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ANTHELMINTIC RESISTANCE IN EQUINE PARASITES: MECHANISMS AND TREATMENT APPROACHES

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TREATMENT APPROACHES

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DISSERTATION

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A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture, Food and Environment
at the University of Kentucky

By

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2019

ABSTRACT OF DISSERTATION

ANTHELMINTIC RESISTANCE IN EQUINE PARASITES: MECHANISMS AND

TREATMENT APPROACHES

Anthelmintic resistance of parasites infecting livestock animals is a global problem resulting in decreased animal welfare and production losses. Horses are not exempt from this issue as wide-spread anthelmintic resistance exists among the equine cyathostomins and *Parascaris* spp. Of the three drug classes available for treating equine intestinal helminths anthelmintic resistance, defined as less than 90-95% drug efficacy, exist to all three. New pharmaceutical control regimens and the elucidation of parasite drug response mechanisms are needed.

Two studies were carried out evaluating combination deworming regimens. A population of cyathostomins with known resistance to the benzimidazole (BZ) and pyrimidine drug classes maintained in a herd of Shetland ponies was used. Fecal egg counts were performed every two weeks and used to evaluate drug efficacy. The first study evaluated the combination of a BZ and pyrimidine drug for four consecutive treatments, and compared the individual drug efficacies before and after combination use. The first combination treatment exhibited an additive effect at 76.6%, but the subsequent three combination treatments decreased to approximately 40%. There was no significant difference between the initial and final efficacies of individual drugs (BZ, $p=0.4421$; pyrimidine, $p=0.8361$). It appears the combination treatment selected for double-drug resistant adult parasites. The timeframe of this study (1 year) and the one year lifespan of adult cyathostomins prevented observations of combination treatment on subsequent generations, however given the sustainability of resistance in this cyathostomin population, it seems unlikely efficacy would improve over time. The second study examined the combination of a BZ drug with a macrocyclic lactone (ML) drug. This parasite population was 100% naïve to the ML drug class. This study was carried out in a similar manner to the first, except only two combination treatments were given. ML exhibited 100% efficacy when it was used alone, or in combination. The initial and final BZ efficacy did not significantly differ ($p=0.9890$). In summary, the results described herein do not support the use of combination treatments where resistance is prevalent, but more long term studies are needed to fully understand the long-term effects on subsequent generations.

The *in vitro* maintenance of *Parascaris* spp. provides opportunity for various molecular analyses. An objective motility scoring assessment allowed for continuous monitoring of worm viability. In this study, several saline solutions, nutrient supplements, environmental conditions, and Roswell-Park Memorial Institute medium 1640 (RPMI-1640) were evaluated for the longevity and viability of adult *Parascaris*

spp. Overall, RPMI-1640 resulted in better longevity (168 hours) and significantly better viability (p<0.0001) than any of the other saline solutions with or without nutrient supplementation. These findings were later used to identify response mechanisms of adult *Parascaris* spp. to *in vitro* drug exposure. Oxibendazole at 10 µg/mL for 24 hours and ivermectin at 1 µg/mL for three hours were employed, and worms were used for transcriptomic analyses to identify drug response mechanisms. The top four genes which were significantly different between drug treated and control groups were*: cyp4504C1, sup-9, frmd4a*, and *klhdc10.* It is hypothesized that *cyp4504C1* and *klhdc10* are drug detox mechanisms, while *sup-9* and *frmd4a* may be indirect response related to the drug effects. Their expression was further evaluated using quantitative RT-PCR, however there was no significant difference in any gene expression between groups. It should be noted that there are several limitations associated with the qPCR method, and the lack of significance should not rule out the possible involvement of these genes and more research on drug response mechanisms is needed.

In summary, there is very little research regarding combination deworming in horses, and their current use is largely due to some success for ruminant parasites, but the current work summarized herein does not support their use. Finally, until now the lack of *in vitro* methods for equine helminths has significantly delayed the elucidation of drug response mechanisms. This was the first whole-transcriptome approach for any ascarid parasite and uncovered proteins with possible involvement in drug metabolism or compensate for the toxic effects Overall, the research surrounding anthelmintic resistance in livestock helminths, particularly in horses, is lacking and the resistance crisis demands further investigation.

KEYWORDS: Equine parasite control, *Parascaris* spp., cyathostomins, anthelmintic resistance, drug mechanisms, combination deworming

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To my loving husband, Brandean Kenealy

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CHAPTER 1. INTRODUCTION

1.1 Helminth parasites of horses

As grazing animals, horses are continuously infected with a variety of intestinal helminths. Most notably, these include nematodes belonging to the super families Strongyloidea (strongyles), Ascaroidea (ascarids), Oxyuroidea (pinworms), Rhabditoidea (threadworm), Habronematidae (stomach worm) and Trichostrongyloidea (stomach worm, lung worm), and cestodes belonging to the Anoplocephalinae family. By way of arthropod intermediate hosts (flies/mosquitoes), horses are also at risk for infection by the super families Spiruroidea and Filarioidea. For the purpose of this literature review, only helminth parasites with strong evidence of anthelmintic resistance will be discussed in detail, namely the Strongyloidea and Ascaroidea.

1.2 Lifecycles and pathogenicity

1.2.1 Strongyloidea

Equine strongyle parasites are ubiquitous among grazing horses and are further classified into two sub-families, the Strongylinae (large strongyles) and Cyathostominae (small strongyles) (Lichtenfels et al., 2008). Over 50 species make up the Cyathostominae sub-family, while the Strongylinae consist of 14 species across 5 genera (*Strongylus, Oesophagodontus, Triodontophorus, Bidentostomum*, and *Craterostomum)*. Aside from the size differences emphasized by the subfamilies' epithet, the large globular buccal capsule differentiates the Strongylinae from the Cyathostominae, which have less prominent buccal capsules. (Lichtenfels et al., 2008). All horses, regardless of age, are at risk for strongyle infections and it is apparent that horses are unable to mount complete immunity to these parasites (Klei, 2000).

All equine strongyle parasites undergo a direct life cycle consisting of free-living stages (Figure 1.1). Adult parasites reside in the lumen of the cecum and large intestine where they undergo sexual reproduction. Female worms lay eggs which are shed with the horse's feces into the environment. All species undergo three larval development stages during the environmental phase. The first stage larva (L_1) develops within the egg,

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hatches, and subsequently matures into the second (L_2) and third (L_3) stage, otherwise known as the infective stage. Under favorable environmental conditions, strongyles can reach the infective stage as soon as 3-4 days after being expelled by the horse (Nielsen et al., 2007). Horses become infected upon ingesting the L_3 larva during grazing. Following ingestion, the larva molt to the L_4 and L_5 stage, where some species require extensive migration through various host tissues. Adult worms reside in the lumen of the large intestine as sexually mature adults (Ogbourne and Duncan, 1985).

Those belonging to the genus *Strongylus* are the only strongylids to undergo extra-intestinal migration through the horse during development from the L_3 to adult stage. These include *S. vulgaris, S. edentatus,* and *S. equinus. Strongylus vulgaris* is considered to be the most pathogenic nematode parasite infecting horses (Kester, 1975; Drudge, 1979) and will be the only *Strongylus* species mentioned herein. Following ingestion, migrating larvae reside within the cranial mesenteric artery (CMA) and its associated branches causing thickening of the arterial walls and emboli/thrombi formation (Duncan and Pirie, 1975). The lack of blood flow causes intestinal infarction which has been associated with peritonitis (Pihl et al., 2018), painful, agitated colic, and death (Duncan and Pirie, 1975; Drudge, 1979). The larvae develop to the L_5 stage within the CMA, before migrating via the blood stream to the submucosa of the large intestine. The larvae emerge and reside in the lumen of the intestine for the remainder of their existence. The pre-patent period for *S. vulgaris* is 5.5-7 months (Ogbourne and Duncan, 1985). Traditional deworming regimens implemented treatments as often as every 4 weeks in order to eliminate this parasite (Duncan, 1982). Although its prevalence and associated disease incidences have been significantly reduced (Herd, 1990), several studies report the presence of *S. vulgaris* on managed horse farms around the world (Nielsen et al., 2012, Singh et al., 2016; Salas-Romero et al., 2017; Lyons et al., 2014; Scare et al., 2018a).

The Cyathostominae have less extensive migration. Once ingested, the infective larvae encyst into the submucosa lining of the large intestine as the early L_3 (EL_3) stage (Love et al., 1999). Within the cyst, maturation continues to the late L_3 stage (LL_3) and then the L_4 stage. Upon development into the L_4, the larva ruptures the cyst and returns to the intestinal lumen, a process known as excystment. In the lumen, the larva matures into

the L_5 and adult stage. During the encysted stage, some larvae may undergo arrested development at the EL_3 stage (Eysker et al., 1984). Gibson (1953) and Smith (1976a,b) have reported arrested stages to persist for at least two years. The horse's immune response is thought to drive the arrestment process, as evidence of the arrested stages are not found in foals or horses naïve to cyathostomin infection (Reinemeyer et al., 1988; Chapman et al., 2002, 2003; Nielsen and Lyons, 2017). A large accumulation of larvae and mass excystment can cause the disease larval cyathostominosis. This disease process is characterized by watery diarrhea, dehydration, hypoproteinemia, and ventral edema (Love et al., 1999). In acute forms, this disease is fatal in 50% of cases (Reid et al., 1995). Deworming with an adulticidal treatment to remove the luminal cyathostomins has been known to 'trigger' mass excystment of the encysted larvae, seemingly to replace the recently removed luminal population (Reid et al., 1995). Younger horses (1-4 years old) and those recently dewormed are also at a higher risk for disease (Reid et al., 1995). While most infections do not manifest with clinical signs, the disease is more frequently reported in Europe (Giles et al., 1985; Love et al., 1992; Mair, 1993; Reilly et al., 1993; Mair, 1994; Mair and Pearson, 1995; Reid et al., 1995; Van Loon et al., 1995; Mair et al., 2000). Some reports also exist in Iran (Oryan et al., 2015), Canada (Peregrine et al., 2006; Wobeser and Tataryn, 2009; Zakrajsek, 2017), and the United States (Lyons et al., 2000).

1.2.2   Ascaroidea

Parascaris spp. are pathogenic and pervasive parasites that are most commonly found in horses <2 years of age (Clayton, 1986). Two species belong to this genus namely *P. equorum* and *P. univalens*, however it is suggested that the latter is the most prevalent (Nielsen et al., 2014; Martin et al., 2018). Regardless of species, infection occurs when foals ingest infective eggs containing a larva (Figure 1.2). The eggs, surrounded by a proteinaceous coat, hatches and upon reaching the small intestine, the newly emerged second-stage larvae penetrate the lining of the small intestine. The larvae travel to the liver where they remain for one week (Clayton and Duncan, 1979a*). Next, the larvae migrate to the lungs via pulmonary circulation (Clayton and Duncan, 1979a). Larval emergence from the local arterioles and capillaries into the alveoli causes

edematous and hemorrhagic inflammation. At this stage of infection, foals may present clinically with coughing and nasal discharge (Clayton and Duncan, 1978; Clayton, 1980). Approximately 2-4 weeks post-infection, the larvae are coughed up and swallowed only to return to the alimentary tract where they mature into dioecious adults within the small intestine. *Parascaris* spp. do not attach to the intestinal wall; instead they ingest digested feed competing with the host for nutrients (Clayton and Duncan, 1979a). This stage of infection can cause stunted growth, diarrhea, rough hair-coat, and impaction colic (Clayton and Duncan, 1978; Clayton, 1980; Cribb et al., 2006). Females lay eggs which are shed in the foals' feces, contaminating the environment and thus repeating the infection cycle. Patent infections occur within 79-110 days post-infection (Lyons et al., 1976; Clayton and Duncan, 1978; 1979a,b). In general, horses acquire immunity to *Parascaris* spp. infections by one year of age (Clayton and Duncan, 1979b; Fabiani et al., 2016), with adult worm burdens peaking around five months of age (Fabiani et al., 2016).

Foals' tendency to orally explore their environment and coprophagic behavior coupled with the prolific nature of the worms put foals at a high risk for infection. Prevalence of this parasite infecting foals (<1 year old) have been reported up to 83% (Laugier et al., 2012; Relf et al., 2013; Armstrong et al., 2014; Fabiani et al., 2016).Worm burdens reaching over 4,000 individuals in the small intestine following experimental infections have been reported (Lyons et al., 1976, 1996; Clayton and Duncan, 1979a), however a 16 year retrospective study examining 83 foal necropsies naturally infected with parasites found them to harbor <1,000 *Parascaris* spp. in the small intestine (Fabiani et al., 2016). Although ascarid impaction is the cause of <0.5% foal colics requiring surgery, the prognosis beyond one year is poor, and has been associated with recent deworming (Southwood et al., 1996; Cribb et al., 2006; Tatz et al., 2012).

1.2.3   Other equine helminth parasites

Three tapeworms infect horses, they are *Anoplocephala perfoliata*, *A. magna,* and *Anoplocephaloides mamillana*, but the former is the most common (Nielsen, 2016). Regardless of species, all equine tapeworms require an intermediate host, the oribatid pasture mite. The mite ingests tapeworm eggs that are present in the horse's feces. Inside the mite, the egg develops into the infective stage, known as the cysticercoid. Both mite

and cysticercoid are passively ingested by the horse during grazing. Some differences do exist between species, where *A. perfoliata* immature stages and adults reside at the ileo-cecal junction and attached to the cecum whereas *A. magna* and *A. mamillana* are found attached to the small intestine (Nielsen, 2016). Gravid proglottids, or body segments containing eggs, are released from the tapeworm. The proglottid is presumed to disintegrate as it passes through the digestive tract, and eggs are expelled with the horse's feces. Unlike strongyles and *Parascaris* spp., eggs are not excreted uniformly throughout the horse's feces (Nielsen, 2016). Some case reports have identified associations between tapeworm infection with colic, intussusception, and cecal rupture (Nielsen, 2016). Case-control studies further support this association as the horses that were diagnosed with various diseases processes (*i.e.* ileocecal colic, ileal impaction) were more likely to have a tapeworm infection (Nielsen, 2016).

The equine pinworm, *Oxyuris equi*, is first deposited into the environment with the horse's feces and the larva develops within the egg. The infective L_3 stage residing inside the egg is ingested. The L_3 invade the mucosal lining of the cecum and ventral colon where they develop in to the L_4 stage. The L_4 excyst and continue development to the adult stage within the lumen. The adult worms are generally found in the dorsal colon. Female worms travel to the rectum of the horse and deposit eggs around the perianal region (Reinemeyer and Nielsen, 2014). Pinworms elicit mild pathology, such as localized inflammation during the larval encystment process, and perianal irritation may occur following egg deposition (Reinemeyer and Nielsen, 2014).

Strongyloides westeri, a rhabditid parasite, is common in young foals (<4 months of age; Lyons, 1994), but rarely causes clinical disease. It has three routes of transmission, lactogenic transmission of L_3 larvae (Lyons et al., 1973), percutaneous penetration of L_3 larvae, or ingestion of L_3 larvae (Lyons, 1994). This parasite is also unique in that it can complete its entire lifecycle outside of the host, and only females are known to be parasitic. It is also one of the parasites that horses seemingly develop protective immunity to and adult horses are rarely found shedding eggs.

1.3   Diagnosis of equine intestinal helminths

Coprological examination for parasite eggs or larvae is the most commonly used method for characterizing intestinal helminth infections in horses (Nielsen et al., 2016). It

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is important to note that coprological detection is only representative of patent infections, but in most cases the pathogenicity of strongyle infections is caused by the migrating larval stages rather than adults (Duncan and Pirie, 1975; Love et al., 1999). There are some methods available for diagnosing the larval stages, as well as molecular based techniques.

1.3.1 Fecal egg counts

Numerous techniques are available for performing a fecal egg count (FEC), however in most cases they all follow the same general procedure. A small sample of fresh fecal material of known quantity is homogenized with a specified volume of flotation media. When passive flotation of the eggs is implemented, then a small sub-sample of the fecal slurry is loaded into a slide or counting chamber, and the sample is allowed to rest for 5-10 minutes for the eggs to float to the surface and subsequently be identified and quantified by microscopic examination (MAFF, 1986; Cringoli et al., 2017). Other techniques implement active flotation using a centrifuge. Here, the fecal slurry is poured into a conical tube, a cover slip placed on top, and centrifugation forces the eggs to the surface of the sample and make contact with the cover slip. The coverslip is then placed on a glass slide for identification and quantification of the eggs (Stoll, 1930; Egwang and Slocombe, 1982). Still, a recent technique does not rely on flotation at all, but rather a series of washes and filtering steps, and then utilizes image analysis for automatic identification and quantification of eggs (Slusarewicz et al., 2016; Scare et al., 2017).

While FECs can be used to identify a variety of equine helminth egg types, they are most commonly used to characterize strongyle infections, and for evaluating anthelmintic efficacy. It is important to note that the eggs of all equine strongyles are virtually identical, and cannot be differentiated to the sub-family, genus, or species to which they belong. Furthermore, a crucial concept to acknowledge is that a horse's FEC is not linearly correlated to their worm burden (Nielsen et al., 2010). Because strongyle infections are a commonality among grazing horses and do not reflect infection burden, the use of a FEC as clinical diagnostic tool is limited and instead it is recommended as a tool to guide parasite control programs (Nielsen et al., 2016; ESCCAP, 2018; Rendle et

al., 2019). Their implementation in control regimens is discussed in detail in section 1.3.1and use for evaluating anthelmintic efficacy in section 1.6.2.

Because of the lack of standardization in FEC methodology and the number of FEC methods currently available, it is important to consider each techniques accuracy, precision, sensitivity, and specificity. These parameters are being increasingly considered when selecting a method (Levecke et al., 2012; Godber et al., 2015; Noel et al., 2017; Scare et al., 2017; Paras et al., 2018; Went et al., 2018). The definitions for precision and accuracy are best illustrated using a bulls-eye target. Accuracy describes how close the obtained value (*i.e.* observed FEC) is to the true/known value (actual number of eggs in a sample). Consider throwing a dart at a target, your accuracy is how close you are to the bulls-eye. Precision describes how replicable repeated FECs are, regardless of the true count. This is relatable to how close together all of your darts are on the target, regardless of their relation to the bulls-eye. Sensitivity is the ability of the test to give a positive result, given the sample is indeed positive. Specificity is the ability of the test to give a negative result, given the sample is indeed negative. A low sensitivity results in a high false negative rate, while a low specificity results in a high false positive rate.

These statistics are largely attributed to sample preparation and the method employed, flotation medium used (if any), operator dependency, egg loss during sample preparation, and uneven egg distribution within the feces (Vidyashankar et al., 2012). Some techniques, such as the Cornell-Wisconsin (Egwang and Slocombe, 1982), the Stoll (Stoll, 1930), and the FLOTAC (Cringoli et al., 2010) utilize a centrifugation step. This serves to concentrate the eggs and improve egg recovery (Lester et al., 2014). However, even when both the Cornell-Wisconsin and FLOTAC techniques involved centrifugation and had a detection limit of 1 EPG, variation in precision between techniques still existed and was affected by the size of the subsample volume examined (i.e. coverslip, size of flotation chamber); Levecke et al., 2012). Despite the lower detection limit, some reports recognize the tendency for the Cornell-Wisconsin to have lower accuracy than other methods (Bosco et al., 2018; Paras et al, 2018). The equipment and time requirement of these centrifuge-based techniques often deter their routine use in diagnostic facilities. Other methods, such as the McMaster (MAFF, 1986) and Mini-FLOTAC (Cringoli et al., 2017) rely on passive ('table top') flotation which occurs within the egg counting

chambers. Currently, the McMaster method is the standard technique for evaluating FECs as recommended by the AAEP (Nielsen et al., 2016) despite several studies proving the Mini-FLOTAC to be more accurate and/or precise (Godber et al., 2015; Lima et al., 2015; Scare et al., 2017; Noel et al., 2017). Flotation media vary in specific gravity, where heavier eggs (*i.e. Trichuris* spp.) require a higher specific gravity (David and Lindquist, 1982), but more delicate ova (*i.e. Strongyloides* spp*., Giardia* spp.) can be distorted by these solutions making diagnosis near impossible. Differences in the specific gravity of common equine intestinal parasite eggs has also been reported (Norris et al., 2018), but it is still common practice to utilize a single flotation method for routine equine parasite diagnostics and FECs. Operator dependency also largely influences the outcome of the FEC (McCoy et al. 2005; Vidyashankar et al., 2012). Finally, precision has been regarded as the most important parameter when evaluating a FEC method as the largest source of variability is attributed to the variation between subsamples and repeated counts (Carstensen et al., 2013). Variation between repeated counts must be minimized in order for drug efficacy by the fecal egg count reduction test (FECRT, described in section 1.6.2) to be appropriately evaluated and avoid the incorrect interpretation of variation as a change in drug efficacy (Vidyashankar et al., 2012). Therefore, it is important to employ the use of replicate counts to obtain an EPG average rather than relying on a single FEC (Vidyashankar et al., 2012; Lester et al., 2014).

1.3.2 Larval cultures

As previously mentioned, the eggs of equine strongyles cannot not be differentiated on a FEC, but culturing the eggs to induce hatching and development to the L$_3$ stage can provide some further differentiation. This is achieved via a coproculture (fecal culture) followed by the Baermann technique and microscopic larval identification (Henriksen and Korsholm, 1983).

The methodology of coprocultures has been described by Henriksen and Korsholm (1983). Briefly, a sub-sample of freshly collected feces is mixed with tap water to achieve a 'dough-like' consistency. The sample is then suspended on a piece of cheese cloth within a home-made humidity chamber consisting of two plastic cups. The sample is incubated at room temperature at 24˚C for approximately two weeks and moistened

with tap water as needed. Subsequently, the sample is transferred to a Baermann apparatus for 12-48 hours for larval recovery. The Baermann apparatus is essentially a wine glass with small basin in the stem. The larvae's swimming nature coupled with gravity pull the larvae into the basin allowing for easy harvest. Larvae that have developed to the L_3 stage can be morphologically identified by their number and shape of intestinal cells, and the length of the larvae. Those belonging to the Strongylinae sub-family can be identified to species, except for *Triodontophorus* spp. which is only identifiable to the genus at this stage. Of the 50+ species belonging to the cyathostomin sub-family, the majority can only be characterized as a cyathostomin, but a few can be further identified to genus (*Poteriostomum* spp.) or species (*Oesophagodontus robustus*, *Gyalocephalus capitatus*) (Russel, 1948). *Trichostrongylus axei*, the stomach worm of horses, ruminants, pigs, and humans, can also be identified.

The Baermann technique alone is also used to recover live larvae, such as the equine lungworm, *Dictyocaulus arnfieldi*, from fecal samples (Mair, 1987). The same principles mentioned above are applied, except fecal culturing is not necessary prior to using the Baermann because the lifecycle of this parasite produces larvae in the feces instead of eggs. Live, immature cyathostomin larvae (L_4) which have recently excysted from the intestinal mucosa can also be harvested in this manner and may be indicative of larval cyathostominosis (Olsen et al., 2003).

As previously mentioned, this technique is primarily used to diagnose the presence of the pathogenic *S. vulgaris* on a farm. However, one limitation is that this method only reflects patent infections, and the pathogenic stage of *S. vulgaris* are the migrating larvae which cannot be diagnosed with this technique. In most cases, *S. vulgaris* is diagnosed at the farm level, as horses housed together are under the same infection pressure, and this can be used to guide anthelmintic treatments of the herd (Nielsen et al., 2016). This is discussed further in section 1.4. It is important to note that the negative predictive value is reportedly only 0.37, and false-negative results are likely to occur (Nielsen et al., 2010). Therefore, a technique capable of identifying the larval stages would be preferred, and is further described in section 1.3.4.

1.3.3 Molecular diagnostics

The use of molecular based diagnostic techniques for equine helminths is limited. At present, only five methods exist. An rDNA-based PCR method (Drögemüller et al., 2004a) is described for detecting the equine tapeworm, *Anoplocephala perfoliata,* in the feces. This method has a detection sensitivity of 500 femtograms, but only 0.5-1 g of fecal material can be used without risk of diluting the DNA due to increasing the volume of reagents. This small amount of feces would likely decrease the sensitivity of this method under field conditions as equine tapeworm eggs are not uniformly distributed in the feces (see section 1.2.3), and sensitivity was only marginally better than traditional coprological examination methods (Traversa et al., 2008). Later, a multiplex PCR assay was developed capable of identifying the DNA of all three equine tapeworms (*A. perfoliata, A. magna,* and *Anoplocephaloides magna*). The assay can detect the DNA from whole worms, and from pure or mixed species infections from eggs in the feces. It has a detection limit of 50 EPG and uses five grams of feces (Bohórquez et al., 2015), which does not provide an advantage over most FEC methods. Regarding strongyles, there is a PCR-ELISA capable of detecting six cyathostomin species (Hodgkinson et al., 2003, 2005), and a reverse line blot assay has been developed and validated for detecting 21 species of cyathostomins and all three *Strongylus* species (Traversa et al., 2007a, Cwiklinski et al., 2012). However, these molecular methods only provide qualitative results and cannot provide information on the proportion of each species present within the sample. Lastly, a *Strongylus vulgaris* specific PCR has been described and validated for the detection and semi-quantification of *S. vulgaris* DNA present in a fecal sample (*i.e.* parasite eggs; Nielsen et al., 2008).

1.3.4 ELISA methods

A serum antibody ELISA (Proudman and Trees, 1996a,b; Kjaer et al., 2007) and saliva antibody ELISA (Lightbody et al., 2016) are available for detecting the tapeworm infections in horses, however it should be noted that horses can remain antibody positive for up to five months after treatment (Proudman and Trees, 1996b). Both tests are

commercially available in Europe. A *Strongylus vulgaris* specific serum antibody ELISA has been validated for detecting the pathogenic migrating larval stages present in the CMA and its associated branches (Andersen et al., 2013), but this technique is not available commercially.

1.3.5 Other diagnostic methods

It is important to note that while equine tapeworms (*Anoplocephala perfoliata*) and pinworms (*Oxyuris equi*) are intestinal helminths, the presence of their eggs in feces is sporadic. Because of the irregular release of tapeworm eggs from the proglottids their presence in a sub-sample examined for diagnostic purposes produces variable results regarding sensitivity. Increasing the amount of feces examined and use of flotation enhanced by centrifugation can greatly improve the diagnostic sensitivity (Meana et al., 1998; Slocombe, 2004; Tomczuk et al., 2014). Pinworm eggs are not excreted in the horse's feces, but rather deposited around the perianal region of the horse and therefore rarely found in the feces unless eggs happen to make contact with feces as they are deposited, or the sample is rectally collected and eggs get onto the collector's glove. A more practical diagnostic technique is to stick a piece of scotch-tape on the perianal region and subsequently microscopically examine it for pinworm eggs (Reinemeyer and Nielsen, 2014).

1.4 Control of equine parasites

Historically, it was recommended to treat horses every 4-6 weeks in order to suppress parasite egg output and pasture contamination, thus reducing the risk of clinical disease. This was known as the interval-dose program (Drudge and Lyons, 1966; Duncan, 1982). As new drugs came to market, they were recommended for use in a rotational deworming manner, where horses were still treated every 4-6 weeks (Duncan, 1982). Traditionally, *S. vulgaris* was the target parasite and the treatment intensity was an attempt to eliminate this parasite (Drudge and Lyons, 1966). However, the frequent anthelmintic treatments led to substantial anthelmintic resistance by other parasites to all three of the available drug classes for equine use (Peregrine et al., 2014), which is further

discussed in section 1.7 The wide-spread anthelmintic resistance status, particularly harbored by cyathostomins and *Parascaris* spp., has led to new approaches for parasite control. These approaches are presented in various guideline papers, namely the American Association of Equine Practitioners (AAEP) Parasite control guidelines (Nielsen et al., 2016), the European Scientific Counsel for Companion Animal Parasites (ESCCAP) A guide to the treatment and control of equine gastrointestinal parasite infections (ESCCAP, 2018), and the UK-Vet Equine deworming: a consensus on current best practice (Rendle et al., 2019). It is important to note that parasite epidemiology varies with regional climatic conditions, and should be considered when implementing a control program. Horse age is also an important component because horses gain some immunity to intestinal helminths which is largely influenced by age and previous exposure. All of the aforementioned guidelines incorporate biological and pharmaceutical control methods. Most of the recommendations are echoed between the guidelines, and these commonalities are outlined below.

1.4.1 Biological control

The importance and means of biological control are primary considerations for parasite control guidelines. Limiting the abundance of parasite infective stages in the environment, otherwise known as the infective pressure, and disruption of the parasitic lifecycle will directly decrease the number of parasites available for infection. There are several components of effective biological control, including manure removal and pasture hygiene, proper manure composting, pasture resting and/or mixed-species grazing, low stocking density, and quarantining new animals. (Nielsen et al., 2016; ESCCAP, 2018; Rendle et al., 2019).

Timely manure removal (*i.e.* before development of the infective stage) from stalls and pastures has proven beneficial in the control of cyathostomin parasites (Herd, 1986a,b; Corbett et al., 2014), but it can be very labor intensive. Pasture vacuum technology does exist and has proven an efficient method for pasture hygiene and parasite control, but is also very costly (Herd, 1986b). The collected manure must also be properly disposed of and not spread back onto the pasture. Manure composting has been shown to kill the parasite environmental stages for equine strongyles and *Parascaris* spp.

when internal compost pile temperatures reached approximately 35-55˚C (Gould et al., 2013). Frequent pasture rotation to disrupt the strongyle lifecycle has been shown to reduce strongyle FECs when implemented on at least a monthly basis (Relf et al., 2013). Mixed or alternate grazing is when another species, such as sheep, cattle, or goats, reside on the pasture with the horses, or are turned out onto the paddocks after the horses have grazed it. This method is useful in disrupting parasite lifecycles, but is only effective if the different hosts do not share the same parasites. It has been proven to reduce strongyle egg shedding in horses, but did increase the infection prevalence of *Trichostrongylus axei*, a common parasite of ruminants not normally found infecting horses (Eysker et al., 1983). In cattle, high stocking rates leading to over-grazed pastures are directly associated with increased signs of parasitism due to high parasite infection pressure and decreased nutritional status (Bransby, 1993). No studies have directly examined these effects in horses, but maintaining lower stocking density is still encouraged. Finally, horses new to the farm especially those with an unknown deworming history, are recommended to be dewormed with a purge dewormer (*i.e.* moxidectin) and then quarantined for 3-4 days before allowed turnout with the other horses. This is to reduce the transmission risk of parasites that are considered more pathogenic, such as *S. vulgaris.*

Because foals are subject infection by other parasite species typically absent from adult horses (i.e. *S. westeri* and *Parascaris* spp.), it is recommended to only turn foals out onto a 'clean' pasture and to rotate the pastures annually between foal crops. *Parascaris* spp. eggs are known to remain infective on pasture for more than a single grazing season, and maintaining foals on the same pasture for consecutive years will continually increase the infection pressure for the subsequent foal crops. *Parascaris* spp. eggs are surrounded by a sticky proteinaceous coat allowing them to reside on vertical surfaces. Proper farm hygiene can help reduce the risk of transmission. Foals are also at risk for infection by *S. westeri,* which can be transmitted vertically through the mare's milk (Lyons et al., 1973). In general, foals are not at risk of serious clinical disease from *S. westeri* infection, but proper hygiene, deworming the mare prior to foaling, can help reduce transmission.

1.4.2   Pharmaceutical control

At present, there are four drug classes approved and available in for the control of equine helminth parasites namely, the benzimidazoles (BZ), pyrimidines, and macrocyclic lactones (ML), while the fourth drug class, praziquantel, is effective only against the equine tapeworm (*Anoplocephala perfoliata*). As opposed to anthelmintic use in other species, equine formulations are only available for oral administration (Nielsen et al., 2016). Because the focus of this literature review is anthelmintic resistance, the praziquantel drug class will not be covered herein as there is no evidence of anthelmintic resistance against it.

1.4.3   Control regimens

While biological regimens can offer effective parasite control, the regular implementation of the aforementioned techniques is cumbersome and rarely implemented frequently enough for adequate control. Therefore, chemotherapy is often used to supplement. However, due to the anthelmintic resistance crisis, the AAEP, ESCCAP, and the UK-Vet equine parasite control guidelines recommend a balance of reduced treatment frequency while still preventing clinical disease. Horse age is a major contributor to parasite management practices, and therefore parasite management for adult horses will be described separately from foals/weanlings/yearlings.

1.4.3.1   Parasite control for adult horses

Strongyles are the primary focus of parasite control of adult horses, where cyathostomins are known to make up 99-100% of the total worm burden (Nielsen et al., 2010). Because of the high levels of anthelmintic resistance among cyathostomin populations and the high prevalence of infection, the overarching goal is not to eradicate a horse's cyathostomin burden, but rather to limit the infection pressure in the environment, maintain anthelmintic efficacy by decreasing the total number of treatments given on a farm, and prevent clinical disease. These strategies can be classified as selective-therapy, strategic-based deworming, or a combination of the two.

1.4.3.2 Selective therapy

Selective therapy regimens focus on decreasing the overall pasture contamination level of strongyle parasites by targeting high-egg shedding horses with anthelmintic treatments. Individual horse egg shedding levels are determined by a FEC, and horses are subsequently categorized as a low, medium, or high egg shedder. The thresholds are arbitrary, but in general most leading experts consider low egg shedders as 0-200 EPG, medium shedders as 201-500 EPG, and high shedders as >500 EPG. Healthy horses generally maintain a consistent egg shedding level throughout their adult life (Döpfer et al., 2004; Nielsen et al., 2006; Wood et al., 2013), but horses can switch egg shedding categories and therefore FECs are recommended on an annual basis. Under the selective therapy regime, it is recommended to treat low-egg shedding horses only once to twice a year, whereas moderate and high egg shedding horses should be treated 3-4 times per year. The parasite control guidelines recommend that all treatments be given around the grazing season when environmental conditions favor larval development on the pasture and infection pressure is the highest. Only drugs with known efficacy on the given farm should be used, and in most cases a ML drug is the most appropriate choice. As discussed in section 1.6.2, drug efficacy can be monitored using FECRT. It is recommended for at least one annual treatment to target tapeworms, bots, and encysted cyathostomins. For tapeworms, the guidelines suggest to give this treatment in the late fall or early winter when the transmission period for tapeworms has ended.

1.4.3.3 Strategic deworming

Strategic deworming can take on many different definitions of approaches that one considers 'strategic,' however the most common definition requires the maintenance of horses based on their age and the current season/region. Foals and young horses (<5 years old) are considered as a group and their control regimens is described in the section below. Healthy, adult horses (≥5 years old) are also considered as a group. Strategic deworming seeks to decrease the overall infection pressure by treating all adult horses 3-4 times per year with an effective dewormer.

