (52.7%) were allocated into treatment groups, and 196 (47.3%) into control groups.

Treatment with PYR was done in the summer, during a period in which most horse owners traditionally would be using this drug. Horses were fully or partly kept at grass, for at least two hours daily, but much longer on most farms. The first sampling (day 0) occurred between June 29th and September 1st. Horses allocated into the treatment groups were treated on the day after the first faecal sample had been taken (day 1). All participating horses were re-sampled on day 14, and faecal samples on day 14 were available from 411 out of 414 horses. The remaining three horses had been sold or moved in the meantime.

4.2.1. Egg Count Reduction two Weeks after Treatment with Pyrantel Embonate

4.2.1.1. Calculations

4.2.1.1.1. Method 1 – as recommended by the WAAVP

FECR was calculated according to the recommendations of the WAAVP (Coles et al., 1992).

On two out of 21 farms (9.5%) the FECR was 100%, with all horses in the treatment group showing a zero egg count on day 14. On seven farms (33.3%) the FECR was between 95% and 99%, and eight farms (38.1%) had a FECR between 90% and 95%. On four farms (19.1%), FECR was <90%. In total, 68 out of 218 horses (31.2%) in the treatment group showed a positive egg count on day 14, and 68.8% had zero EPG.

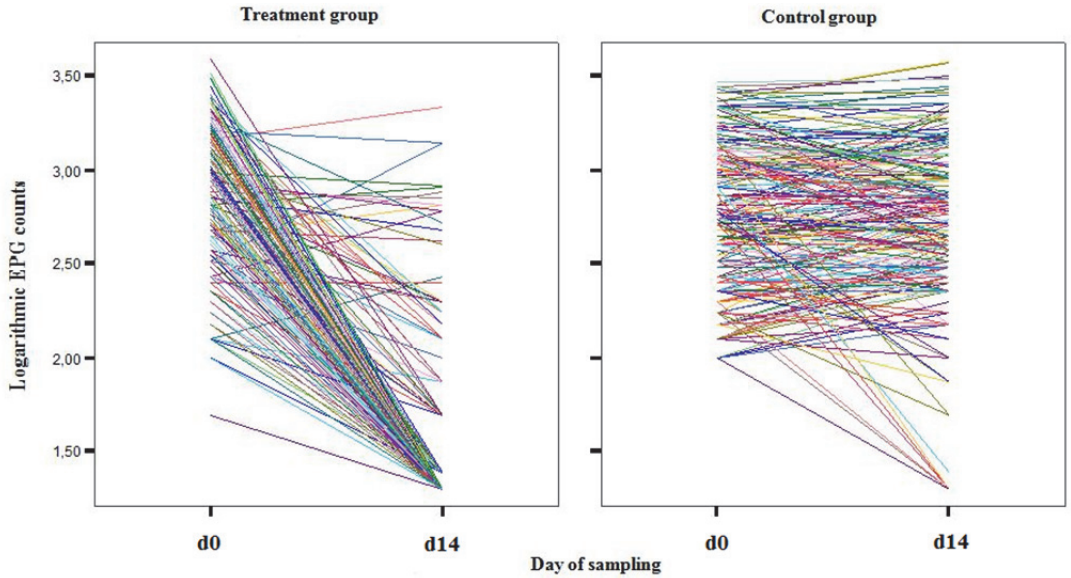

Figure 3: Logarithmic visualization of FECR after treatment with PYR in the individual animals, showing FEC on days 0 and 14.

Table 5 shows the number of horses in the treatment and the control groups, the arithmetic mean egg count pre and post treatment, and the FECR for each farm.

Table 5: Egg count reduction on equine premises in the Federal State of Brandenburg following treatment with PYR (Banminth®) in 2008, calculated according to the recommendations of the WAAVP (Coles et al., 1992). Premises with an FECR of less than 90% are marked in grey.

Farm	Nº of horses		EPG: arithmetic mean (range)		Egg count
Nº	control group	treatment group	control group	treatment group	Reduction in %
1	**6**	**7**	**921** (150-3100)	**0**	**100**
2	**15**	**17**	**718** (175-1875)	**234** (0-2125)	**65.3**
3	**6**	**7**	**479** (0-1050)	**43** (0-150)	**91.1**
5	**17**	**17**	**794** (50-2725)	**94** (0-1400)	**88.1**
6	**5**	**6**	**980** (325-1800)	**117** (0-650)	**88.1**
7	**6**	**6**	**808** (275-2250)	**21** (0-50)	**97.4**
8	**13**	**14**	**879** (150-3725)	**13** (0-50)	**98.6**
9	**4**	**5**	**1069** (700-1325)	**10** (0-25)	**99.1**
11	**7**	**8**	**546** (300-1500)	**28** (0-200)	**94.9**
12	**2**	**5**	**500** (225-775)	**10** (0-50)	**98**
13	**6**	**5**	**292** (125-375)	**0**	**100**
14	**3**	**5**	**408** (250-500)	**40** (0-175)	**90.2**
15	**13**	**18**	**733** (0-2275)	**57** (0-525)	**92.2**
16	**7**	**9**	**432** (0-950)	**92** (0-600)	**78.8**
17	**13**	**15**	**1017** (0-3800)	**18** (0-275)	**91.7**
18	**5**	**5**	**465** (150-1225)	**31** (0-100)	**93.3**
19	**20**	**20**	**1003** (0-3175)	**51** (0-825)	**94.9**
20	**13**	**13**	**1083** (100-2800)	**17** (0-75)	**98.4**
22	**12**	**12**	**727** (100-2025)	**16** (0-175)	**97.7**
23	**14**	**17**	**627** (0-1325)	**38** (0-425)	**93.9**
24	**6**	**7**	**1308** (400-2975)	**29** (0-175)	**97.8**

On farm Nº 2, an egg count reduction of 65.3 was observed. This showed a large difference to the results obtained on the remaining farms. Thus, the FECRT on farm Nº 2 was repeated after 10 weeks in order to validate this result. Following the second treatment, a FECR of 51.1% was observed.

Table 6: Egg count reduction on farm Nº 2 following the second treatment with PYR (Banminth®) in 2008, calculated according to the recommendations of the WAAVP (Coles et al., 1992).

Farm Nº	Nº of horses control group	Nº of horses treatment group	EPG: arithmetic mean (range) control group	EPG: arithmetic mean (range) treatment group	Egg count Reduction in %
2	**19**	**20**	**539** (50-1600)	**264** (0-1400)	**51.1**

All FECR values obtained for each farm ranged within the respective CL. Confidence intervals were rather large. Only the two farms with FECR of 100% had narrow confidence intervals, ranging from 99.99 to 100%.

Table 7: Upper and Lower 95% confidence intervals for all farms treated with PYR (Banminth®) in 2008, calculated according to the recommendations of the WAAVP (Coles et al., 1992) (where the LCL was a negative value, zero was used in this table).

Farm Nº	FECRT%	LCL (95%)	UPL (95%)
1	**100**	**99.99**	**100**
2	**65.3**	**0**	**89.6**
3	**91.1**	**63.4**	**97.8**
5	**88.1**	**29.7**	**98**
6	**88.1**	**15.5**	**98.3**
7	**97.4**	**90.8**	**99.3**
8	**98.6**	**95.5**	**99.6**
9	**99.1**	**96.6**	**99.7**
11	**94.9**	**65.5**	**99.2**
12	**98**	**79.3**	**99.8**
13	**100**	**99.99**	**100**
14	**90.2**	**41.3**	**98.4**
15	**92.2**	**73.3**	**97.7**
16	**78.8**	**0**	**95.9**
17	**91.7**	**56.9**	**98.4**
18	**98.3**	**60.3**	**98.9**
19	**94.9**	**72.8**	**99**
20	**98.4**	**95.9**	**99.4**
22	**97.7**	**85.3**	**99.6**
23	**93.9**	**74.5**	**98.5**
24	**97.8**	**85.2**	**99.7**

The second FECRT on farm Nº 2 showed a repeated decline in efficacy of PYR; the FECR was 51.1% (Table 8).

Table 8: Upper and Lower 95% confidence intervals for farm Nº 2 after 2nd treatment with PYR (Banminth®) in 2008, calculated according to the recommendations of the WAAVP (Coles et al., 1992).

Farm Nº	FECRT%	LCL (95%)	UCL (95%)
2	**51.1**	**0**	**76.4**

When applying the definition of resistance as recommended by Coles et al. (1992) and taking into account the LCL as suggested by Lester et al. (2013) and Relf et al. (2014), resistance was present on four farms (Nº 2, 5, 6 and 16), as the mean FEC was < 90%, and the LCL 95% was < 80%. Farms Nº 6 and 16 use anthelmintic drugs more than four times a year; farms Nº 2 and 5 twice per year or less often.