1.4.3.4 Parasite control regimens for foals, weanlings and young horses

Young horses, including foals, weanlings, and yearlings, require a different parasite management approach because *Parascaris* spp. is considered ubiquitous among foals, and yearlings are presumed to be high strongyle egg shedders. It is recommended to give the first anthelmintic treatment around two months of age to remove immature stages of *Parascaris* spp. and prevent an immediate high worm burden. A BZ drug is the drug of choice as it does not have a paralytic mode of action (see section 1.5.1). Paralytic drugs, when given in the presence of a high *Parascaris* spp. worm burden, have been associated with impaction colic of the small intestine (Southwood et al., 1996; Cribb et al., 2006; Tatz et al., 2012). It is suggested for foals to be treated again around the time of weaning, and to use a FEC to determine if the target parasite is *Parascaris* spp. or strongyles and justify the choice of drug class to ensure effective treatment. The last two treatments are given at nine and 12 months of age targeting strongyles. Similar to the adult horses, it is recommended for foals to receive a treatment for tapeworms around 9-12 months of age. Yearlings are assumed to have minimal immunity to strongyle infections, and are considered high-egg shedders and at a higher risk for parasitic disease. Therefore, the guidelines suggest for yearlings to receive 3-4 treatments targeting strongyles, and to monitor their egg–shedding status thereafter. Only drugs that have been previously determined efficacious on that farm should be used. If the efficacy status is unknown, a macrocyclic lactone drug is recommended as it is considered the most efficacious (see section 1.7.1).

1.5 Anthelmintic drug classes

1.5.1 Benzimidazoles

1.5.1.1 Formulations and uses

The BZ drug class, the first broad spectrum anthelmintic with high efficacy, was introduced in 1961 (Brown et al., 1961). Since its introduction, numerous BZs have been

developed, including but not limited to, thiabendazole, mebendazole, cambendazole, oxibendazole (OBZ), luxabendazole, parabendazole, albendazole sulphoxide, fenbendazole (FBZ), oxfendazole, flubendazole, febantel, netobimin, thiophanate, and triclabendazole (McKellar and Scott, 1990; Martin, 1997). Febantel and netobimin are considered pro-benzimidazoles, meaning that they are converted to the active drugs, FBZ and albendazole, respectively, once metabolized in the animal (McKellar and Scott, 1990; Martin, 1997). The drugs used, routes of administration, targeted parasite species, and efficacies vary across the domestic animal host. Preparations vary by species and drug formulation, but include drenches (oral suspension), pastes, powder, capsules, granules, pour on, pellets, boluses, and intra-ruminal injectors (McKellar and Scott, 1990).

For equine intestinal parasites, the efficacies have been evaluated for mebendazole (8.8 mg/kg; Colglazier et al., 1977; Drudge et al., 1974), cambendazole (20 mg/kg; Colglazier et al., 1977), FBZ (5, 15, 30, and 60 mg/kg; Colglazier et al., 1977; Drudge et al., 1975; Duncan et al., 1977; McBeath et al., 1978), albendazole (2.5, 5, and 10 mg/kg; Colglazier et al., 1977; Drudge et al., 1984), OBZ (10 mg/kg and many other dosages; Kates et al., 1975; Drudge et al., 1979, 1981, 1984; Tolliver et al., 1993), oxfendazole (many dosages; Lyons et al., 1977; Tolliver et al., 1993), thiabendazole (44 mg/kg; Drudge et al., 1984), and febantel (6, 12, and 24 mg/kg; Drudge et al., 1984). The majority of these drugs were considered efficacious against luminal stages of large strongyles, cyathostomins, *Parascaris* spp., and *O. equi*. However, FBZ (5 mg/kg) exhibited variable efficacy against *Parascaris* spp. and immature *O. equi* (Drudge et al., 1975). Oxibendazole (10 mg/kg) also reduced the number of *S. westeri* eggs found in the feces (Drudge et al., 1981). Oxfendazole and FBZ were reported efficacious against *T. axei* and *D. arnfeldi*, respectively, whereas mebendazole was effective against both (McKellar and Scott, 1990). None of the aforementioned drugs exhibited efficacy against *Gasterophilus* spp. or *A. perfoliata.* Only elevated dosages of FBZ exhibited some larvicidal efficacy, where 7.5 mg/kg administered for five consecutive days reduced the number of encysted cyathostomins by 95.3% (Duncan et al., 1998). Single dosages of 30 or 60 mg/kg exhibited some efficacy against migrating large strongyles (Duncan et al., 1977) and 10 mg/kg has also reduced the number of migrating *Parascaris* spp. by 99.8% (Vandermyde et al., 1987). Presently, only OBZ (10 mg/kg) and FBZ single dose (5

mg/kg) or a five-day regimen 10 mg/kg/day is available for equids in the United States (Nielsen et al., 2016).

1.5.1.2   Mode of action

The mode of action of BZs is to disrupt microtubule polymerization (Lacey, 1988). Microtubules are composed of heterodimers consisting of α- and β- tubulin subunits. Microtubules are considered to have a '+' and '-' end, where polymerization occurs at the '+' end. Growth occurs in a conveyor-belt mechanism, where heterodimers dissociate from the microtubules at the '-' end and then are added back to the '+' end (Mandelkow and Mandelkow, 1990). The BZ binds to nematode β-tubulin preventing microtubule polymerization resulting in shortening of the molecule (Dawson et al., 1984; Lacey, 1988, Martin, 1997). Microtubules are essential components for cellular structure, the mitotic spindle, and for transporting molecules across the cell membrane. The inhibition of microtubule formation disrupts cell structure and energy metabolism essentially causing cellular disequilibrium and leading to parasite death (Lacey, 1988; Martin, 1997).

1.5.2   Cholinergic agonists

1.5.2.1   Formulations and uses

The cholinergic agonists can be further divided into three subgroups, namely the imidazothiazoles which consists only of levamisole, the tetrahydropyrimidines (pyrimidines, PYR) which consists of pyrantel, morantel, and oxantel, and lastly the amino-acetonitrile derivatives consisting of only monepantel (Martin, 1997; Abongwa et al., 2017; Lecová et al., 2014). Levamisole was brought to market in 1970 and is widely used in livestock, but is not marketed for use in horses or small animals. Monepantel is the newest cholinergic agonist drug. It acts on a different nAChR sub-family than the other cholinergic agonists (Lecová et al., 2014) and is proven to be effective against ruminant parasitic nematodes resistant to other anthelmintics (Kaminsky et al., 2011). Monepantel is not marketed for use in horses.

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The only cholinergic agonist marketed for equine use is pyrantel, which was developed following the construction of the intermediate compound, tetrahydropyrimidine (Austin et al., 1966; McFarland et al., 1972). Pyrantel exists in three salt formulations, including pamoate (embonate), hydrochloride, and tartrate, but only pamoate (embonate) and tartrate are used in horses (Sheehan et al., 2016; Reinemeyer, 2016). Pyrantel is poorly absorbed across the intestinal wall, which increases safety margins and luminal efficacy, but prevents the opportunity for larvicidal efficacy (Gokbulut et al., 2001).

Pyrantel pamoate (embonate) is available for horses as both a suspension and paste formulation (6.6mg/kg) (Reinemeyer, 2016). The suspension is labeled for removal of *S. vulgaris* and cyathostomins (Reinemeyer, 2016) with 100% efficacy (Lyons et al., 1974; Slocombe and Smart, 1975; Boersema et al., 1996). It is also labeled against *S. edentatus* (Reinemeyer, 2016), but with efficacy <90% (Lyons et al., 1974). The paste formulation removed *S. vulgaris*, *S. edentatus*, and small strongyles at 100% efficacy (Drudge et al., 1984). Luminal stages of *Parascaris* spp. have been sufficiently reduced by the suspension (Lyons et al., 1974) and paste formulation (Reinemeyer et al., 2010a). Both formulations are labeled for the control of pinworms where the oral suspension administered by stomach tube has exhibited 100% efficacy against the L_4 and adult stages present in the lumen (Lyons et al., 1974). Later, Reinemeyer et al. (2010b) found administration of the paste formulation at double the labeled dose (13.2mg/kg) to reduce adult and L_4 pinworms by >90%. Administration of a double-dose (13.2 mg/kg) is also effective against *A. perfoliata* (Lyons et al., 1989).

Pyrantel tartrate (2.64 mg/kg) is administered as a daily feed additive to horses and serves as prophylactic rather than therapeutic treatment (Reinemeyer, 2016). It was effective against large strongyles and cyathostomins (Lyons et al., 1974) and both luminal immature and adult stages of *Parascaris* spp. (Valdez et al., 1995). It is also labeled for efficacy against immature and adult pinworms, but studies report <90% efficacy when given at the labeled dose (2.64 mg/kg) as a daily feed additive for 30 days (Valdez et al., 1995), or at an elevated dose (7.2 mg/kg, 4.6 mg/kg) via stomach tube (Lyons et al., 1974). Valdez et al. (1995) also reported pyrantel tartrate to effectively eliminate luminal stages of *Parascaris* spp. Pyrantel tartrate has prevented accumulation

of *S. vulgaris* larvae present in the CMA (Reinemeyer et al., 2014), but is still labeled for larvicidal efficacy. Efficacy against equine tapeworms is also reported (Greiner and Lane, 1984; Lyons et al., 1997), but label claims have not been made.

Morantel exists as two salt formulations, namely morantel citrate and morantel tartrate. Morantel citrate is only available in Australia for porcine and small ruminant parasites (Reinemeyer, 2016). In the United States, morantel tartrate is only marketed for use in cattle and goats. Australia and New Zealand have approved morantel tartrate for use in horses as a paste or pelleted feed additive, and it exhibits very similar efficacies to that of pyrantel pamoate (Reinemeyer, 2016). Oxantel only exhibits efficacy against the canine whipworm (Howes, 1972; Rim et al., 1975).

1.5.2.2 Mode of action

Drugs of the cholinergic agonists drug class act as agonists of the nicotinic acetylcholine receptors (nAChR). These neurotransmitters are present at the neuromuscular junction of the parasite's somatic and pharyngeal muscle cells. The drug, coupled with the natural ligand, acetylcholine, opens this ligand-gated ion channel for an extended period of time allowing an increased flow of cations (Na^+ and Ca^{2+}). The influx of cations causes depolarization of the neuronal cell membranes and causing paralysis of the parasite rendering them unable to swim against peristalsis and subsequently expelled by the host (Harrow and Gration et al., 1985; Aceves et al., 1970; Aubry et al., 1970; Robertson and Martin, 1993).

Three nAChR subtypes have been identified, namely the L-subtype which is most sensitive to levamisole and pyrantel, the N-subtype conferring most sensitivity to nicotine, oxantel, and methyridine, and lastly the B-subtype which is most sensitive to bephenium (Martin et al., 2004; Levandoski et al., 2005; Qian et al., 2006). Regardless, nAChRs consist of five subunits, which may be homomeric or heteromeric, and subunit composition varies across nematode species. Subunit composition affects the binding properties of the receptor resulting in varying sensitivities to the anthelmintic agonists (Robertson et al., 2000; Bartos et al., 2006; Williamson et al., 2009; Boulin et al., 2011; Buxton et al., 2014; Sloan et al., 2015; Duguet et al., 2016; Whittaker et al., 2016; Blanchard et al., 2018). Additionally, genetic diversity among subunit orthologues of

different species exist (Neveu et al., 2010). The ligand-binding site occurs at the junction of two adjacent receptor subunits (Hibbs and Gouaux, 2011). The junctions are further individualized by the presence of aromatic residues which select different ligands, or anthelmintic drugs (Beene et al., 2004; Habibi et al., 2018). A glycine at position 153 (Rayes et al., 2004) is necessary for levamisole sensitivity, while pyrantel requires a glutamine at position 57 in addition to the glycine at 153 (Bartos et al., 2006) on subunit *unc-38* and *unc*-63. The residues present also differ across animal species (Lynagh and Pless, 2014). These variations present further challenges for studying drug pharmacology and anthelmintic resistance mechanisms (Kotze et al., 2014), but also allow for the possibilities of combination deworming to combat anthelmintic resistance by targeting receptors with alternative subunit compositions (Qian et al., 2006; Buxton et al., 2014).

In *C. elegans,* 29 nAChR subunits and 32 nAChR-like subunits are described (Jones et al., 2007), and it is well established that the alpha subunits UNC-38, UNC-63, LEV-8*,* non-alpha subunits UNC-29 and LEV-1 (Fleming et al., 1997; Richmond and Jorgensen, 1999; Culetto et al., 2004; Towers et al., 2005; Boulin et al., 2008), coupled with three ancillary proteins (UNC-50, UNC-74, and RIC-3; Boulin et al., 2008) form the L-nAChR. Contrastingly, the levamisole insensitive receptor (N-subtype) is a homopentamer, consisting of only ACR-16 subunits (Touroutine et al., 2005).

Neveu et al. (2010) characterized the sequences of *unc-38, unc-63, lev-1,* and *unc-29* in three strongylid parasites infecting small ruminants (*H. contortus, T. circumcincta* and *T. colubriformis*) and found high sequence similarity to the *C. elegans* and *A. caninum* (canine strongylid) orthologues. Diversity of the *unc-29* orthologue (L-nAChR) was identified in the ruminant parasites, where both *H. contortus* and *T. circumcincta* had four gene copies, and three genes were found for *T. colubriformis (*Neveu et al., 2010). Furthermore, the LEV-8 subunit is missing in several parasite species (Williamson et al., 2007; Neveu et al., 2010; Blanchard et al., 2018), including the small ruminant parasite *H. contortus* (Neveu et al., 2010; Laing et al., 2013). However, a closely related subunit, ACR-8 was identified in the L-nAChR of *H. contortus* (Fauvin et al., 2010) and later found to also be present in *C. elegans*, but not functionally necessary for the receptor (Hernando et al., 2012). The ACR-8 subunit is able to form a functional receptor when co-expressed with homolog subunits from *H. contortus* (*Hco-unc-38, Hco-unc-63, and*

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Hco-unc-29; Boulin et al., 2011) and the swine strongyle, *Oesophagostomum dentatum* (*Ode-unc-38, Ode-unc-63, and Ode-unc-29*; Buxton et al., 2014) in the *Xenopus* oocyte expression system. Both receptors were highly sensitive to levamisole, but not pyrantel or nicotine. Removal of the ACR-8 subunit resulted in sensitivity to pyrantel and nicotine, but not levamisole (Boulin et al., 2011; Buxton et al., 2014). The role of ACR-8 in levamisole sensitivity was confirmed in *O. dentatum* and revealed to be necessary for increased receptor calcium permeability upon levamisole binding (Buxton et al., 2014). Contrastingly, for *A. suum* only the *unc-38* and *unc-29* genes are necessary to form a levamisole sensitive receptor in the *Xenopus* oocyte system (Williamson et al., 2009). Finally, the *lev-1* homologue is reportedly missing in *H. contortus, T. circumcincta,* and *T. colubriformis* (Neveu et al., 2010).

While both levamisole and pyrantel target the same receptor subtype (L-nACh), their mode of action and binding properties are different and heavily dependent upon subunit composition and residues (Bartos et al., 2006; Habibi et al., 2018). This summary illustrates the complexity of cholinergic receptors and drug sensitivity across different species, and the limitations for making conclusions based on the *C. elegans* model. More studies are needed to explore the preferential binding sites and subunit compositions of pyrantel in parasitic nematodes.

1.5.3 Macrocyclic lactones

1.5.3.1 Formulations and uses

The MLs can be subdivided into the avermectins and milbemycins (Davies and Green, 1986), where the former group consists of avermectin (Stapley and Woodruff, 1982), ivermectin (IVM; Campbell, 1983), eprinomectin (Shoop et al., 1996), doramectin (Goudie et al., 1993), and selamectin (Banks et al., 2000), and the latter consists of milbemycin oxime and moxidectin (MOX; McKellar and Benchaoui, 1996). Avermectin was the first to be discovered in 1979 (Burg et al., 1979; Egerton et al., 1979), followed shortly by IVM, a derivative of avermectin (Campbell, 1983). The milbemycins were originally used as insecticides and milbemycin oxime was the first anthelmintic developed (Takiguchi et al., 1983). Moxidectin was then developed as a derivative of nemadectin, a fermentation product of milbemycin oxime (Carter et al., 1988). The

avermectins and milbemycins are considered to have a wide-safety margin as they have high potency and require only micrograms of drug to achieve desirable efficacy as opposed to the milligrams required of other drug classes (Shoop et al., 1995; McKellar and Benchaoui, 1996). The term 'endectocide' was coined upon the development of the MLs and discovery of efficacy against both endo- and ecto- parasites for both avermectins (Campbell, 1981; Geary and Moreno, 2012) and milbemycins (Ranjan et al., 1992; Williams et al., 1992; Webb et al., 1991; Lyons et al., 1992; Stansfield and Hepler, 1991).

In general, the MLs are available for use in livestock and companion animals, and are available as chewable tablets, liquid suspensions, drenches, boluses, injectable, pour-on/topical, paste, and oral gel (Vercruysse and Rew, 2002). Macrocyclic lactones (selamectin, IVM, MOX, milbemycin oxime) are the only drugs used for the prevention of heartworm in cats and dogs (Wolstenholme et al., 2015). In the United States, the only MLs approved for horses are IVM (paste, 0.2 mg/kg) and MOX (gel, 0.4 mg/kg). Abamectin is also available for horses in Australia and New Zealand, and IVM in a liquid suspension is available in Canada. Both IVM and MOX have exhibited efficacy against luminal cyathostomins and large strongyles (Torbert et al., 1982; Xiao et al., 1994; Costa et al., 1998), *Parascaris* spp. (Torbert et al., 1982; French et al., 1988; Lyons et al., 1992), and *O. equi* (Torbert et al., 1982; Lyons et al., 1992; Reinemeyer et al., 2010b). They were reported to be efficacious against migrating stages of *S. vulgaris* (Slocombe and McCraw, 1981; Lyons et al., 1992; Monahan et al., 1995) and *Parascaris* spp. (French et al., 1988; Monahan et al., 1995). Only MOX is labeled for larvicidal efficacy against encysted cyathostomins (Xiao et al., 1994; Reinemeyer et al., 2015; Bellaw et al., 2018). Specifically, in Australasia, Europe, and South America, MOX is marketed for efficacy against all encysted stages, whereas it is not labeled for efficacy against EL_3s in North America. Larvicidal efficacies at 2-3 weeks post-treatment for the EL_3 and LL_3/L_4 stages have been respectively reported at 0% and 62.6% (Xiao et al., 1994), 63.6% and 85.3% (Reinemeyer et al., 2015), and 73.8% and 74.6% (Bellaw et al., 2018). These findings indicate some differences in anthelmintic susceptibilities depending on the stage

of development and study design, however, further studies are warranted to elucidate the reasons behind this phenomenon.

1.5.3.2 Mode of action

Macrocyclic lactones act on glutamate-gate chloride (GluCl) channels, in either a direct manner or to potentiate the effects of the natural ligand, glutamate (Martin, 1997; Wolstenholme, 2012; Abongwa et al., 2017). These channels are specific to invertebrates and are highly expressed in nematode sensory and motor neurons making them excellent drug targets. They regulate locomotion, feeding behavior, and mediate sensory inputs (Wolstenholme, 2012). Ivermectin is also known to influence nematode reproduction and fecundity (Wolstenholme, 2012). The exact location of drug-neuronal interaction is unknown, but some evidence suggests that drug action may exist on major neurons located in the parasite amphids. Amphids are sensory organs used by nematodes to gather information about their environment, such as changes in the chemical surroundings (Guerreror and Freeman, 2004). The GluCl channel is a homologous pentamer, consisting of either α or β subunits (Cully et al., 1994). The α channel is IVM sensitive (Cully et al., 1994). Six GluCl genes have been identified in *C. elegans*, namely *glc-1, glc-2, glc-3, glc-4, avr-14,* and *avr-15*, where both *avr-14* and *avr-15* can produce at least eight additional subunits through alternative splicing events (Yates et al., 2003). While *avr-14* and *glc-2* are present in all GluCl gene families of parasitic nematodes studied, but the presence of other and additional genes varies between species (Wolstenholme, 2012). This variability complicates the unveiling of drug and resistance mechanisms. The gamma-aminobutryic acid (GABA) receptors are a secondary target of MLs (Martin, 1997; Yates et al., 2003; Prichard et al., 2012).

Macrocyclic lactone binding to the GluCl and GABA receptors causes an influx of chloride ions (Cl⁻) into the cell leading to hyperpolarization, or negative membrane potential. This inhibits neurotransmission leading to flaccid paralysis and subsequent expulsion of the parasite with peristalsis (Martin, 1997; Wolstenholme, 2012). Binding is essentially irreversible (Wolstenholme, 2011, 2012). These receptors are also known to be present on the pharyngeal muscle of *Ascaris suum* and application of IVM inhibits

pharyngeal pumping (Brownlee et al., 1997). It is interesting that inhibition of the pharyngeal muscle requires much lower concentrations of IVM than the drug concentrations needed to observe effects on the somatic musculature. This observation has been reported in *A. suum* (Brownlee et al., 1997), *Haemonchus contortus* (Geary et al., 1993), and *C. elegans* (Avery and Horvitz, 1990).

While all MLs are hydrophobic molecules consisting of a 16-member macrocyclic lactone ring, there are several differences between the structures of avermectins and milbemycins (Prichard et al., 2012), most notably is the presence of a disaccharide group at carbon-13 of avermectins (Davies and Green, 1986). This structural difference affects the affinity for the GluCl receptors; three of the proposed binding sites for IVM are not available for MOX. It is known that MOX requires a higher concentration than IVM to achieve the same anthelmintic efficacy (Ardelli et al., 2009; Prichard et al., 2012), which is reflected by the different labelled dosages for horses (0.2 mg/mL for IVM and 0.4 mg/mL for MOX). Both drug groups are highly lipophilic and readily absorbed into the fat of the host awarding them some larvicidal efficacy, but MOX is known to have higher concentrations in fat tissues than IVM (McKellar and Benchaoui, 1996) which is associated with MOX having persistent efficacy up to 2-3 weeks post treatment (Vercruysse et al., 1998).

1.6   Anthelmintic resistance
1.6.1   General overview of anthelmintic resistance

As previously discussed, anthelmintic resistance is an ever-increasing problem affecting parasites of small animals, livestock, and horses. Despite it being a world-wide issue, the definition of resistance is debated as some consider it as total drug failure while others define it as any decrease in efficacy, or perhaps when the maximum dose is no longer effective (James et al., 2009). It is widely accepted that anthelmintic resistance developed as a consequence of frequent treatment regimens, and is exasperated by the current failure to condemn these traditional practices and implement new routines (Van Wyk, 2001). Furthermore, the lack of knowledge pertaining to the pharmacological properties of anthelmintic drugs and misinterpretation of their interactions with host-related factors leads to incorrect use (Lanusse et al., 2014).

Historically, it was proposed that the genes driving resistance were present at low levels in susceptible populations, but were associated with a fitness loss (Prichard, 1990). Fitness loss is commonly represented by a decrease in fecundity to represent a decrease in the overall heredity of the resistance alleles. However, under the selection of anthelmintic treatment, worms harboring these genetics had a genetic advantage and the resistance alleles within the population would increase in frequency (Prichard, 1990). Recent work suggests that there are many possible mechanisms at work, which may occur concurrently. Some of these theories are that mutations may occur spontaneously and provide an advantage at the time of treatment, resistance is due to recurrent mutations, or the so-called 'resistance gene' is brought in from another source due to animal trafficking (Gilleard and Beech, 2007). These genetic changes may be due to heritable changes at the genetic/epigenetic level following drug exposure, and/or due to changes in the drug-target interactions (James et al., 2009). More specifically, it is theorized that resistance occurs by a mutation or deletion of an amino acid in the target gene, due to a reduction in the number of target receptors, decreased affinity of the drugs to the receptor, and/or perhaps the absence of bioactivating enzymes (Abongwa et al., 2017). Biochemical mechanisms may include insufficient intracellular drug concentrations, cellular defense mechanisms which neutralize the drugs' toxic effects and/or increase the concentration of drug antagonists, and/or altered availability and structure of the target receptors (Martin and Robertson, 2007; Lanusse et al., 2014). Drug efflux mechanisms involving ATP-binding cassette transport proteins, such as P-glycoproteins (Pgps), multi-drug resistance proteins, and breast-cancer resistance proteins have also been identified (Kotze et al., 2014). Regarding the different drug classes available, it is unknown if anthelmintic resistance is spread between the actives within the same drug class, known as side resistance, or if the mechanisms are shared between different drug classes, otherwise known as cross resistance (Abongwa et al., 2017). Overall, the conflicting conclusions about resistance mechanisms suggest that resistance is a complex quantitative trait and the multiple loci involved likely have an additive effect, otherwise known as a quantitative trait locus (Gilleard, 2013; Kotze et al., 2014).

1.6.2   Diagnosis of anthelmintic resistance

1.6.2.1   Critical test

This method begins with the treatment of animals, then recovery of parasites expelled from the host in the feces for the following for 5-7 days (Drudge et al., 1963, 1974, 1975, 1979b, 1984). Subsequently, animals are necropsied, and the larvae and adult parasites are enumerated allowing the direct evaluation of the drug effects on the parasite burden. In this method, each animal serves as its own control allowing for a smaller sample size than other methods. It is, however, very labor and time intensive. It is obviously not an option for production farms as it requires the elimination of numerous animals (Drudge et al., 1963, 1974, 1975, 1979b, 1984; Johansen, 1989).

1.6.2.2   Controlled anthelmintic efficacy test

This *in vivo* technique is considered the gold standard method for determining anthelmintic efficacy. Animals are infected with known susceptible isolates or an isolate with suspect resistance. The animals are treated and necropsied for enumeration of parasites. The known time of infection allows for determining drug efficacy against the various parasite stages by necropsying at different time points. A dose response rate can also be established by testing a range of drug dosages. As expected, this procedure requires highly skilled personnel, and requires a substantial amount of resources (*i.e.* money, animals, time) (Johansen, 1989). A major limitation of this method is the requirement of pure isolates. Attempts for maintaining an isolate of cyathostomins have yet to be successful, and therefore the only method used for equine cyathostomins is natural infection and drug efficacies are evaluated in this way.

1.6.2.3   Fecal egg count reduction test

The fecal egg count reduction test (FECRT) is commonly used for evaluating drug efficacy against a variety of livestock helminths, and it is the only recommended method for *in vivo* anthelmintic efficacy evaluations in horses (Matthews et al., 2012; Nielsen et al., 2016; ESCCAP, 2018; Rendle et al., 2019). In horses, the FECRT is only useful for strongyles and *Parascaris* spp. It is not recommended for efficacy evaluations for other equine helminths because of their unevenly distributed egg shedding. Currently,

no standardized approach for performing a FECRT exists, but the following summary is based upon the common recommendations given by the AAEP (Nielsen et al., 2016); ESCCAP (ESCCAP, 2018), UK-Vet (Rendle et al., 2019), and the World Association for the Advancement of Veterinary Parasitology (WAAVP; Coles et al., 1992).

In general, fecal samples are collected pre-treatment and 10-14 days post-treatment. Drug efficacy is determined by the percent reduction in eggs present in the feces, and there are variety of methods for performing this calculation. The number of animals used and their current egg shedding level must also be considered for appropriate efficacy representation. Presently, the recommendations are to evaluate 6-10 horses with FECs >200 EPG on any given farm, and drug resistance is interpreted at the farm level. Each drug class has a reduction threshold for it to be considered efficacious. The AAEP (Nielsen et al., 2016), UK-Vet (Rendell et al., 2019), and WAAVP (Coles et al., 1992) provide percent reduction thresholds for diagnosing drug resistance in strongyle populations. In general, a percent efficacy <90% for the BZ, <95% for the MLs, and <85-90% for the PYR (Nielsen et al., 2016; Rendell et al., 2019) is considered indicative of resistance. These recommendations are primarily made based on equine strongyles, but resistance evaluation for *Parascaris* spp. generally follows the same guidelines (Osterman-Lind and Christensson, 2009; Armstrong et al., 2014; Martin et al., 2018) while others report a general decrease in egg shedding or drug efficacy (Craig et al., 2007; Slocombe et al., 2007; Lyons et al., 2008a; Molento et al., 2008).

The FECRT formula is as follows:

$$FECRT = \left(\frac{(pre\ treatment\ FECs - post\ treatment\ FECs)}{pre\ treatment\ FECs} \right) x100\%$$

This formula can be applied in a 'herd-based' approach where the herd total pre-treatment and total post-treatment EPG values are calculated and then implemented into the formula. Or, it can be applied in an 'individual-based' approach where the percent efficacy is calculated for each animal and then a mean efficacy for the entire herd is obtained. The first method provides equal weight for each egg counted whereas the second method gives equal weight to each animal. It must be noted that when using the

second method, some animals may present with a 'negative efficacy' which could bias the end result.

1.6.2.4 Egg reappearance period

The egg reappearance period (ERP) is defined as the amount of time it takes for parasite eggs to begin reoccurring in the feces following anthelmintic treatment (Sangster et al., 1999; Nielsen et al., 2016). Shortened ERPs are considered evidence of emerging resistance before a decrease in efficacy has been observed (Sangster et al., 1999), but can only be defined when initial efficacy is 100% (*i.e.* egg disappearance) (Nielsen et al., 2016). However, ERPs are only practical when the original ERP was known at the time of drug development. Most notably, the ERP is implemented for ML drugs.

Presently, an established definition of how to determine the ERP does not exist. Several researchers define it as the number of weeks it takes for egg counts to reach ≥10% of the pre-treatment EPG (Borgsteede et al., 1993; von Samson-Himmelstjerna et al., 2007a; Rossano et al., 2010; Larsen et al., 2011; van Doorn et al., 2014; Relf et al., 2014; Kooyman et al., 2016; Rosanowski et al., 2017; Tzelos et al., 2017). Other investigators are less conservative and use a threshold of 20% the pre-treatment EPG (Nielsen et al., 2016; Kyvsgaard et al., 2011). Osterman-Lind et al. (2007) described the ERP as when the herd mean exceeded 100 EPG. Finally, others consider the ERP when the first egg is observed in the feces after treatment (Little et al., 2003; Lyons et al., 2008b; Molento et al., 2008; Relf et al., 2014; Tzelos et al., 2017).

1.6.2.5 Egg hatch assay

The egg hatch assay is based on the viability of strongyle eggs, and is only used to evaluate BZ efficacy (Le Jambre, 1976; Muchiut et al., 2018). Thiabendazole is most commonly used because of its high water solubility. Serial concentrations of BZ are used and eggs isolated from the feces are incubated within the well plate. The percentage of eggs that hatch per concentration is calculated. Control samples must also be performed in order to correct for natural egg mortality. It is important that eggs are from freshly

collected samples and used within three hours of collection or stored in a manner to prevent embryonation as embryonated eggs are less sensitive to the drugs and will cause variability in the results (Le Jambre, 1976; Coles et al., 2006). Additionally, this test does not allow for the differentiation between strongyle genera and/or species as all strongyle eggs are virtually identical. The EHA is commonly used for evaluating small ruminant parasites, mostly *Haemonchus contortus* (Coles et al., 2006). Variable results are reported for the reliability of this assay and detecting BZ resistance in cyathostomins, and therefore it remains to be validated. Furthermore, because of the wide-spread BZ resistance in equine strongyles, this assay provides little benefit as this drug class is rarely used for treatment (Matthews et al., 2012).

1.6.2.6   Larval development assay

The larval development assay evaluates anthelmintic efficacy of the BZ, levamisole (only used in ruminants), and ML drug classes (Muchiut et al., 2018) based on the ratio of development from egg to L_3 between susceptible and resistant isolates. A liquid-based test using Earle's balanced salt solution supplemented with yeast and bacteria (Hubert and Kerboeuf, 1992) and agar-based test (Gill et al., 1995; Coles et al., 2006) are used. The agar-based test is commercially available (DrenchRite®). Eggs are isolated and placed on agar in micro-well plates provided with water, nutrient, and anti-mycotics. Serial dilutions of the drug are added to the wells. The well-plates are incubated at room temperature for six days to facilitate hatching and larval development. Subsequently, the number of larvae are enumerated (Gill et al., 1995). This test allows for the comparison of multiple drug classes, and some genera and species differentiation, which can provide information for which genera/species are harboring anthelmintic resistance (Coles et al., 2006; Matthews et al., 2012). This assay has been successfully used for nematodes infecting small ruminants (Gill et al., 1995; Coles et al., 2006; Kaplan et al., 2007; Howell et al., 2008), and cattle (Demeler et al., 2010b), but unreliable for detecting ML resistance in field strains of *O. circumcincta* (Lloyd, 1998; Besier, 1998; Palmer et al., 1998). Recently, it has been proven to be a better indicator of avermectin resistance in *Cooperia* spp. and *H. contortus* than other *in vitro* assays described below (George et al., 2018).

Several studies have examined the application of the larval development assay for detecting anthelmintic resistance in cyathostomins. Pook et al. (2002) found a relatively strong correlation (-0.704) between the assay using thiabendazole and *in vivo* efficacy of OBZ by FECRT, whereas Tandon and Kaplan (2004) were unable to detect the resistance ratio for BZ due to the lack of BZ susceptible cyathostomin populations. A confirmed threshold for diagnosing cyathostomin OBZ resistance using this assay has not been determined. This assay does not assess pyrantel resistance, and one might assume that levamisole efficacy evaluations using this assay would translate to pyrantel *in vivo* efficacy because the drugs are in the same drug-class. However, Tandon and Kaplan (2004) confirmed that the levamisole assay does not provide comparable results to pyrantel resistance in field-based FECRT studies. In fact, Osterman-Lind et al. (2005) used modified assay plates impregnated with pyrantel. Comparisons between pyrantel and levamisole plates did not identify a significant correlation. Therefore, extrapolations between these two drugs cannot be made. Finally, it was also concluded that consistency between replicates provided substantial variability in the assay (Tandon and Kaplan, 2004), and overall larval development is poor where even as many as a quarter of the larvae cease development for unknown reasons (Osterman-Lind et al., 2005). Without a pre-determined threshold established for cyathostomin BZ resistance, and the assay not being designed for use with pyrantel, the larval development assay does not appear useful for cyathostomin resistance in its current form.

1.6.2.7 Larval feeding inhibition assay

The larval feeding inhibition assay is useful for detecting anthelmintic resistance against IVM and levamisole in some species of trichostrongylids infecting small ruminants. This method observes the feeding habits of first stage larva by detecting their intestinal fluorescence after being fed *E. coli* mixed with fluorescein (Álvarez-Sánchez et al., 2005). Resistant isolates required a significantly higher drug concentration to inhibit feeding than susceptible isolates (Álvarez-Sánchez et al., 2005). To date, no published studies have applied this technique to equine cyathostomins, but preliminary experiments show wide variation in feeding activities between isolates (Matthews et al., 2012).