With the criteria suggested by Lyndal-Murphy et al. (2014), one (Nº 2) out of 21 farms (4.8%) was classified as “Resistant”, six farms (28.6%) as “Susceptible” and on 14 farms (66.6%) the data were inconclusive.

4.2.1.1.2. Bootstrapping

While employing four different equations for calculation, the “BootStreat” programme by Antoine and Cabaret was utilized for calculating FECR (Cabaret and Antoine, 2008). Table 9 shows a comparison between the outcomes of the bootstrapping with these four equations: calculations were made according to Coles et al. (Coles et al., 1992), Kochapakdee et al. (Kochapakdee et al., 1995), Dash (Dash, 1988) and Presidente (Presidente, 1985).

Table 9: Egg count reduction on all farms following the treatment with PYR (Banminth®) in 2008, calculated with BootStreat, using four different equations.

	FECR in % calculated with BootStreat			
Farm N°	Coles et al.	Kochapakdee et al.	Dash et al.	Presidente
1	99	99	99	99
2	64	53	65	97
3	89	95	92	93
5	88	94	92	99
6	87	76	86	97
7	97	98	98	99
8	98	98	97	99
9	99	99	99	99
11	94	98	98	99
12	97	98	98	99
13	100	100	99	99
14	86	90	93	98
15	88	90	88	92
16	71	64	63	75
17	97	96	96	99
18	93	88	90	97
19	94	96	95	99
20	98	97	98	99
22	97	98	97	99
23	92	93	91	99
24	97	98	97	99

The second FECRT performed on farm Nº 2 yielded values ranging from 34% (according to Kochapakdee et al.) to 86% FECR (according to Presidente) (Table 10).

Table 10: Egg count reduction on farm Nº 2 following the second treatment with PYR (Banminth®) in 2008, calculated with BootStreat, using four different equations.

	FECR in % calculated with BootStreat			
Farm N°	Coles et al.	Kochapakdee et al.	Dash	Presidente
2	49	34	58	86

Tables 11 shows the FECR and upper (UCL) and lower (LCL) 95% confidence limits for the four different equations, calculated with BootStreat (Cabaret and Antoine, 2008).

Table 11: FECR and 95% confidence intervals on all farms following treatment with PYR (Banminth®) in 2008, calculated with BootStreat, using four different equations (where the LCL was a negative value, zero was used in this table).

		FECR in % according to Method			
Farm Nº		**Coles et al.**	**Kochapakdee et al.**	**Dash**	**Presidente**
1	**FECR**	**99**	**99**	**99**	**99**
	CI	100-100	100-100	100-100	100-100
2	**FECR**	**64**	**53**	**65**	**97**
	CI	12-94	0-95	2-97	86-100
3	**FECR**	**89**	**95**	**92**	**93**
	CI	49-100	85-100	52-100	0-100
5	**FECR**	**88**	**94**	**92**	**99**
	CI	56-100	78-100	69-100	98-100
6	**FECR**	**87**	**76**	**86**	**97**
	CI	51-100	0-100	34-100	75-100
7	**FECR**	**97**	**98**	**98**	**99**
	CI	91-100	95-100	94-100	96-100
8	**FECR**	**98**	**98**	**97**	**99**
	CI	95-100	95-100	91-100	96-100
9	**FECR**	**99**	**99**	**99**	**99**
	CI	98-100	96-100	96-100	95-100
11	**FECR**	**94**	**98**	**98**	**99**
	CI	80-100	89-100	83-100	97-100
12	**FECR**	**97**	**98**	**98**	**99**
	CI	91-100	90-100	89-100	98-100
13	**FECR**	**100**	**100**	**99**	**99**
	CI	100-100	100-100	99-100	99-100
14	**FECR**	**86**	**90**	**93**	**98**
	CI	58-100	49-100	60-100	78-100
15	**FECR**	**88**	**90**	**88**	**92**
	CI	69-99	75-100	65-99	47-100
16	**FECR**	**71**	**64**	**63**	**75**
	CI	0-100	0-100	0-100	0-100
17	**FECR**	**97**	**96**	**96**	**99**
	CI	90-100	86-100	84-100	94-100
18	**FECR**	**93**	**88**	**90**	**97**
	CI	74-100	64-100	51-100	72-100
19	**FECR**	**94**	**96**	**95**	**99**
	CI	83-100	88-100	85-100	98-100
20	**FECR**	**98**	**97**	**98**	**99**
	CI	96-100	95-100	96-100	97-100
22	**FECR**	**97**	**98**	**97**	**99**
	CI	91-100	92-100	88-100	98-100
23	**FECR**	**92**	**93**	**91**	**99**
	CI	79-100	80-100	71-100	94-100
24	**FECR**	**97**	**98**	**97**	**99**
	CI	89-100	88-100	84-100	94-100

Table 12: FECR and 95% confidence intervals for the second FECRT on farm Nº 2 following treatment with PYR (Banminth®) in 2008, calculated with BootStreat, using four different equations (where the LCL was a negative value, zero was used in this table).

Method	Coles	Kochapakdee	Dash	Presidente
FECR	**49%**	**34%**	**58%**	**86%**
CI	6-80%	0- 74%	12-86%	60-98%

The four different equations gave varied results, as with equation 1, a FECR <90% was obtained on seven farms, with equations 2 and 3 on four farms, and with equation 4 on one farm. Table 13 illustrates the detection of resistance on all farms for the four different equations.

Table 13: Frequency of detection of resistance by the four different equations employed for bootstrapping. "X" marks the detection of resistance. Resistance was declared when the FECR was < 90% and the LCL95% was < 80% (Coles et al., 1992; Lester et al., 2013; Relf et al., 2014). Farms that use anthelmintics frequently are marked in grey.

Farm N°	Coles et al.	Kochapakdee et al.	Dash	Presidente
1				
2	**X**	**X**	**X**	
3	**X**			
5	**X**			
6	**X**	**X**	**X**	
7				
8				
9				
11				
12				
13				
14	**X**			
15	**X**		**X**	
16	**X**	**X**	**X**	**X**
17				
18		**X**		
19				
20				
21				
22				
23				

Resistance was detected on seven farms (Nº 2, 3, 5, 6, 14, 15 and 16) while bootstrapping with the equation recommended by Coles et al. (Coles et al., 1992). This means that three additional farms were detected in comparison to applying the equation to the raw data, without re-sampling with BootStreat®. Of these seven farms, three farms (Nº 6, 15 and 16) use anthelmintics more than four times a year; four farms (Nº 2, 3, 5 and 14) twice per year or less often. For the second FECRT on farm Nº 2, resistance was detected with all four equations.

With criteria 2, as recommended by Lyndal Murphy et al. (2014), and equation 1, one out of 21 (4.8%) farms was categorized as “Resistant”. With equations 2, 3 and 4, none of the farms was rated as “Resistant”.

4.2.1.1.3. Markov Chain Monte Carlo

A novel Markov Chain Monte Carlo (MCMC) method was employed in order to calculate confidence intervals. FECR was >95% on one farm (4.8%) and between 90% and 95% on three farms (14.3%). On 17 farms (80.9%) FECR was <90%. Table 14 shows the FECR and confidence intervals on all yards.

Table 14: FECR and 95% confidence intervals on all farms following treatment with PYR (Banminth®) in 2008, calculated with a MCMC method (where the LCL was a negative value, it was replaced by zero). Data courtesy of Dr. Matthew Denwood.

Farm N°	LCL	FECR	UCL
1	0	55.9	100
2	21.3	60.1	88.8
3	0	29.2	99.4
5	0	68.9	98.4
6	39.3	76.9	98.1
7	0	75.4	99.9
8	26.5	93.8	99.8
9	15.5	91.3	99.9
11	0	67.9	99.4
12	0	73.7	99.9
13	0	53.6	100
14	0	64.3	99.7
15	10.4	80.4	98.5
16	5.3	63.5	97.4
17	13.6	79.8	99.6
18	10.4	73.8	99.7
19	47.1	90.1	98.8
20	65.5	96.2	99.8
22	0	82.3	99.7
23	11.7	82.8	98.8
24	0	70.7	99.8

Table 15: FECR and 95% confidence intervals on farm Nº 2 following the second treatment with PYR (Banminth®) in 2008, calculated with a MCMC method. Data courtesy of Dr. Matthew Denwood.

Farm N°	FECR %	LCL (95%)	UCL (95%)
2	51.8	22.5	78.3

Using criteria 1 as advocated by the WAAVP and including the LCL as proposed by Lester et al. (2013) and Relf et al. (2014), one of the farms was classified as “Susceptible” and three as “Suspect susceptible”, with the remaining 17 farms (76.2%) classified as “Resistant”. However, using the second criteria derived from Lyndal-Murphy et al. (2014), one out of 21 farms (Farm N°2) was classified as "Resistant", a further farm was classified as "Susceptible" and the remainder as "Inconclusive".

FECR in % and size of the CI can be seen in Figure 4.

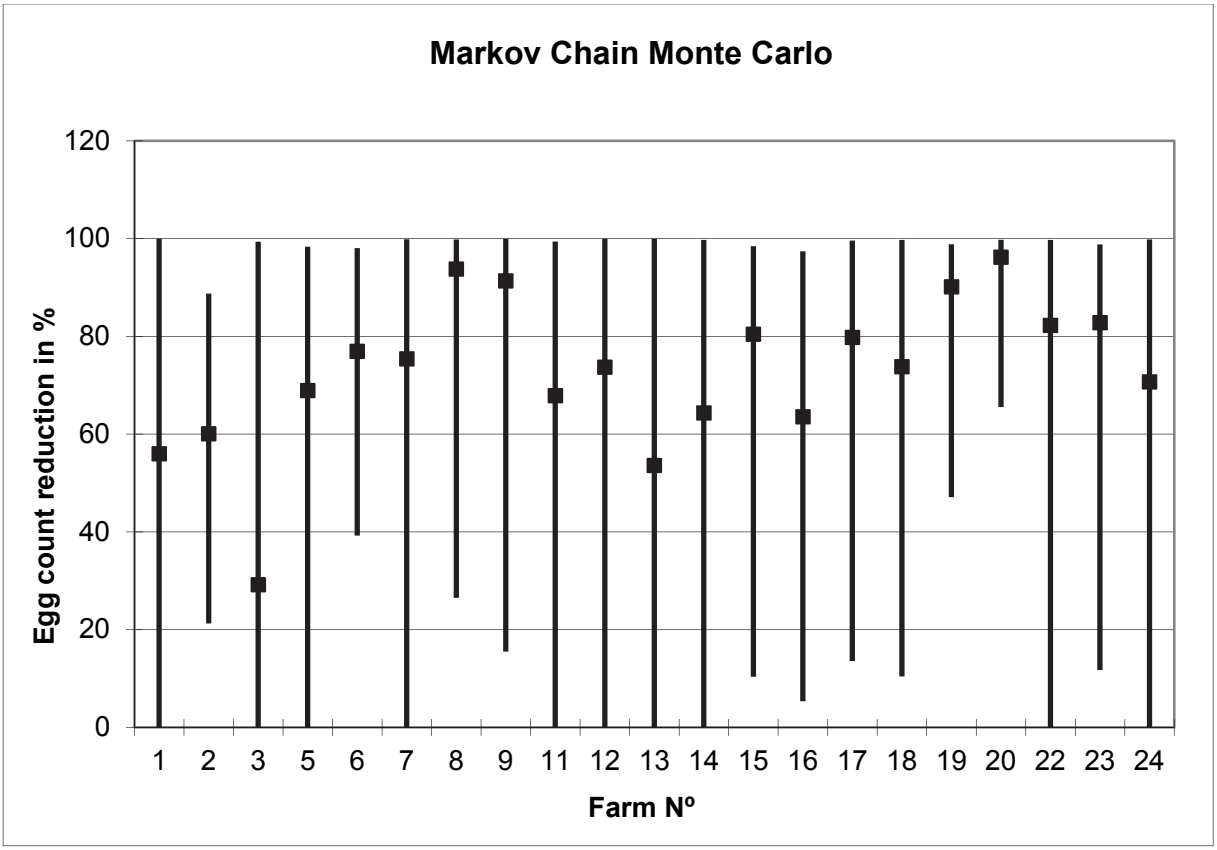

Fig. 4: Egg count reduction calculated with a MCMC method (Denwood et al., 2009).

Figure 5 shows the egg count reduction in % as well as the CIs for the two FECRTs performed on farm N° 2.

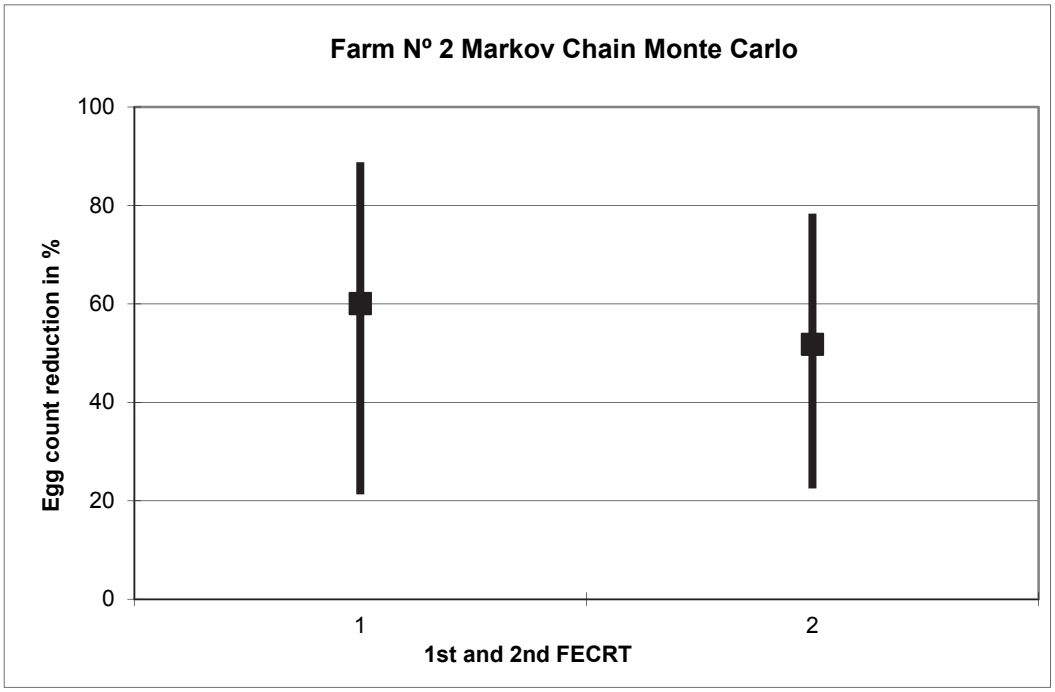

Fig. 5: Egg count reduction on farm Nº 2 calculated with a MCMC method (Denwood et al., 2009)

4.2.1.2. Interpretation of Results

Criteria 1:
When applying the criteria recommended by the WAAVP (Coles et al., 1992), the results show that the efficacy of PYR on horse farms in the Federal State of Brandenburg is generally acceptable. However, on four farms the presence of PYR resistant CYA is presumed (FECR of 88.1% on two farms, 78.7% and 65.3% (51.1% on the second occasion)).

Criteria 2:
When including the UCL, as suggested by Lyndal-Murphy et al. (2014), farm N° 2 was the only one detected as “Resistant”. This result was repeated with all three methods of calculation.

4.2.2. Larval Cultures

In 2008, larval cultures were obtained from the faecal material taken on the day before the administration of PYR (Day 0) in order to assess the prevalence of large strongyles. The individual samples from each farm were mixed in order to obtain a pooled sample per farm. Larval cultures were obtained as described in 3.6. All third stage larvae that could be designated were larvae of CYA, and no larvae could be identified as *Strongylus* spp.
In addition to this, two individual faecal samples showing an unusually high positive egg count on day 14 after treatment with PYR were used to obtain larvae for further differentiation. This included one horse from farm Nº 5 (1400 EPG 14d post treatment) and one horse from farm Nº 6 (650 EPG 14d post treatment). Furthermore, a pooled sample from all horses showing an EPG count ≥250 on day 14 post treatment with PYR on farm Nº16 was used for larval cultures.
On farm Nº 2, on which the FECRT was repeated, pooled samples were taken on day 0 and day 14, on both occasions.

Courtesy of Dr. Donato Traversa of the Department of Comparative Biomedical Sciences, Faculty of Veterinary Medicine, University of Teramo, Italy, genetic analyses of theses larvae were performed. The results were the following:

Table 16: Results of the genetic analysis of three different samples on three different equine premises 14d post treatment. Data courtesy of Dr. Donato Traversa.

Sample	Species found
Farm Nº 5, Horse Nº 22 14 d post treatment	*Cylicocyclus leptostomum* *Cylicostephanus longibursatus* *Cylicoyclus nassatus* *Cyathostomum pateratum*
Farm Nº 6, Horse Nº 12 14 d post treatment	*Cylicostephanus goldi* *Cylicocyclus leptostomum* *Cylicostephanus longibursatus* *Cylicostephanus minutes*
Farm Nº16, pooled sample, 14 d post treatment	*Cylicocyclus ashworthi* *Cylicostephanus calicatus* *Cyathostomum catinatum* *Coronocyclus coronatus* *Cylicostephanus goldi*

On farm Nº 2, a pooled sample from all horses in the treatment group was taken on day 0, and again, including all horses in the treatment group that showed an EPG count ≥ 250, on day 14. The results are stated in Table 17.