1.6.2.8 Larval migration inhibition assay

The larval migration inhibition assay is used for evaluating the ability of L$_3$ larvae to move through a migration apparatus (*i.e.* mesh filter) following anthelmintic exposure (Boisvenue et al., 1983; Wagland et al., 1992; Kotze et al., 2006). It is useful for detecting resistance to MLs (Kotze et al., 2006), LVM (Wagland et al., 1992) and thiabendazole (Muchiut et al., 2018). The larvae are incubated with the anthelmintic prior to relocation onto the migration apparatus, and anthelmintic exposure is continued throughout the allotted time larvae are given to migrate, which varies from 2-72 hours. Subsequently, larvae which moved through the mesh filter and presumed to be resistant are enumerated. The percent of larvae which migrated in the treated and control groups are compared (Kotze et al., 2006). It is very effective for identifying IVM resistant *H. contortus*, even in mixed-species infections where some species are ML sensitive, but it did not identify resistant strains of *T. circumcincta* and *T. colubriformis* (Kotze et al., 2006). However, according to Demeler et al. (2013c) the larval migration inhibition assay is a better indicator for MOX resistance in all three of these species than the larval development assay. This method has previously been optimized for use with ruminant nematodes of susceptible and resistant isolates (Demeler et al., 2010a,b), and Zhao et al. (2017) reported success for several drug classes against various isolates of *A. suum* larvae.

A few studies have evaluated the application of the larval migration inhibition assay using IVM with cyathostomins. First, van Doorn et al. (2010) used this assay to identify cyathostomin larvae that were either sensitive or resistant to IVM. The larvae before and after drug selection were determined to species using the reverse-line blot assay, and it was evident that a shift in the species composition had occurred following drug selection. Therefore, it must be considered if the resistance is due to true lack of efficacy, or the variation of drug tolerance by the cyathostomin species examined. Later, McArthur et al. (2015) found significant differences in the drug concentrations to inhibit migration between IVM resistant and IVM susceptible cyathostomin populations. The authors also noted that larval storage time before use in the assay and the time since the

last anthelmintic was administered to the equids introduced sources of variability that must be considered when performing this assay (McArthur et al., 2015). Most recently, Beasley et al. (2017) optimized the larval migration inhibition assay to increase the migration success of control larvae. This study found that an increase in temperature to 37°C and an increase in incubation time to 24 hours significantly improved the migration. Analysis of replicates provided adequate repeatability when they were from the same batch of larvae and processed on the same day. Variability increased with the age of the sample. Overall, the IC_{95} values were more stable than the IC_{50} (Beasley et al., 2017). It appears that this assay may be the most favorable *in vitro* technique of the aforementioned methods for assessing resistance, but several considerations must be taken into account and validation of this method in comparison to *in vivo* susceptible and resistant populations still needs to be completed.

1.6.2.9   Motility assays

Several assays examining the motility of adult parasites as an assessment of anthelmintic sensitivity have been developed, but the majority of these are labor intensive and subject to operator variability. Such methods have been developed for *H. contortus* (Eguale et al., 2007a,b; O'Grady and Kotze) *Ancylostoma duodenale, Trichuris trichuria, Ascaris lumbricoides* (Hu et al., 2013)*, Necator americanus* (Richards et al., 1995; Hu et al., 2013), *Ancylostoma caninum* (Richards et al., 1995), and *A. suum* (Dmitryjuk et al., 2014). The WormAssay (Marcellino et al., 2012) combats the variability and tediousness of the previous motility assays. It is a high-throughput system utilizing a camera and computer software program to create objective, quantitative motility scores for macroparasites. The technique utilizes microtiter plates varying from 6-96 wells allowing evaluation of multiple samples in a single reading. Similarly, the Worminator was later developed by Storey et al. (2014), but focuses on the evaluation of microparasites. None of these techniques have been evaluated for equine parasites. While the WormAssay is designed for macroparasites, the microtiter plate size limitations prevent its application for the large adults of *Parascaris* spp.

1.6.2.10 Diagnosis of anthelmintic resistance: Summary

In regards to equine parasites, more studies are needed to evaluate and optimize the utility of these methods. This is especially complicated by the 50+ species of cyathostomins, and the differing drug sensitivities between species. Overall, the FECRT remains the most applicable method for evaluating drug resistance *in vivo* without sacrificing the animal.

1.7 Prevalence of anthelmintic resistance

1.7.1 Anthelmintic resistance of equine helminths

1.7.1.1 Anthelmintic resistance in *Parascaris* spp.

As described above, *Parascaris* spp. is the most pathogenic parasite infecting foals. Decades of routine treatment has resulted in significant anthelmintic resistance. Presently, *Parascaris* spp. has wide-spread resistance to the ML drug class, and cases of failed efficacy to the PYR and BZ drug classes exist providing early indications of resistance. A thorough summary of anthelmintic resistance reports in the literature for *Parascaris* spp. can be found in Table 1.1

1.7.1.2 Anthelmintic resistance in cyathostomins

Cyathostomins are well known for their wide-spread resistance to the BZ drug class, and resistance to the PYR is becoming more common. While the ML drug class still exhibits acceptable efficacy, shortened ERPs have been reported and are considered evidence for developing resistance. A thorough summary of literature reports on anthelmintic resistance and shortened ERPs for cyathostomins can be found in Table 1.2.

As previously described, administration of a double-dose (10 mg/kg) of FBZ for five consecutive days exhibits some larvicidal efficacy against the encysted (EL3, LL3/L4) stages of cyathostomins (Vandermyde et al., 1987). As described in section 1.2.1, the cyathostomin larvae encyst into the mucosal wall of the large intestine where they mature to the L4 stage (Eysker et al., 1984). Killing the encysted stages using a drug

with larvicidal efficacy will help decrease the risk of disease associated with mass excystment. Reduction in the efficacy of FBZ (10 mg/kg for five days) against luminal and encysted stages has been reported (Lyons and Tolliver, 2003; Lyons et al., 2007; Rossano et al., 2010; Reinemeyer et al., 2015; Bellaw et al., 2018). Failure to remove the encysted larvae and/or immature larvae (L4) within the lumen will likely affect the ERP, as has been demonstrated by the failure to IVM to remove immature luminal stages (Lyons et al., 2009). This introduces a new challenge when observing the emergence of resistance based on ERP and/or determining anthelmintic efficacy against adults. The exact rate of development from encysted stages or luminal immature L4 to patent infections are not known, and introduces variability for determining accurate efficacy.

1.7.1.3   Anthelmintic resistance in other equine parasites

As described above, diagnosis of the equine pinworm, *O. equi*, can be particularly challenging and provides difficulties for diagnosing resistance. As described by Reinemeyer and Nielsen (2014) there have been numerous anecdotal reports of reduced ML efficacy against pinworms. This topic has been somewhat controversial as the FECRT does not suffice for diagnosing resistance, MLs were never deemed 100% effective at the labeled dosages (Reinemeyer and Nielsen, 2014), and not all studies carry out the recommended method of the critical test (Drudge and Lyons, 1977; Reinemeyer et al., 2010b). Nonetheless, the number of reports of failed ML efficacy against *O. equi* is increasing, and currently exists in the United States (Lyons et al., 2009; Reinemeyer, 2012), New Zealand (Rock et al., 2013), Germany (Wolf et al., 2014), Brazil (Felippelli et al., 2015) and France (Sallé et al., 2016). Additionally, there have been anecdotal reports for treatment failure against some equine stomach worms, *Habronema* spp., which are known to aberrantly infect open wounds via the fly intermediate host. Persistent infection prevents healing of the wound as deposited larvae continuously inflame the wound. Oral administration of IVM is common treatment, but several veterinarians have reported lack of efficacy.

1.7.2 Anthelmintic resistance in small ruminant helminths

There are numerous gastrointestinal nematodes (GIN) infecting small ruminants, most notably are those belonging to the genera *Haemonchus, Teladorsagia, Trichostrongylus, Cooperia,* and *Oesophagostomum.* The clinically and economically most important GIN infecting small ruminants are *Haemonchus contortus, Ostertagia* spp., and *Trichostrongylus axei,* all of which reside in the abomasum, as well as those dwelling in the small intestine, namely *Trichostrongylus* spp., *Nematodirus* spp., and *Cooperia* spp. (Vlassoff and McKenna, 2010). Parasite control regimens have relied heavily on anthelmintics, which inevitably led to overuse and anthelmintic resistance (Kaplan, 2004). Anthelmintic resistance has resulted significant economic losses due to GIN depleting meat, milk, and fiber production (Fitzpatrick, 2013; Kenyon et al., 2017; Craig, 2018).

As reviewed by Kaplan (2004), *H. contortus* was the first GIN among livestock and horse parasites to develop anthelmintic resistance. Shortly after the introduction of BZs in 1961, reports of decreased efficacy began as early as 1964. The same trend was observed upon introduction of dewormers from the cholinergic agonists and ML drug classes where resistance was noted within ten years of the drugs being brought to market. By the 1990s, *H. contortus* had gained resistance to all three major anthelmintic drug classes, and multi-drug resistant strains are reported (Kaplan, 2004; Wolstenholme et al., 2004). Resistance developed at an alarming rate among *H. contortus* populations and is now considered a world-wide issue. Resistance harbored by sheep GIN to all three drug classes has been reported in Australia (Playford et al., 2014), Canada (Falzon et al., 2013), Europe (Papadopoulos et al., 2012), Ireland (McMahon et al., 2013), New Zealand (Waghorn et al., 2006; Hodgson and Mulvaney, 2017), the United States (Howell et al., 2008; Crook et al., 2016), and numerous other countries of the American continent (Torres-Acosta et al., 2012). Multi-drug resistant strains exist for other GIN species as well, including *T. circumcincta* and *T. colubriformis* (Papadopoulos et al., 2012; Torres-Acosta et al., 2012; Geurden et al., 2014). Within the last 30 years, two new anthelmintics, monepantel and derquantel, have been brought to market for control of GIN in sheep. Within two years, resistance reports of sheep GIN to monepantel began to

surface, first in New Zealand (Scott et al., 2013), then Uruguay (Mederos et al., 2014), the Netherlands (Van den Brom et al., 2015), Brazil (Cintra et al., 2016; Ciuffa et al., 2017), Australia (Sales and Love, 2016; Lamb et al., 2017), and the United Kingdom (Hamer et al., 2018). Derquantel exists only in combination with abamectin and initially achieved >99% reduction of strongyle eggs in sheep (Geurden et al., 2012). Currently, only one report of reduced efficacy for this combination against sheep GIN exists, where treatment resulted in 93% total strongyle egg reduction following FECRT and 90% reduction in *H. contortus* following coproculture and larval identification (Sales and Love, 2016).

<p style="text-align:center">1.7.3   Anthelmintic resistance in cattle helminths</p>

Similar to the sheep and goat industry, cattle producers are at a significant risk for economic loss due to GIN infections and rely heavily on the use of anthelmintics for parasite control (Sutherland and Leathwick, 2011). The most clinically relevant GINs of cattle include *Ostertagia ostertagia, Cooperia* spp*., Haemonchus placei, Trichostrongylus* spp. and *Oesophagostomum* spp*. (Vercruysse and Dorny, 1999). Emergence of anthelmintic resistance among cattle GIN occurred more slowly than in small ruminants, but nevertheless it has become a world-wide issue (Sutherland and Leathwick, 2011). While *O. ostertagi* is considered the most pathogenic GIN (Bairden and Armour, 1981), treatment regimens have been focused on *Cooperia* spp. as it is the dose-limiting species for efficacious ML treatment and now harbors substantial resistance (Sutherland and Leathwick, 2011). Sutherland and Leathwick (2011) reviewed numerous cases of resistance to all drug classes among cattle GIN, and it should be noted that the reports described in the current section are not an exhaustive representation.

Initial reports of AR among cattle GIN were attributed to the survival of *Cooperia* spp. following ML treatment, which has been documented in the United States (Gasbarre et al., 2009; Edmonds et al., 2010; Gasbarre, 2014), Brazil (Borges et al., 2015; Felippelli et al., 2014), Europe (Geurden et al., 2015), the United Kingdom (Bartley et al., 2012), New Zealand (Waghorn et al., 2006), South America (Suarez and Cristel, 2007), Australia (Lyndal-Murphy et al., 2009), and numerous other countries (Sutherland and

Leathwick, 2011). Later, it was also discovered that *O. ostertagi* (Edmonds et al., 2010; Geurden et al., 2015; Waghorn et al., 2016) and *H. placei* (Soutello et al., 2007; Costa Mdo et al., 2011; Borges et al., 2015) were surviving ML treatment as well. Resistance is not limited to the ML drug class; several reports exist of cattle GIN populations harboring resistance to BZ drugs (Sutherland and Leathwick, 2011; Furtado et al., 2016b). Although fewer in number, reports of resistance to levamisole are emerging among mixed GIN species populations in Mexico (Becerra-Nava et al., 2014), *O. ostertagi* in Australia (Rendell, 2010; Cotter et al., 2015), *Trichostrongylus* spp. in Ireland (McMahon et al., 2013) and New Zealand (Mason and McKay, 2006). Finally, multidrug resistance to all three drug classes has been documented in *Haemonchus* spp., *Ostertagia* spp., and *Cooperia* spp. (Sutherland and Leathwick, 2011).

1.7.4 Anthelmintic resistance in small animal helminths

Presently, the only helminths of domestic cat and dogs harboring resistance are the canine hookworm, *Ancylostoma caninum*, and the heartworm, *Dirofilaria immitis.* Resistance reports to pyrantel by *A. caninum* are limited and appear to be isolated to the Australasia region (Jackson et al., 1987; Hopkins et al., 1989; Hopkins and Gyr, 1991 Kopp et al., 2007, 2008a). The limited diagnoses may not be a true reflection of the resistance status, but rather reflected limited surveillance opportunities as the FECRT is not a reliable method for *A. caninum* (Kopp et al., 2007, 2008a) and true efficacy evaluations require the sacrifice of animals (Kopp et al., 2008b). The same reasoning is true for the lack of surveillance for other hookworms (*A. brasiliense, A. tubaeforme*) and ascarids (*Toxocara* spp.) infecting companion animals (Kopp et al., 2008b). Presently, there are no reports of decreased anthelmintic efficacy with the ML or BZ drug classes against these intestinal helminths.

The topic of anthelmintic resistance among *D. immitis* to the ML drug class is a matter of great debate in the parasitology community. Arguments in support of this include that MLs are the only drugs used against this parasite, and it is recommended that all dogs be administered year-round prevention. This places a heavy selection pressure on *D. immitis* (Wolstenholme et al., 2015). Because heartworm preventatives act against the

L$_3$ larvae and not the adult or microfilaria stages (McCall et al., 2004), it is possible for L$_3$s harboring resistance genetics to survive to adulthood and produce microfilariae. The microfilariae may harbor the genetics for resistance, assuming it is heritable, and contribute resistance alleles to the population (Bowman, 2012). Another problem is that circulating microfilariae require a higher dose of ML drug than the L$_3$ stage and subsequently what is provided for prophylactic treatment, therefore microfilariae existing in a dog on prophylactic treatment is continuously exposed to sub-lethal doses of ML, which places selection-pressure on the parasite for resistance. The microfilariae can then be transmitted to another host via mosquitoes and may mature into potentially resistant L$_3$ stages. Presently, it is unknown if the resistance status of microfilariae translates to resistance of the L$_3$ stage and vice-versa (Bowman, 2012). However, others previously argued that the risk for anthelmintic resistance in *D. immitis* was unlikely (Prichard, 2005) due to the perfect (100%) efficacy required by the FDA for drug approval (Hampshire, 2005), the opportunity for a large refugia population in feral dogs and coyotes (Prichard, 2005), and the long (7 month) life cycle.

Nonetheless, reports of failed efficacy and resistant cases do exist (Hampshire, 2005), but it was later suspected that this may be due to a lack of owner compliance and improper prophylactic administration (Atkins et al., 2014, Drake and Wiseman, 2018). Laboratory studies have confirmed the presence of ML-resistant strains by evaluating motility of the infective L$_3$ (Evans et al., 2013), *in vitro* monitoring of the motility of the circulating microfilariae (Geary et al., 2011; Storey et al., 2014), and identification of molecular mechanisms (Bourguinat et al., 2011, 2015; Ballasteros et al., 2018). Recently, however, Evans et al. (2017) and Maclean et al. (2017) have indicated that the motility assays for the L$_3$ and microfilariae, respectively, are not good indicators of ML-resistance. *In vivo* studies under laboratory conditions remain the gold standard of detection (Geary et al., 2011; Blagburn et al., 2011; Snyder et al., 2011; Pulaski et al., 2014). The sacrifice of animals, long life-cycle of the parasite, requirement of the mosquito intermediate host, and financial expense hinder the progression of resistance studies in *D. immitis* (Wolstenholme et al., 2015). The conflicting views and urgent need for effective preventative treatment warrant future research focused on this field.

1.7.5 Anthelmintic resistance in other domestic animals

The BZ drugs are heavily relied on for the control of parasitic nematodes in poultry. Presently, only one report of anthelmintic resistance exists in the United States, where resistance to FBZ was found in *Ascaridia* spp. and *H. gallinarum* infecting chickens and turkeys (Yazwinski et al., 2013). The lack of reports may not reflect the true prevalence of resistance, but is likely affected by the absence of guidelines for identifying resistance in poultry nematodes (Coles et al., 1992).

The common swine parasites (*Ascaris suum, Trichuris suis, Oesophagostomum* spp.) can be largely controlled through proper sanitization and environmental control practices coupled with anthelmintic treatment (ivermectin, pyrantel, levamisole, fenbendazole; Roepstorff et al., 2011). Only a few reports of resistance exist, all of which are based upon the swine nodular worm, *Oesophagostomum* spp. Resistance to pyrantel citrate (Roepstorff et al., 1987; Bjørn et al., 1990) and levamisole (Bjørn et al., 1990) have been reported in Denmark, whereas resistance to levamisole and flubendazole exists in Germany (Gerwert et al., 2002). The limited number may be due to the lack of surveillance as these parasites do not cause near the pathology as ruminant parasites. However, the high number of carcass condemnations due to parasite infections warrant further investigation (Roepstorff et al., 2011).

1.8 Mechanisms of anthelmintic resistance

1.8.1 General overview

The mechanisms driving anthelmintic resistance in parasitic nematodes are poorly understood. Parasitic nematodes make challenging research subjects as the lifecycles cannot be completed *in vitro* and maintenance of specific stages *in vitro* compromises their viability (Geary et al., 1999). Nematode parasite populations are largely heterogenous resulting in varying drug responses causing resistance to develop quickly, and variations are found between isolates (James et al., 2009). They also have a high capacity to develop new mutations when under selection, have complex population structures with geographically distributed mutations (Gilleard, 2013), and their extensive

genetic diversity leads to varying drug responses (James et al., 2009; Prichard et al., 2012). The lack of genetic resources due to limited finances and large genomes with tendencies for high polymorphisms present further challenges for advancing knowledge in the parasitology field (Gilleard, 2013), however recent advances in annotated genomes and transcriptomes are creating more opportunities for study (Coghlan et al., 2019). Alternatively, *C. elegans* has been used as a model organism for *in vitro* studies of anthelmintic resistance and drug mechanisms (Grant, 1992; Kwa et al.*,* 1995; Yan et al., 2012; Burns et al., 2015). Likewise, the extensive knowledge base for *H. contortus* makes it the primary model parasitic nematode for both *in vitro* and *in vivo* studies (Gilleard, 2013). However, one must bear in mind that the results obtained from lab isolates are not consistently found in field derived isolates (Beech et al., 2011; Kotze et al., 2014).

Because of the limited knowledge, gene studies for better understanding AR mechanisms are generally focused on the presumed drug targets and the few identified resistance mechanisms which limits the possibilities for characterizing novel mechanisms (Gilleard, 2013). In order to identify mechanisms of anthelmintic resistance, the assembled and annotated genome for the parasite of interest must first be generated, then identify genome-wide genetic markers, and characterize the variation between field and lab strains as their sensitivity to anthelmintics and environmental fitness differ (Beech et al., 2011). Currently, there are 31 published and 45 draft genomes of parasitic helminths (Coghlan et al., 2019). Developing a better understanding of anthelmintic resistance will aid in delaying the rate of resistance of the current drug classes, developing methods for monitoring resistance, and identifying new drug targets (Yan et al., 2012), as well as further understanding of parasite defense mechanisms to xenobiotics, diagnostic methods for resistance, and the effects of combination drug regimens on resistant populations (Kotze et al., 2014).

Described in the sections below it will be become apparent that resistance mechanisms are most clearly understood for the BZ drug class, and less so for the cholinergic agonists and ML. Across the drug classes, studies have focused largely on single nucleotide polymorphisms (SNPs), altered receptor subunit expression, changes in

the drug-receptor binding site, and non-specific mechanisms such as drug efflux and metabolism.

A SNP can change the amino acid and alter the affinity of a drug for its target receptor (James et al., 2009). A large focus of this research has been to characterize the SNP differences between resistant and susceptible parasite populations, and identify a loss of polymorphism in genes of interest following drug selection (Gilleard, 2013). Furthermore, it is likely that the SNPs are only present after resistance is established, leaving limited to no opportunity for further anthelmintic preservation (James et al., 2009). Single nucleotide polymorphisms can be detected by restriction enzyme digestion, sequencing of a PCR product, pyrosequencing, and diagnostic PCR primers which overlap the SNP of interest (Beech et al., 2011). Some of these methods have been implemented in resistance mechanism studies and are described below.

Changes in ligand-gated ion channels, such as the L-nACh and GluCl channels, can be identified using the patch-clamp technique (Sakmann and Neher, 1984). The flow of ions, or lack thereof, through a channel is measured which provides insight to the channel's functionality and response to a ligand, such as an anthelmintic. This method can be performed as the whole cell (multi-channel) or single-channel level. A glass micropipette is placed onto the cell membrane, and applied suction sucks a small amount of membrane into the pipette to create a seal. The patch-clamp measures the membrane potential, or electrical current, that occurs as a result of the flow of ions through the channel. This can be performed under various conditions and provide insight to the channel mean open-time, probability of channel opening, and the number of receptors present (Sakmann and Neher, 1984). Studies using this technique are described below. Once changes in the receptors have been identified, other molecular methods, such as PCR or western blot, can be used to identify the changes within the receptor and its subunits.

Non-specific resistance mechanisms describe generalized drug efflux or metabolism pathways. The ABC transporters are commonly investigated for drug efflux properties, and consist of numerous proteins, but those most commonly associated with drug resistance are the Pgps. Of the drug metabolizing enzymes, one of the most

commonly investigated families is the cytochrome P450 monooxygenases (CYP) due to their ability to confer insecticide resistance (Ffrench-Constant et al., 2004). Originally, it was assumed these enzymes were not present in nematodes (Barrett, 1998), but the work outlined below provides ample evidence of their existence and function in several parasitic nematodes. These drug efflux proteins and metabolizing enzymes are commonly studied *in vitro* using functional inhibitors, such as verapamil for Pgps and piperonyl butoxide for CYPs. Other methods include transgenic expression of parasite Pgps in model organisms, such as *C. elegans.* Implementation of these techniques for understanding resistance mechanisms are described in more detail below.

1.8.2   Mechanisms of benzimidazole resistance

The first case of resistance to the BZs was reported in 1964, just three years after their introduction to the market. To date, the mechanisms of anthelmintic resistance is most widely understood for the BZ drug class (von Samson-Himmelstjerna et al., 2007b). It is well established that a primary mechanism of BZ resistance is due to polymorphisms within the β-tubulin gene (von Samson-Himmelstjerna et al., 2007b; James et al., 2009; Beech et al., 2011; Abongwa et al., 2017). There are two isoforms of this gene, but most of the findings associated with resistance lie within isotype one. Three SNPs leading to amino acid changes have been identified in the β-tubulin isotype one gene (BEN-1). The most commonly identified SNP across numerous parasite species described below occurs at codon 200, where phenylalanine is changed to tyrosine (F200Y). Changes of both phenylalanine to tyrosine (F167Y) and phenylalanine to histidine (F167H) have been observed at codon 167 in BZ resistant nematode populations. Lastly, a SNP at 198 changing glutamine to alanine (E198A) has been associated with BZ resistance (von Samson-Himmelstjerna et al., 2007b; James et al., 2009; Beech et al., 2011; Abongwa et al., 2017). These amino acid changes lead to structural alterations in the drug target which presumably decreases drug affinity (James et al., 2009; Furtado et al., 2016b), and may lead to improved microtubule stabilization (Kwa et al., 1995).

1.8.2.1   Benzimidazole resistance mechanisms in strongylids of small ruminants

Because of the production losses and economic impacts caused by anthelmintic resistance in small ruminants, *H. contortus* has been the most widely studied nematode for BZ resistance and evidence for all aforementioned SNPs are reported in this species (von Samson-Himmelstjerna et al., 2007b). It is presumed that F200Y is a fundamental component for BZ resistant *H.* contortus populations (Kwa et al., 1994, 1995), as several studies report a strong correlation with its presence and BZ resistance (von Samson-Himmelstjerna et al., 2009; Kotze et al., 2014). More recently, two other mutations were identified, F167Y (Silvestre and Cabaret, 2002) and E198A (Ghisi et al., 2007). Interestingly, one study found only the F200Y genotype to increase when selection pressure of repeated albendazole treatment at increasing dosages was applied, while the F167Y genotype decreased and no polymorphisms at codon 198 were identified (Barrère et al., 2012). It has been suggested that mutations at codons 200 and 167 are mutually exclusive (Mottier and Prichard, 2008). In contrast, Zhang et al. (2016) examined eight different geographical isolates of *H. contortus* and found the E198A polymorphism to occur more frequently than the F200Y, and F167Y was not detected in any of the populations. Furthermore, a study observing the effects of increasing thiabendazole concentrations on L$_3$ larvae found the E198A allele to increase upon drug exposure, but the F200Y mutation frequency decreased (F167Y was not examined in this study). These two polymorphisms were not observed in the same individuals, suggesting that they are also mutually exclusive (Kotze et al., 2012).

The F200Y polymorphism is also known to convey BZ resistance in other small ruminant trichostrongylid parasites, including *T. circumcincta* and *T. colubriformis* (Furtado et al., 2016b; Ramünke et al., 2016). Ramünke et al. (2016) examined the prevalence of all three SNPs in several European countries, and only very low prevalence of polymorphisms at codon 198 for *Trichostrongylus* spp. and codon 167 for *Teladorsagia* spp. were reported. Avramenko et al. (2019) validated a new technique for SNP identification, deep amplicon sequencing. Using this method, the investigators explored the prevalence of all three SNPs in seven parasitic nematode species from ewes on 95 UK farms and lambs from 69 UK farms. Overall, the F200Y SNP was most commonly identified, and highest in *T. circumcincta,* while *H. contortus* had nearly equal prevalence of the F200Y and F167Y SNPs.

1.8.2.2 Benzimidazole resistance mechanisms in strongylids of cattle

Polymorphisms related to resistance in cattle nematodes have also been reported, but reports are lacking likely due to the lower prevalence of resistance. BZ resistance in *Cooperia oncophora* has been associated with the F200Y polymorphism (Winterrowd et al., 2003; Demeler et al., 2013a) and E198A, but not F167Y (Demeler et al., 2013a). All three polymorphisms have been identified in BZ resistant *Ostertagia ostertagi* (Demeler et al., 2013a), while Encalada-Mena et al. (2014) did not find any changes in codons 167 and 198 in *H. placei* populations. The F200Y SNP can accumulate at high levels in *O. ostertagi* populations within just one grazing season if inaccurate BZ doses are administered (Knapp-Lawitzke et al., 2015).

Numerous molecular tests are available for identifying these SNPs in trichostrongylid nematodes, the majority of which are PCR-based (von Samson-Himmelstjerna et al., 2007b). An allele-specific PCR was developed for the detection of the F200Y polymorphism of adult *H. contortus* and other small ruminant nematodes, and L3 larvae of mixed species (Silvestre and Humbert, 2000). The allele specific PCR for detecting the F200Y mutation is a more sensitive diagnostic method than the egg hatch assay for diagnosing BZ resistance (Čudeková et al., 2010). Later, RT-PCR technology was employed to genotype homo- and heterozygous individuals, and is capable of estimating resistant allele frequencies in pooled samples (Walsh et al., 2007). Amplification-refractory mutation system (ARMS) PCR utilizes four primers to detect both mutant and non-mutant alleles which reduces the risk of primer-template mismatch due to allelic differences. Likewise, four fragments are produced and can be distinguished between homozygous and heterozygous individuals (Niciura et al., 2012). Most recently, pyrosequencing technology has been employed and offers accurate allele frequencies from individual and pooled samples of trichostrongylids infecting small ruminants (Troell et al., 2003; Ramünke et al., 2016) and cattle (Demeler et al., 2013a). This method has proven useful for detection of polymorphisms at codons 167, 198, and 200 and is comparable with the FECRT and larval development assay (Barrére et al., 2013).

1.8.2.3 Benzimidazole resistance mechanisms in cyathostomins

As previously mentioned, cyathostomins have substantial resistance to the BZ drug class. The challenge of detecting SNPs related to resistance is dramatically increased by the 50+ species of cyathostomins. Presently, the β-tubulin sequence is known for eight species (Pape et al., 1999; von Samson-Himmelstjerna et al., 2001; Drögemüller et al., 2004b; Lake et al., 2009). It was originally presumed that cyathostomins had only one isotype of this gene (Pape et al., 2002), but Clark et al. (2005) reports a second isotype. Still, the only evidence of SNPs associated with BZ resistance were present on isotype one. The F200Y was first identified in *Cyclicocyclus nassatus* (von Samson-Himmelstjerna et al., 2001) which promoted the development of an allele-specific primer capable of identifying this polymorphism in other cyathostomin species (von Samson-Himmelstjerna et al., 2002). Later, a *Taq*Man assay targeting the F200Y polymorphism in L_3 and adults was developed (von Samson-Himmelstjerna et al., 2003). Most recently, Lake et al. (2009) developed a pyrosequencing assay for genotyping L_3 cyathostomin larvae of unknown species at codon 200, however, further optimization of this method remains necessary.

Several studies have reported the prevalence of the F200Y polymorphism in BZ resistant populations. Pape et al. (2003) reports a phenotypically FBZ resistant population where the majority was genotypically susceptible, but Drögemüller et al. (2004b) later identified the F167Y mutation in this same population. In contrast, Hodgkinson et al. (2008) reported a significantly higher frequency of the F200Y mutation in a FBZ and OBZ resistant population in comparison with a FBZ susceptible population. Likewise, the F167Y codon was also identified at significantly higher frequencies in the FBZ population, however, no significant differences of this polymorphism were reported within the OBZ resistant population. Further examination of genotypes failed to identify 'double mutant' cyathostomins (*i.e.* homozygous resistant alleles at both codon 167 and 200) (Hodgkinson et al., 2008). Most recently, Blackhall et al. (2011) reported the genotypes of six small strongyle species from BZ resistant and BZ susceptible populations. All specimens were examined for polymorphisms at codons 167, 198, and 200. F167Y was found at a significantly higher frequency among the resistant population

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than the susceptible, while F200Y was not significantly different between populations. No amino acid changes were identified at codon 198. Overall, cyathostomins have a higher frequency of the F167Y polymorphism than the F200Y, which contrasts the aforementioned studies of BZ resistance in trichostrongylids of small ruminants. This supports the speculation that there are likely other SNPs or entirely different mechanisms related to BZ resistance in cyathostomins.

1.8.2.4 Benzimidazole resistance mechanisms in hookworms

Reports of BZ resistance in hookworm populations of domestic cats and dogs (*Ancylostoma* spp.) is lacking, however the intense prophylactic treatment frequencies raise the concern of selecting for anthelmintic resistance alleles (Humphries et al., 2012). The lack of resistance allows for the opportunity to detect its presence before it becomes prevalent. Therefore, molecular tests have been developed. Real-time PCR assays are described for detecting mutations in *A. caninum* at codons 167 and 200 (Schwenkenbecher et al., 2007), and 198 (Schwenkenbecher and Kaplan, 2009). Later, ARMS-PCR assays were developed for F200Y (Furtado and Rabelo, 2015a) and F167Y (Furtado and Rabelo, 2015b) for *A. caninum*, followed by a tetra primer ARMS-PCR assay for E198A (Furtado et al., 2016a). These methods have allowed numerous investigations of the aforementioned SNPs in various populations of *A. caninum*, however no polymorphisms at codon 167 (Furtado and Rabelo, 2015b; Schwenkenbecher and Kaplan, 2009) nor codon 198 (Schwenkenbecher and Kaplan, 2009; Furtado et al., 2014; Furtado et al., 2016a) have been reported. One study did identify the F200Y mutation at a frequency of less than 1% (Furtado et al., 2014). Similarly, the F200Y polymorphism was not identified in the human hookworms, *A. duodenale* and *N. americanus,* even in areas were BZ resistance was suspected (Albonico et al., 2004), whereas Diawara et al. (2013) identified this polymorphism at a low frequency of 2.3% where the BZ drug was still considered efficacious.

1.8.2.5 Benzimidazole resistance mechanisms of *Ascaris* species

While strongyle type parasites are known to have two β-tubulin isotypes, five isotypes have been identified in the ascarid parasites *Ascaris suum* and *A. lumbricoides,* both of which infect humans (Demeler et al., 2013b). Interestingly, there are very few one-to-one orthologs existing between the β-tubulin strongyle and ascarid isotypes (Demeler et al., 2013b). The F167Y polymorphism has been detected at frequencies between 40-97.7% where the majority of treatments were still considered efficacious (Diawara et al., 2013). In contrast, Krücken et al. (2017) reported reduced BZ activity against *Ascaris* spp.*,* but did not identify any of the polymorphisms associated with BZ resistance. Benzimidazoles are also used extensively in poultry, however, few studies have examined current BZ efficacies. Tarbiat et al. (2017) found BZ treatment against *Ascaridia galli* in laying hens to be efficacious via the FECRT and larval development assay, and no polymorphisms at codons 167, 198, and 200 were found.

1.8.2.6   Benzimidazole resistance mechanisms of *Parascaris* spp.

Presently, no studies exist which examine these polymorphisms in *Parascaris* spp.

1.8.2.7   Benzimidazole resistance mechanisms: Summary

While it is widely accepted that these SNPs are associated with BZ resistance, little is known about the related mechanisms (Beech et al., 2011). It is speculated that the F200Y mutation aids in stabilization of the microtubules during polymerization (Kwa et al., 1995). To further complicate matters, the presence of a SNP in one species with known resistance may not convey resistance in other species. There is also conflicting evidence pertaining to the presence of these SNPs in field isolates of the same species with known resistance (James et al., 2009; Beech et al., 2011). This implies that while these SNPs may be causative of significant drug resistance, they are not they only mechanism and further research is warranted. Overall, it appears that the same polymorphisms that are associated with BZ resistance in strongyles, are not associated with BZ resistance in ascarid parasites, and other resistance mechanisms are likely at work.

1.8.3 Mechanisms of cholinergic receptor agonists resistance

As described above, the anthelmintics belonging to the cholinergic receptor agonist group (*i.e.* levamisole, pyrantel, monepantel) act as agonists of the nAChR. Their binding increases the flow of cations into neuromuscular channels causing muscle contraction and paralysis. The varying composition of receptor subunits, genetic composition, and different binding-site residues between parasite species and ligand-binding requirements complicate the elucidation of resistance mechanisms. Largely, resistance to this class of anthelmintics has been associated with decreased expression of the subunits and/or genetic mutations leading to non-functional subunits, such as truncated subunits (Whittaker et al., 2016). It is also unknown if particular subunits are involved with resistance mechanisms, and if resistant parasite isolates are capable of altering or replacing the subunits conferring anthelmintic sensitivity (Martin and Robertson, 2007).