Table 17: Results of the genetic analysis of pooled samples of the treatment group on farm Nº 2, pre and 14d post 1st treatment. Data courtesy of Dr. Donato Traversa.

Sample	Species found
Farm Nº 2, pooled sample, pre 1st treatment	*Cylicostephanus calicatus* *Cylicostephanus goldi* *Cylicostephanus longibursatus* *Cylicocyclus insigne* *Cyathostomum labiatum* *Cyathostomum catinatum*
Farm Nº 2, pooled sample, post 1st treatment	*Cylicostephanus goldi* *Cylicocyclus insigne*

As a FECR of only 65.3% was achieved on farm Nº 2, the FECRT was repeated 10 weeks after the first treatment to exclude operational mistakes. Again, a pooled sample from all horses in the treatment group was taken on day 0. On day 14, a pooled sample from all horses in the treatment group that showed an EPG count ≥ 100 was taken. The results are shown in Table 18.

Table 18: Results of the genetic analysis of pooled samples of the treatment group on farm Nº 2, pre and 14d post 2nd treatment. Data courtesy of Dr. Donato Traversa.

Sample	Species found
Farm Nº 2, pooled sample, pre 2nd treatment	*Cylicostephanus goldi* *Cylicostephanus longibursatus* *Cylicocyclus insigne*
Farm Nº 2, pooled sample, post 2nd treatment	*Cylicostephanus goldi* *Cylicocyclus insigne*

4.2.2.1. Interpretation

On farm Nº 2, the pooled sample taken before the first treatment showed six species of CYA: *C. calicatus*, *C. catinatum*, *C. goldi*, *C. insigne*, *C. labiatus* and *C. longibursatus*.
The pooled sample before the second treatment (and ten weeks after the first treatment with PYR) showed only three species: *C goldi*, *C. insigne* and *C. longibursatus*.
On both occasions, the only two species present 14d post treatment were *C. goldi* and *C. insigne*.

5. Discussion

5.1. Selection of Horse Farms and Time of Sampling

The farms included in this study had been pre-selected by a study that established the prevalence of helminths on horse farms in the Federal State of Brandenburg (Hinney et al., 2011).
Two groups are being compared: horses on premises that de-worm twice a year or less often, and horses on farms on which de-worming is performed four times per year or more often. The study design follows the hypothesis that frequent worming enhances the development of resistance against anthelmintic drugs (Uhlinger, 1991; Herd, 1993; Herd and Coles, 1995). Therefore, it was deducted that premises that use wormers four times per year or more often ran a comparatively higher risk of showing resistance to anthelmintic drugs, if compared to premises in which worming was carried out two times per year or less often. The horse population included in this study therefore is not representative for the horse population of the Federal State of Brandenburg, but constitutes a proportion of animals of this population.

Only farms where horses had not been wormed for at least 8 weeks previously were included in the study, to allow for the establishment of patent infections since the previous treatment. Considering that the prepatent periods of the different CYA species span over 5.5-14 weeks, it cannot be excluded that prepatent infections with CYA existed on day 0, and a rise in FEC between days 0 and 14 in the control group could have been caused by the maturation of these worms. However, FECs of the control groups provided no considerable changes in egg output. Therefore, ECR was associated with the application of the anthelmintic drugs.

Two FECRTs were conducted in two consecutive years, the first one with IVM and the second with PYR. This set-up is based on the fact that the yards, according to their own statement, would have used either of the respective drugs anyhow at the time of year, irrespective of FEC of their horse. Under the field conditions faced in this study, it was not possible to determine the anthelmintics previously used for dosing. In most cases it was not exactly known which drugs had been used in the preceding years, as the majority of horse owners and farm managers do not keep accurate records.

Sampling was performed during the summer and autumn period to allow for re-infection on pasture; all horses had access to pasture for at least several hours per day or were kept on grass day and night. By allocating the respective eligible animals into treatment and control groups with the aid of random numbers, representativeness of the composition of both groups was provided for.

5.2. Method used for the FECRTs

The McMaster method is the widely accepted method of choice for epidemiological studies. However, it is based on dilution and was originally intended for the use in samples with high egg counts (>500 EPG) (Kraemer, 2005). One of its shortcomings is the relatively low sensitivity (Kraemer, 2005) when only one McMaster slide is used per sample. The use of two slides (with a total of four chambers) per sample, as done in the present study, lowers the threshold to 25 EPG; however, false negative EPG counts were still possible. The combined sedimentation-flotation method is more sensitive (Kraemer, 2005), but more difficult to quantify. To improve sensitivity, a method such as the combined sedimentation-flotation should be quantified (Hinney, 2008), and in the future used in studies that evaluate anthelmintic efficacy.

Another factor influencing sensitivity and repeatability is that egg distribution throughout the faeces may vary, and the particular part taken for the egg count may not be representative of the sample. An average dropping of an adult horse weighs about 2-3 kg. By using only 4g of a faecal sample, the representativeness is always at risk. Those 4g may have been taken from a portion of the sample which contained fewer eggs than the average, or rather, from a portion in which eggs were highly abundant if compared to the remaining sample. Even by thoroughly mixing the sample, this problem cannot be avoided completely. Also, egg excretion depends on a variety of factors, such as the time of day, fecundity of the female adult worms, age and immune status of the host. One has thus to consider that the results obtained with a modified McMaster technique as used in the present study possibly suffer from high variances, and can only be indicative of the egg output of the host. Nonetheless, despite its disadvantages, the McMaster method still constitutes the gold standard worldwide for the conduction of FECRTs and is the method recommended by the WAAVP (Coles et al., 1992).

Ideally, only horses with an egg count of >200 EPG would have been incorporated in the study, as the McMaster method was originally designed for higher egg counts. However, it was necessary to recruit enough horses on each farm to conduct the FECRT with a minimum of ten horses, and consequently the cut-off was set at 150 EPG.

Egg shedding itself depends on the fecundity of the female adult worms present in the gut rather than being representative of the overall worm number. A FEC can be low, even if large numbers of parasites are present, but fecundity is low (Uhlinger, 1993). The FEC also does not give any estimate of the burden of larvae encysted in the intestinal mucosa, or the prepatent stage. Yet, it is very convenient to use FECRTs in the field, as opposed to the running of critical or controlled tests, and the FECRT is the widely accepted method for field studies testing efficacy of anthelmintic drugs. The details of the molecular mechanisms of resistance to both tetrahydropyrimidines and the ML remain unknown, and hence no PCR

based tests can be expected to be developed in the near future. However, as they would be a means for identifying genotypic changes before therapeutic failure could detect phenotypic resistance, further research into bio-molecular diagnostic tools is greatly desirable (Kaplan, 2002).

The possible error of underdosing the anthelmintic drugs was minimised by estimating the weight of each horse, and validating the estimation with a commercially available girth measuring tape (Tarigo-Martinie et al., 2001, Pook et al., 2002).

5.3. Outcome of FECRTs

In this study, the first FECRT was calculated according to the recommendations of the WAAVP (Coles et al., 1992), and, in analogy to Lester et al. (2013) and Relf et al. (2014), the 95%LCL of 80% was included for interpretation of the data. The results showed excellent efficacy of IVM, with FECR of 100% on 21 farms (91.3%). On the remaining two farms (8.7%), one individual horse each had a FEC of 25 EPG on day 14. These are small yards, hence horse numbers incorporated in the study on both farms were small, and the presence of a positive egg count in one single horse lowered the FECR to 99.7% and 98.3%, respectively. Both farms used anthelmintic drugs infrequently.

The data of the second FECRT was subjected to a number of different methods of calculation. The aim was to establish if different outcomes would be obtained with these different methods. The interpretation of data from FECRT is controversial, for sample sizes are mostly small, the distribution of data is not a Poisson distribution, and data are often skewed (Denwood et al., 2009). Obtaining meaningful CIs is one of the difficulties of FECRT data, as they tend to be large, and results in the present study gave undesirably broad CIs.

According to the recommendations of the World Association for the Advancement of Veterinary Parasitology (WAAVP), the arithmetic mean (AM) rather than the geometric mean (GM) should be used to calculate FECR (Coles et al., 1992). The GM constitutes the antilog of the mean of the log transformed egg counts and provides the advantage that the impact of extreme values of individuals is reduced; at the same time FECR calculated with GM tends to be higher than when calculated with AM. Presidente used the GM in order to reduce the impact of extreme values of individuals in the group (Presidente, 1985). As expected, this method could be distinguished as more conservative than the other equations used in this study, due to the fact that the GM is used in the calculation.