1.8.3.1 Cholinergic receptor agonists resistance mechanisms in strongylids of small ruminants

Changes in expression patterns of the receptors and ancillary proteins between drug susceptible and resistant isolates have been obvious study targets. Initial investigations of levamisole resistance in small ruminant parasites identified reductions in the number of receptors when compared to susceptible parasites (Sangster et al., 1988). Neveu et al. (2007) identified a novel protein (HA17) that is highly expressed in numerous *H. contortus* resistant isolates from various geographical locations, but is weakly expressed in susceptible isolates. The function of this gene remains unknown. Sarai et al. (2014) found decreased expression of the ancillary proteins UNC-74*,* RIC-3*,* and UNC-50 in a resistant isolate of *H. contortu*s. Neveu et al. (2010), however, examined the expression levels of the L-nAChR subunits in isolates for *H. contortus, T. colubriformis,* and *T. circumcincta*, and significant differences were not found between susceptible and resistant isolates. Non-specific drug resistance mechanisms have also been explored and are further described in section 1.8.4. Briefly, Raza et al. (2016)

observed the effects of *in vitro* levamisole exposure to a drug-susceptible and multi-drug resistant isolates of *H. contortus* larvae. An increase in expression of two Pgps (*pgp-1* and *pgp-9.1*) was identified in the resistant isolate. Contrastingly, a thorough gene expression evaluation of numerous Pgp genes failed to identify any consistent changes between resistant and susceptible isolates of *H. contortus* (Sarai et al., 2014). To further complicate matters, it is known that parasite life stage (*i.e.* larva vs adult; Sarai et al., 2013) and drug selection method (*i.e. in vitro* larvae exposure vs. *in vivo* isolates; Sarai et al., 2014) influences the subunit expression patterns.

Truncated isoforms have been a recent focus of levamisole resistance mechanism studies. A truncated form of the *H. contortus acr-8* orthologue (*Hco-acr-8b)* was identified. The full gene (*Hco-acr*-8) was expressed in both resistant and susceptible isolates of *H. contortus,* but only the abbreviated form was found in resistant isolates (Fauvin et al., 2010). This suggests a novel role of truncated isoforms in resistance mechanisms. Similarly, Neveu et al. (2010) performed a thorough investigation of the cDNAs between levamisole resistant and susceptible isolates for *H. contortus*, *T. colubriformis* and *T. circumcincta.* The subunits *unc-29, unc-28, lev-1,* and *unc-63* were expressed in all isolates, however, an additional yet truncated form of *unc-63* was identified in levamisole resistant isolates of all three species (Neveu et al., 2010). Larval stages from a multi-drug resistant *H. contortus* isolate also had significantly higher expression of the truncated *Hco-acr*-8 subunit than the susceptible strain (Williamson et al., 2011). Furthermore, Rufener et al. (2009) identified truncated forms of the gene *Hco-mptl-1* in mutant lab isolates of *H. contortus* expressing reduced sensitivity to monepantel. It is hypothesized that the truncated isoforms may express a dominant negative effect on the parent subunit (Rufener et al., 2009). Presently, only one study has examined this hypothesis. The dominant negative effect of the *Hco-acr-8* truncated isoform (*Hco-acr-8b)* was confirmed in the *Xenopus* oocyte model (Boulin et al., 2011) and in larvae of a triple-resistant *H. contortus* isolate when larvae were exposed to levamisole *in vitro* (Williamson et al., 2011).

1.8.3.2   Cholinergic receptor agonists resistance mechanisms in

Oesophagostomum dentatum

In levamisole resistant isolates of *Oesophagostomum dentatum*, resistance was associated with a decreased number of active channels, a lower probability of channel opening, and shorter channel open times when compared to an anthelmintic susceptible isolate when exposed to levamisole (Robertson et al., 1999). These changes would decrease the influx of cations and reduce the parasite's risk of flaccid paralysis. A similar study was performed for pyrantel resistant isolates of *O. dentatum* where levamisole was also used for drug exposure (Robertson et al., 2000). Again, resistant isolates had a decreased number of active channels and a lower probability of channel opening, however, the channels of the pyrantel resistant isolate had a significantly longer mean open time than the levamisole resistant and susceptible isolates. Observations of channel conductance using the patch-clamp technique revealed that while the pyrantel resistant isolate consisted of the same subunit types at the susceptible isolates, the subunits were present at different proportions. Furthermore, the pyrantel resistant isolate included a subunit type that was not present in the levamisole resistant isolate. Therefore, it can be concluded that not only do the drug targets of anthelmintics within this drug class differ, but the mechanisms of resistance do as well (Robertson et al., 2000). Later, Romine et al. (2014) compared numerous genes associated with nAChR in levamisole resistant and susceptible isolates of *O. dentatum*. Specifically, two genes, *acr-21* and *acr-25*, had increased expression in the resistant isolate, and decreased expression of *unc-63*. A number of SNPs were also identified as a difference between the two isolates. No truncated isoforms, as observed in small ruminant GIN, were identified in this study.

1.8.3.3 Cholinergic receptor agonist resistance mechanisms of cyathostomins

Presently, no studies exist examining the resistance mechanisms of cyathostomins to this drug class.

1.8.3.4 Cholinergic receptor agonists resistance mechanisms in hookworms

Presently, the only small animal helminth species known to harbor resistance against the PYR drug class is the canine hookworm, *A. caninum*. Kopp et al. (2009) performed transcriptional analyses on *A. caninum* isolates that exhibited either high or low levels of resistance. The highly resistant isolate had significantly lower expression levels of three receptor subunits orthologous to *unc-29, unc-38*, and *unc-63* (*aar-29, aar-38, aar-63*) than the isolate with low levels of resistance. Additionally, three additional subunits (AAR-8, AAR-15, AAR-19) had significantly higher expression in some of the highly-resistant isolates. This was the first study to associate the latter subunits with pyrantel activity. Unlike the case for BZ resistance, no SNPs were identified between the two populations (Kopp et al., 2009).

1.8.3.5   Cholinergic receptor agonists resistance mechanisms of *Ascaris* species

Although resistance to this drug class has not been identified for *Ascaris* species, Williamson et al. (2009) investigated the subunit composition of nAChR in the body wall muscle of A. *suum*. The investigators found that altering the proportion of subunits affects the binding site and sensitivity to the anthelmintics pyrantel, oxantel, and levamisole.

1.8.3.6   Cholinergic receptor agonist resistance mechanisms of *Parascaris* species

Presently, no studies exist examining the resistance mechanisms of *Parascaris* spp. to this drug class.

1.8.3.7   Cholinergic receptor agonist resistance mechanisms: Summary

The cholinergic receptor agonist drug class consists of a variety of drugs, and in many cases it is presumed that these drugs act in similar ways and therefore must have similar modes of action. The aforementioned evidence of altered binding sites and receptor subunit composition effecting drug sensitivity and resistance disproves these

assumptions. It was previously assumed that resistance to this drug class was due to changes in the expression of subunits or altered transcript products (Whittaker et al., 2016). The compilation of references herein speaks to the complicated nature of anthelmintic resistance within this drug class and the likelihood of its polygenic nature. Further research is warranted, especially to examine the resistance mechanisms associated with decreased pyrantel sensitivity as resources are lacking.

1.8.4   Mechanisms of macrocyclic lactone resistance

As previously described, the MLs consist of the avermectins and milbemycins. They act on the GluCl and GABA receptors causing an influx of chloride ions (Cl^-). The primary target, the GluCl, is a homo-pentamer consisting of alpha subunits. The alpha subunit genes vary between parasite species. Inhibition of motility and pharyngeal pumping is ensued, and the parasite is subsequently expelled by the host through peristalsis. Although within the same drug class, stark differences have been observed between the avermectins and milbemycins (see section 1.5.3, Prichard et al., 2012). Resistance mechanisms for this drug class have focused primarily on altered receptor subunit expression, mutations in the receptor-drug binding site causing changes in binding affinity, as well as non-specific mechanisms, such as drug efflux via ATP binding cassette transport proteins and drug uptake. The transport proteins of primary interest for ML resistance have been Pgps, multi-drug resistant associated proteins, and the breast cancer resistance proteins (Kotze et al., 2014; Whittaker et al., 2016). While numerous *in vitro* studies have been carried out, including utilization *C. elegans,* many of the findings fail to explain the extreme levels of resistance observed in field trials (Kotze et al., 2014).

1.8.4.1   Macrocyclic lactone resistance mechanisms in strongylids of small ruminants

Altered receptor subunits

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Blackhall et al. (1998) examined the GluCl alpha subunit constructed by the *glc-1* gene between two susceptible and three drug selected isolates (two IVM, one MOX) of *H. contortus*. Significant differences in the frequency of five alleles were identified between the drug-selected and non-drug selected strains. The role of these polymorphisms with drug resistance mechanisms, if any, is unknown (Blackhall et al., 1998). Dent et al. (2000) identified that point mutations within three alpha subunit genes, namely *avr-14, avr-15* and *glc-1,* conferred IVM resistance in *C. elegans.* This was later confirmed by Ardelli et al. (2009). However, Ardelli et al. (2009) also noted that these same mutations led to only partial resistance to MOX, and this suggests that MOX may act on different receptors than IVM and/or have different modes of resistance mechanisms in *C. elegans* (Ardelli et al., 2009). Of these three subunit genes, only one (*avr-14*) is orthologous in *H. contortus* (McCavera et al., 2007). An amino acid substitution (L256F) in the *avr-14* subunit gene is known to induce IVM resistance in *H. contortus* (McCavera et al., 2009). Likewise, there is a subunit gene in *H. contortus* that is not present in *C. elegans*, that is *HcoGluClα,* and it is known to be IVM sensitive (McCavera et al., 2007). The differences in subunit presences between species introduce further challenges to elucidating the resistance mechanisms directly associated with the IVM-ligand receptors. As described above, the GABA-receptors are a secondary target of ML drugs. One study investigated these receptors for their role in ML resistance of *H. contortus* (Blackhall et al., 2003). This study identified a total of 13 alleles of the HG1 GABA-receptor gene between IVM selected and non-selected isolates, some of which were differentially expressed between the different strains, however it remains unknown if these changes are a direct cause of the resistant phenotype (Blackhall et al., 2003).

Redman et al. (2012) and Rezansoff et al. (2016) identified the microsatellite *Hcms8a20* as a reliable marker linked to IVM resistance in separate backcrossing experiments utilizing the same parental strains. However, the mutation(s) conveying resistance which are linked to this microsatellite marker remain unknown.

Drug uptake

In *C. elegans*, the *dyf* (defective dye filling) gene family is responsible for amphid (chemosensory organ) development (Li et al., 2001; Heiman and Shaham, 2009). Dent et

al. (2000) found four alleles of *dyf* genes that constituted decreased uptake of dye from the environment and these *C. elegans* mutants also had low-level resistance to IVM. Urdaneta-Marquez et al. (2014) found that a *dyf-7* gene rescued IVM sensitivity in a resistant strain, and *dyf-7* mutants were selected for upon IVM exposure. Therefore, mutations in *dyf-7* are suggested to be directly related to a ML resistance mechanism. More recently, Page (2018) confirmed the association between IVM resistance and *dyf* when numerous strains of IVM resistant *C. elegans* failed to intake dye via their amphid organs, and the *dyf* mutant *C. elegans* (*dyf-3, dyf-4, dyf-7*) had decreased sensitivity to IVM (Page, 2018).

Studies of amphids in *H. contortus* noted structural changes between IVM susceptible and resistant isolates of *H. contortus*, where resistant isolates had degenerated amphids (Freeman et al., 2003; Guerrero and Freeman, 2004). Urdaneta-Marquez et al. (2014) further explored this in ML resistant *H. contortus.* Resistant and susceptible isolates derived from the same parental strain were sequenced and 15 SNPs characterized two highly divergent haplotypes, namely *Hco_dyf-7(r)* and *Hco_dyf-7*(s), respectively. Significantly fewer animals harboring the resistant haplotype were capable of dye uptake, supporting the role of reduced amphids in anthelmintic resistance. Finally, resistant haplotypes, identified by some, but not all of the 15 SNPs was consistently found at high frequencies in ML resistant *H. contortus* populations across five continents, while the susceptible haplotype was consistent with susceptible populations (Urdaneta-Marquez et al., 2014). In contrast, Elmahalawy et al. (2018) explored the prevalence of three SNPs in the *Hco_dyf-7(r)*, previously identified by Urdaneta-Marquez et al. (2014), in field isolates identified to IVM resistant by FECRT, and did not find any significant differences in SNPs in larva harvested before and after IVM treatment. Another study found no association of the *Hco_dyf-7(r)* haplotype with IVM resistance in a backcross experiment (Rezansoff et al., 2016). Laing et al. (2016) also did not identify different haplotypes between IVM-treated and non-treated sheep farms in the UK.

Furthermore, a genome-wide scan of SNPs in resistant and susceptible *H. contortus* strains was carried out by Luo et al. (2017). Several SNPs were identified that exhibited significant differences in allele frequencies between the two populations. Of these, the function of eight genes were recognized as potentially playing a role in ML

resistance, such as functioning as an IVM target receptor or participating in receptor formation. None of the candidate genes identified in previous studies, such as various ATP binding cassette transporters (described below) or *dyf-7*, were identified in this study (Luo et al., 2017). Mutations in the *dyf-7* gene were not identified in a multi-drug resistant strain of *T. circumcincta* (Choi et al., 2017). The differences in these reports may be due to the known high genetic diversity of *H. contortus* populations, including laboratory isolates (Gilleard and Redman, 2016).

<p align="center">*Drug efflux and metabolism*</p>

The association of protein transporters, especially Pgps, with anthelmintic resistance has been a popular research topic. P-glycoproteins serve as an efflux mechanism to transport toxic molecules, such as xenobiotics, across the cell membrane thus lowering their intracellular concentration (Higgins, 1992; Broeks et al., 1995). This prevents the drug from reaching its target site (Lanusse et al., 2014). However, a general consensus of their involvement in anthelmintic resistance mechanisms has not been reached (Lespine et al., 2012).

The first report of Pgps associated with IVM resistance was in *H. contortus* in 1998 (Xu et al., 1998). Similarly, Le Jambre et al. (1999) associated *hc-pgp-1* with resistance in an IVM-selected strain of *H. contortus*. When a Pgp inhibitor, such as verapamil, was applied to ML resistant *H. contortus strains,* IVM sensitivity reversed (Molento and Prichard, 1999), confirming the role of Pgps in drug resistance. Currently, at least ten Pgp sequences have been identified in *H. contortus* (Williamson and Wolstenholme, 2012; Laing et al., 2013), and 14 identified in *C. elegans* (Sheps et al., 2004). The role of Pgps as a protective mechanism has also been identified in *C. elegans,* where animals with inhibited Pgps were significantly more sensitive to IVM treatment (Janssen et al., 2013a).

Probably the most consistently identified Pgp to be associated with ML resistance is PGP-2. Godoy et al. (2015a) proved the interaction of ML drugs with PGP-2*,* and PGP-2 is unable to translocate fluorophores in the presence of drug due to its paralyzing effects. James and Davey (2009) identified increased expression of *pgp-2* in an IVM resistant strain of *C. elegans.* Later, Yan et al. (2012) confirmed the necessity of this gene

in conferring IVM resistance in *C. elegans* through a gene knockout study using RNAi techniques. Both Lloberas et al. (2013) and Maté et al. (2018) reported increased expression of *pgp-2* in a ML resistant strains of adult *H. contortus* exposed to IVM *in vivo* (Lloberas et al., 2013; Maté et al., 2018). Larvae obtained from a multi-drug resistant *H. contortus* strain showed increased expression of *pgp-2* and *pgp-9*, but further *in vitro* drug exposure of this isolate produced inconsistent changes in Pgp expression levels (Williamson et al., 2011).

Many other Pgp genes have been studied in small ruminant GIN, but results are inconsistent and sometimes contradictory (Williamson et al., 2011; Williamson and Wolstenholme, 2012; Rezansoff et al., 2016; David et al., 2018; Maté et al., 2018). Of course, the role of these transporters may not be limited to changes in expression levels. The presence of SNPs and allelic changes in these genes have associated with resistance in *T. cicumcincta* (Prichard and Roulet et al., 2007; Dicker et al., 2011a,b; Lespine et al., 2012; Turnbull et al., 2018) and *H. contortus* (Blackhall et al., 1998; Prichard and Roulet et al., 2007; Williamson et al., 2011; Lespine et al., 2012).

Differing interactions of Pgps between IVM and MOX exposure have been reported in *H. contortus* (Godoy et al., 2015a,b, 2016) and in a pig kidney epithelial cell line overexpressing *Pgps* (Lespine et al., 2007). Similar findings have been presented in *C. elegans,* where no significant differences were found in expression of *pgp-2* between the IVM resistant and IVM susceptible strain of *C. elegans* when exposed to MOX (Bygarski et al., 2014). This suggests different mechanisms of anthelmintic resistance between the avermectin and milbeymicin groups, and may reflect the lower potency of MOX and the differences in molecular structures as described in section 1.5.3.

Drug metabolizing enzymes are another non-specific mechanism presumably leading to ML resistance. The CYPs are known to confer insecticide resistance (Ffrench-Constant et al., 2004), and treatment of *C. elegans* with anthelmintics has been associated with increased expression of enzymes in the *cyp* family (Laing et al., 2010, 2012). Currently, 23 CYP subfamilies have been described for *C. elegans* (Menzel et al., 2001), of which CYP31, CYP33, CYP34, and CYP35 have been identified as inducible following xenobiotic exposure (Menzel et al., 2001; Laing et al., 2010; Jones et al.,

2013). In *H. contortus,* 73 partial CYP sequences were found, 68 of which were confirmed by PCR (Laing et al., 2015). Increased expression of *cyps* was found within adult worm intestine (Laing et al., 2015). Recently, Yilmaz et al. (2017) identified CYPs of the CYP34/35 subfamily having increased expression in a multi-drug resistant *H. contortus* isolate compared to a susceptible isolate.

<div align="center">

1.8.4.2   Macrocyclic lactone resistance mechanisms in strongylids of cattle

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Two subunits of the GluCl receptor from *C. oncophora* have been sequenced and identified to be highly similar the corresponding subunits of *H. contortus* and *C. elegans* (Njue et al., 2004a). Further investigation of two UK *C. oncophora* isolates, one being IVM resistant and the other IVM susceptible, identified significantly different frequencies in nine alleles of the *glucla3* subunit gene between the two populations. Later, Njue et al. (2004b) used the *Xenopus* oocyte expression system to model a GluClα3 homomeric channel and attributed differences in IVM sensitivity to be due to a single amino acid substitution (L256F) in the GluClα3 subunit. However, the L256F substitution was not present in neither resistant nor susceptible isolates of *C. oncophora* and *O. ostertagi* from Belgium (El-Abdellati et al., 2011). The resistant *C. oncophora* and *O. ostertagi* isolates showed a decreased expression of *avr-14*, and resistant *C. oncophora* also exhibited increased expression of *glc-2*. Some differences in expression were also noted between parasite life stages (El-Abdellati et al., 2011).

As in *H. contortus* it is presumed that Pgps constitute drug efflux and play a role in ML resistance for GIN of cattle. Using the larval development assay, both Demeler et al. (2013d) and AlGusbi et al. (2014) restored IVM susceptibility in IVM resistant isolates of *C. oncophora* by applying the Pgp inhibitor, verapamil. The same finding was reported for *O. ostertagi* (AlGusbi et al., 2014). Interestingly, the addition of verapamil and IVM to IVM resistant *C. oncophora* during the larval migration inhibition assay increased IVM sensitivity even beyond that of the IVM susceptibility isolate. The different magnitude of responses between the two *in vitro* assays examined by AlGusbi et al. (2014) are likely due to fluctuations of drug uptake at different life stages *(i.e.* the

larval development assay observes $L_1, L_2,$ and L_3 whereas the larval migration inhibition assay utilizes only L_3s). However, another study suggests that diminished development of *T. circumcincta* observed in the larval development assay may be caused by verapamil itself, and not IVM (Sargison et al., 2010). A CYP inhibitor, piperonyl butoxide, when co-administered with IVM, restored IVM sensitivity in IVM resistant larval isolates of *C. oncophora in vitro* (AlGusbi et al., 2014). The gene for *pgp-9* has been identified in *C. oncophora*. Expression levels of this gene were examined between IVM-selected and non-selected isolates, however no significant differences were identified. Overall, it seems feasible to assume that non-specific mechanisms may also play a role for anthelmintic resistance in cattle GIN, but the exact contributors are unknown.

1.8.4.3 Macrocyclic lactone resistance in *Dirofilaria immitis*

As described above, the existence of anthelmintic resistance by the canine heartworm, *D. immitis*, to the ML drug class is a topic of great controversy, but reports of failed efficacies do exist and have been the driving force behind studies of these resistance mechanisms.

An extensive study was undertaken to identify all the ion-channels existing in the current *D. immitis* genome, and any SNPs within (Mani et al., 2016b). Five genes encoding GluCl subunits were identified, namely *glc-2, glc-3, glc-4, avr-14* and *avr-15*. Of these, SNPs were identified in *glc-2, glc-4,* and *avr-14*. Those in *avr-14* and *glc-2* were predicted to change the secondary structure of the subunit. Polymorphisms in other GluCl channels were identified, such as *gab-1*, a GABA-gated ion channel. The polymorphism I20T was identified only in anthelmintic susceptible populations. The *unc-49* gene had a SNP (N57D) that was specific to populations with suspected resistance. As previously discussed, the GluCl channels are known targets of the MLs, and therefore it can be theorized that these mutations causing structural changes may alter the drug binding site and confer resistance (Mani et al., 2016b).

The first study to examine Pgps in *D. immitis* was carried out by Bourguinat et al. (2011). In this study, they identified two SNPs located at positions 11 and 618 of a fragment orthologous to *pgp-11* of several nematode species. The genotypes of these

SNPs were combined to identify the genotype of *D. immitis* isolates with lowered IVM susceptibility. The Pgp genotype with GG at both positions 11 and 618 (GG-GG) was at a significantly higher frequency in an isolate with decreased IVM susceptibility both *in vivo* and *in vitro* (Bourguinat et al., 2011). Later, a study sought to identify all of the Pgps within *D. immitis* and a total of five ATP-binding cassette transport genes were identified, three of which are Pgps, namely *Dim-pgp-3, Dim-pgp-10,* and *Dim-pgp-11* (Bourguinat et al., 2016). It has been identified that avermectins interact with the *Dim-pgp-11* and are capable of inhibitory effects in susceptible isolates, but the milbeymicin drugs exhibit a much weaker effect and act in a dose-dependent manner (Mani et al., 2016a). Continuing with the trend of ML resistance, the mechanisms of ML resistance in *D. immitis* remain largely unknown. In order to better monitor and control resistant isolates of any parasite, markers of resistance need to be identified. This work has recently begun for *D. immitis* which identified 40 SNPs that differed between ML susceptible and resistant isolates (Bourguinat et al., 2015).

1.8.4.4 Macrocyclic lactone resistance mechanisms in *Parascaris* species

Despite the wide spread resistance of *Parascaris* spp. to the ML drug class, only two studies have examined this at the molecular level. Only two Pgps have been confirmed in *Parascaris equorum*, namely *pgp-11* and *pgp-16* (Janssen et al., 2013b). In the *pgp-11* gene, three missense mutations have been identified in IVM resistant populations. Using 3D modelling, it is presumed that these amino acid substitutions occur near residues involved with drug binding sites. The *pgp-11* was found to be more highly expressed in the worm intestine than other tissues, while *pgp-16* was more highly expressed in body wall tissues. Despite these findings, differences in expression of these genes were not identified in parasite eggs isolated from farms with differing susceptibility status to IVM. Differences were also not identified between adult worms exposed to IVM *in vitro* and those without treatment (Janssen et al., 2013b). In contrast, worms isolated from a farm with suspected IVM resistance had an increased frequency of all three SNPs when compared to worms collected from farms with an unknown IVM treatment/status history (Janssen et al., 2013b). Later, Janssen et al. (2015) successfully expressed the

Parascaris spp. *pgp-11* in a *C. elegans* model. In doing so, the ability of *pgp-11* to decrease *C. elegans* susceptibility to IVM was discovered. Both studies provide support that Pgps, specifically *pgp-11,* are likely involved in anthelmintic resistance mechanisms for *Parascaris* spp., but it is unlikely that this is the sole mechanism conveying the extreme levels of ML resistance observed world-wide.

1.8.4.5 Macrocyclic lactone resistance mechanisms in cyathostomins

Resistance to the ML drug class among cyathostomin populations is not yet definite, but rather is considered emerging. Given the resistance status and the 50+ species of cyathostomins, it has been and continues to be a daunting task to identify potential mechanisms associated with the emergence of resistance. Only two putative Pgps have been identified across nine cyathostomin species (Drögemüller et al., 2004c). Later, Kaschny et al. (2015) transgenically expressed *pgp-9* isolated from *Cylicocyclus elongatus* in a yeast assay. The investigators found *pgp-9* to protect yeast from various fungicides, suggesting a drug efflux mechanism was at play. Recently, Peachey et al. (2017) confirmed the presence of *pgp-9* in mixed species cyathostomin populations from an equine herd with heavy ML use and a herd naïve to anthelmintics. Larvae (L_3) from both populations were exposed to IVM *in vitro*, and the cyathostomins with previous ML exposure had significantly higher expression of *pgp-9* than the anthelmintic naïve population. The phenotypic characterization was confirmed using the larval migration inhibition assay and Pgp inhibitors. When a Pgp inhibitor was applied with IVM, the efficacy of IVM increased in the cyathostomin isolates from previous ML exposure. This suggests that *pgp-9* can reduce sensitivity to IVM (Peachey et al., 2017).

1.8.4.6 Macrocyclic lactone resistance mechanisms: Conclusion

The majority of work for ML resistance has been focused on non-specific resistance mechanisms. The Pgps are continuously being extensively studied, but the evidence presented above suggests that their involvement varies between species and isolates. Additionally, the studies have focused largely on the avermectin group over the

milbemycins, and mostly on *H. contortus* and *C. elegans.* While this is a promising start, significant challenges exist that prevent broad assumptions from being made. As mentioned several times, the avermectins and milbemycins have structural differences that affect their binding affinities to the target receptors (Prichard et al., 2012). Evidence described above proves that these differences influence the drug-specific resistance mechanisms and extrapolations between the two drug groups should be interpreted with caution. Secondly, the majority of work presented herein involves *H. contortus* and *C. elegans.* Both of these nematodes are phylogenetically classified into clade V of the phylum nematoda (Blaxter et al., 1998), and therefore presumably have similar characteristics and some extrapolations regarding drug and anthelmintic resistance mechanisms between the two can be made. However, other parasites harboring ML resistance, such as *Parascaris* spp. and *D. immitis* are not closely related to these nematodes (Clade III). Although some similarities in ML resistance mechanisms have been identified, more studies are needed to elucidate the mechanisms for these parasites.

1.9   Parasite management in the presence of anthelmintic resistance

1.9.1   Refugia maintenance

The rate of development for anthelmintic resistance is largely dependent upon the ability of parasites surviving treatment to genetically contribute to the next generation (Barnes et al., 1995). Refugia populations offer the opportunity to dilute the resistant alleles with the susceptible alleles. Refugia is defined as the portion of the parasite population left unexposed to anthelmintic treatment, and is recognized as a key component of herd based (i.e. livestock, equine) parasite control programs in order to preserve anthelmintic efficacy (Martin et al., 1981; Van Wyk, 2001; Waghorn et al., 2008; Kaplan and Nielsen, 2010; Leathwick, 2012; Leathwick et al., 2012; Leathwick and Besier, 2014; Cornelius et al., 2016; Muchiut et al., 2018). Those, which escape treatment, include the environmental stages (*i.e.* larvae on pasture), migrating and encysted larvae within the host when a larvicidal drug is not used (*i.e.* encysted cyathostomins), and those within untreated animals (Van Wyk, 2001). Selective treatment approaches (see section 1.4.3) offer the opportunity to leave some animals

untreated, and thus leaving some parasites unexposed (Kenyon et al., 2009; Kaplan and Nielsen, 2010). Maintenance of refugia is presumably only effective when the refugia population largely consists of susceptible parasites in order to dilute the resistant alleles (Martin et al., 1981; Van Wyk, 2001; Muchiut et al., 2018), and the seasonal timing of treatments must be considered so that the environmental conditions favor larval development on pasture (Gaba et al., 2006, 2010). Several computer modelling and field studies have examined the impact of refugia on the development and persistence of resistance. These are described below.

1.9.2   Computer modelling studies of refugia

1.9.2.1   Modelling studies of small ruminant trichostrongylids

A computer model developed by Gaba et al. (2006) emphasized the importance of implementing individual sheep parameters within the model, such as the unequal disbursement of egg shedding between the hosts. This approach highlighted the significant effect of refugia size on the development of anthelmintic resistance. The maintenance of refugia decreased the rate of resistance development when stocking densities were low, treatments were given outside of the dry season, and the overall number of treatments was reduced (Gaba et al., 2006). Similarly, a mathematical model found that treating only 20-30% of the flock when environmental conditions favored larval development resulted in a higher number of larvae in environmental refugia. This proposed treatment regimen predicted the frequency of resistance alleles to be below phenotypic detection (*i.e.* FECRT; Gaba et al., 2010). The influence of treatment frequency and proportion of sheep treated on resistance frequency was again examined by Laurenson et al. (2013). First, a short-term scenario evaluated the proportion of lambs treated in a single treatment throughout the grazing season. As expected, increasing the number of lambs treated reduced the refugia population on pasture and increased the resistance frequency by the end of the grazing season. In a second, but long-term (20 years) simulation, the model predicted the resistance frequency when all, 50%, or 20% of lambs were treated. Even when 50% of the lambs were left untreated, the resistance frequency ended up being the same at the end of the 20 years as when all lambs were treated. However, the resistance frequency was reduced by over half when 80% of the

lambs were left untreated (Laurenson et al., 2013). Another modelling study examined the development of resistance in presence of refugia based on the number of lambs untreated in two different environments when two different drugs (99.99% or 97% efficacious) were used (Cornelius et al., 2016). When hot, dry summers were experienced, the rate of resistance significantly decreased as the portion of untreated animals increased when the lesser efficacious drug was used. When the more highly efficacious drug was used, resistance developed significantly slower when at least 10% of the animals were left untreated when compared to all animals treated. In the environment with high rainfall, no significant differences for either drug were found in the rate resistance when at least 10% of the animals were left untreated. This study confirms the necessity of parameters, such as environment and initial drug efficacy, when considering the number of susceptible parasites present in refugia and the overall effect on the rate of resistance development (Cornelius et al., 2016). Proper use of a new anthelmintic to achieve the maximum efficacious lifespan is an important topic of discussion. Leathwick (2012) has modelled different treatment regimens and refugia proportions to predict sustainability of a new drug. This model continues to emphasize the importance of refugia to delay the initial onset of anthelmintic resistance as low-refugia scenarios resulted in an increased rate of development.

While the aforementioned studies provide useful information for the implementation of refugia and delaying resistance, they are assuming that resistance alleles are not already present in the population, and this is rarely the case. Other factors that must be considered when modelling the influence of refugia on resistance are the initial frequency of resistance alleles in a given population and the associated fitness loss of the parasite (see section 1.8.1). Leathwick (2013) accounted for these variables and the effect of refugia on resistance development. However, in this model refugia was not considered as the number of animals untreated according to physiological parameters, but rather as total number of eggs excreted onto the pasture. In all simulations of initial anthelmintic frequency and fitness loss, increasing the FEC output slowed the development of resistance (Leathwick, 2013).

1.9.2.2   Modelling studies of equine parasites

Parascaris spp. offers a challenging perspective on refugia maintenance as this parasite is generally not present in horses beyond two years of age (Clayton, 1986), and it is recommended for foals to receive two treatments within their first six months of life (Nielsen et al., 2016; Reinemeyer and Nielsen et al., 2017; ESCCAP, 2018; Rendle et al., 2019). Leathwick et al. (2017) modelled the effects of different treatment regimens on the rate of resistance development. Monthly treatments and use of a drug with larvicidal efficacy resulted in resistance more quickly than the when only two treatments were given. This is likely due to the minimized refugia population. The current recommendations are to provide foals with two treatments in their first six months of life (section 1.4.3). When treatments were given at two and five months of age resistance was delayed compared to treatments given at three and four months. The latter targets immature luminal stages and diminishes the possibility of egg shedding thus reducing the refugia population. Furthermore, the use of IVM as the first treatment also increased the rate of IVM resistance. The larvicidal efficacy of IVM prevented the development of patent worms and thus negated the opportunity of IVM-based refugia, again driving resistance. Overall, the timing of treatments must be considered in order to not completely diminish egg shedding as this will reduce the genetic contribution of susceptible parasites in refugia (Leathwick et al., 2017).

Another recent modeling study examined the influence of treatment frequency, timing of treatments, and seasonality on anthelmintic resistance in cyathostomin parasites (Nielsen et al., 2019). While this exercise did not specifically focus on refugia maintenance, some of the regimens employed implemented the maintenance of refugia. Overall, this study found selective therapy implementing only two annual treatments to reduce the rate of resistance. In turn, the reduction of treatment frequency and reducing the number of animals treated reflects an increase in refugia. Furthermore, the seasonal timing of treatments also significantly impacted resistance development. When treatments were administered during the grazing season (*i.e.* climates favoring larval development), the rate of resistance increased compared to when treatments were given outside of the grazing season (i.e. when environmental conditions did not favor larval development). Resistant worms surviving treatment would primarily contribute to egg shedding and thus the environmental refugia population. If this occurs during favorable

environmental conditions, then resistant larval will dominate the refugia. If eggs are shed during unfavorable conditions, then obviously the resistant larval will be less likely to survive and contribute less to subsequent adult populations (Nielsen et al., 2019). Overall, the number and timing of treatments must be considered in order to support a refugia population with minimal resistant alleles.

1.9.3   Field studies of refugia
1.9.3.1   Field studies of small ruminant trichostrongylids

Martin et al. (1981) published one of the first reports showing the direct association between the number of parasites in refugia and the rate of development for anthelmintic resistance. This was done by artificially infecting sheep with various numbers (0-10,000) of *H. contortus* larvae before and after anthelmintic treatment. The larvae after treatment represented the refugia population as they were not exposed to treatment. After six generations, results indicated that the rate of development for anthelmintic resistance was slower with larger refugia populations. A field study of four different treatment regimens for controlling trichostrongylids in sheep found targeted selective treatment (<2.2 treatments per year) or strategic deworming (2 treatments/year) approaches to maintain better IVM efficacy over a given period than regular drenching of the entire herd every four weeks. Presumably, this was likely due to the increased refugia population (Kenyon et al., 2013). Refugia maintenance is a large part of the 'best practice parasite management program' presented by Rhodes et al. (2011) to maintain anthelmintic efficacy on working sheep farms. Leathwick et al. (2015a) identified that implementation of this program resulted in an overall increase in anthelmintic efficacy over five years. While equal efforts in all areas of this program are important, the authors conclude that the increased efficacy is largely due to the use of combination anthelmintics in the presence of a large refugia population (Leathwick et al., 2015a).