The first method employed for the calculation of the FECRT with PYR was identical with the one used in the first FECRT with IVM. Resistance was detected on four farms when calculated according to Coles et al. (Coles et al., 1992). Of these farms, two use anthelmintic drugs frequently, and two infrequently.
For the second procedure of calculation, FECR was determined applying a bootstrapping programme to the data. Bootstrap analysis is an approach for evaluating confidence intervals on non-Gaussian distributions (Cabaret and Antoine, 2008). In this study, four different equations for calculating FECR were used while employing the BootStreat® programme (Cabaret and Antoine, 2008) for re-sampling. These four different equations gave varied results: with equation 1, a FECR <90% was obtained on seven farms, with equations 2 and 3 on four farms, and with equation 4 on one farm.

The third method was a MCMC method as suggested by Denwood et al., which is based on re-sampling and generates more accurate CIs while knowledge of the distribution of the data is not required (Denwood et al., 2010). This method proved to be the most sensitive and detected resistance in 17 out of 21 farms (81%). Table 19 illustrates the detection of resistance on all farms for the method recommended by the WAAVP (Coles et al., 1992), four different equations using bootstrapping, and the MCMC method (data courtesy of Dr. Matthew Denwood).

Table 19: Frequency of detection of resistance by three different methods employed for the calculation of the FECRTs. "X" marks the detection of resistance. Resistance was declared when the FECR was <90% (Coles et al., 1992) and the LCL95% was <80% (Lester et al. (2013), Relf et al. (2014)). Farms with frequent use of anthelmintic drugs are marked in grey.

Farm N°	Coles et al.	Bootstrap 1	Bootstrap 2	Bootstrap 3	Bootstrap 4	MCMC
1						X
2	X	X	X	X		X
3		X				X
5	X	X				X
6	X	X	X	X		X
7						X
8						
9						
11						X
12						X
13						X
14		X				X
15		X		X		X
16	X	X	X	X	X	X
17						X
18			X			X
19						
20						
21						X
22						X
23						X

Table 20 illustrates that resistance was detected with all methods of calculation for the second FECRT on Farm N° 2.

Table 20: Frequency of detection of resistance for the second FECRT on Farm N° 2 by three different methods employed for the calculation of the FECRTs. “X” marks the detection of resistance. Resistance was declared when the FECR was <90% (Coles et al., 1992) and the LCL95% was <80% (Lester et al. (2013), Relf et al. (2014)).

Farm N°	Coles et al.	Bootstrap 1	Bootstrap 2	Bootstrap 3	Bootstrap 4	MCMC
2	X	X	X	X	X	X

Different authors claim different levels as a cut off point for resistance against PYR in CYA (see 2.3.2.1.2.), and the outcome of FECRTs varies depending on which criteria for resistance are applied to the data. However, to date there is no consensus on when to draw the line and call diminished efficacy “resistance”. These criteria should differ for each drug or drug class and have to take into account the efficacy achieved when the substances were first introduced (Kaplan, 2002; Pook et al., 2002).
The cut-off used in the present study was defined as <90% for PYR, as recommended for BZ in horses by the WAAVP (Coles et al., 1992), with a LCL95% of <80% (Lester et al. (2013) and Relf et al. (2014)). With this method, resistance was detected on four farms, two of which had a FECR of 88.1%. From a more conservative point of view, it could be argued that 88.1% is close enough to 90% to consider these results as merely indicative of resistance. The other two farms showed FECRs of 78.8% (Farm N° 16) and 65.3% (Farm N° 2) and it can be concluded that resistance is present on these two farms. The particularly low FECR on Farm N° 2 was yet again confirmed when the FECRT was repeated, as FECR was lowered to 51.1%.

When including the UCL, as suggested by Lyndal-Murphy et al. (2014), farm N° 2 was the only one detected as “Resistant”. This result was confirmed by all three methods of calculation. This approach therefore proved to be the most consistent method.

Table 21 shows a comparison of the results for all three methods of calculation and the two methods for interpretation.

Table 21: Frequency of detection of resistance by three different methods employed for the calculation of the FECRTs, showing the difference in conclusions elicited by different interpretations of the same data. “X” marks the detection of resistance declared based on (1) when the FECR was <90% (Coles et al., 1992) and the LCL was <80% (Lester et al. (2013), Relf et al. (2014)) or (2), when also the UCL was <95% (Lyndal-Murphy et al., 2014). Farms with frequent use of anthelmintic drugs are marked in grey.

Sample N°	**Farm N°**	**Coles et al.**		**Bootstrap 1**		**MCMC**	
		(1)	**(2)**	**(1)**	**(2)**	**(1)**	**(2)**
1	1					X	
2	2	X	X	X	X	X	X
3	3			X		X	
4	5	X		X		X	
5	6	X		X		X	
6	7					X	
7	8						
8	9						
9	11					X	
10	12					X	
11	13					X	
12	14			X		X	
13	15			X		X	
14	16	X		X		X	
15	17					X	
16	18					X	
17	19						
18	20						
19	22					X	
20	23					X	
21	24					X	

As shown, the outcome of FECRT depends hugely on the method(s) for calculation employed. In order to generate comparable data it is therefore necessary to come to a universal agreement on how to calculate FECR, to establish a standardized statistical technique for analysing this type of data and to set cut-off points for resistance for the different anthelmintic drugs.

The results concerning the ERP were unequivocal, as 198 out of 203 horses (97.5%) showed zero EPG on day 42. The five remaining horse had very low EPG, ranging from 25 (four horses) to 75 (one individual horse). No shortening of the ERP can be concluded, and hence the results are not indicative for a loss of efficacy for IVM.

5.4. Larval Cultures and Species Differentiation

Only small strongyles were found in this study, a result that corresponds to the findings of Hinney, who found large strongyles on only one out of 126 farms sampled (Hinney, 2008). However, it is possible that low numbers of eggs of large strongyles were present in the faeces that were not detected in the larval cultures. Out of the 52 species of CYA, about twelve species have been detected as the most frequent (reviewed by Kaplan, 2002). Eleven of these were found after treatment on farms Nº 2, 5, 6 and 16 in the present study.
One cannot conclude that the species found in the larval cultures were really the only ones present, as a species with very low prevalence might not have been detected due to the relatively small amount of faecal material that each individual horse contributed to the pooled samples. All the same, it can be concluded that the species present after treatment have developed resistance to PYR.
A total of twelve species were present in the samples taken 14 days post treatment. On farm Nº 2, six species were found in the first sample, taken before the first treatment with PYR. On day 14, two species were present in a pooled sample of all horses with a FEC >250: *Cylicostephanus goldi* and *Cylicocyclus insigne*. Ten weeks later, only three species were detected pre treatment: *Cylicostephanus goldi, Cylicostephanus longibursatus* and *Cylicocyclus insigne*. On day 14 post treatment, the same two species remained as after the first application of PYR: *Cylicostephanus goldi* and *Cylicocyclus insigne*.

It is of huge value to be able to identify the presence of large strongyles that might be present alongside CYA in a mixed infestation, because large strongyles have a high pathogenic potential. It can also be important to know which species of CYA are present, as in mixed infections only one or a few species might be resistant (Prichard et al., 1980).

5.5. Situation in the Federal State of Brandenburg

In this study, efficacy of IVM was excellent, and resistance of CYA to PYR is not a widespread problem in the Federal State of Brandenburg. However, the fact that it was found on four farms, when calculated with the currently recommended method, should be seen as a forewarning and calls for close monitoring of the efficacy of PYR in the future.

It is arguable if the FECRT should have been repeated on farm Nº 2, as using the same anthelmintic drug repeatedly on a population that is already showing a reduced FECR probably was not an ideal procedure. However, operational mistakes had to be excluded, and the repetition of the FECRT was therefore esteemed necessary. On this farm, young stock is treated four times per year, and animals over 3 years of age twice a year, in fast rotation, i. e., a different product is used every time, changing between BZs, IVM and PYR. A FECRT should be performed on this farm to establish the efficacy of BZs, and the efficiency of the worm control programme should be monitored by routine FECRTs.

The hypothesis that frequent worming (four times per year or more often) contributes to the development of AR in equine strongyles could not be confirmed in this study. However, though frequencies of more than four anthelmintic treatments per year can be considered as relatively high, much higher numbers, of up to twelve times per year, have been reported (O'Meara and Mulcahy, 2002) and seem to be common practice in many countries. Ideally, farms with an even higher frequency should have been compared with farms that use anthelmintic drugs twice a year or less often. On the other hand, the frequencies of worming in the Federal State of Brandenburg are generally low, and a number of four or more treatments per year can already be considered as relatively high for this area.