A great challenge to overcome is when resistance is established on a pasture and in the refugia population. Attempts can be made to reestablish susceptible parasites within the refugia population by administering effective treatment to remove the resistant

worms infecting the hosts, pasture resting allowing time for the infective stages to die, and/or by seeding a clean pasture with susceptible worms. The latter can be accomplished by infecting parasite-free animals with susceptible parasites and turning them out on the pasture, or by bringing in animals harboring parasites with known susceptibility (Muchiut et al., 2018). Muchiut et al. (2018) has summarized the studies employing this practice to date, where all have involved trichostongylids of sheep except one that examined *Cooperia* spp. of cattle. While a number of these studies were either not successful at all or limitedly sustainable, several reports were successful. One of the most important factors is to consider the length of time between introducing the susceptible parasites and re-evaluating the anthelmintic efficacy of the drug in question. For initial success, this may require at least two years to fully dilute out the resistant alleles in the refugia population (Muchiut et al., 2018).

1.9.3.2 Field studies in equine parasites

Currently, no studies exists examining the direct association between the presence and/or level of refugia and the rate of anthelmintic resistance development in equine strongyles. The implementation of refugia maintenance in equine herds is largely based on the evidence obtained from ruminant strongylid parasites. Evidence for managing resistance by reducing treatment intensity (frequency and number of animals treated), such as in selective treatment regimens, would seemingly increase the number of parasites in refugia. Therefore, the maintenance of a refugia population among equine strongyles is recommended by leading experts (Nielsen et al., 2016; ESCCAP, 2018; Rendle et al., 2019). Long-term studies are needed to evaluate the impact of selective deworming strategies on anthelmintic resistnace before direct conclusions can be made.

1.9.4 Combination deworming

Combination deworming in the current context is described as combining two or more drugs with different modes of action and targeting a single type of parasite (Leathwick et al., 2009). The theory is that parasites resistant to one of the compounds

will be removed by the other compound, and multi-drug resistant parasites will have a reduced ecological fitness and unable to pass their genetics onto future generations.

When combining drugs there may be a risk or benefit for the associated drug-drug interactions. These interactions can be broadly classified into two types, pharmacokinetic (PK) and pharmacodynamic (PD) (Lanusse et al., 2014). Pharmacokinetic interactions occur when one drug affects the concentration of another drug at the target site. This is generally related to drug absorption, distribution, metabolism, and excretion. These interactions may occur at the host or target parasite level. Pharmacodynamic interactions refer to when one drug alters the receptor and/or effector responses of the other drug (Lanusse et al., 2014). The PD interactions can be further described as additive, synergistic, antagonistic, and indifference. In general, two drugs having different modes of action should result in either an additive or synergistic effect. By definition, an additive effect occurs when the combined effect is equal to the sum of the effects for each individual drug. A synergistic effect is when the combined effect is significantly greater than the sum of the individual drug effects. While a synergistic effect is the ideal achievement for combination anthelmintics, most studies report an additive effect (Lanusse et al., 2014). On the other hand, antagonistic effects may occur when one drug blocks the effects of the other drug. Some examples of these interactions include inhibition of drug metabolism, drug interactions at the site of drug efflux transporters, or inhibition or alteration of drug absorption (Lanusse et al., 2014). Understanding the drug interactions of a combination product is crucial prior to its implementation, however, elucidation of these mechanisms are not widely understood, and there are many unknown variables such as, anthelmintic resistance status/mechanisms present, seasonality of parasite infection, changes and presence of drug metabolites, and route of drug administration. Despite this, there are several combination products, containing 2-4 different compounds available for ruminants and horses in South America, Australia, and New Zealand. There are no combination products available in the United States, but they are commonly used extra-label and in some cases even encouraged by parasitology experts, such as the American Consortium for Small Ruminant Parasite control (Kaplan, 2017).

Furthermore, combination deworming is heavily dependent upon the maintenance of an adequate refugia population as described above (Leathwick, 2012) and is most beneficial when at least one of the actives is new to the population and/or exhibits 100% efficacy (Barnes et al., 1995; Leathwick, 2012). When resistance exists to both actives used (in a two active compound), then their use is not recommended as it will likely select for multi-drug resistant parasites (Lanusse et al., 2014).

1.9.4.1 Modelling studies of combination deworming in ruminants

The majority of modelling studies in ruminants have focused on helminths infecting sheep due to the major resistance crisis surrounding these hosts. Learmount et al. (2012) modelled UK sheep farms and the effects of introducing a new anthelmintic belonging to the spiroindole drug class, derquantel in combination with the ML, abamectin. The model assumed a minimum resistance allele frequency corresponding to near complete efficacy (99.9%) for derquantel, while abamectin was modelled having either a low resistance allele frequency with 95% initial efficacy, or a high allele frequency with only 50% efficacy. Anthelmintics were administered either individually in the traditional rotation method, or in combination. Furthermore, two farm management scenarios were examined. The first was to implement recommendations by the committee for the sustainable control of parasites in sheep (SCOPS) including increased refugia and decreasing treatment intensity, while the other farm did not maintain these practices. Over the course of 40 years, the model found that introducing a new drug in combination slowed the development of resistance to the new drug (derquantel) and slowed the rate of resistance to the current drug used (abamectin) regardless of initial resistance allele frequency or management style. It also found that implementation of SCOPs delayed resistance to both drugs compared to non-SCOPs management practices. Finally, the lower the initial level of resistance allele frequency, the slower resistance developed. A similar simulation study was performed by Leathwick (2012) modelling management practices implemented by New Zealand sheep farmers and the effects of introducing a new anthelmintic (monepantel or derquantel) in combination with abamectin. Again, the combination product greatly delayed the development of resistance to the new drug and

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slowed the rate of resistance development to abamectin. Management scenarios which maintained an adequate refugia population further improved these results and provided more flexibility for the initial frequency of the abamectin resistance alleles in the population (Leathwick, 2012). The benefit of combination products and maintaining a refugia population was echoed again in a modelling study with levamisole and IVM, and this model was developed following findings of a field study which produced similar results (Leathwick et al., 2012). Later, Leathwick (2013) prepared a generalized model utilizing two drugs in order to demonstrate the association of different refugia proportions, levels of fitness loss associated with resistance alleles, and varying the initial frequency of resistance alleles in a given population. Again, regardless of all other variables, increasing the size of the refugia population slowed the development of resistance. The same conclusion was found when the drugs were used in combination rather than in annual rotation. A new finding was that the higher the fitness cost associated with the resistance alleles resulted in slower development of resistance, and this was more pronounced when a combination was used. It is suggested that this is because the combination leaves fewer resistant worms surviving treatment. In summary, the aforementioned modelling studies provide preliminary evidence supporting the use of combination deworming products against drug-resistant trichostrongylids infecting sheep. However, these benefits are largely dependent on the refugium size and preexisting factors, such as the current level of resistance.

1.9.4.2   Field studies of combination deworming in ruminant parasites

The first *in vivo* study examining the benefits of combination deworming against anthelmintic resistant sheep nematodes occurred just over four decades ago (Anderson et al., 1991). As previously described, combination treatments are most effective when at least one of the drugs administered are new to the parasite population of interest and/or have complete efficacy (Barnes et al., 1995; Leathwick, 2012). Therefore, release of the newest anthelmintic drug for treating sheep GIN, derquantel, a member of the spiroindole drug class, was released in combination with abamectin. This combination awarded 99.8-100% efficacy against a variety of GIN infecting sheep of the hypobiotic larval stage,

fourth larval stage, and adult stages of both resistant and unknown status (Little et al., 2011). Later, Geurden et al. (2012) performed a controlled efficacy study and confirmed the ability of the derquantel-abamectin combination to totally (100%) reduce the worm burden following experimental infection with a MOX resistant isolate of *T. cicumcincta*. Single active MOX and abamectin provided only 12.4% and 71.8% reduction, respectively. This study also examined treatments against naturally acquired mixed-nematode infections from farms harboring multi-drug resistance. The derquantel-abamectin combination had 99.7% efficacy, while MOX and abamectin exhibited 42.6% and 96.9%, respectively (Geuerden et al., 2012). In contrast, George et al. (2012) determined the efficacy derquantel-abamectin combination against a ML resistant *Teladorsagia* spp. isolate to only reduce egg counts by 94.8% whereas monepantel single active and abamectin single active awarded 98.5% and 34%, respectively.

Other work has explored the combination of anthelmintics when resistance already exists. Entrocasso et al. (2008) administered IVM and albendazole either individually or in combination against sheep GIN with resistance to both actives. When given intravenously, the combination had a FECR of 91.9%, compared to the 73.4% and 79% efficacy of albendazole and IVM given alone, respectively. When albendazole was administered intraruminally it exhibited 43.5% efficacy, and IVM given subcutaneously had 79.8% efficacy, however the combination of these two drugs and the same routes of administration exhibited only 70.8% efficacy. The authors concluded that the decrease in albendazole efficacy was due to the inability for the drug to reach the target site at the necessary concentrations. Therefore, the proper route of administration of each drug class must be considered when using combination drugs (Entrocasso et al., 2008). Edmonds et al. (2018) explored the benefits of combination therapy against a known ML-resistant cattle GIN population. The combination consisted of an injectable ML drug with 28-day activity (doramectin) with an oral BZ (albendazole), which was presumed to still be efficacious. The combination was compared to an oral dose of doramectin only or a 100-day extended release injectable ML (eprinomectin). Overall, the doramectin-albendazole combination exhibited higher FECR than the other groups where efficacy was maintained at 98.8% or higher until day 32 post-treatment. At 14-days post-treatment the doramectin and eprinomectin single actives were 47% and 71.3%, respectively. Combination

deworming against another IVM resistant cattle GIN population was targeted using the BZ drug, ricobendazole and levamisole. This was compared to IVM injectable single active, ricobendazole single active, and no treatment. At 21-days post treatment, the IVM only group had a FECR of only 18%, while the ricobendazole only was 96% and the ricobendazole-levamisole combination was 100%. Overall, combination deworming against ruminant parasites award a synergistic or additive effect, but several management practices must also be considered, such as route of administration, hygiene practices, and the proportion of parasites in refugia. The large majority of the aforementioned studies utilized an untreated control group to calculate the FECR which also provides a source of refugia.

1.9.4.3 Modelling studies of combination deworming in equine parasites

Currently, the only equine modelling study investigating combination deworming was against *Parascaris* spp. (Leathwick et al., 2017). The primary focus of this study was evaluating different treatment regimens and their effect on anthelmintic resistance. In the model, combination deworming with IVM, FBZ, and PYR significantly delayed the development of resistance when administered twice in the foal's first year, however resistance to IVM developed more quickly than the other actives. This was likely due to the larvicidal efficacy of IVM resulting in minimal IVM-based refugia. However, even in the presence of IVM resistance, the use of IVM did not affect the efficacy of FBZ and PYR (Leathwick et al., 2017). Therefore, a combination of at least FBZ and PYR should be considered in future research for treatment against *Parascaris* spp.

1.9.4.4 Field studies of combination deworming in equine parasites

Presently, only four studies exist evaluating the *in vivo* effects of combination deworming against equine parasites. The first combination treatment evaluation in horses was by Rolfe and Dawson (1994). They utilized a combination of dichlorvos (an organophosphate not approved for use in horses), OBZ, and morantel tartrate to target

Parascaris spp. and suspected OBZ resistant strongyles. Against *Parascaris* spp., the combination demonstrated 98% efficacy at 14 days post-treatment. Against the strongyles, the combination had 99% efficacy at 14 days post-treatment, whereas morantel, dichlorvos, and OBZ had single active efficacies of 96%, 42%, and 84%, respectively (Rolfe and Dawson, 1994). Later, Lyons et al. (2016) tested the combination of OBZ and PYR, and OBZ with piperazine (no longer marketed for use in horses) against *Parascaris* spp. and strongyles. Drug efficacies using the FECRT were not calculated in this study, but the number of horses positive/negative for each parasite before and after treatment was reported. The single active OBZ decreased the number of horses positive for *Parascaris* spp. by 100%, whereas PYR did not change the number of positive horses, and piperazine decreased the positive horses by approximately 50%. Similarly to the OBZ-single active, the OBZ-PYR and OBZ-piperazine combinations reduced the number of *Parascaris* spp. positive horses to zero. For the strongyle infections, the OBZ single active treatment failed to reduce the number of positive horses, whereas PYR single active decreased from three positive horses to two, and the piperazine single active reduced strongyle positive horses from 86% to 12%. The OBZ-PYR combination reduced the strongyle positive horses from three to two, and the OBZ-piperazine combination reduced the number of strongyle positive horses from 94% to 15%. Wilkes et al. (2017) examined the efficacy of a single treatment of morantel-abamectin combination against *Parascaris* spp. with known resistance to IVM and abamectin in foals in Australia. The combination provided >99% efficacy whereas an increase in ascarid egg counts followed the treatment with abamectin single active. IVM single active had an efficacy of only 49.71% (Wilkes et al., 2017). Another study examined a single treatment of OBZ-PYR combination against equine strongyles on 11 different horse farms (Kaplan et al., 2014). On all farms, OBZ-single active exhibited ≥80% efficacy, but the majority of farms had ≥90% efficacy. For PYR-single active, six farms exhibited ≥90% efficacy and the other farms ranged from 46.4% to 89.7%. For the combination treatment, six farms exhibited ≥99% efficacy, and the other farms ranged from 90.0%-97.9%. The study showed that a single combination treatment can be beneficial when at least one of the actives is efficacious. Of all the aforementioned combination deworming studies, this was the only study to evaluate the additive effect

formula for predicting the efficacy of a combination anthelmintic based on the individual drug efficacies, and found it to be a reliable indicator of expected efficacy (Kaplan et al., 2014).

1.9.4.5   Combination deworming: Summary

Overall, it appears that the results of computer modelling studies for combination deworming in ruminants is supported by the field data, in that combination deworming, when applied under appropriate conditions can be beneficial. In horses, the data is much more limited and studies exploring the common scenario of multi-drug resistant cyathostomins have not been done. In all cases, the field data of long-term studies with repeated combination deworming is lacking and more studies are needed to evaluate real long-term consequences.

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Table 1.1 Reports of drug resistance in *Parascaris* spp.

Country	Macrocyclic Lactones	Drug Class Tetrahydropyrimidines	Benzimidazoles
Australia	Armstrong et al., 2014; Beasley et al., 2015; Wilkes et al., 2017	Armstrong et al., 2014	Armstrong et al., 2014
Brazil	Molento et al., 2008		
Canada	Hearn and Peregrine, 2003; Slocombe et al., 2007		
Denmark	Schougaard and Nielsen, 2007		
Estonia	Lassen and Peltola, 2014		
Finland	Näreaho et al., 2011		
France	Laugier et al., 2012; Geurden et al., 2013		
Germany	von Samson-Himmelstjerna et al., 2007a		
Italy	Veronesi et al., 2009, 2010		
The Netherlands	Boersema et al., 2002		
New Zealand	Bishop et al., 2014		
Saudi Arabia	Alanazi et al., 2017		
Sweden	Lindgren et al., 2008; Osterman-Lind and Christensson, 2009	Martin et al., 2018	Martin et al., 2018
Turkey		Cirak et al., 2010	
United Kingdom	Relf et al., 2014	Stoneham and Coles, 2006	
United States	Craig et al., 2007; Lyons et al., 2008a	Lyons et al., 2008a, 2011a	Lyons et al., 2008a, 2011a

Table 1.2 Reports of drug resistance and shortened egg reappearance periods in cyathostomins. Reports using fenbendazole (10 mg/kg, 5 days) are designated with an asterisk.

Country	Macrocyclic Lactones	Drug Class Tetrahydropyrimidines	Benzimidazoles
Australia		Pook et al., 2002	Rolfe and Dawson, 1994; Pook et al., 2002
Brazil	Molento et al., 2008	Molento et al., 2008; Canever et al., 2013	Molento et al., 2008; Canever et al., 2013; Saes et al., 2016
Canada		Slocombe and de Gannes, 2006	Slocombe and Cote, 1977; Slocombe et al., 2008
Chile			von Samson Himmelstjerna et al., 2002
Cuba			Salas-Romero et al., 2018
Denmark		Craven et al., 1998; Nielsen et al., 2013	Bjørn et al., 1991 Craven et al., 1998
England		Lester et al., 2013*	Kelley et al., 1981 Chandler et al., 2000; Lester et al., 2013;
Estonia		Lassen and Peltola, 2014	
Ethiopia			Seyoum et al., 2017
Finland		Näreaho et al., 2011	
France		Traversa et al., 2012	Geurden et al., 2013; Traversa et al., 2012
Germany	von Samson-Himmelstjerna et al., 2007a**	Traversa et al., 2009	Bauer et al., 1986; Wirtherle et al., 2004; Traversa et al., 2009;
India			Kumar et al., 2016
Italy	Milillo et al., 2009; Traversa et al., 2009	Milillo et al., 2009; Traversa et al., 2007b, 2009	Mililllo et al., 2009; Traversa et al., 2007b, 2009
Netherlands	van Doorn et al., 2014**; Kooyman et al., 2016**		
New Zealand	Rosanowski et al., 2017**		
Nicaragua	Kyvsgaard et al., 2011**		
Norway		Ihler, 1995	Ihler, 1995
Romania			Buzatu et al., 2015; Cernea et al., 2015
Slovakia			Várady et al., 2000; Čerňanská et al., 2009
South Africa			Van Wyk and Van Wijk, 1992
Sweden	Osterman-Lind et al., 2007**	Osterman-Lind et al., 2007	Nilsson et al., 1989; Osterman-Lind et al., 2007
Switzerland		Meier and Hertzberg, 2005	Meier and Hertzberg, 2005
Ukraine			Kuzmina and Kharchenko, 2008
United States	Woods et al., 1998**; Little et al., 2003**; Rossano et al., 2010**; Bellaw et al., 2018**	Chapman et al., 1996; Woods et al., 1998; Tarigo-Martini et al., 2001; Little et al., 2003; Kaplan, 2004; Brazik et al., 2006; Lyons et al., 2001, 2003, 2008a; Smith et al., 2016; Nielsen et al., 2018	Herd et al., 1981; Chapman et al., 1996; Woods et al., 1998; Tarigo-Martini et al., 2001; Little et al., 2003; Kaplan, 2004; Lyons et al., 2001, 2003, 2008a; Rossano et al., 2010*; Garcia et al., 2013; Reinemeyer et al., 2015*; Smith et al., 2015; Bellaw et al., 2018*; Nielsen et al., 2018
UK	Traversa et al., 2009; Relf et al., 2014**; Tzelos et al., 2017**	Traversa et al., 2009; Relf et al., 2014	Stratford et al., 2014
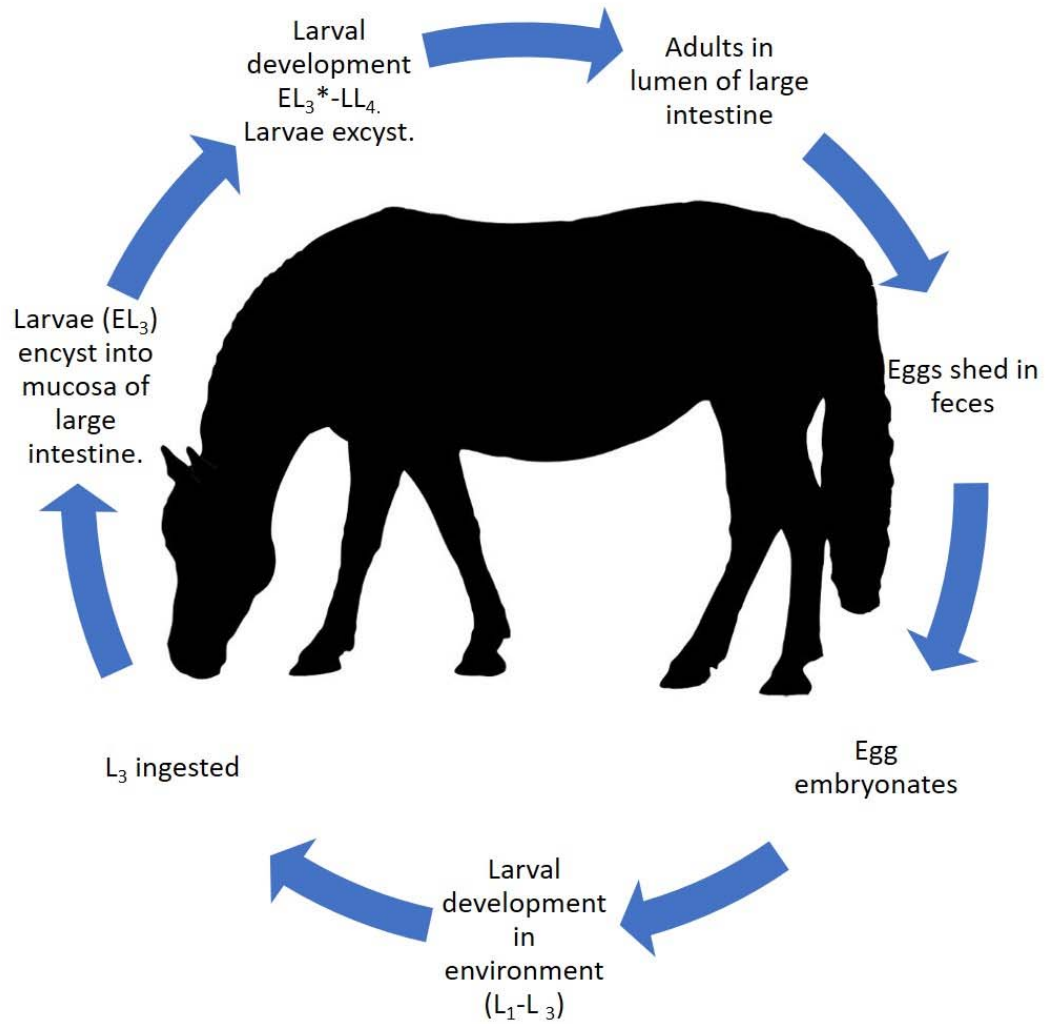
Figure 1.1 Illustration of the cyathostomin life cycle. (*EL$_3$ may arrest).
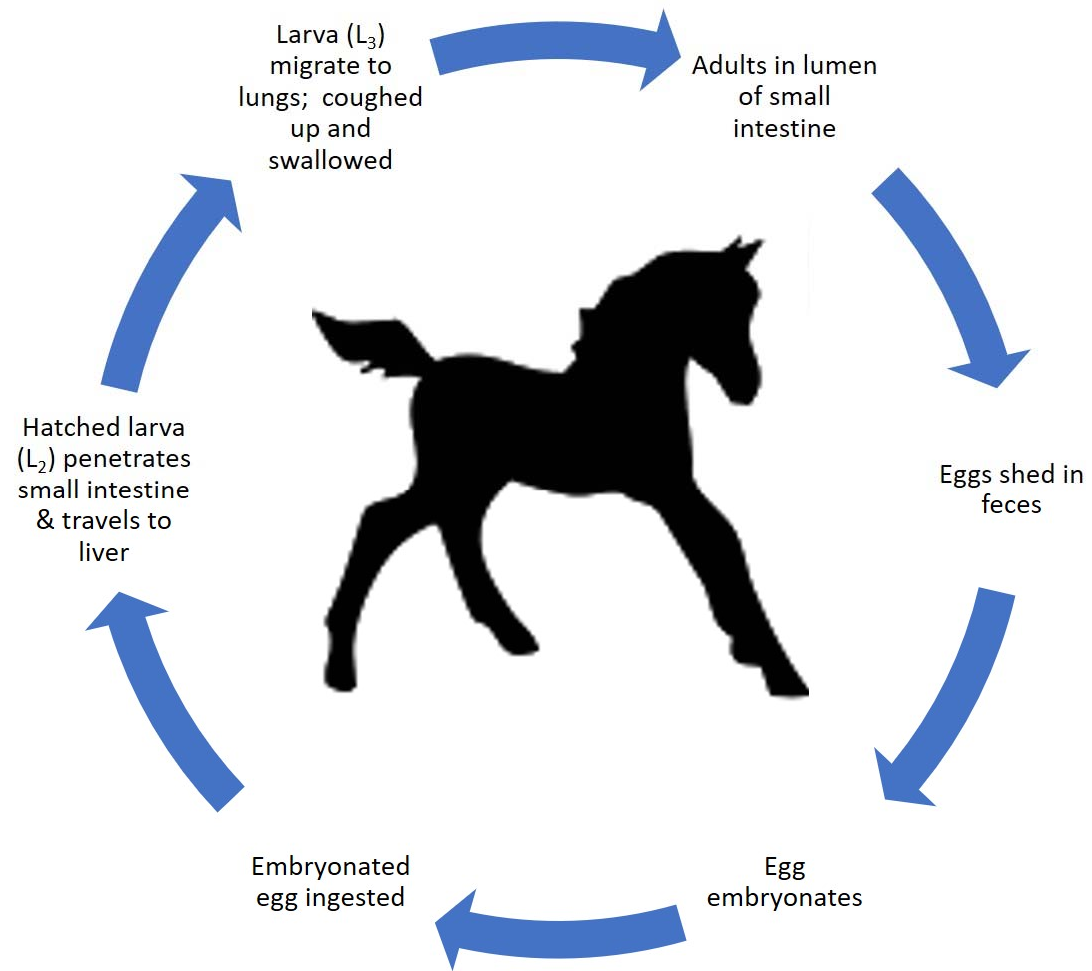
Figure 1.2 Illustration of the *Parascaris* spp. life cycle

CHAPTER 2. COMBINATION DEWORMING FOR THE CONTROL OF DOUBLE-
RESISTANT CYATHOSTOMIN PARASITES-SHORT AND LONG TERM
CONSEQUENCES

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2.1   Introduction

Cyathostomins are clinically important helminth parasites of the horse, and typically comprise 99-100% of the total worm burden (Nielsen et al., 2010). The early third larval stage (EL3) can enter a hypobiotic state as they encyst into the mucosal lining of the large intestine (Eysker et al., 1984). Most horses do not exhibit signs of infection, however, the disease larval cyathostominosis may occur in rare cases. Simultaneous excystment of larvae from the mucosal lining of the cecum and colon can result in an array of clinical signs, including weight loss, diarrhea, dehydration, subcutaneous edema, and pyrexia (Love et al., 1999; Peregrine et al., 2006). The disease has been reported fatal in 50% of diagnosed cases (Reid et al., 1995).

Presently, there are three anthelmintic drug classes approved for controlling equine helminth parasites; the benzimidazoles, the tetrahydropyrimidines, and the avermectin/milbemycins (also known as macrocyclic lactones). Originally, parasite control regimens were based on frequent treatments with benzimidazole drugs, and development of additional drug classes resulted in the rotation between drug classes (reviewed by Kaplan and Nielsen, 2010; Nielsen, 2012). A proposed benefit warranting rotational deworming methods was to avoid over-exposure of a single drug-class to a parasite population in hopes of preventing anthelmintic resistance (Prichard et al., 1980), but this has not proved to be a sustainable approach. Unfortunately, the frequent use of anthelmintics has driven the development of anthelmintic resistance in cyathostomins. Resistance to the benzimidazole and tetrahydropyrimidine drug classes is reported world-wide, and there are increasing reports of shortened egg reappearance periods and decreased efficacy following treatment with the macrocylic lactones (reviewed by Peregrine et al., 2014). Furthermore, some cyathostomin populations are observed harboring multi-drug resistance (reviewed by Peregrine et al., 2014).

It was originally proposed that genes associated with anthelmintic resistance likely occurred at very low levels in a naïve parasite population, and therefore may be associated with an ecological fitness disadvantage (Prichard, 1990). Under the selection pressure of anthelmintic use, however, these genetic mutations would offer an advantage and worms surviving treatment allow for the resistance alleles to increase in frequency within the parasite population (Prichard, 1990). Recent work has established that anthelmintic resistance may arise in a population in one of four ways; (1) pre-existing alleles are present prior to anthelmintic exposure, (2) spontaneous mutations occur immediately before or at the time of anthelmintic exposure, (3) frequent mutation events may allow alleles to appear recurrently, or (4) resistant alleles may have arisen elsewhere and were brought into the population through host migration (Gilleard and Beech, 2007). Gastrointestinal nematodes of small ruminants, and likely horses as well, are presumed able to acquire resistance at such a high rate because of their high fecundity and ability to undergo rapid rates of nucleotide sequence evolution, contributing to a high level of genetic diversity (Blouin et al., 1995; Anderson et al., 1998; reviewed by Gilleard, 2013). The trichostrongylid nematodes of small ruminants, *Haemonchus contortu*s and *Teladorsagia circumcincta*, have been the most widely studied species due to their high infection prevalence and the extremely high levels and rates of resistance to multiple anthelmintic actives. A recent study regarding the emergence of anthelmintic resistance among populations of these species supports the latter two theories mentioned above. This model proposes that resistance occurs from multiple independent mutations recurrently arising and are spread by host migration (Redman et al., 2015).

It is interesting to note that even in the absence of a selection pressure, resistant alleles appear to remain within a cyathostomin population. Lyons et al. (2007) reported sustained resistance in a benzimidazole resistant cyathostomin population after remaining unexposed to anthelmintic treatment for 22 years. Another cyathostomin population, known as Population S, developed resistance to the benzimidazole drug class over a 18 year period from repeated use of cambendazole for four years (Drudge et al., 1983) followed by treatment with oxibendazole for 14 years (Drudge et al., 1985a,b; Lyons et al., 1994). Lyons et al., 2001 reported that after the subsequent seven years, in which

pyrantel pamoate was used and pyrantel resistance was documented. The resistance to the benzimidazole drug class was unaffected despite the change in drug class use.

Computer modelling studies suggest that combination deworming, defined as using different drug actives to target the same parasite, may preserve drug efficacy and slow the development of resistance (Smith, 1990; Barnes et al., 1995; Leathwick, 2012). Leathwick (2012) used a computer model to observe the effects of combining a new active with an active to which resistance existed. They found the development of resistance to the new active to be delayed when used in combination, but this effect was decreased in scenarios with lower starting efficacies and in populations with low parasite refugia. Leathwick (2013) performed another modelling study to observe the rate of resistance development during 40 years of selection when sheep were treated with two drugs used in either annual rotation or in combination. Overall, the rate of resistance development was slowed when the drugs were used in combination. These results suggest that a combination of actives may increase the probability of killing parasites harboring the genetics for resistance to either one of the actives used. Furthermore, field studies performed in lambs infected with drug resistant trichostrongylids found a combination of multiple actives to have an additive effect (Bartley et al., 2004, 2005; Entrocasso et al., 2008; Le Jambre et al., 2010). Combination deworming has been found to be most successful when resistance does not exist to either of the drug classes used. Modelling studies performed by Barnes et al. (1995) and Leathwick et al. (2012) found that combining drugs, when one or both are 100% effective, slows the rate of resistance. Once resistant alleles become prevalent in a population, however, this strategy is unlikely to be successful. Even with low levels of resistance, it is presumed that combining multiple actives may result in a synergistic effect (reviewed by Fleming et al., 2006). However, efficacy is unlikely to be beneficial once high frequency of resistant alleles to both actives are present (reviewed by Fleming et al., 2006; reviewed by Bartram et al., 2012).Presently, combination treatments are increasingly recommended to combat anthelmintic resistance in nematodes infecting ruminants (Bartram et al., 2012; Geary et al., 2012; Ramos et al., 2016) and against equine nematodes (Scott et al., 2015). Combination products are currently marketed in New Zealand, Australia and South America. However, the lack of effective anthelmintic drug classes available for equine

cyathostomins questions whether combining actives would be effective against these parasites. To date, only one equine study has been performed evaluating combination therapy against cyathostomins. The results illustrated an additive effect against drug resistant cyathostomins after a single treatment with a combination of oxibendazole and pyrantel pamoate (Kaplan et al., 2014).

Presently, it is unknown how repeated combination treatments will affect a cyathostomin population harboring double-drug resistance. The aims of this study were (1) to evaluate the combined drug efficacy of oxibendazole and pyrantel pamoate for treatment of a herd naturally infected with a cyathostomin population with known drug resistance to both actives; (2) to observe changes in the efficacies of the single actives after four repeated combination treatments; (3) to test the additive effect formula proposed by Bartram et al. (2012) for estimating the efficacy of a combination treatment; and (4) to characterize the strongyle population before and after treatment using coprocultures.

2.2   Materials and Methods

2.2.1   Ponies

A band of 21 Shetland ponies housed at the University of Kentucky was used in this study. The herd consisted of 20 mares and 1 stallion, ranging from ages 3 to 20 years. The herd harbors a population of cyathostomin parasites with documented resistance to the benzimidazole and tetrahydropyrimidine drug classes, otherwise known as Population S (Lyons et al., 2003). The ponies are maintained outside year-round. During the warmer months (March to October), the ponies were kept in dry lot with restricted access to striped grazing and were provided grass hay, consisting of either timothy or orchard grass. During the winter months, the ponies continued to receive hay and also had access to pasture which was comprised of clover, blue grass, and an assortment of weeds. Salt and mineral blocks were available ad libitum. The research was conducted under the approval from the University of Kentucky's Institutional Animal Care and Use Committee (IACUC) under protocol number 2012-1046.

2.2.2   Study design

This study took place between April, 2015 and June 2016. All ponies were weighed on an electronic scale prior to each treatment and treated at 110% of their body weight every eight weeks. The 110% dosage was used to account for any drug loss that may have occurred during or following drug administration. Ponies were ranked by pre-treatment fecal egg count (FEC) and blocked into groups of two. Within each block, ponies were randomly assigned to a single active treatment of either oxibendazole (OBZ) or pyrantel pamoate (PYR) for the first treatment. Fecal egg counts (FEC) were determined at the day of treatment and every two weeks post-treatment. Eight weeks later, the single active treatments were repeated with the groups reversed. Following this, all ponies received four combination treatments with both drugs, eight weeks apart. Before concluding the study, single active efficacies were determined again following the same protocol as before. A timeline of the study design can be found in Figure 2.1. Given this is the first study to examine repeated combination treatments in horses against cyathostomins, there are no current recommendations to guide the number of treatments. We chose to carry out the study over the course of 14 months, which allowed the examination of the single actives before and after the four consecutive combination treatments, and this is the reason why we chose this treatment interval.

Fecal egg count reduction tests (FECRTs) were carried out at 2 week intervals to monitor anthelmintic efficacy using the following formula:

$$FECRT = \frac{(pre\ treatment\ FEC - post\ treatment\ FEC)}{pre\ treatment} \times 100\%$$

The additive effect formula was used to compare the expected combination efficacy based on the single active efficacies to the observed combined drug efficacy (Bartram et al., 2012).

$$FECR\%_{A+B} = 1 - [(1\text{-}FECR\%_A) \times (1\text{-}FECR\%_B)]$$

2.2.3   Anthelmintics

Paste formulations of oxibendazole (OBZ; Anthelcide EQ, Zoetis, Kalamazoo, MI, USA) and pyrantel pamoate (PYR; Strongid P, Zoetis, Kalamazoo, MI, USA) were used in this study to represent the benzimidazole and tetrahydropyrimidine drug classes, respectively. Anthelmintics were administered according to the labeled doses, at 10 mg/kg bodyweight for OBZ and ad 6.6 mg base/kg bodyweight for PYR. Anthelmintics were prepared by weighing the dose on an electronic scale and placing in a second syringe for administration.

2.2.4   Fecal egg counts

All FECs in this study were performed in triplicate using the Mini-FLOTAC technique which has a detection limit of 5 eggs per gram (EPG) (Cringoli, et al., 2017). Samples were prepared as described by Noel et al. (2017). Briefly, 5 g of sample were placed in the Fill-FLOTAC and homogenized with 45 mL of glucose-NaCl flotation medium with a specific gravity of at least 1.24. The fecal slurry was loaded into both chambers of the Mini-FLOTAC slide and allowed to rest for 10 minutes to allow for adequate flotation of the eggs before being analyzed using a microscope. Three slides were prepared per sample and used to obtain an average egg count.

2.2.5   Coprocultures, Baermann procedure, and larval identification

Coprocultures were performed to characterize the strongyle population. Ten ponies were randomly selected using a random number generator (Random.org) to represent the pony herd, and only fecal samples collected at the time of treatment and at two weeks post-treatment were used for the coprocultures. The cultures were set up individually as described by Henriksen and Korsholm (1983) using 10 grams of feces. The cultures were placed in an incubator at 24 ºC for 14 days and moistened with tap water every other day. Following incubation, the samples were then sedimented in a Baermann apparatus for 48 hours at room temperature. After this, the sediment was collected and were stored at 4°C for no more than two weeks. For larval identification and counting, the pellet was re-

suspended and placed into a nematode counting chamber (Chalex Corp. Ketchum, ID, USA). The slide was heated to 55°C for approximately 3 minutes in order to inactivate the larvae. All larvae present in the sample were then examined at 100X and identified to stage, genus, and species where applicable, as described by Russel (1948).

2.2.6   Statistical analyses

Using the triplicate counts, mean FECs were determined for each sample at all time points. Pre and post-treatment FECs were used to determine the percent strongyle fecal egg count reduction (FECR) at each time point, and any negative FECRs were replaced with 0%. Individual pony FECRs were used to calculate a herd mean FECR, standard deviation, and 95% confidence intervals ($\alpha=0.05$) at each time point. The mean OBZ FECR was calculated for Treatments 1 and 2 in 2015, and then again for Treatments 7 and 8 in 2016. Likewise, mean PYR FECRs were also determined. These single active OBZ and PYR efficacies were used to estimate each pony's predicted combination efficacy using the additive effect formula, and a herd mean predicted FECR was calculated.

All statistical analyses were performed using SAS software (version 9.4, SAS Institute, Cary, North Carolina, USA). A mixed linear model analysis was used to compare the FECs following the single active treatments with OBZ and PYR before and after the four combination treatments. The FECs were first log-transformed to achieve a normal distribution. In the model, 'Horse ID' and 'Year' were kept as random variables, while 'treatment' and 'weeks post-treatment' were included as fixed effect categorical variables. Whenever the interaction term 'Year*Treatment*Weeks post-treatment' was found significant, a 'least squares means' analysis was used for a Tukey's pair-wise comparison ($\alpha=0.05$). A second mixed linear model analysis was used to compare the single active efficacies by analyzing FECRs. Here, 'horse ID' and 'season' were kept as random variables, and 'Weeks post treatment' was included as a fixed effect categorical variable. Whenever the interaction term 'Year*Treatment*Weeks post-treatment' was found significant, a 'least squares means' analysis was used for a Tukey's pair-wise comparison ($\alpha=0.05$). Efficacies of the combination treatments were evaluated using

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combination therapy FECRs in a mixed linear model analysis. Again, 'horse ID' and 'season' were kept as random variables, and 'Weeks post treatment' was included as a fixed effect categorical variable. Whenever the variable 'weeks post treatment' was found significant, a 'least squares means' analysis was used for a Tukey's pair-wise comparison ($\alpha=0.05$). Lastly, a mixed linear model analysis was carried out to compare FECR between the observed and predicted (additive effect formula) combination drug efficacies. Here, 'Year' and 'Horse ID' were kept as random variables, while 'test' (observed or predicted) was included as a fixed effect categorical variable. Whenever the variable 'test' was found significant, a 'least squares means' analysis was used for a Tukey's pair-wise comparison ($\alpha=0.05$). In all analyses, all covariates were kept in the model regardless of p-value.

2.3 Results

All treatment efficacies determined at two weeks post treatment are presented in Appendix 1.

2.3.1 Single active treatments

Fecal egg counts for the single active treatments occurring before (2015) and after (2016) the four combination treatments are presented in Figure 2.2A. Overall, the 2015 pre-treatment (time point 0) FECs were significantly higher than the 2016 pre-treatment FECs for both treatment groups ($p<0.0001$). Figure 2.2B shows the efficacy of the single active treatments occurring before (2015) and after (2016) the combination treatments. No significant differences were found at two weeks post-treatment between treatment groups within the same year (2015, p=1.0000; 2016, p=1.000), nor within the same treatment group between different years (OBZ, p=0.4421; PYR, p=0.8361).

2.3.2 Combination treatments

The percent efficacies of the four combination treatments (Treatments 3, 4, 5, and 6) are presented in Figure 2.3. The efficacy at 2 weeks post-treatment of the first

combination treatment was not significantly different from the single active starting efficacies of OBZ (p=0.8588) and PYR ($p=$ 0.5537). The FECR for the first combination treatment was significantly greater than the second ($p=$0.0454), third ($p=$0.0318), and fourth ($p=$0.0372) combination treatments. The observed efficacies of the first (Treatment 3) and fourth (Treatment 6) combination treatments and the corresponding additive effect formula predicted efficacies are presented in Figure 2.4. There was no significant difference between the observed and predicted efficacies for the first combination treatment (Treatment 3) ($p=$0.9592). The predicted efficacy of the fourth combination treatment (Treatment 6), however, was significantly greater than the observed efficacy ($p=$0.0058).

2.3.3 Larval identification

Larval counts from the coprocultures are presented in Table 2.1. *Strongylus edentatus* was the only large strongyle species found and represented less than 1% of the larvae identified from the pre-treatment samples of Treatment 1 and 2. All other strongyle larvae were identified as within the sub-family cyathostominae.

2.4 Discussion

This study is the first to evaluate the efficacy of a combination of anthelmintics targeting cyathostomin parasites over the course of repeated treatments. The results suggest that this approach may not be sustainable against cyathostomins with resistance already developed to both actives included in the combination. This is important as benzimidazole and pyrantel resistance is widely reported in cyathostomin populations across the world (reviewed by Peregrine et al., 2014).

The successful use of combined actives for slowing the development of resistance was previously observed in sheep parasites when a novel anthelmintic was introduced in combination with a pre-existing drug class (Leathwick, 2012). This method is not possible for equine helminths as new anthelmintics have not been developed in the last 30 years. Leathwick (2012) further concluded that the benefits of combination deworming were depleted in scenarios where drug resistance to both actives already existed. The

single active starting efficacies used in this study were 60% (Figure 2.2B), which is not an unusual occurrence found on horse farms around the world, and their combination did not offer a sustained additive effect (Figure 2.3). Rather, the efficacy of the latter three treatments dropped significantly and remained consistent as seen in Figure 2.3. As previously mentioned, this cyathostomin population (Population S) has a long history of drug resistance to both actives used in this study (Lyons et al., 2003). It is important to note that the study presented here is different from previous studies regarding combination therapy in small ruminants (Barnes et al., 1995; Entrocasso et al., 2008; Leathwick, 2012) in three ways; 1) resistance was well established to both of the actives used in this study, 2) a selection pressure was implemented due to the four treatments administered with 8-week intervals, and 3) all ponies were treated at every time point minimizing parasite refugia. In a simulation study evaluating anthelmintic treatment regimens against sheep nematodes over 40 simulation years, Leathwick et al. (2013) reported increased efficacies of combination treatments compared to using the same actives in annual rotation. The greatest effect, however, was seen when initial starting efficacies were high and a portion of the parasite population was kept in refugia. As previously discussed, cyathostomin parasites encyst into the mucosal lining where they may enter a hypobiotic state before maturing into the fourth larval stage (L4) (Eysker et al., 1984). Effective removal of luminal stages following deworming has been associated with recruitment of L4 larvae into the intestinal lumen (Love et al., 1999). Since neither of the actives examined in this study possess larvicidal efficacy, the encysted stages in principle remained in refugia and protected from the deworming treatment. The permanently present adult luminal burden, however, likely prevented this refugia population from entering the luminal stage. Without this event, the encysted burden did not provide a source of refugia within the time frame of this study. Thus, it is presumed that the reduced efficacies observed in this study allowed for a substantial adult worm burden to persist, and possible effects of parasite refugia to be minimal or absent in this study."

In the present study, the predicted efficacy of the final combination treatment was significantly greater than the observed efficacy. This is surprising since the predicted efficacy is calculated based on the final two single active efficacies. Given the fact that

the additive effect formula was an accurate predictor of the first combination treatment efficacy, one would have expected that the final single active efficacies would be even lower since the observed efficacy of the last combination treatment was a mere 40.67%, as shown in Figure 2.4. Adult cyathostomins in the intestinal lumen are presumed to have a maximum life span of one year, so it is possible that a new pool of adults were recruited from the mucosal walls towards the end of the study, which took place over the course of 14 months. It is plausible that the adult burden was gradually replaced somewhere between the last combination treatment, and the subsequent re-evaluation of the single active efficacies. This could potentially explain two observations made in this study: 1) The consistent observed efficacies of the second, third, and fourth combination treatment (Treatments 3-6), as the luminal adult cyathostomin burden likely remained relatively unchanged, and 2) The apparent discrepancy between the low observed efficacy of the last combination treatment (Treatment 6), and the somewhat higher single active efficacies observed subsequently. Furthermore, given the fact that OBZ and PYR resistance has remained unchanged in the population for decades during times of both intense or no selection pressure, we speculate that the resistant alleles may arise from recurrent mutations and/or spontaneous mutations occurring near the time of treatment, as proposed by Gilleard and Beech, 2007.

The initial drug efficacies observed in this study are similar to those found on managed horse farms (reviewed by Peregrine et al., 2014), and 8 week treatment intervals are also a common component of treatment regimens (Smith et al., 2000; Earle et al., 2002; O'Meara and Mulcahy, 2002; Robert et al., 2015), and the presented results suggest that combination therapy applied in the present treatment regimen is not sustainable. Future studies including an active with larvicidal activity and maintaining a larger refugia population are necessary to further examine the utility of alternative combination treatment regimens. Future studies including an active with larvicidal activity and maintaining a larger population in refugia are necessary to further examine the utility of combination treatment regimens. Treatment with a larvicidal active will remove the encysted and luminal stages allowing new parasites being ingested from the environment to infect the horse and be present in both the encysted and luminal stages. This new

infection will allow for an effect of keeping parasites in refugia, by leaving a proportion of the herd untreated, for example.

We do not believe an interaction between the two actives administered affected the results as they act on different physiological components of the parasites. The benzimidazole drug class affects the worms by acting as a blocking agent for microtubules. This hinders glucose absorption, and essentially slowly starves the parasites. The pyrantel drug class, on the other hand, works as an inhibitor of acetylcholinesterase, a neurotransmitter molecule. This causes near immediate paralysis of the worms allowing them to be expelled by peristalsis (reviewed by Martin, 1997). Additionally, if there were drug interactions at play, we would expect to see consistent results reflecting their interaction at each combination treatment, which we did not. The initial additive effect suggests the removal of susceptible and single-drug resistant worms, while the subsequent decrease in efficacy suggest survival of multi-drug resistant worms.

In summary, this study illustrated that combination of single actives with starting efficacies below 70% resulted in an initial increase in efficacy, followed by a significant decrease over the subsequent three treatments. The use of combination anthelmintic therapy for the control of double resistant cyathostomin populations needs further evaluation to determine if it can be recommended as an effective viable approach for equine parasite control.

Table 2.1 Total larval counts (percent of total number of larvae recovered) following coprocultures of ten individual samples collected at pre- and two weeks post-treatment. Treatments were with oxibendazole (10 mg/kg) and pyrantel pamoate (6.6 mg base/kg). Numbers 1-8 represent the type treatment given (i.e. single active or combination of actives)[a].

	1 (Pre)	1 (Post)	2 (Pre)	2 (Post)	3 (Pre)	3 (Post)	4 (Pre)	4 (Post)	5 (Pre)	5 (Post)	6 (Pre)	6 (Post)	7 (Pre)	7 (Post)	8 (Pre)	8 (Post)
Total Larvae	**2355**	**2188**	**5362**	**61**	**240**	**409**	**2280**	**254**	**640**	**287**	**1037**	**473**	**571**	**4255**	**1512**	**3062**
Cyathostominae	2166 (92%)	2021 (92.4%)	5216 (97.3%)	58 (95%)	239 (99.6%)	403 (98.5%)	2257 (99%)	249 (98%)	640 (100%)	287 (100%)	1037 (100%)	473 (100%)	564 (98.8%)	4253 (99.9%)	1509 (99.8%)	3054 (99.7%)
L1 Strongyles	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L2 Strongyles	129 (5.5%)	123 (5.62%)	123 (2.3%)	3 (5%)	1 (0.4%)	6 (1.5%)	23 (1%)	5 (2%)	0	0	0	0	7 (1.2%)	2 (0.1%)	3 (0.02%)	8 (0.3%)
Strongylus edentatus	8 (0.33%)	0	2 (0.04%)	0	0	0	0	0	0	0	0	0	0	0	0	0
Strongylus vulgaris	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gyalocephalus capitatus	14 (0.59%)	22 (1%)	6 (0.11%)	0	0	0	0	0	0	0	0	0	0	0	0	0
Poteriostomum spp.	38 (1.6%)	22 (1%)	15 (0.28%)	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] Treatments 1 and 2 were the initial single active treatments, while Treatments 7 and 8 were the final single active treatments. Treatments 3-6 were a combination of the two actives
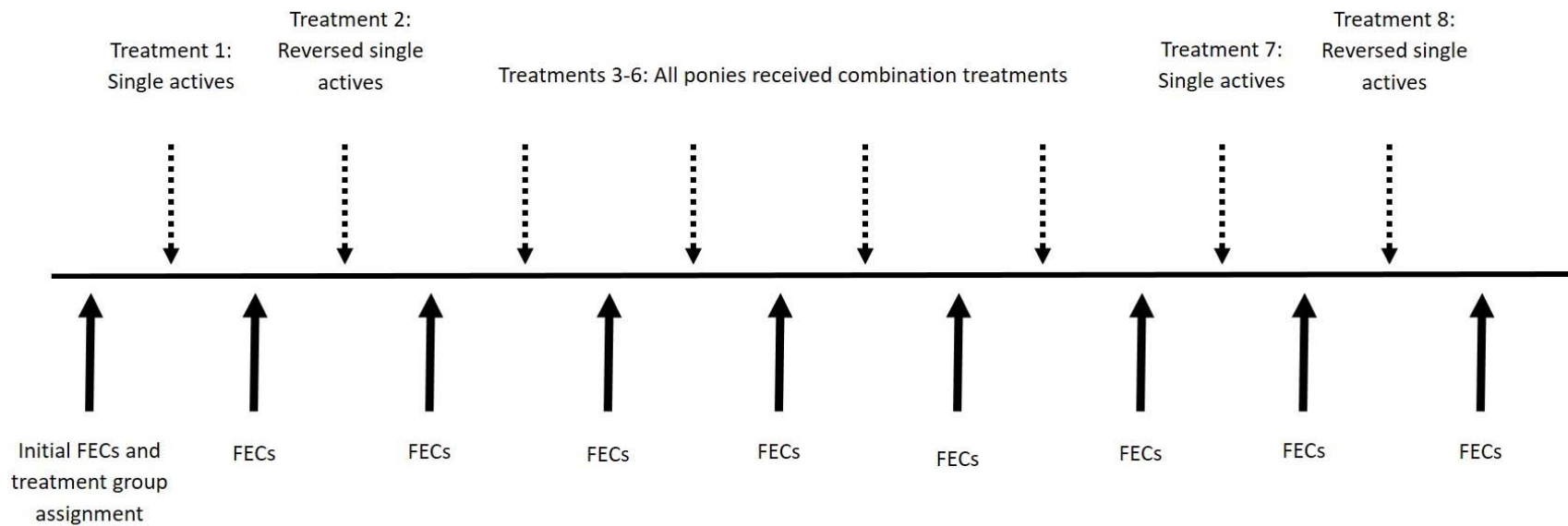
Figure 2.1 A pictorial representation of the study design. Fecal egg counts (FECs) occurred bi-weekly for 8 weeks following each treatment. For the single active treatments (Treatments 1, 2, 7, and 8), the ponies were divided into two groups. For the combined active treatments (Treatments 3, 4, 5, and 6) all ponies were treated. The treatments used in this study were oxibendazole at 10 mg/kg body weight and pyrantel pamoate at 6.6 mg base/kg bodyweight.
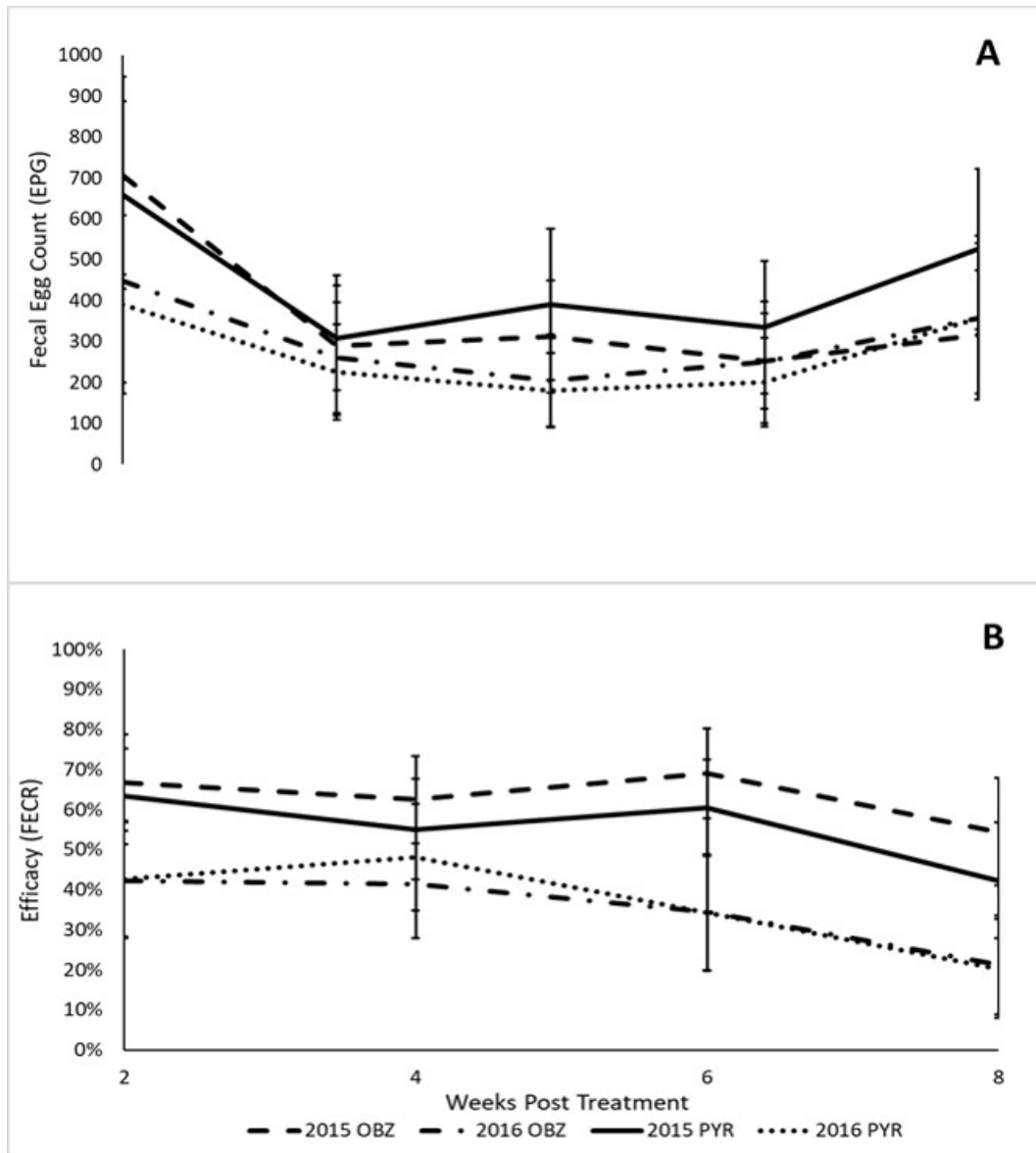
Figure 2.2 A graphical representation showing the effects of single active treatments with either oxibendazole (OBZ) or pyrantel pamoate (PYR). Figure A shows fecal egg counts as eggs per gram (EPG) pre- and post-treatment with either (OBZ) or (PYR). Figure B shows the percent efficacies of the single active treatments calculated using the Fecal Egg Count Reduction Test. The 2015 treatments occurred prior to exposure of the combination therapy, while the 2016 treatments occurred after the final combination treatment. The error bars represent 95% confidence intervals (α=0.05).
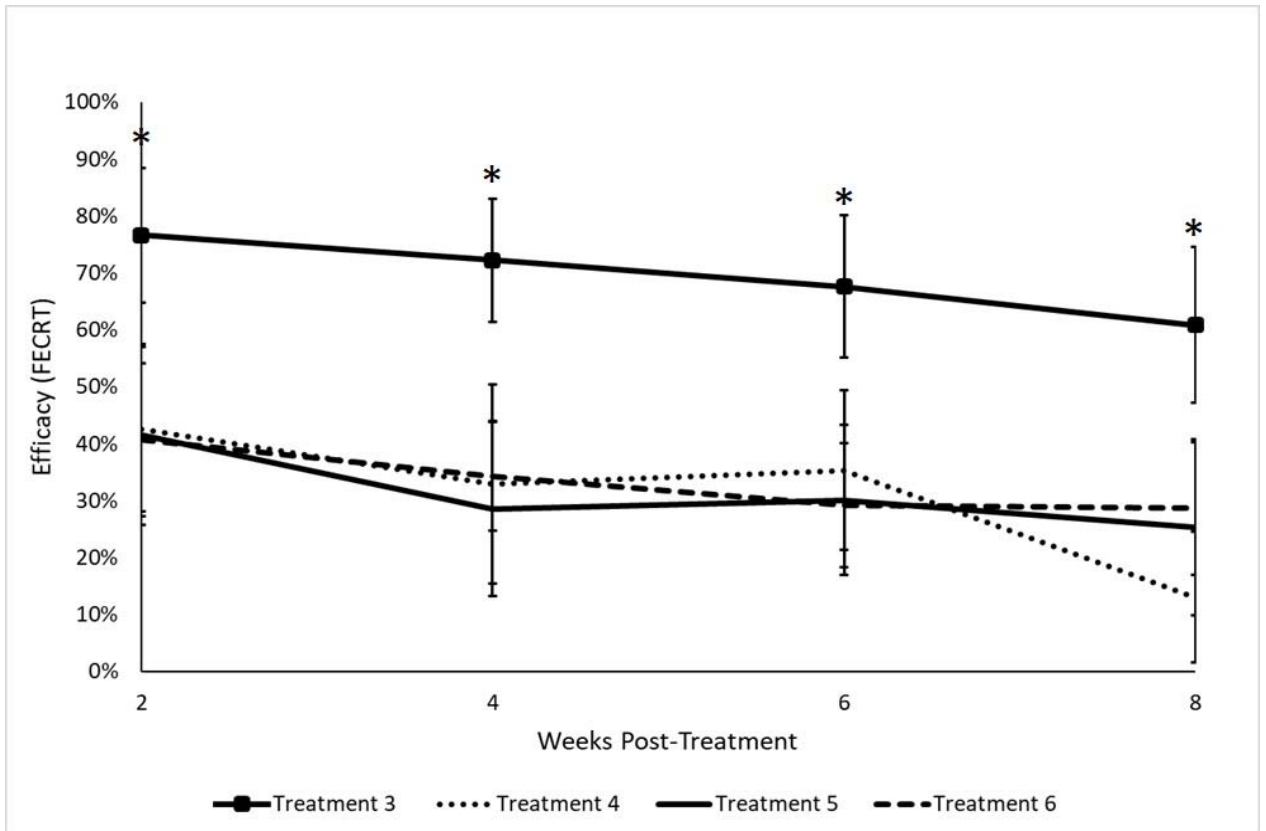
Figure 2.3 . A graphical representation of the percent efficacies of the four combination treatments of oxibendazole and pyrantel pamoate (Treatments 3-6) calculated using the Fecal Egg Count Reduction Test (FECRT). The error bars represent 95% confidence intervals ($\alpha=0.05$). Asterisks indicate significant differences at each time point between Treatment 3 and all other treatments.
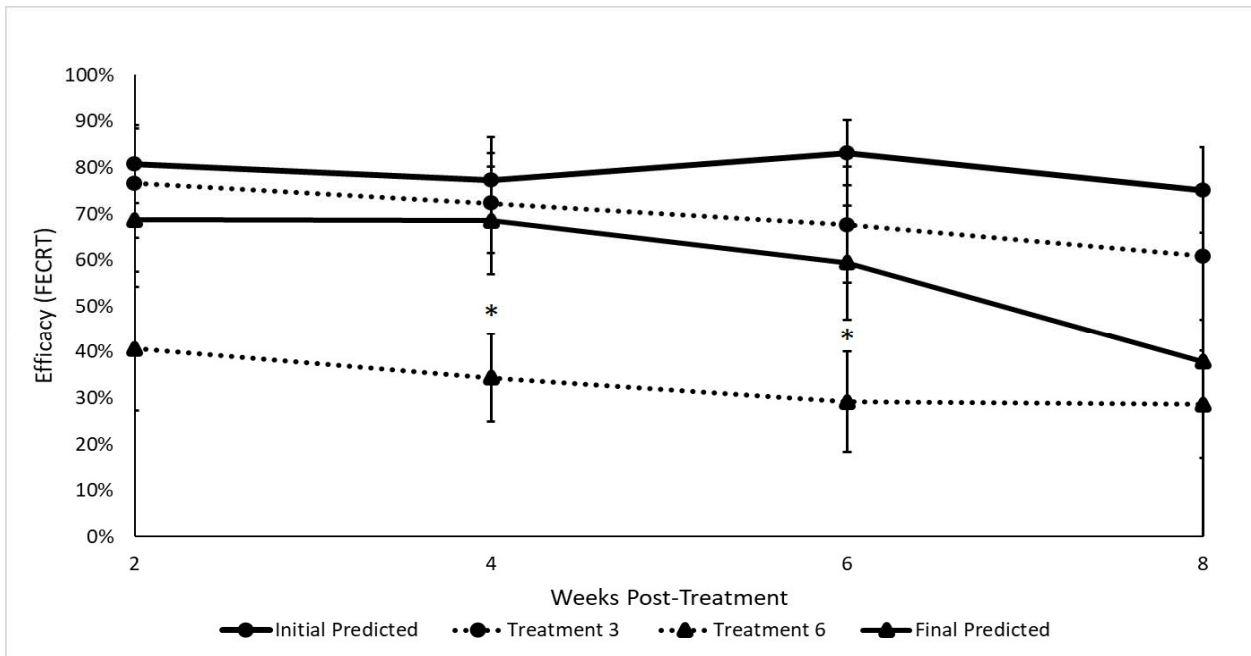
Figure 2.4. A graphical representation of the observed and predicted percent efficacies of the combination treatments of oxibendazole and pyrantel pamoate. The initial predicted efficacy was calculated using the efficacies of the initial single active treatments and corresponds with the first combination treatment (Treatment 3). The final predicted efficacy was calculated using the efficacies of the final single active treatments and corresponds with the final combination treatment (Treatment 6). The error bars represent 95% confidence intervals (α=0.05). Asterisks indicate significant differences between Treatment 6 and the final predicted efficacy.

CHAPTER 3. DEALING WITH DOUBLE TROUBLE: COMBINATION
DEWORMING WITH MOXIDECTIN AND OXIBENDAZOLE AGAINST
DOUBLE-DRUG RESISTANT CYATHOSTOMINS TO MACROCYCLIC
LACTONES

3.1   Introduction

Cyathostomins are the most prevalent (Herd, 1990) and abundant (Nielsen et al., 2010) helminth parasite infecting horses, and can cause the disease larval cyathostominosis. Most horses do not show clinical signs of infection, but the disease has been reported to be fatal in 50% of cases (Reid et al., 1995). Presently, three anthelmintic drug classes are available for treating equine cyathostomins, namely the benzimidazoles (BZ), tetrahydropyrimidines (TP), and the macrocyclic lactones (ML) which on the Northern Hemisphere are comprised of ivermectin (IVM) and moxidectin (MOX), where the latter exhibits larvicidal efficacy (Nielsen et al., 2016). Cyathostomins have wide-spread resistance to the BZ and resistance to the TP drug class is common, and some farms report multi-drug resistance (Peregrine et al., 2014). Reports of shortened egg-reappearance periods (ERP) following ML treatment exist, indicating that resistance is developing to this last remaining drug class (Peregrine et al., 2014).

Combination deworming, or the simultaneous administration of two drugs with different modes of action targeting the same parasite, is proposed as an alternative method for parasite control (Leathwick and Hosking, 2009), and has proven useful against trichostrongylid parasites in sheep (Bartley et al., 2004, 2005; Entrocasso et al., 2008; Le Jambre et al., 2010). Combination products are most sustainable for parasite control and preserving anthelmintic efficacy when little or no resistance exists to the drug classes combined (Barnes et al., 1995; Leathwick, 2012), and is heavily dependent upon a large refugia population, or a portion of the parasites that are not exposed to treatment (Leathwick, 2012). The maintenance of refugia provides a source of susceptible alleles to dilute resistant alleles (Leathwick et al., 2012, Muchiut et al., 2018). It is also

hypothesized that resistant worms have ecological disadvantages, otherwise known as fitness loss, in comparison to drug susceptible worms (Prichard et al., 1980). Resistance develops more slowly when a greater fitness loss is associated with resistance (Leathwick, 2013). Single-drug resistant worms would be targeted by the opposite drug, while multi-drug resistant worms would have such an extreme fitness loss that they would not be capable of passing on these alleles. Collectively, these studies suggest that combining a new and presumably effective drug in combination with a drug, to which resistance exists, may 1) decrease the rate of resistance development to the new drug, and/or 2) improve the efficacy of the drug to which resistance already exists by reducing the number of individuals carrying the resistant genotype by use of the effective drug.

Despite minimal scientific evidence, combination products are marketed for cyathostomin treatment in some countries and used off label in others (Bartram et al., 2012; Scott and Pomroy, 2015; Lyons et al., 2016; Wilkes et al., 2017). Kaplan et al. (2014) found an additive effect for drug efficacy against equine cyathostomins when oxibendazole (OBZ) and pyrantel (PYR) were used in combination for a single treatment, where the starting efficacies were >80% for both drugs. In contrast, Scare et al. (2018a) observed the effects of repeated combination treatments of OBZ and PYR against a cyathostomin population with known resistance to both the BZ and TP drug classes (Lyons, 2003). Starting efficacies of each drug were much lower in the latter study than those reported by Kaplan et al. (2014). Scare et al. (2018) reported the first combination treatment to demonstrate an additive effect with an efficacy of 76%, however, three subsequent combination treatments resulted in significantly lower efficacies around 40%. Thus, this study suggested that combination treatments using actives with low starting efficacies is not a sustainable approach for cyathostomin control.

Resistance exists to all actives available for equine use , and it is unknown if combining an active to which resistance exists with a new active would provide any benefit for control against drug-resistant equine cyathostomins. The purpose of this study is to observe the effects of OBZ combined with MOX to target a cyathostomin population (Population *S*) harboring resistance to the BZ drug class, but has never before been exposed to an ML drug. Specifically, the aims were to 1) evaluate the efficacy of MOX against a cyathostomin population with established resistance to both BZ and TP

products, 2) evaluate the efficacy of a combination of MOX and OBZ over the course of two consecutive treatments against this cyathostomin population, and 3) evaluate OBZ efficacy before and after administering the two consecutive combination treatments.

3.2   Materials and Methods
3.2.1   Ponies

A band of 20 Shetland ponies housed at the University of Kentucky that was originally established in 1974 was used in this study. The herd currently consists of 20 mares, ranging from ages 5 to 23 years. The herd harbors a population of cyathostomin parasites, otherwise known as Population *S,* with documented resistance to the BZ and TP drug classes, (Lyons et al., 2003). The ponies are maintained outside year-round. During the warmer months (March to October), the ponies were kept in a dry lot with restricted access to striped grazing and were provided grass hay, consisting of either timothy or orchard grass. Hay was continuously provided during the winter months in addition to pasture access. Salt and mineral blocks were available ad libitum. The research was conducted under the approval from the University of Kentucky's Institutional Animal Care and Use Committee (IACUC) under protocol number 2012-1046.

3.2.2   Study Design

This study took place between August 2016 and December 2018. Fecal samples were collected at the time of each treatment and every two weeks thereafter. Ponies were weighed on an electronic scale prior to each treatment and anthelmintics were orally administered at 110% of their body weight. Treatments were administered when ten ponies exceeded 100 eggs per gram (EPG) or at 40 weeks post treatment. A total of five treatments were administered and all ponies received the same treatments. In order to establish the single active baseline efficacies, all ponies were first treated with OBZ on June 21, 2016 and then with MOX on August 24, 2016. All ponies were then administered a combination of MOX and OBZ for treatments three and four, which

occurred on March 28, 2017 and January 4, 2018, respectively. All ponies received OBZ for the fifth treatment on October 8, 2018 to observe any potential changes in its efficacy after the combination treatments. Drug efficacies were determined every two weeks by the fecal egg count reduction (FECR) test using the following formula:

$$FECR = \left(\frac{(pre\ treatment\ FECs - post\ treatment\ FECs)}{pre\ treatment\ FECs} \right) x 100\%$$

The FECR test was performed in two ways, 1) using the individual horse pre- and post-treatment FECs and then calculating the mean efficacy, and 2) using the total herd pre- and post-treatment FECs. Egg reappearance periods were determined when the total herd efficacy was <85% calculated by the latter method.

3.2.3 Anthelmintics

A paste formulation of OBZ (Anthelcide EQ, Zoetis, Kalamazoo, MI, USA) and a gel formulation of MOX (Quest, Zoetis, Kalamazoo, MI, USA) were used in this study to represent the BZ and ML drug classes, respectively. Anthelmintics were administered according to the labeled doses, at 10 mg/kg bodyweight for OBZ and at 0.4 mg/kg bodyweight for MOX. Anthelmintics were prepared by weighing the dose on an electronic scale and placing in a second syringe for administration to eliminate the existence of air bubbles within syringes.