The fact that large strongyles were not found in the present study correlates with the findings of Hinney, who reported large strongyles on one out of 126 horse farms in the Federal State of Brandenburg (Hinney, 2008); and prevalence on German horse farms is generally low (Wirtherle, 2003; Fritzen, 2005). However, the low prevalence of *Strongylus* sp. is most likely due to the frequent use of anthelmintic drugs. It is therefore necessary to routinely perform larval cultures and/or DNA-based tests to monitor the prevalence and theoretical reappearance of large strongyles when a targeted treatment scheme is implemented on a farm. Nielsen et al. conducted a study in Denmark, suggesting that reduced treatment frequencies may have contributed to higher prevalences of *S. vulgaris* (Nielsen et al., 2012).

Farm N° 2 is a long established large scale stud farm. Young stock is wormed four times per year, all horses over three years of age are wormed twice per year. The fields are rotated between summer and winter pasture. Fields are rarely rested for more than six months.

Yearlings, two- and three-year olds are divided into groups by age and sex. They spend the winter in large open barns, with access to pasture if weather permits, and the rest of the year on pasture. Older horses in work are stabled during the night and generally have daily access to pasture or sand paddocks at least for a few hours. Brood mares live in a herd of heterogeneous age distribution. They are kept on pasture with access to a large open barn for feeding and during night time. They are also confined to large open barns with weather-dependent access to pasture during the winter months. A possible reason for the apparent resistance of CYA to PYR on this farm is the fact that large numbers of horses have been kept on the same land for a long period of time and the use of PYR for many decades. This probably led to a selection towards resistance despite the fact that anthelmintic drugs are used in moderate frequencies.

5.6. Conclusions

In this study, the determination of resistance from the FECRT with PYR very much depended on the criteria used to assess resistance, and to a lesser extent the statistical methods used to generate 95% confidence intervals. Resistance to PYR was detected on four farms with 95% CI calculated according to the recommendations of the WAAVP, with the cut-off set at 90% FECR and the LCL at 80% (Lester et al., 2013; Relf et al., 2014) . However, only one of these results was still interpreted as resistant when considering the upper 95% CL as recommended by Lyndal-Murphy et al. (2014). Using the parametric bootstrapping approach BootStreat® to generate 95% CI, the equation recommended and the interpretation provided by WAAVP, seven of the farms were classified as resistant - but when considering the upper 95% CL this was reduced to the same one farm as for the WAAVP generated confidence intervals. The third method employed to generate 95% CI was a MCMC method (Denwood et al., 2010). Using the WAAVP method interpretation, 17 of the farms would be classified as resistant. However, using the Lyndal-Murphy definition of resistance, this method detected resistance in only 1 out of 21 farms. There was huge variation in inference between statistical methods when using the WAAVP interpretation, but when using the Lyndal-Murphy interpretation all three statistical methods produced very similar recommendations in that there was conclusive evidence for resistance on yard No 2, but none of the other yards.

High egg counts are not necessarily indicative of parasitic disease; but they do reflect the potential for contamination that the horse poses for its environment. High shedding leads to high contamination of fields and thus implies a high possibility of ingestion of larvae. It has been shown that helminthic burdens are distributed unevenly within a herd of horses, and a small proportion of animals usually carry the majority of worms (Matthee and McGeoch, 2004, Nielsen et al., 2006a). Identification of those horses that shed larger quantities than the

herd average would give the owners an opportunity to target their treatment towards these animals, thus avoiding high contamination of fields. At the same time, overuse of anthelmintic drugs in animals that really do not need them could be prevented and refugia would be created. The creation and maintenance of parasite refugia is a corner stone to managing anthelmintic resistance (Nielsen et al., 2010).

However, to selectively treat a proportion of the herd which has been identified as "high shedders" also entails the risk of potentially not detecting all animals that need treatment. FECs are merely indicative for the presence of egg laying worms in the host. They do not confer exact information about to the magnitude of the worm burden. Faecal samples show high variations in egg count, as egg shedding can be intermittent, and sexually immature stages or a high proportion of male worms can lead to low egg counts (Osterman Lind et al., 2003; Uhlinger, 1993). Also, eggs are distributed unevenly within the faeces (Kraemer, 2005; Uhlinger, 1993). Because a standardized weight is used, a sample with high water content will appear to have a lower egg count than a drier sample, as it contains less faecal material per gram (Uhlinger, 1993). The age (Herlich, 1960) and immune status (Michel, 1986; Roberts et al., 1951) of the host also influence the faecal egg output. Another factor influencing the consistency of FECs is that techniques based on dilution inherently are subjected to large variance (Peters and Leiper, 1940), as they rely on the even suspension of the faecal mass. Repeatability is further negatively influenced by the necessity to select an aliquot of the mixture (Uhlinger, 1993).
There is no public information on the development of new drug classes in the near future (Kaplan et al., 2004). Consequently, the development of resistance by CYA to anthelmintic drugs is a worldwide problem and constitutes a growing threat to equine health. But even if new drugs were introduced onto the market, resistance to these might develop yet again. To be sustainable in the future, the control of helminths in grazing animals cannot be based on drugs alone. On the contrary, these need to be used in combination with alternative approaches and rather to support other control measures, such as grazing management and pasture hygiene (Barger, 1999; van Wyk et al., 2006).

Modern control schemes should be aimed to reduce the number of anthelmintic treatments, as a high frequency of treatments is the most important of the influencing factors for the development of resistance (Samson-Himmelstjerna, 2012), beside underdosing and the off-label use of drugs. To date, the efficacy of control programmes is rarely monitored by faecal egg counts (Herd, 1993; Nielsen et al., 2006b), and it is probably not an easy task for veterinarians to overcome traditional believes and practices, as some horse owners will refuse to skip an anthelmintic treatment, even if they are shown zero egg counts (Kaplan, 2002). Also, Little et al. dealt with a farm with multiple-resistant CYA and highly recommended pasture hygiene measures, but the farm management did not implement these recommendations (Little et al., 2003).

The excellent efficacy and wide use of the ML has led to high levels of complacency in the equine industry (Kaplan, 2002), and a possible development of resistance to these drugs will mean a definite impact on horse management as known to date.
Kaplan advocates that it is not advisable to rely on drug treatment as the sole means of controlling nematodes; he poses the question, however, if "the message of change in parasite control methods (...) will be heard (...) until Ivermectin resistance becomes common" (Kaplan, 2004).

Sustainable parasitic control programmes further require knowledge of seasonal larval ability, origin of larvae contributing to any peaks and climatic requirements for worm egg hatching, larval development and survival (Barger, 1999). It is important to raise awareness of these problems amongst equine practitioners as well as farm managers and horse owners (Herd, 1993), and veterinary surgeons should play a more important role in advising on worm control.
It is not to be expected that horse owners will trust in the innate or acquired resistance to helminths or even in the resilience their animal might develop if anthelmintic drugs are applied only rarely. Also, sport horses or brood mares, and even most hobby and leisure horses, need to be kept with only minimal exposure to worms, as their uses require a certain level of fitness and for means of animal welfare they cannot be exposed to the stress of worm burden. Hence, the transfer of information from parasitologists to practising veterinarians to horse owners is of great importance for the successful management of worm control.

A problem of the concept of the annual rotation is that those parasites not targeted by the drug used over the period of a year would need to be addressed separately. However, the use of e. g. IVM to treat larvae of bot flies (*Gasterophilus* spp.), on which PYR has no effect, in a PYR year would probably defeat the purpose of the programme. Owners should be encouraged to manually remove the eggs of bot flies, which are clearly visible, from the horses´ hair. Considering the high numbers and the relatively low level of handling involved, such measures are probably not feasible on large stud farms, but could be applied by individual horse owners. For the treatment of tapeworms, one could recur to Praziquantel in a non-PYR year, and double the PYR dose in a year when treatment with this drug is scheduled.

For the past years, researchers have been calling for a new approach to worm control that does not entirely rely on the use of anthelmintic drugs, including worming schemes based on FECs, FECRTs to monitor anthelmintic efficacy, and pasture hygiene and grazing management (Herd, 1993; Osterman Lind et al., 2007b). With the advent of developing resistance to the last remaining drug class, the ML, the time has come that these recommendations have to sternly be brought across to horse owners and to veterinarians in the field who are responsible for the management of the efficacy of the remaining drugs. Practising veterinarians need to become more involved in parasite control programmes (Kaplan et al., 2004; Stratford et al.,

2011), as their expertise is needed to develop viable treatment options tailored to the needs of each individual horse keeping farm.

6. Summary

Grazing horses in intensive management face pastures contaminated with infective stages of a number of endoparasites on a daily basis. The management of equine premises as well as the veterinarians have in the past heavily relied on readily available and affordable drugs to treat infestations with parasitic helminths. Due to this fact, the prevalence of the formerly very common Large Strongyles has declined, and the Cyathostomins (CYA) have become the main intestinal parasites of the horse. Their distribution is worldwide, and they carry a high potential of clinical disease. Traditionally, control programmes have been based on anthelmintic drugs, to which CYA are becoming increasingly resistant. Three different drug classes with different modes of action are commercially available, the benzimidazoles (BZ), the tetrahydropyrimidines and the macrocyclic lactones (ML). Resistance of CYA has been detected to all three classes. The objective of this study was to investigate the efficacy of two commonly used anthelmintic drugs, in the German Federal State of Brandenburg: Ivermectin (IVM), a ML, and Pyrantel embonate (PYR), a tetrahydropyrimidine. The efficacy of both drugs was investigated with faecal egg count reduction tests (FECRTs) in two consecutive years. For IVM, the aim was also to detect a possible shortening of the egg reappearance period (ERP), which is considered a first warning sign for the development of resistance.

A pre-selected population of horses kept on 24 farms scattered over the Federal State of Brandenburg was considered. These farms had shown a FEC for CYA higher than the average in a previous study, the horses had daily access to pasture or were kept in the field during the summer months. Premises that use anthelmintics infrequently, i.e. twice per year or less often (n=7), were compared with premises that worm their horses frequently, i.e. at least four times per year (n=17), since a risk factor associated with the development of resistance to anthelmintics is the frequent application of these drugs. In 2007, a FECRT with IVM was conducted in late summer/autumn on 23 of these 24 premises, with a total of 428 horses. In 2008, a FECRT with PYR was performed during the summer months on 21 of these 24 premises, and 414 horses were included.

Faecal samples were analysed using a modified McMaster technique with a detection threshold of 25 eggs per gram (EPG). The weight of each animal was estimated, and a girth measuring tape was used to validate the estimation. The anthelmintic drugs were then applied according to weight. Horses of all ages with the exception of foals younger than six months were included in the study.

The first FECRT (with IVM) was calculated in analogy to the recommendations of the WAAVP, which for cattle considers a FECR of <95% with a 95% LCL of <90% as ”Resistant”, and a result where only one of the two criteria is met as “Suspect resistant”

(Coles et al. 1992). These recommendations propose a value of <90% as indicative of resistance to BZ in horses but give no value for the 95% LCL. Hence, in line with other recent equine AR-studies (Lester et al. 2013; Relf et al. 2014) the cut off was set at 90% FECR, and the 95% LCL was set at 80% in this study. The LCL was calculated according to WAAVP recommendations.
For IVM, a FECR of 100% can be reported for 21 out of 23 farms (91.3%). On the remaining two farms (8.7%), one individual horse each had a faecal egg count (FEC) of 25 EPG, lowering total FECR to 99.73% and 98.33%, respectively. Anthelmintic drugs were used infrequently on both farms. A shortening of the ERP for IVM was not detected.

Controversy exists among parasitologists concerning the calculation and interpretation of FECRTs, with regards to a) at what % of FECR the threshold for resistance should be set, and b), as to which method of calculation should be used.
In this study, resistance was defined (i) in analogy to the recommendations for BZ by the WAAVP (mean FECR < 90%), with a lower 95% confidence limit (LCL) of <80% (Lester et al. 2013; Relf et al. 2014); and (ii) with the criteria as above, but also including the upper 95% limit (UCL) – the cut-off was set at UCL <95%, as suggested by Lyndal-Murphy et al. In order to assess the impact of the statistical method used, a total of three methods for the calculation of anthelmintic resistance were applied to the data sets of the FECRT for PYR:

Firstly, the method for calculating FECRTs as currently recommended by the WAAVP was used, and resistance to PYR was detected on four farms (19%). Secondly, a bootstrapping method was employed together with four different equations for the calculation of FECRTs. These four different equations gave varied results: with equation 1, a FECR <90% was obtained on seven farms, with equations 2 and 3 on four farms, and with equation 4 on one farm. Thirdly, a Markov Chain Monte Carlo (MCMC) method was applied to the data. Resistance was detected on 17 farms (73.9%). This accounts for the WAAVP criteria of interpretation. When applying the criteria suggested by Lyndal-Murphy et al., only Farm N° 2 was detected as “Resistant”, by all three methods of calculation.

There was huge variation in inference between statistical methods when using the WAAVP interpretation, but when using the Lyndal-Murphy interpretation all three statistical methods produced very similar recommendations in that there was conclusive evidence for resistance on yard No 2, but none of the other yards.
Of the four farms on which resistance was detected according to the method of calculation as recommended by the WAAVP, two use anthelmintics frequently, and two infrequently. The hypothesis that frequent worming might enhance the development of resistance was therefore not confirmed. However, it was not possible to determine the frequencies in which each drug class had been used in the past, as the horse owners generally did not keep exact records. It is

recommended that on these four farms routine FECRTs should be performed to monitor anthelmintic efficacy.

A general agreement needs to be established on the calculation of FECRTs, and thresholds for the detection of resistance need to be created for each drug or drug class. Reports of resistance of equine helminths to all classes of anthelmintics available from all over the world have brought to attention non-chemical approaches to nematode control, as the reliance on anthelmintic treatments alone is not considered sustainable for parasite control in the future. New drug classes are momentarily not expected on the market, as costs for the development of a new drug are high, and the pharmaceutical industry does not regard the market for horses and livestock large enough as to invest in research for new anthelmintic drugs. This implies that the preservation of anthelmintic efficacy has to be of priority. However, it is necessary to involve the expertise of the veterinarian in the adoption of new parasite control programmes, as epidemiological principles need to be incorporated for sustainable control.

7. Zusammenfassung (German Summary)

Untersuchungen zur Wirksamkeit ausgewählter Anthelminthika gegen Strongyliden-Infektionen bei Pferden im Bundesland Brandenburg

Pferde, denen unter intensiven Haltungsbedingungen Weidegang gewährt wird, sind täglich den infektiösen Stadien von Endoparasiten ausgesetzt und nehmen diese zusammen mit dem Weidegras auf. Pferdehalter, Stallbetreiber und auch Tierärzte haben in den letzten Jahrzehnten für die Wurmkontrolle stark auf die Wirksamkeit kommerziell erwerblicher Anthelminthika gesetzt. Dies führte zu einer Abnahme im Auftreten der vormals weit verbreiteten Großen Strongyliden und der mit ihnen assoziierten Kolik-Symptomatik. Mittlerweile sind Cyathostominae (CYA) die wichtigsten Endoparasiten des Pferdes; sie treten weltweit bei allen Equiden auf, und auch von ihnen geht ein hohes Pathogenitätsrisiko aus. Bekämpfungsmaßnahmen basieren traditionell auf dem regelmäßigen Einsatz von Anthelminthika; allerdings treten mittlerweile vermehrt Anthelminthika-Resistenzen auf. Derzeit sind auf dem Markt drei verschiedene Klassen von Anthelminthika erhältlich, die alle über einen unterschiedlichen Wirkmechanismus verfügen: Benzimidazole (BZ), Tetrahydropyrimidine und Makrozyklische Laktone (ML). Resistenzen der CYA sind allen drei Klassen gegenüber beschrieben worden. Ziel der vorliegenden Arbeit war es, die Wirksamkeit zwei verschiedener Anthelminthika im Bundesland Brandenburg zu überprüfen: Ivermectin (IVM), ein ML, und Pyrantel (PYR), ein Tetrahydropyrimidin. Zur Überprüfung der Wirksamkeit dieser Mittel wurden in zwei aufeinanderfolgenden Jahren zwei Eizahlreduktionstests (EZRTs) durchgeführt. Für den Wirkstoff Ivermectin war es außerdem Ziel der Arbeit, eine eventuelle Verkürzung der sogenannten Egg Reappearance Period (ERP), also der Zeit zwischen der Entwurmung und dem Wiederauftreten von Wurmeiern im Kot, festzustellen.

Auf Grundlage der in einer Prävalenzstudie aus dem Jahr 2006 im Bundesland Brandenburg erhobenen Daten wurden für die vorliegende Studie 24 pferdehaltende Betriebe ausgewählt. Diese wiesen im Vergleich zu anderen Höfen Brandenburgs eine überdurchschnittlich hohe Eiausscheidung von CYA auf. Die Pferde werden stundenweise bzw. ganztägig auf der Weide gehalten. Es wurden Betriebe, die höchstens zweimal pro Jahr entwurmen, mit Betrieben verglichen, die mindestens viermal pro Jahr entwurmen. Dieser Unterteilung liegt die Annahme zugrunde, dass häufiges Entwurmen die Entstehung von Resistenzen gegen Anthelminthika fördert. Im Jahr 2007 wurde im Spätsommer und Herbst mit IVM ein EZRT auf 23 der 24 Betriebe und mit insgesamt 428 Pferden durchgeführt. Im Sommer 2008 wurde mit PYR auf 21 der 24 Betriebe mit insgesamt 414 Pferden ein EZRT durchgeführt.