3.2.4 Fecal egg counts

The Mini-FLOTAC technique, with a detection limit of 5 EPG, was used to perform all FECs in this study (Cringoli et al., 2017). Counts were performed in triplicate to obtain a mean egg count. Samples were prepared as described by Noel et al. (2017). Briefly, 5 g of feces homogenized within the Fill-FLOTAC containing 45 mL of glucose-NaCl flotation medium with a specific gravity of at least 1.24. Both chambers of the Mini-FLOTAC slide was filled with the fecal slurry and allowed to rest for 10 minutes to allow for adequate flotation before microscopic examination and enumeration.

3.2.5 Coprocultures, Baermann procedure, and larval identification

To characterize the strongyle population, ten ponies were randomly selected using a random number generated (Random.org) and fecal samples were collected for coproculture and subsequent larval identification. Only fecal samples collected at the time of treatment and at two weeks post-treatment were used. The cultures were set up individually as described by Henriksen and Korsholm (1983) using 10 grams of feces. The cultures were placed in an incubator at 24 ºC for 14 days and moistened with tap as needed. Subsequently, samples were placed in a Baermann apparatus for 48 hours at room temperature for sedimentation. After this, the sediment was collected and were stored at 4°C and processed within two weeks. The harvested larvae were placed in a nematode counting chamber (Chalex Corp. Ketchum, ID, USA). The slide was heated to 55°C for approximately 3 minutes in order to inactivate the larvae. All larvae present in the sample were then examined at 100X and identified to stage, genus, and species where applicable, as described by Russel (1948).

3.2.6 Statistical analyses

Using the triplicate counts, mean FECs and 95% confidence intervals ($\alpha=0.05$) were determined for each sample at all time points using Microsoft Excel 2016 (Redmond, WA, USA). Drug efficacies were determined at each timepoint using the two FECRT methods as previously described in section 2.2.

Further statistical analyses were performed using SAS software (version 9.4, SAS Institute, Cary, North Carolina, USA). The individual horse FECs and FECR tests were used for these analyses. Any negative efficacies were replaced with 0%, and horses that had 0 EPG pre-treatment were excluded from the FECR analyses. All FECs and FECR tests were first log-transformed to achieve a normal distribution. All statistical analyses were interpreted at $\alpha=0.05$. Because the shortest treatment interval was 30 weeks when ten horses reached the >100 EPG threshold, the analyses run did not include data beyond 30 weeks for the other treatments.

3.2.6.1   Analyses for fecal egg counts

Two mixed linear models with repeated measures over time were used to evaluate individual horse FECs pre- and post-treatment. In both models, the terms 'replicate' and 'date' were kept as random effects. The first model evaluated FECs following the single active MOX treatment and both combination treatments, while the second evaluated FECs following the two OBZ treatments. The categorical variables for both models were 'treatment,' 'weeks post-treatment,' and an interaction term of 'weeks post treatment' and 'treatment.' Whenever the interaction term of 'treatment' and 'weeks post-treatment' was found significant, a 'least squares means' analysis was used for a Tukey's pairwise comparison (α=0.05).

3.2.6.2   Analyses for efficacies

Two mixed linear models with repeated measures over time were used to evaluate the drug efficacies per horse. 'Date' was kept as the random effect for both models. The first model compared the efficacies of MOX and the two combination treatments, and the second model compared the efficacies of the two OBZ treatments. For both models, the terms 'treatment,' 'weeks post-treatment,' and the interaction term 'weeks post treatment' and 'treatment' were the categorical variables. Whenever the interaction term 'weeks post treatment' and 'treatment' was found significant, a 'least squares means' analysis was used for a Tukey's pairwise comparison (α=0.05).

3.3   Results

3.3.1   Fecal egg counts

The mean strongyle egg counts prior to each treatment were higher at the beginning of this study and declined over the subsequent treatments, however these differences were not significant (Fig. 3.1 and Table 3.1).

Fecal egg counts at two weeks post-treatment following MOX and both combination treatments were 0 EPG. The ERPs and associated efficacies can be found in

Table 3.2. Both OBZ treatments resulted in significantly lower FECs at two weeks post-treatment (p<0.0001, Table 3.1).

3.3.2   Anthelmintic efficacy

The efficacy of MOX alone and both combination treatments were 100% (Fig. 3.1). The efficacy of the first and last single active OBZ treatments were 46.7% and 40.1%, respectively, and these were not significantly different (p=0.9890) (Table 3.1). There were no significant differences between the MOX and combination treatments at any common timepoint.

3.3.3   Larval counts

Larval counts from the coprocultures are presented in Table 3.3. Anytime MOX was used, whether alone of in combination, the larval counts were reduced to zero. On the other hand, larval counts increased after the first OBZ treatment, and only decreased 76.7% after the final OBZ treatment.

3.4   Discussion

Within the time frame of this study, the combination of MOX and OBZ did not provide evidence of an improved control regimen against cyathostomins with substantial BZ drug resistance compared to when either active was used alone. Moxidectin was 100% efficacious throughout all treatments it was used (Fig. 3.1). Given the long-term resistance status of Population S cyathosomins to the BZ and TP drug classes, it appears that these resistance mechanisms did not affect the efficacy of MOX and no evidence of cross resistance was observed. The use of MOX for three treatments over a 22 month period did not affect the efficacy of OBZ, which did not change significantly over the course of the study (Table 3.1).

The ERP estimates for the three treatments using MOX were variable, ranging from 12 to 18 weeks (Fig. 3.1). Historic ERPs reported for MOX were between 16 and 22 weeks (Jacobs et al., 1995; Demeulenaere et al., 1997; DiPietro et al., 1997), but a couple aspects must be considered. First of all, previous studies lacked concensus in

methodology used for determining ERP, and secondly the Population *S* ponies utilized in the present study demonstrated moderate to low fecal egg counts and a declining trend over the course of the study. The latter may well be a consequence of efficacious MOX treatments and lowered reinfection pressure which was also influenced by the grazing restrictions the ponies were under. However, low starting egg counts are likely to add variability to the ERP determination, which could explain the findings made in this study. Seasonality is also known to significnatly affect strongyle egg shedding, and this likely added a source of variability to the ERPs as well (Chapman et al., 2003; Wood et al., 2012).

Combining OBZ with MOX did not appear to affect efficacy. This was expected, as starting efficacy was already 100%. A measurable effect would have been expected, if OBZ had been combined with another active with a starting efficacy of less than 100%. Although the last combination treatment had longer ERP than the two previous treatments, more studies are needed to investigate whether OBZ can positively affect ERP. If such an effect is real, it might be due to changes in cyathostomin species composition effected by the OBZ treatment, and this could be investigated further using molecular identification of cyathostomin species present.

Finally, we did not observe any significant changes in OBZ efficacy after the two combination treatments. There are several possible reasons for this. First of all, it may well be that just two combination treatments administered over the course of one year are far from enough to affect single active efficacy. For example, Leathwick (2013) performed a simulation model over 40 years of selection in order to see the effects of combination treatments where the initial frequency of resistance was high. Secondly, BZ resistance has existed within this population since the 1970s (Lyons et al., 2003), and although the MOX treatments would remove all luminal stages and also exhibit larvicidal efficacy, a considerable proportion of encysted larvae would still survive every treatment. The next generation of adults in the intestinal lumen would be recruited from these surviving mucosal larvae, which all would carry genetic alleles conferring OBZ resistance. Again, this study only involved two combination treatments, which is unlikely to be enough to observe any shifts in OBZ efficacy over time. The long ERP following MOX treatment and the general trend towards lowering egg count levels over

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time necessitated very long treatment intervals (30 weeks or more), which must have slowed down any selection process, should it have occurred. Nevertheless, this study speaks to the challenges of sustainable control programs due to the hypobiotic stages, and evidence for combination deworming in other parasite species can not be extrapolated to managing multi-drug resistant cyathostomins. For future studies, it may be of value to substitute MOX with ivermectin as the expected ERP would be considerably shorter, allowing for more treatments within a given year.

Furthermore, the benefits of combination treatments are contingent upon the size of the refugia population (Leathwick et al., 2012) . Regarding cyathostomins, there are three possible sources of refugia, which are the free-living environmental stages, luminal stages in untreated horses, and the encysted stages when a larvicidal drug is not used. In this study, the refugia population was minimal as all ponies received treatment simultaneously, and MOX provides some larvicidal efficacy. While this situation is not ideal, this study modeled similar situations on managed horse farms in the United States where it is common practice to treat all horses simultaneously for general prevention (Nielsen et al., 2018; Scare et al., 2018b). While it is proposed that anthelmintic resistance may be associated with a fitness loss (Prichard et al., 1980), this study continues to support the finding that double-drug resistant cyathostomins do not appear to be affected by any apparent fitness loss as resistance is maintained even in the absence of selection pressure by the BZ drug class (Lyons et al., 2003).

In summary, this study did not identify any clear consequences of combining MOX and OBZ for treatment of a cyathostomin population with resistance to both benzimidazoles and pyrimidines. However, a shift of MOX ERP towards 18 weeks was noted during the study, which warrants further investigation. Similarly, it would be of value to evaluate the consequences of such combinations over longer time periods and allowing more treatments to fully establish what the outcomes may be.

Table 3.1 Results of OBZ treatments administered before (Treatment 1) and after (Treatment 5) a single MOX and two MOX/OBZ combination treatments. The top portion of the table shows results as the mean of individual pony FECs, and the bottom portion shows results of the FECRT. 95% confidence intervals are included in parenthesis (α=0.05).

Mean of individual EPG		
	Initial	2 weeks PT
Treatment 1-OBZ	447.8 (200.4-695.2)[a]	260.6 (125.2-396.1)[a]
Treatment 5-OBZ	169.6 (31.7-307.5)[b]	110.3 (19.1-201.5)[b]
Mean herd efficacy (FECRT)		
	2 weeks PT (%)	
Treatment 1-OBZ	46.7	
Treatment 5-OBZ	40.1	

Abbreviations: OBZ, oxibendazole; MOX, moxidectin; EPG, eggs per gram feces; PT, post treatment; FECRT, fecal egg count reduction test
Superscript letters indicate significant differences between time points (α=0.05).

Table 3.2 Egg reappearance periods following moxidectin single active and two combination treatments. The ERPs are defined when the mean herd efficacy was <85%. The actual percent efficacies are included in parentheses.

Treatment	Time of ERP in weeks
MOX, single active	16 (67.2%)
1st Combination Treatment (MOX/OBZ)	12 (80.4%)
2nd Combination Treatment (MOX/OBZ)	18 (82.5%)

Abbreviations: MOX, moxidectin; OBZ, oxibendazole; ERP, egg reappearance period

Table 3.3Total larval counts (percent of total number of larvae recovered) following coprocultures of ten individual samples collected at pre- and two weeks post-treatment. Treatments were with oxibendazole (10 mg/kg), moxidectin (0.4 mg/kg), or a combination of the two. No strongylin species were encountered.

	OBZ-1 (Pre)	OBZ-1 (Post)	MOX (Pre)	MOX (Post)	Combo-1 (Pre)	Combo-1 (Post)	Combo-2 (Pre)	Combo-2 (Post)	OBZ-2 (Pre)	OBZ-2 (Post)
Total Larvae	**1512**	**3062**	**1350**	**0**	**246**	**0**	**216**	**0**	**43**	**10**
Cyathostominae	1509	3054	1340	0	242	0	196	0	43	10
L1 Strongyles	0	0	0	0	0	0	0	0	0	0
L2 Strongyles	3	8	10	0	4	0	20	0	0	0
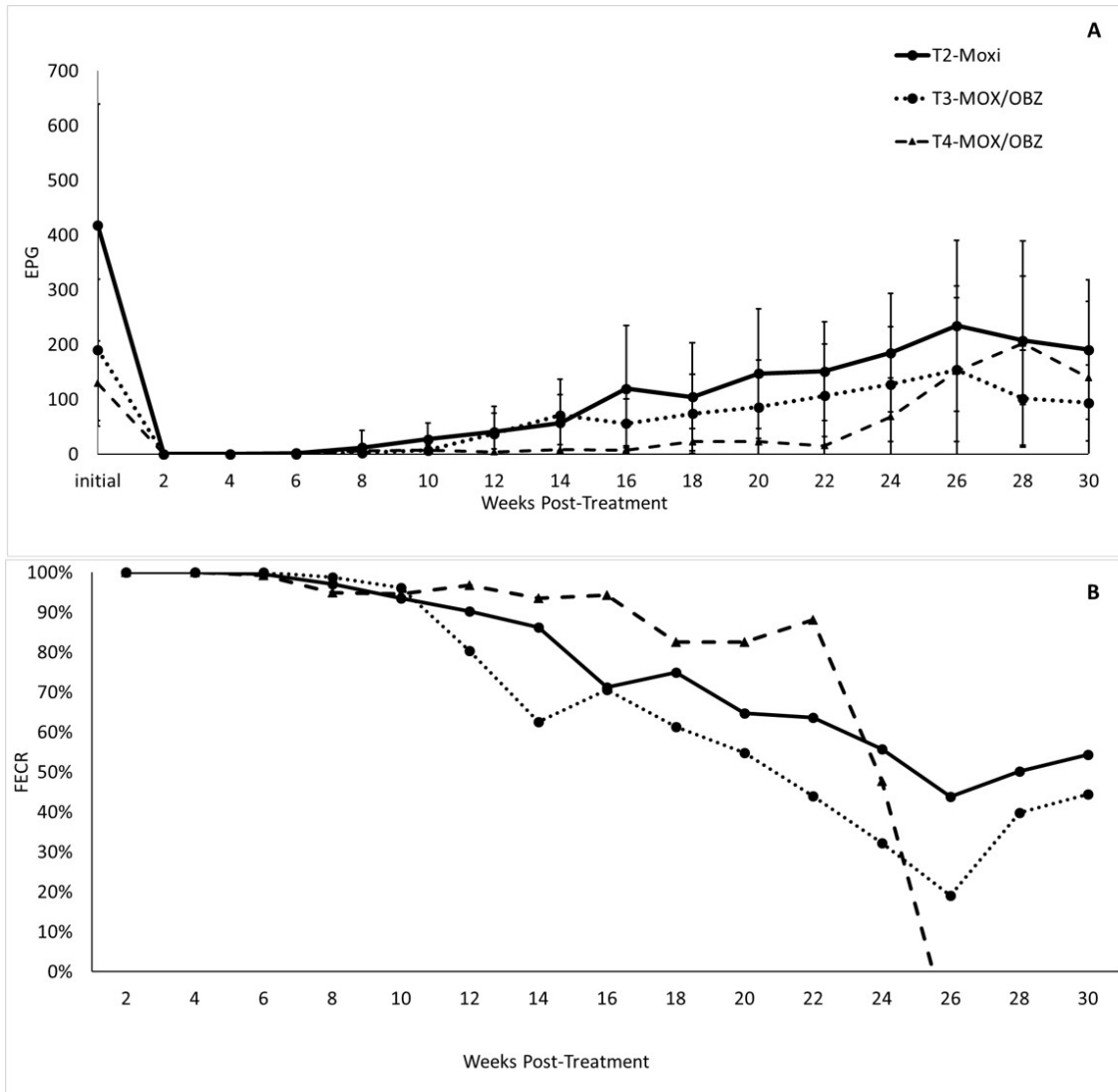
Figure 3.1 Graphical representations of the single active moxidectin (MOX) treatment and the combination treatments of oxibendazole (OBZ) and MOX. Figure A shows fecal egg counts as eggs per gram (EPG). Error bars represent 95% confidence intervals (α=0.05). Figure B shows the percent efficacy of the treatments using the fecal egg count reduction (FECR) test calculated using the total herd fecal egg counts pre- and post-treatment.

CHAPTER 4. LONG LIVE THE WORMS: METHODS FOR MAINTAINING AND ASSESSING THE VIABILITY OF INTESTINAL STAGES OF *PARASCARIS* SPP. *IN VITRO*

4.1   Introduction

Parascaris spp. is a clinically important helminth parasite infecting foals (Clayton and Duncan, 1978; Cribb et al. 2006; Tatz et al. 2012) with anthelmintic resistance reported world-wide (Peregrine et al. 2014). Anthelmintic resistance has not been described for other mammalian ascarid species. The ability to maintain gastro-intestinal helminths in vitro would enhance the experimental tractability of nematode parasites by facilitating the application of a range of molecular and biochemical tools and analyses in clinically relevant species. Such an advance would prompt a paradigm shift in parasitology research permitting progress in key areas including evaluation of anthelmintics and natural products with anthelmintic properties, (Rapson et al. 1985; Brownlee et al. 1997; O'Grady and Kotze, 2004; Janssen et al. 2013b), application of transcriptomics to investigate the genetic mechanisms driving anthelmintic resistance (Janssen et al. 2013b), induction of RNAi interference for the identification of novel drug targets (McCoy et al. 2015), analysis of excretory and secretory products (Young et al. 1995; Geldhof et al. 2000, Islam et al. 2004; Cribb et al. 2006; Burk et al. 2014; Thomas et al. 2016), and interrogation of host-parasite interactions (Kotze and McClure, 2001).

Most of the literature on *in vitro* culture and maintenance of ascarid parasites has focused on the pig nematode, *Ascaris suum*, where a variety of culture conditions have been employed. Some reports describe *in vitro* maintenance of larval stages (Douvres and Urban, 1983, 1986), however the size and nutrient requirements of the intestinal stages introduce new challenges to *in vitro* maintenance. Chehayeb et al. (2014) maintained adult *A. suum* collected from the small intestine of pigs for 24 hours in Locke's solution where glucose was provided as the main nutrient. Weisblat and Russel (1976) described culturing *A. suum* in artificial perienteric fluid (APF), and Brownlee et al. (1997) maintained worms in APF for five days. Islam et al. (2004) maintained adult *A. suum* under both aerobic and anaerobic conditions to observe changes in proteome expression patterns. Worms were maintained in Roswell Park Memorial Institute- 1640 (RPMI-1640) medium, and viability was maintained in both systems for over two weeks. Dmitryjuk et al. (2014) sustained adult *A. suum* in Ascaris ringer's solution (ARS) for 20 hours without any nutrient, while McCoy et al. (2015) maintained *A. suum* for eight days in ARS without any nutrient. In contrast, only two studies have reported the *in vitro* maintenance of *Parascaris* spp. Burk et al. (2014) reported culturing of larval stages and maintenance of adult stages to investigate the production of excretory-secretory products.

In that study, two adult worms were maintained in RPMI-1640 medium at 37°C for five days. Janssen et al. (2013b) maintained an undisclosed number of adult worms in APF for 30 hours at 37°C for *in vitro* ivermectin exposure. No attempts have been made to evaluate the requirements for long term in vitro maintenance of *Parascaris* spp. intestinal stages, nor to characterize their preferred environment and nutrient requirements.

In order to determine the optimum *in vitro* requirements and monitor the effects of *in vitro* drug exposure, it is necessary to ascertain helminth longevity and viability. *In vitro* evaluation of anthelmintic efficacy in adult worms has been done by determining worm longevity by classifying them on an alive or dead basis (Eguale et al. 2007a,b; Hu et al. 2013). While Hu et al. (2013) implemented a scoring system on a 0-3 scale to assess worm movement, it was still largely subjective and the results considered worms only on an alive (score 1-3) or dead (score 0) basis. Similarly, Richards et al. (1995), described a simple method to monitor drug sensitivity of *Necator americanus* and *Ancylostoma caninum* based on the observation of worm motility of treated versus control worms. Worms were characterized as either active or inactive after gentle prodding. Neither the method proposed by Hu et al. (2013) nor Richards et al. (1995) allows for the objective evaluation of worm viability over a series of time points. A similar subjective method was reported by Dmitryjuk et al. (2014) to monitor the effects of *in vitro* anthelmintic exposure to adult *A. suum*. Later, a motility assay was developed by O'Grady and Kotze (2004) that utilized a scoring system to monitor anthelmintic efficacy against *Haemonchus contortus*. While the scoring system allows one to observe a decline in viability over time, the definition of each score is subjective as scores are assigned based on the investigators definition of significant movement, and a set amount of time for each observation was not described. Marcellino et al. (2012) developed the WormAssay, a high throughput screening method to assess the anthelmintic efficacy against macroparasites based on motility. The WormAssay uses an open source computer software program and a camera to automatically assess worm movement and provide a quantitative measurement. Worms must be placed in microtiter plates, and the system is compatible with plates of either 6, 12, 24, 48, or 96 wells. The *Parascaris* species, however, are still too large for the well plates used in this system. Even the largest wells (6-well plate) measuring approximately 3.48 cm in diameter are not large enough for a mature *Parascaris* spp., which are commonly over 10 cm long (Clayton and Duncan, 1978). The Worminator uses a similar method but is specifically designed for determining the motility of microscopic nematode stages (Storey et al. 2014).

The purpose of this study was to characterize appropriate *in vitro* conditions for maintaining intestinal *Parascaris* spp., and to establish a scoring system to monitor worm viability over several time points.

4.2 Materials and methods

4.2.1 Parasite sources

The study took place over the course of eight foal necropsies from October 2016 to October 2017. The foals were born in a herd housed at the University of Kentucky that has not been treated with any anthelmintics since 1979 and has been documented to harbor a variety of equine parasites through natural infection (Lyons *et al.* 1990). The foals employed in the study consisted of five colts and three fillies. Foals were humanely euthanized when they reached 4.5-5 months old and subsequently necropsied. The research was conducted following approval from the University of Kentucky's Institutional Animal Care and Use Committee (IACUC) under protocol number 2012-1046.

4.2.2 Study design

During the first phase of this study (necropsies 1-3) worms were monitored on an alive/dead basis in order to make initial observations on the necessary conditions for *in vitro* maintenance and nutrient requirements of *Parascaris* spp. specimens. The second phase (necropsies 4-8) commenced following the development of a scoring system to objectively assess the viability of *Parascaris* spp. specimens under various environmental and nutrient conditions.

A variety of different media types, nutrient supplements and environmental conditions were examined (see Section 2.4.1 and 2.4.2). The number of worms evaluated for each media, nutrient and environmental condition (CO_2 and platform rocker) is described in Table 4.1.

4.2.3 Collection of *Parascaris* spp.

Following necropsy, the small intestine was detached from the stomach and cecum. The intestinal contents were milked out onto a 425μ mesh sieve. Room temperature (RT) tap water was slowly added to the sieve to dilute the contents to better visualize the worms. Intestinal stages of *Parascaris* spp. (adult and fourth larval stage, L4) specimens were recovered using a spay hook and placed in a container of RT media

of either ARS (see Table 4.2 for composition) (necropsies 1-6) or RPMI-1640 (R8758, Sigma-Aldrich, St. Louis, MO, US) (necropsies 7 and 8). The container was placed into a water bath maintained at 37˚C for transport to the laboratory. Worms were classified as adult or L_4, and adult worms were further characterized by sex. Worms were considered adults when gonads were visible as white material in the mid-section of the worm. Males were differentiated from females by being smaller and having less gonad material than females, and occasionally presented with a curved hook in the tail. Immature worms (L_4) did not have any visible gonad material.

4.2.4 *In vitro* maintenance of *Parascaris* spp.

Worms were maintained in vented TPP tissue culture flasks (300 cm^2, MidSci, St. Louis, MO) containing 200 mL of the pre-assigned medium. Media were changed every 12 hours. This was done by placing a cell strainer of 400 µm pore size (pluriSelect Life Science, Leipzig, Germany) over the mouth of the flask and allowing the old media to flow through while keeping the worms in the flask to limit handling and subsequent damage. New media, pre-warmed to 37˚C, were then added to the flask. The flasks were kept in the pre-determined incubator with or without CO_2 (5%) supplementation at 37˚C.

In the first phase of the study (necropsies 1-3) worms were maintained in groups of four or five, containing two males and at least one female and one L_4 worm. In the second phase of the study (necropsies 4-8) a total of five worms were placed in each culture flask consisting of either two males, one female, and two immatures, or three males, one female, and one L_4 worm. The variation in worm stage/sex within each cohort was due to the number of worms per category collected at each necropsy.

4.2.4.1 Preparation of culture media

Media (ARS, APF, ARS 3x Tris, APF 2x NaCl, physiological saline (PS) (Hospira Inc, Lake Forest, IL, US), homemade physiological saline (HMPS), and RPMI-1640; see Table 4.2) were freshly prepared, stored at 4˚C, and then warmed to 37˚C prior to adding to the culture flasks. Streptomycin (1mg /1L), Penicillin (1000 U/1L) and

Amphotericin-B (10 µg/1L) were added to all media types, except when *Escherichia coli* was added as a nutrient (see 2.4.2). All media types were employed within 24 hours of preparation.

4.2.4.2 Nutrient supplementation

A list of the nutrients and their respective concentrations can be found in Table 4.2. *Escherichia coli* OP50 (University of Kentucky) was prepared in the following manner. LB (lysogeny broth) (Miller formulation, ThermoFisher Scientific, Waltham, MA) and LB-agar (Fisher Scientific, Hampton, NH) were prepared according to the manufacturer's instructions. *Escherichia coli* OP50 (University of Kentucky) were cultured in 15 mL of LB broth overnight at 37°C in a shaking incubator at 225 rpm. Following incubation, cells were pelleted by centrifugation at 3220g for eight minutes. After centrifugation, the supernatant was decanted and pelleted. *E. coli* were re-suspended in 15 mL of filter-sterilized culture media. Colony forming units (CFUs) were determined for the *E. coli* suspension by plating ten-fold serial dilutions to determine the starting culture concentration (*i.e.* input). The remaining suspension was equally divided and added to the assigned flasks. One flask was kept without worms as a control. Prior to the media changes, an aliquot of the media from the culture flasks, including the flask without worms, was plated to determine the final concentration (*i.e.* output) of surviving *E. coli*.

4.2.4.3 Environmental conditions

The environmental conditions assessed were the use of a 5% CO_2 incubator and platform rocker. The number of flasks assigned to each condition can be found in Table 4.1. Pre-assigned flasks were placed in a 5% CO_2 incubator at 37°C for the entirety of their survival. Flasks assigned to the platform rocker (Hofer Scientific Instruments, San Francisco, CA model PR70) were maintained at approximately 60 rpm within the air-only incubator at 37°C for the entirety of their survival.

4.2.5   Longevity and viability assessment of *Parascaris* spp.

For the first phase of the study (necropsies 1-3), worms were monitored on an alive or dead basis and the number of worms surviving per flask at each time point/media change was recorded (*i.e.* longevity). Worms were considered dead when they became flaccid and/or displayed signs of decay. Flaccidity was determined by placing the worm over a pair of forceps at midpoint and carefully lifting it out of the medium. If the worm draped loosely over the forceps and appeared as an acute angle, it was considered flaccid. Decay was noted visually and determined as breakdown of the exterior cuticle. The second phase of the study (necropsies 4-8) began with the development of an objective scoring system to monitor worm viability. Prior to each medium change, worm viability was assessed and awarded a score according to the descriptions in Table 4.3. Each worm was observed for 15 seconds for movement while remaining in the flask. If no movement occurred during the 15 second observatory period, forceps were used to gently stimulate the worm in an attempt to initiate movement. If still no movement was observed, the forceps were used to assess flaccidity and check for decay as previously described. Dead worms were removed from the flask and discarded.

4.2.6   Statistical analyses

4.2.6.1   Phase one: Longevity

For the first phase of the study (necropsies 1-3), a percent reduction in the number of worms in each flask was calculated at each time point. The final time of longevity was considered when all worms in a flask had died. Mean longevity with 95% confidence intervals (CI), and the range for media, nutrient, and incubator type were calculated using Microsoft Excel 2016 (Redmond, WA, USA). These values can be found in Table 4.4.

Further statistical analyses were performed using SAS software (version 9.4, SAS Institute, Cary, North Carolina, USA). Here, four mixed linear models with repeated measures across time were constructed to determine which media, nutrient supplementation profile, and incubator type significantly affected worm longevity. 'Percent loss' was the response variable for all analyses. The first model assessed the

longevity of worms maintained in the different media types without nutrient supplementation or CO_2 incubator. The covariates were 'Time' and the interaction term 'media ID*none', where 'none' implied an air incubator and no nutrients were used. 'Necropsy date' was kept as a random effect. The second analysis was used to analyze the supplementation with glucose in all types of media because it was the only nutrient tested across all media types. The interaction term 'Media ID*glucose' was the covariate analyzed and 'necropsy date' was kept as the random effect. The third model examined worm longevity when maintained in ARS media supplemented with either glucose, gelatin, *E. coli*, yeast, FBS, cholesterol, or gelatin and glucose. ARS was the only medium supplemented with all the nutrients and therefore was the only medium examined in this model. 'Nutrient' and 'time' were the covariates examined. 'Necropsy date' and 'CO_2' were kept as random effects. The fourth model examined the use of the CO_2 incubator across all media and nutrient supplements. The covariates examined were 'time' and 'CO_2'. 'Necropsy date', 'Media ID' and 'nutrient' were kept as random effects. The fifth analysis analyzed the stage (L_4 or adult) and sex (adult worms only) over time, regardless of media, nutrients used, or the use of the CO_2 incubator. The covariates analyzed were 'stage' and 'sex'. 'Media ID' and 'necropsy date' were kept as random effects. Any time a significant covariate ($\alpha=0.05$) was observed, a 'least squares means' analysis was performed for a Tukey's pair-wise comparison.

4.2.6.2   Phase two: Viability

For the second phase of the study (necropsies 4-8), the scoring system (see Table 4.3) was used to monitor worm viability. Mean worm viability per flask at each time point was calculated. Worms that had died continued to receive a score of zero and were included in the mean calculation until all the worms within the same flask had died. Mean values and 95% confidence intervals (CI) were calculated using Microsoft Excel 2016 (Redmond, WA, USA). The percent viability per flask was calculated in Microsoft Excel for each time point using the following formula, where 'X' refers to each time point:

$$\% \, Viability = 100 - \left(\frac{(initial \; score - score \; at \; time \; 'X')}{initial \; score} \times 100\% \right)$$

Further statistical analyses were performed using SAS software (version 9.4, SAS Institute, Cary, North Carolina, USA). Here, a total of six mixed linear models with repeated measures across time were performed to determine which media, nutrients, and environmental conditions significantly affected worm viability. For all models, 'percent viability' was the response variable. The first model assessed the viability of worms maintained in the different media without nutrient supplementation, CO_2 incubator, or platform rocker. The covariates were 'time' and the interaction term 'media ID*none', where 'none' implied that no nutrients or environmental conditions were implemented. 'Necropsy date' was kept as a random effect. The second model analyzed worm viability when maintained in one of the saline-based media (*i.e.* ARS, APF, ARS 3x Tris, APF 2x NaCl, PS, HM PS) with glucose compared to worm viability maintained in the same saline-based media without glucose. Glucose was the only nutrient added across all saline-based media types and therefore was the only nutrient analyzed in this model. The covariates examined were 'time' and the interaction term 'media ID*glucose'. 'Necropsy date' was kept as a random effect. The third model examined worm viability when maintained in APF media supplemented with either glucose, FBS, cholesterol, a combination of FBS and cholesterol, Tween only control, or as a no nutrient control. APF was the only medium supplemented with all the nutrients and therefore was the only medium examined in this model. 'Nutrient' and 'time' were the covariates examined. 'Necropsy date' and 'environment' (i.e. CO_2 incubator or platform rocker) were kept as random effects. The fourth model examined the use of the platform rocker and CO_2 incubator across all media and nutrient supplements. The covariates examined were 'time' and 'environment'. 'Necropsy date', 'Media ID' and 'nutrient' were kept as random effects. The fifth model analyzed the use of RPMI against all media, nutrients, and environmental conditions. The covariate tested was 'RPMI,' and 'necropsy date' was kept as random effect. The last model analyzed the stage (L_4 or adult) and sex (adult worms only) over time, regardless of media, nutrients used, or the use of the CO_2 incubator or platform rocker. The covariates analyzed were 'stage' and 'sex'. 'Media ID', and 'necropsy date' were kept as random effects. Any time a significant covariate (α=0.05) was observed, a 'least squares means' analysis was performed for a Tukey's pair-wise comparison.

4.3   Results

A total of 212 cultures were performed and a total of 1045 *Parascaris* spp. worms were used. The number of cultures and worms per media type, nutrient supplementation, and environmental condition (incubator type and/or platform rocker) can be found in Table 4.2.

4.3.1   Phase one: Longevity

For the first phase of the study pertaining to worm longevity (necropsies 1-3), a total of 210 worms were used consisting of 98 adult males, 54 adult females, and 58 L$_4$s. During this phase of the study, the worms lived a maximum of 84 hours. The media type employed when considered without nutrient supplementation or CO_2 did have a significant effect on worm longevity (p=0.0100), however the least squares means pairwise comparison did not identify any significant differences between media. ARS was the only media type significantly affecting worm viability with the addition of glucose. Worms maintained in ARS supplemented with glucose lived significantly longer than worms maintained in ARS alone (p<0.0001). There were no significant differences observed in any of the other media types supplemented with glucose compared to when glucose was not added. Regarding the various types of nutrient supplementation with the ARS media, worms maintained with glucose (p<0.0006) or a combination of glucose and gelatin (p<0.0001) had significantly better longevity than worms maintained without any nutrient. Worms maintained with glucose had significantly better longevity than worms maintained with *E. coli* (p=0.0008), yeast (p<0.0001), FBS (p=0.0013), or cholesterol (p=0.0279). Similarly, worms maintained with a combination of glucose and gelatin had significantly better longevity than those maintained with gelatin only (p=0.0484), *E. coli* (p<0.0001), yeast (p<0.0001), FBS (p<0.0001), or cholesterol (p=0.0008). The mean longevity, 95% confidence intervals, and range of longevity for the different nutrients and incubator type can be found in Table 4.4. The use of a CO_2 incubator did not significantly affect worm longevity (p=0.2854). 'Adult male (p=0.0021) and female (p<0.0001) worms had significantly better longevity than immature worms, however there was no significant difference between males and females (p=0.5780). The mean longevity, 95%

confidence intervals, and range of longevity for immatures, males, and females can be found in Table 4.4.

4.3.2   Phase two: Viability

For the second phase of the study pertaining to worm viability (necropsies 4-8), a total of 835 worms were used, consisting of 350 adult males, 215 adult females, and 270 L4s. The RPMI-1640 media resulted in significantly better worm viability than any of the other media ($p<0.0001$) (Figure 4.1). APF 2x NaCl had significantly better viability than ARS ($p=0.0002$). APF ($p=0.0005$), ARS 3x Tris ($p=0.0169$), and APF 2x NaCl ($p<0.0001$) had significantly better viability than the homemade physiological saline. The addition of glucose to the saline-based media did not significantly affect worm viability compared to those maintained in the saline-based media without glucose ($p=0.3048$). The addition of a nutrient to the APF medium did significantly decrease worm viability ($p=0.0413$), however the least squares means pairwise comparison did not identify any significant differences (Figure 4.2). The use of the platform rocker resulted in significantly better worm viability than worms maintained without the rocker ($p=0.0305$), while there were no significant differences in worm viability between the use of an air or CO_2 incubator ($p=1.0000$) (Figure 4.3). Overall, worms maintained in RPMI-1640 had significantly better viability than worms maintained with any other method regardless of media, nutrient, or environmental condition ($p<0.0001$) (Figures 4.1 and 4.2). In regards to worm stage and sex, adult worms regardless of sex had significantly better viability than L4s ($p<0.0001$) and females had significantly better viability than males ($p<0.0001$) across all media types, nutrient supplementation, and environmental conditions.