Die Analyse der Kotproben erfolgte mithilfe einer modifizierten McMaster-Technik. Die Nachweisgrenze lag bei 25 Eiern pro Gramm Kot (EPG). Die Bestimmung des Körpergewichtes erfolgte bei allen Pferden zunächst durch Schätzung, deren Ergebnis mit Hilfe eines handelsüblichen Maßbandes überprüft wurde. Die Dosierung des Anthelminthikums erfolgte nach Herstellerangaben gemäß Gewicht. Pferde aller Altersstufen ab einem Alter von sechs Monaten wurden in die Studie aufgenommen.

Der im Jahr 2007 mit IVM durchgeführte EZRT wurde analog zu den Empfehlungen der World Association for Advancement in Veterinary Parasitology (WAAVP) berechnet. Diese Empfehlungen sehen bei Rindern eine EZR <95% und ein unteres 95% Konfidenzintervall von <90% als einen Hinweis für das Vorhandensein von Resistenzen an. Für Pferde wird bei BZ eine EZR von <90% als auf Resistenz hinweisend angesehen, und es wird kein Konfidenzintervall genannt. In Analogie hierzu wurde in der vorliegenden Studie eine EZR von <90% mit einem unteren 95% Konfidenzintervall von <85% als Grenzwert angesehen. Für IVM wurde auf 21 der 23 Betriebe eine EZR von 100% festgestellt. Auf den übrigen beiden Betrieben (8,7%) wurde bei jeweils einem Pferd eine Eiausscheidung festgestellt. Diese war minimal und betrug jeweils 25 EPG. Dadurch wurde die EZR auf den Betrieben auf 99,7%, bzw. 98,3% gesenkt. Die Entwurmung erfolgte auf diesen zwei Betrieben zweimal im Jahr oder seltener. Eine Verkürzung der ERP wurde für IVM nicht festgestellt.

Der Grenzwert, ab dem von einer Resistenz gesprochen werden kann, wird in der Literatur kontrovers behandelt, ebenso, wie die Frage, welche Methode zur Berechnung der EZR angewendet werden sollte. In der vorliegenden Studie wurde das Vorhandensein von Resistenz nach zwei verschiedenen Kriterien definiert: 1. analog zu den Empfehlungen der WAAVP für BZ bei Pferden: EZR < 90% mit einem unteren 95% Konfidenzintervall von <80% (in Analogie zu Lester et al. (2013) und Relf et al. (2014)), und 2. zusätzlich mit dem oberen 95% Konfidenzintervall von <95% (von Lyndal-Murphy et al. empfohlen). Um die Auswirkungen verschiedener Berechnungsmethoden auf das Ergebnis beurteilen zu können, wurden drei verschiedene Methoden für die Berechnung der EZR auf die Daten des EZRT mit PYR im Jahr 2008 angewandt:

Zunächst wurde die EZR nach der von der WAAVP empfohlenen Methode berechnet, und es wurden auch deren Kriterien für die Detektion einer Resistenz angewandt; hiermit wurde auf vier Betrieben (19%) eine Resistenz festgestellt. Desweiteren wurde eine bootstrapping-Methode angewendet, mit der die Berechnung mit vier verschiedenen Formeln durchgeführt wurde. Diese vier Formeln ergaben sehr unterschiedliche Ergebnisse: mit Formel 1 wurde eine EZR <90% auf sieben Betrieben festgestellt, mit den Formel 2 und 3 auf jeweils vier Betrieben und mit Formel 4 auf einem Betrieb. Drittens wurde eine Markov Chain Monte Carlo (MCMC) Methode für die Berechnung verwendet, mit der auf 17 Betrieben (73,9%)

eine Resistenz festgestellt wurde. Mit den Kriterien nach Lyndal-Murphy et al. wurde für alle drei Berechnungsmethoden lediglich auf Hof N°2 eine Resistenz festgestellt.
Es gab große Unterschiede zwischen den Ergebnissen der statistischen Methoden mit den Kriterien der WAAVP; bei Einbeziehung des oberen 95% Konfidenzintervalls hingegen fielen die Ergebnisse aller drei Methoden sehr ähnlich aus, da jeweils ausschließlich Hof N°2 als resistent erkannt wurde. Daher sollte in Zukunft auch das obere Konfidenzintervall für die Interpretation der Daten von FECRT herangezogen werden.

Die Ergebnisse nach Berechnung gemäß den Empfehlungen der WAAVP lassen darauf schließen, dass die Wirksamkeit von PYR bei Pferden in Brandenburg im Allgemeinen als akzeptabel anzusehen ist. Dagegen kann bei vier Betrieben davon ausgegangen werden, dass eine nicht sensitive Population kleiner Strongyliden besteht. Von diesen vier Betrieben entwurmen zwei Betriebe höchstens zweimal pro Jahr und zwei Betriebe mindestens viermal pro Jahr. Die These, dass häufiges Entwurmen eine Resistenzentwicklung fördert, konnte in der vorliegenden Studie somit nicht bestätigt werden. Allerdings war es nicht möglich festzustellen, welche Wirkstoffklassen in der Vergangenheit eingesetzt wurden, da keiner der Betriebe exakte Aufzeichnungen hierüber führt. Es ist dennoch zu empfehlen, dass auf diesen Betrieben im Rahmen eines Programms zur Kontrolle des Endoparasitenbefalls regelmäßige EZRTs durchgeführt werden.

Es besteht die Notwendigkeit einer grundsätzlichen Übereinkunft zur Berechnung von EZRTs; außerdem sollten für die einzelnen Wirkstoffgruppen Grenzwerte definiert werden, ab denen vom Vorhandensein einer Resistenz ausgegangen werden kann. Berichte aus mehreren Ländern über vermehrt auftretende Resistenzen bei Helminthen des Pferdes haben alternative Bekämpfungsmaßnahmen in den Fokus gerückt. Da der Marktanteil der Anthelminthika für Pferde von der pharmazeutischen Industrie nicht als wichtig genug erachtet wird, um in die Entwicklung neuer Wirkstoffklassen zu investieren, kann eine zukunftsfähige Wurmkontrolle nicht ausschließlich auf der Verwendung von Anthelminthika basieren. Dem Erhalt der Wirksamkeit der vorhandenen Anthelminthika ist eine ebenso hohe Bedeutung beizumessen wie der Einbindung tierärztlicher Expertise in die Planung und Durchführung der Wurmkontrolle unter Berücksichtigung der Epidemiologie der Parasiten.

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9. Annex

9.1. List of Abbreviations

AM	Arithmetic Mean
AR	Anthelmintic Resistance
BZ	Benzimidazole
CI	Confidence Interval
CL	Confidence Level
CYA	Cyathostominae
ECR	Egg Count Reduction
EL	early larva(e)
EPG	Eggs per Gram
ERP	Egg Reappearance Period
FEC	Faecal Egg Count
FECRT	Faecal Egg Count Reduction Test
GM	Geometric Mean
IVM	Ivermectin
LCL	Lower Confidence Limit
LL	late larva(e)
MCMC	Markov Chain Monte Carlo
ML	Macrocyclic Lactones
PYR	Pyrantel
PZQ	Praziquantel
UCL	Upper Confidence Level
WAAVP	World Association for the Advancement of Veterinary Parasitology

9.2. Materials used

Arm-length examination gloves, Kruuse, Langeskov, Denmark
Capped bottles, Hecht-Assistent, Sondheim, Germany
Cooling box, and cooling packs, manufacturer unknown
Densitometer, manufacturer unknown
Digital scales, Sartorius, Göttingen, Germany
Examination gloves, Kimberley Clark, Roswell, USA
Funnels, plastic, manufacturer unknown
Glass jars with lids, manufacturer unknown
Graduated cylinders, 100ml, Duran, Hirschmann, Deutschland
Incubator, Heraeus, Hanau, Germany
Lubricant, VWR Darmstadt, Germany
McMaster slides, Chalex, Wallowa, USA
Microscope, “Axiostar”, Carl Zeiss Jena, Germany
$NaCl^-$, Honeywell, Riedel-de Haen, Seelze, Germany
Petri dishes, plastic, 9cm diameter, VWR Darmstadt, Germany
Pipettes, plastic, VWR Darmstadt, Germany
Tea strainers, mesh ca.0.8mm, manufacturer unknown
Tweezers, metal, manufacturere unknown
Vermiculite, Rajapack, Birkenfeld, Germany
Wash bottles, plastic, manufacturer unknown
Wooden spatulas, Hecht-Assistant, Sondheim, Germany

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11. Declaration of authorship

I hereby truthfully declare that I have carried out this thesis myself and without the help of any third party and that all literal quotations and other authors' ideas have completely been accounted for.

Berlin, 20-01-2015

Juliane Fischer