4.4   Discussion

This is the first study to determine the preferred *in vitro* conditions for the intestinal stages of *Parascaris* spp., and to describe a reliable and objective method for assessing their viability. Worm motility and the presence of muscle tone appears to be reliable indicator for assessing *in vitro* conditions. This study is the first to report a difference in *in vitro* worm viability for *Parascaris* spp. between L4 and adult stages, as well as between male and female adult worms.

Intestinal stages of *Parascaris* spp. must be active swimmers against the flow of intestinal contents in order to maintain their position in the host and avoid being expelled by peristalsis (Drudge and Lyons, 1983). Therefore, worm responses to *in vitro* conditions should be judged based on activity level, where a decrease in activity likely reflects a decrease in overall worm viability. Other scoring systems for gastrointestinal nematodes have been developed, but these did not provide strict parameters of movement per score (Richards *et al.* 1995; O'Grady and Kotze, 2004). While *Parascaris* spp. intestinal stages are not compatible with the current size restrictions of the WormAssay (Marcellino *et al.* 2012), a modification of this technique to accommodate larger macroparasites should be a target for future research.

The use of RPMI-1640 media resulted in significantly better worm viability than all other media types regardless of nutrient supplementation and/or environmental condition (Figures 4.1 and 4.2). Worms lived a maximum of 168 hours in RPMI-1640 (Figures 4.1 and 4.2), which is well above the 84 and 96 hours achieved in phase 1 and phase 2, respectively, with the addition of glucose (Table 4.3 and Figure 4.2). At this time, it is unknown which components of the RPMI-1640 media caused this improvement in viability and longevity, but it is likely due to the combination of vitamins and amino acids that were missing from the other media evaluated. This finding is in agreement with Urban *et al.* (1984) who found improved growth and survival of L_4 *A. suum* when cultured in RPMI-1640 rather than a saline medium supplemented with glucose.

The use of sugar (glucose or dextrose) as a nutrient is reported in several other studies maintaining adult stages of *A. suum* (Weisblat and Russel, 1976; Brownlee *et al.* 1997; Chehayeb *et al.* 2014)*, and one study used dextrose for maintaining adult *P. equorum* (Janssen *et al.* 2013b). While it is assumed that sugar is necessary for the *in vitro* cultivation of *Ascaris* and *Parascaris* species, this had not previously been evaluated in a published study. In phases one and two of this study, *Parascaris* spp. survived a maximum of 84 and 96 hours, respectively, when glucose was added as a nutrient and it did not significantly affect worm viability. The success of the RPMI-1640, but not the glucose provides evidence that *Parascaris* spp. intestinal stages require

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different and/or additional nutrients beyond glucose for sustainment *in vitro*. It is interesting that *A. suum* can be maintained for eight days in ARS without any nutrient supplementation (McCoy *et al.* 2015). In the current study, *Parascaris* spp. did not live more than 168 hours in any of the media regardless of the media type or nutrient provided. This may suggest that adult *A. suum* and *Parascaris* spp. worms have very different nutrient and metabolic requirements, however direct conclusions cannot be made at this time. A comparative study could be performed to determine the viability of *Parascaris* spp. and *A. suum* when supplemented with different nutrients, and analyses of the media after a nutrient has been provided could determine if the worms successfully ingested the nutrient. If so, the effectiveness of the worm to generate energy from the given nutrient could be assessed using metabolic techniques. Such findings would provide significant advances toward *in vitro* techniques of the parasitic stages.

Douvres and Urban (1983, 1986) described methods for culturing larval stages of *Ascaris* species utilizing various gaseous stages, including 5% CO_2. Several studies report the maintenance of adult *A. suum* worms without CO_2 (Weisblat and Russel, 1976; Brownlee *et al.* 1997; Chehayeb *et al.* 2014; McCoy *et al.* 2015). Janssen *et al.* (2013b) maintained *P. equorum* adult worms without 5% CO_2 while Burk *et al.* (2014) cultured second and third larval stages of *P. equorum* under 5% CO_2 conditions, but not the adult worms. Based on these reports, it appears that adult worms may not require CO_2, but this had not been specifically evaluated for *Parascaris* spp. The current study did not find the use of 5% CO_2 to significantly affect worm longevity or viability (Figure 4.3). However, this study did not investigate the impact of CO_2 on worms maintained in RPMI-1640 and this should be evaluated in future studies.

The use of a platform rocker for *in vitro* maintenance of ascarid parasites had not been evaluated prior to this study. In this study, the use of the rocker significantly improved worm viability (Figure 4.3), however no firm conclusions can be made at this time. The platform rocker could not be tested simultaneously with CO_2 due to limited space in the incubator. Furthermore, this study did not evaluate RPMI-140 media with the use of the rocker, and this should be investigated in future studies.

It is also known that nematodes are unable to synthesize cholesterol *de novo* (Dutky *et al.* 1967; Cole and Krusberg, 1968), however this study did not find the addition of cholesterol to improve worm longevity or viability. Additionally, the addition of FBS did not significantly improve viability. These findings are interesting because Urban *et al.* (1984) found the addition of cholesterol (50 µg/mL) and serum (10%) to RPMI-1640 to have an additive effect on the growth of L4 *A. suum.* Urban *et al.* (1984) also found that an increase in cholesterol concentration to 250 µg/mL from 50 µg/mL reversed this effect. While the aforementioned study examined the development of larval stages, it is possible that a similar scenario was observed in the current study where the *Parascaris* spp. intestinal stages were negatively impacted by the cholesterol concentration examined herein. Future studies should investigate varying concentrations of cholesterol to determine if there is an optimum concentration and/or a tolerance threshold.

The varying sample sizes between the nutrient trials are a limitation to this study, particularly in regards to the number of worms used for evaluating the RPMI-1640 media and the saline-based media supplemented with cholesterol, FBS, yeast, and *E. coli* (Table 4.1)*.* Variations occurred due to the number of worms harvested at each necropsy. While the results of this study clearly support the recommendation for using RPMI-1640 for maintaining intestinal stages of *Parascaris* spp., the conclusions should be interpreted with caution and warrant further investigation. The effects of stocking density and keeping male, female, and immature worms together would also provide interesting points for future studies.

It is important to note that the *in vivo* immune responses exhibited by the foal prior to necropsy may also affect worm viability *in vitro*. Foals typically gain immunity to *Parascaris* spp*.* worms around nine months of age (Clayton and Duncan, 1979). Some response by the immune system to the present parasites is expected and it is unknown how the parasites were affected prior to harvest and culturing. This variability was controlled for by using foals which were all born into the same herd, and harvesting the worms when the foals were between 4.5-5 months of age which is the peak age for

Parascaris spp. burden (Fabiani *et al.* 2016) and thus minimizing the potential influence of host immunity.

In summary, the scoring system proved to be a useful method for monitoring L_4 and adult worm viability *in vitro*, and should be considered for future studies. This study found RPMI-1640 media to significantly improve worm viability. The use of a 5% CO_2 incubator did not significantly affect worm viability, but a platform rocker significantly increased viability. The viability of adult worms was also significantly better than that of L_4s. Further investigations should be performed to examine the effects of a platform rocker and CO_2 incubator when RPMI-1640 is used as the culture media.

Table 4.1 . Distribution of intestinal stages of *Parascaris* spp. specimens among the different media, nutrients, and environmental conditions (*i.e.* CO_2 incubator, platform rocker) for *in vitro* maintenance. The number of worms is listed followed by the number of cultures in parenthesis. The top table is from phase one of the study (necropsies 1-3) for initial observations regarding worm longevity. The bottom table is from phase two of the study (necropsies 4-8) when worm viability was assessed. Cultures were kept at 37˚C.

Medium	None[a]	Glucose (5 mM)	Gelatin	Glucose & Gelatin*	Cholesterol (50µg/mL)	FBS (10%)	*E. coli OP50*	Yeast (1%)	CO_2 (5%) Incubator	Glucose & CO_2*	Gelatin & CO_2*	Total*
ARS	30 (8)	28 (6)	10 (2)	8 (2)	15 (3)	15 (3)	10 (2)	15 (3)	17 (4)	4 (1)	5 (1)	140 (31)
APF	10 (2)	5 (1)	0	0	0	0	0	0	0	0	0	15 (3)
ARS 3x Tris	10 (2)	10 (2)	0	0	0	0	0	0	0	0	0	20 (4)
APF 2x NaCl	10 (2)	10 (2)	0	0	0	0	0	0	0	0	0	20 (4)
PS	10 (2)	5 (1)	0	0	0	0	0	0	0	0	0	15 (3)
Total	70 (16)	58 (12)	10 (2)	8 (2)	15 (3)	15 (3)	10 (2)	15 (3)	17 (4)	4 (1)	5 (1)	210 (45)

Medium	None[a]	Glucose (5 mM)	Cholesterol (50µg/mL)	FBS (10%)	Cholesterol & FBS*	Tween (5%) control	Platform rocker	CO_2 (5%) Incubator	Glucose & CO_2*	Glucose & Rocker*	Total*
ARS	25 (5)	35 (7)	0	0	0	0	15 (3)	10 (2)	5 (1)	5 (1)	85 (17)
APF	65 (13)	195 (39)	60 (12)	60 (12)	30 (6)	20 (4)	15 (3)	90 (18)	85 (17)	5 (1)	505 (101)
ARS 3x Tris	25 (5)	35 (7)	0	0	0	0	15 (3)	10 (2)	5 (1)	5 (1)	85 (17)
APF 2x NaCl	15 (3)	35 (7)	0	0	0	0	15 (3)	5 (1)	5 (1)	5 (1)	70 (14)
PS	10 (2)	15 (3)	0	0	0	0	5 (1)	0	0	0	30 (6)
HM PS	10 (2)	10 (2)	0	0	0	0	5 (1)	0	0	0	25 (5)
RPMI-1640	35 (7)	0	0	0	0	0	0	0	0	0	35 (7)
Total*	185 (37)	325 (65)	60 (12)	60 (12)	30 (6)	20 (4)	70 (14)	115 (23)	100 (20)	20 (4)	835 (167)

[a] 'None' implies an air incubator and no nutrient was used.

* Nutrient combinations with other nutrients or environmental conditions were not included in the total values because these were already accounted for in the individual nutrient, CO_2, and platform rocker columns.

Abbreviations: FBS, fetal bovine serum; ARS, ascaris ringers solution; APF, artificial perienteric fluid; ARS 3x Tris, ARS with triple Tris buffer concentration; APF 2x NaCl, APF with double NaCl concentration; PS, physiological saline (0.9% NaCl); HM PS, homemade physiological saline (0.9% NaCl); Roswell Park Memorial Institute- 1640, RPMI-1640

Table 4.2 Components of the media tested and nutrients provided for the in vitro maintenance for intestinal stages of Parascaris spp.

| Media | Concentration of media components (mM) | | | | | | pH[c] |
	NaCl	CaCl$_2$	MgCl$_2$	KCl	NaC$_2$H$_3$O$_2$	C$_4$H$_{11}$NO$_3$/Tris	
ARS[a]	13.14	9.47	7.83	19.64	100	12.09	7.8
APF[b]	23	6	5	24	110	12.09	7.8
ARS 3x Tris	13.14	9.47	7.83	19.64	100	36	7.8
APF 2x NaCl	46	6	5	24	110	12.09	7.8
PS	154	0	0	0	0	0	NA
HM PS	154	0	0	0	0	0	NA
RPMI-1640[d]	-	-	-	-	-	-	NA

Nutrient	Concentration	Manufacturer/Source
(D+)-Glucose monohydrate	5 mM	Acros organics, Fischer Scientific, Hampton, NH)
Food grade unflavored gelatin	2 g/L	Kroger, Cincinnati, OH
E. coli OP$_{50}$	8.55E+10 CFU/ml[g]	University of Kentucky
FBS	10%	Millipore Sigma, St. Louis, MO
Cholesterol[e]	50 µg/mL[f]	Millipore, Sigma, St. Louis, MO
Yeast	1%	BD Biosciences, San Jose, CA
Tween only control[h]		

[a] McCoy et al., 2015
[b] Weisblat and Russel, 1976
[c] pH adjusted with Hydrochloric Acid, the pH was not adjusted for PS, HM PS, or RPMI-1640
[d] The components remained as provided by the manufacturer (Millipore Sigma, St. Louis, MO)
[e] Prepared as at 0.1% stock solution in 5% aqueous Tween 80 (Bolla et al. 1972)
[f] Urban and Douvres, 1984
[g] Average number of CFU calculated from all input concentrations
[h] Included not as a nutrient, but as a control because cholesterol was prepared by dissolving it in 5% aqueous Tween 80 (Bolla et al. 1972).

Abbreviations: ARS, Ascaris ringers solution; APF, artificial perienteric fluid; ARS 3x Tris, ARS with triple the Tris buffer concentration; APF 2x NaCl, APF with double the NaCl concentration; PS, physiologic saline; HM PS, homemade physiologic saline; RPMI, Roswell Park Memorial Institute; CFU, colony forming units

Table 4.3 Scoring system used to assess the *in vitro* viability of *Parascaris* spp. intestinal stages. Scores were assigned following individual observation for 15 seconds.

Score	Description
0	Dead, no movement observed independently nor when stimulated with forceps. Lack of muscle tone/flaccid over forceps when lifted out of the solution. Signs of decay may be present.
1	No movement observed independently nor when stimulated with forceps. Muscle tone is apparent.
2	Movement only when stimulated with forceps.
3	Movement of head only without stimulation.
4	1-3 whole body movements without stimulation.
5	4-6 whole body movements without stimulation.
6	7 or more whole body movements without stimulation.

Table 4.4 Mean longevity of intestinal stages of *Parascaris* spp. *in vitro* with various nutrients and CO_2 incubator use, and of different stages and sex (necropsies 1-3). Worms were maintained in tissue culture flasks (300 cm^2) in groups of four or five. All worms were kept in 200 mL of *Ascaris* ringer's solution and incubated at 37˚C. The time of longevity was considered the hour when all worms in a flask were dead. Flasks were checked every 12 hours. 95% confidence intervals are included in parenthesis (α=0.05).

Nutrient/Incubator	Mean Longevity (hours)	Range of longevity (hours)
None[a]	42 (34.7-49.3)	12-60
Glucose (5 mM)	72 (63.7-80.3)	48-84
Gelatin	72 (63.7-80.3)	60-84
Glucose & Gelatin	78 (69.7-86.3)	72-84
Cholesterol (50 µg/mL)	56 (49.6-62.4)	48-60
Fetal Bovine Serum (10%)	40 (33.6-46.4)	36-48
E. Coli OP50	36	36
Bacto Yeast Extract (1%)	36	36
5% CO_2 Incubator	67.2 (54.6-79.8)	48-84
Glucose & CO_2 Incubator	84	84
Gelatin & CO_2 Incubator	60	60
Total	46 (42.4-49.7)	
Stage/Sex	Mean Longevity (hours)	Range of longevity (hours)
Immature	38 (34.9-41.2)	12-84
Male	43 (3.3-39.7)	24-84
Female	46.5 (42.3-50.7)	24-84

[a] *'None' implies an air incubator and no nutrient was used.*
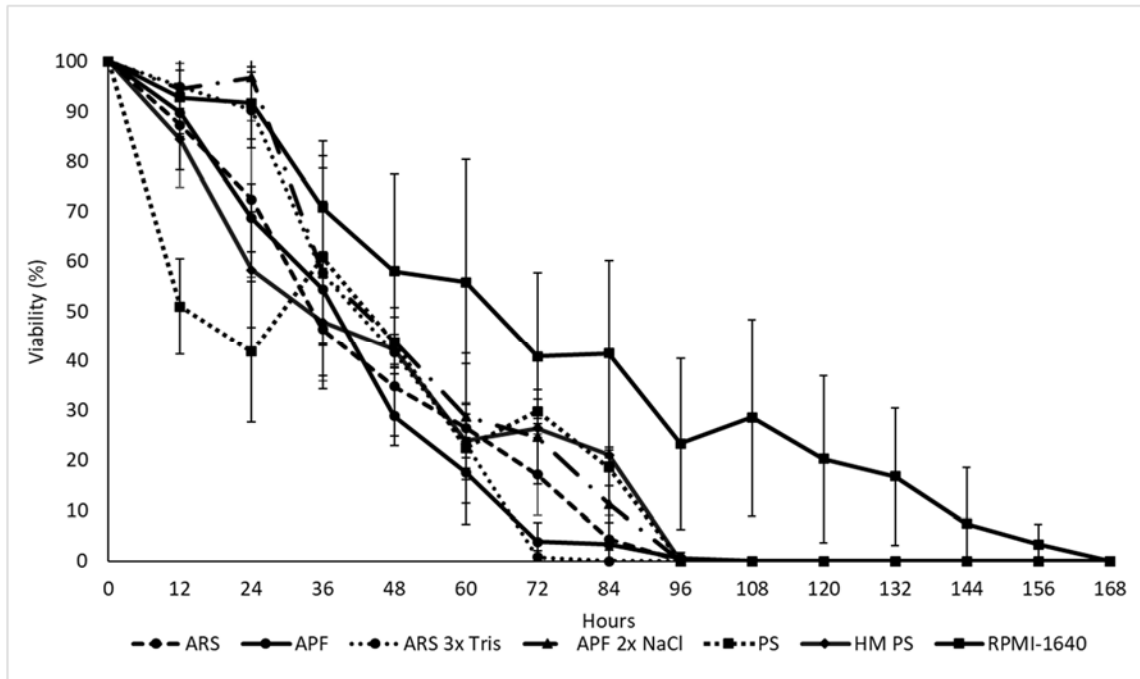
Figure 4.1A graphical representation of mean viability of *Parascaris* spp. intestinal
stages when maintained in various media types (ARS: *Ascaris* Ringer's solution;
APF: artificial perienteric fluid; ARS 3x Tris; ARS with triple the amount of Tris
buffer; APF 2x NaCl: APF with double the amount of NaCl; PS: physiologic
saline; HM PS: homemade physiologic saline, and RPMI: Roswell Park Memorial
Institute). Error bars represent 95% confidence intervals ($\alpha=0.05$).
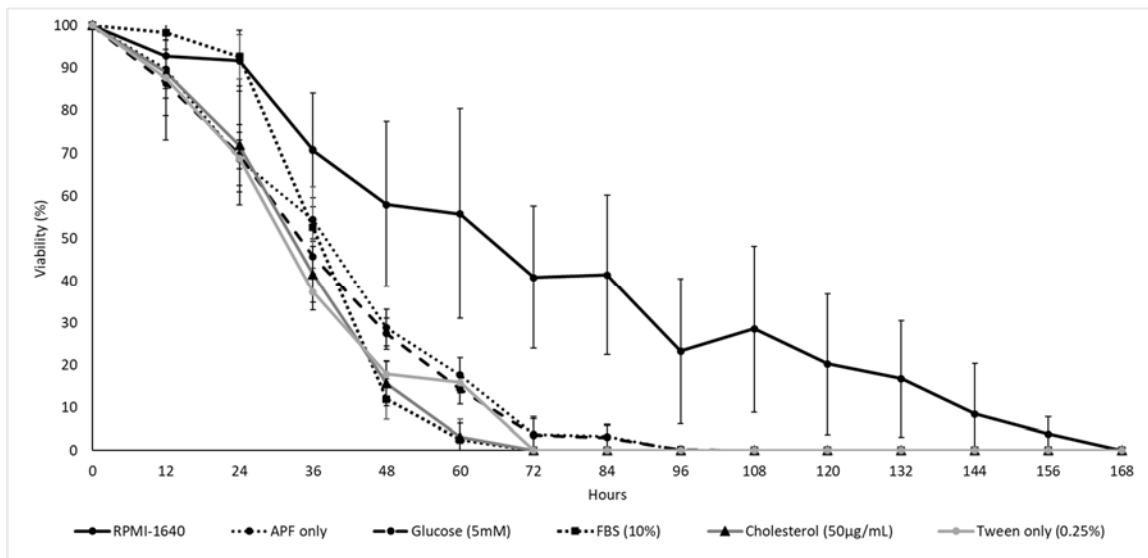
Figure 4.2A graphical representation of mean viability of *Parascaris* spp. intestinal
stages when maintained in either artificial perienteric fluid (APF) medium only,
APF medium supplemented nutrients (glucose, fetal bovine serum (FBS),
cholesterol, cholesterol and FBS, tween), or Roswell Park Memorial Institute-
1640 (RPMI-1640) medium only. Error bars represent 95% confidence intervals
($\alpha=0.05$).
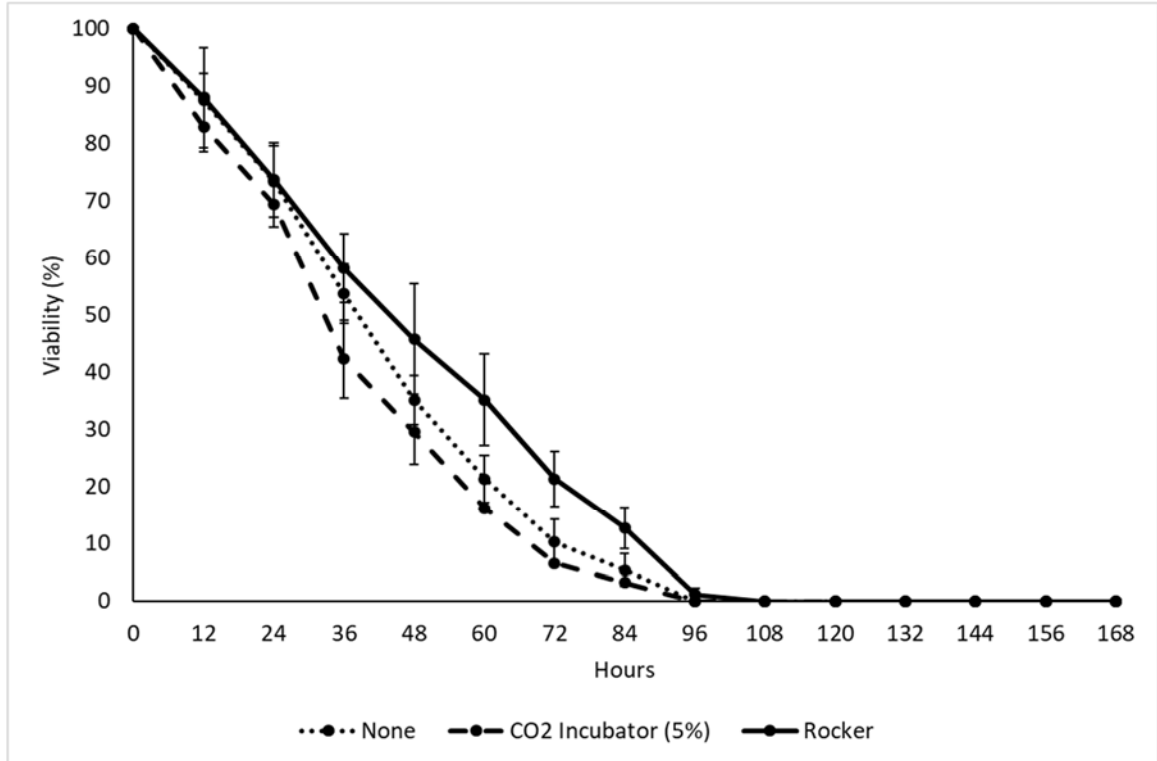
Figure 4.3A graphical representation of mean viability of *Parascaris* spp. intestinal stages maintained with environmental conditions of a platform rocker or a 5% CO_2 incubator across all media and nutrient types. 'None' implies stationary culture flasks in an air incubator. Error bars represent 95% confidence intervals ($\alpha=0.05$).

CHAPTER 5. ASCARIDS EXPOSED: GENE EXPRESSION ANALYSIS OF ANTHELMINTIC NAÏVE *PARASCARIS* SPP. FOLLOWING *IN VITRO* DRUG EXPOSURE

5.1   Introduction

Ascarid parasites infect a variety of hosts, including humans (*Ascaris lumbricoides* Jourdan et al., 2018), swine (*A. suum,* Thamsborg et al., 2013), companion animals (*Toxocara* spp., Overgaauw and Nederland, 1997), poultry (*Ascaridia galli*, Kilpinen et al., 2005), and horses (*Parascaris* spp., Nielsen, 2016), where *Toxocara* spp. and *A. suum* can result in zoonotic transmissions. Infections are commonly associated with wasting disease in children (Jourdan et al., 2018), decreased productivity in livestock (Kilpinen et al., 2005; Thamsborg et al., 2013), and stunted growth and intestinal obstruction in companion animals (Overgaauw and Nederland, 1997; Nielsen, 2016). This array of clinical diseases warrants the routine use of anthelmintics for therapeutic and preventative measures.

Anthelmintic resistance is prevalent and of grave concern among strongylid parasite species (Kaplan, 2004), but the Ascarididae family has received less attention on this issue. *Parascaris* spp. is the only example of an ascarid parasite with major issues of anthelmintic resistance with world-wide drug resistance documented to the macrocyclic lactone drug class (ML; Peregrine et al., 2014) and case reports of treatment failure exist for the pyrantel and fenbendazole anthelmintics (Lyons et al., 2008, 2011; Armstrong et al., 2014; Martin et al., 2018). Only single case reports of failed anthelmintic efficacy exist for other ascarid species (Yazwinski et al., 2013; Krücken et al., 2017). The unique but troubling status of *Parascaris* spp. provides opportunity for identifying possible resistance mechanisms and/or alternative drug targets that could be extrapolated to other ascarid parasites before resistance levels rise to become a major problem.

The mode of action of benzimidazole (BZ) drugs is to bind to parasite β-tubulin and interrupt microtubule formation, ultimately disrupting cell structure and energy metabolism processes (Lacey, 1988). It is widely accepted that BZ resistance is largely conferred through single nucleotide polymorphisms (SNP) leading to amino acid changes

within the β-tubulin gene (Beech et al., 2011), however the proportion at which these SNPs exist in resistant populations vary between ascarids and strongylids, between strongylid species, and between different isolates of the same species (Hodgkinson et al., 2008; von Samson-Himmelstjerna et al., 2009; Diawara et al., 2013; Kotze et al., 2014; Avramenko et al., 2019). The conflicting and lacking evidence of BZ resistance in ascarid species demand further investigations.

Presumably, ML drugs act as ligands for glutamate-gated chloride (GluCl) channels and gamma-aminobutyric acid (GABA) receptors (Martin, 1997). Binding causes an influx of chloride ions leading to hyperpolarization of the cellular membrane. This causes paralysis of the parasite, allowing expulsion from the host (Martin, 1997). Studies of ML resistance have focused on changes in receptor subunits (Blackhall et al., 1998; Dent et al., 2000; McCavera et al., 2009), drug metabolism (AlGusbi et al., 2014; Yilmaz et al., 2017) and drug efflux mechanisms (Xu et al., 1998; Janssen et al., 2013b, 2015; Chelladurai and Brewer, 2019). ATP-binding cassette transport proteins, such as P-glycoproteins (Pgps) have been widely studied, including in *Parascaris* spp. (Janssen et al., 2013b, 2015; Chelladurai and Brewer, 2019), but findings are inconsistent between parasite species and isolates (Xu et al., 1998; Molento and Prichard, 1999; Lloberas et al., 2013; AlGusbi et al., 2014; Maté et al., 2018). While it appears that Pgps may be associated with anthelmintic resistance, their exact role in resistance mechanisms has yet to be determined (Lespine et al., 2012).

The inter- and intra-species variation of mechanisms associated with BZ and ML resistance presents challenges for elucidating how parasites gain anthelmintic resistance, devising methods for preventing or slowing the development of resistance, identifying genetic markers for diagnosing resistance, and identifying future drug targets which may evade these mechanisms. Leading researchers have suggested that studying drug responses at the transcriptomic level in known susceptible isolates will enhance the understanding of anthelmintic resistance (Beech et al., 2011; Kotze et al., 2014). Utilizing susceptible isolates may reduce challenges due to genetic variation as resistant isolates have increased genetic diversity (Beech et al., 2011; Kotze et al., 2014). Xenobiotic defense mechanisms and/or drug responses of parasites are not widely known, and it is uncertain if these defense mechanisms are naturally occurring or developed as a result of

drug selection and anthelmintic resistance. To date, no studies have examined the transcriptomic response of parasites belonging to the order Ascarididae following *in vitro* drug exposure. The unique albeit wide-spread ML resistance status of *Parascaris* spp. urges the need for uncovering this parasite's response to xenobiotics, which may provide valuable insight to preserve anthelmintic efficacy for other ascarid species and beyond.

The purpose of this study was to examine the responses at the transcriptomic level of drug susceptible adult *Parascaris* spp. to ivermectin (IVM), a ML drug to which resistance is widely established on managed horse farms, and to oxibendazole (OBZ), a drug that remains effective.

5.2 Materials and methods

5.2.1 Study design

This study consisted of three parts. Part 1 was to perform *in vitro* drug exposures at varying concentrations and observe the worm responses. Part 1 ended with the determination of the sub-lethal concentration for each drug type and time point where worm viability decreased to approximately $\geq 25\%$. In Part 2, these pre-determined concentrations were used for *in vitro* drug exposure and worms were snap frozen at these designated time points. Subsequently, RNA sequencing and gene expression analysis was used to identify genes of interest in response to the drug exposures. In Part 3, the *in vitro* drug exposure was repeated with five worms per group. The previously identified genes of interest were further evaluated using qPCR.

5.2.2 Parasite sources

The study took place over the course of four foal necropsies from December 2017 to September 2018. The foals were born in a herd housed at the University of Kentucky that has not been treated with any anthelmintics since 1979 and have been documented harboring a variety of equine parasites (Lyons et al. 1990). Foals were humanely euthanized following the American Veterinary Medical Association guidelines for the euthanasia of animals when they reached 4.5-5 months old and subsequently necropsied.

The research was conducted under the approval from the University of Kentucky's Institutional Animal Care and Use Committee under protocol number 2012-1046.

5.2.2.1 Collection of *Parascaris* spp.

Collection of live worm specimens at necropsy occurred as previously described (Scare et al., 2018). Brief details are provided in Appendix 2. For Parts 2 and 3 of this study (see Supplementary Figure 5.1 in Appendix 2 and sections 2.6 and 2.7), additional worm specimens for *in situ* controls were obtained by leaving numerous worms within a 30 cm section of the jejunum. Intestinal content was allowed to remain in the segment and both ends were tied shut with string. The segment was placed in a closed container and then into the water bath to maintain its temperature at 37˚C. The purpose of the *in situ* controls was to mimic the natural environment of the worms while minimizing disturbances. Therefore, this *in situ* control was used as a comparison for the *in vitro* non-drug treated controls.

5.2.3 *In vitro* maintenance and viability assessment of *Parascaris* spp.

Worms for *in vitro* drug exposure were maintained in RPMI 1640 medium (R8758, Sigma-Aldrich, St. Louis, MO, US) within TPP tissue culture flasks (300 cm^2, MidSci, St. Louis, MO) at 37 ˚C as described by Scare et al. (2018). Media were changed every 12 hours (Scare et al., 2018). Worm viability was assessed at regular time intervals (see section 2.5) using a motility-based objective scoring system on a 0-6 scale as previously described (Scare *et al.,* 2018).

5.2.4 Anthelmintics

The anthelmintics employed in this study were powder formulations of ivermectin (IVM, 22,23-dihydroavermectin B1, Sigma-Aldrich, St. Louis, MO, USA) and oxibendazole (OBZ, methyl carbamate, Sigma-Aldrich, St. Louis, MO, USA). Stock solutions of both drugs were individually prepared as described by Hu et al. (2013), where a concentration of 100 µg/mL was dissolved in 100% dimethyl sulfoxide (DMSO). Ten-fold serial dilutions were carried out using 10% DMSO to achieve concentrations of

0.1, 1.0, 10.0 and 100.0 μg/mL. These concentrations were based on previous studies using similar concentrations (Hu et al., 2013; Janssen et al., 2013b).

Four mL of the prepared drug suspensions were added to the pre-assigned flasks containing 196 mL of RPMI-1640 media so that the final concentration of DMSO in the flask was 0.2% and the drug suspension was 1/50 of the final volume (Hu et al., 2013). Control flasks containing only 0.2% DMSO were also prepared. Worms were allowed a 24-hour acclimation period before the anthelmintics were added. Drug treatments were applied at every medium change thereafter.

5.2.5   Part 1: Initial assessment of parasite responses to *in vitro* drug exposure

Part 1 was dedicated to observing worm viability in response to *in vitro* drug exposure at varying concentrations of IVM and OBZ anthelmintics to determine optimal sub-lethal drug concentrations and length of exposure. Worms harvested from two necropsies on separate occasions were used to evaluate the effects of various anthelmintic concentrations over time. The number of worms evaluated for each drug concentration per necropsy can be found in Table 5.1. The time points that viability assessments occurred following each necropsy can be found in the supplementary files in Appendix 2.

The drug concentrations and time points for future snap freezing and gene expression analysis (Parts 2 and 3) were determined when mean worm viability decreased by approximately ≥25%, but remained sub-lethal. The final determined duration of exposure was extended by two hours for IVM and 12 hours for OBZ to ensure that the decrease in viability was stable.

5.2.6   Part 2: RNA-sequencing analysis

In Part 2, a third drug trial was performed using worms harvested from a third necropsy and subsequently were used for RNA-seq analysis. Only adult worms were used due to the lack of immature worms present. Worms were maintained *in vitro* in groups of four, consisting of two adult males and two adult females. The drug exposure parameters determined from Part 1 were applied. One male and one female for each drug

treated/control group were used for further analysis. At the predetermined time points, these worms were snap frozen live in liquid nitrogen and kept at -80°C until use.

5.2.6.1 RNA isolation, library preparation, and RNA sequencing

Frozen whole worms were ground into a fine powder using a mortar and pestle while continuously adding small amounts of liquid nitrogen. Approximately 100 mg of worm powder was used for RNA isolation which was carried out using TRIzol RNA isolation reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Next, DNase treatment was performed (DNA-free DNA removal kit, Thermo Fisher Scientific, Waltham, MA, USA). RNA quantity and quality was determined at the University of Kentucky Genomics Core Lab using the Agilent bioanalyzer (Agilent, Santa Clara, CA, USA).

RNA samples were sent to the University of Louisville CGeMM DNA Core Facility (http://louisville.edu/genetics/gemm-dna-facility-core) for library preparation and sequencing. Libraries were prepared with Illumina's TruSeq stranded total RNA library kit (Illumina, San Diego, CA, USA) with Ribo-Zero Gold depletion. Libraries were sequenced using the NextSeq 500 High Output v2 75 cycles kit. Samples were run in 1x75 base pair configuration, generating up to 400 million reads total, approximately 40 million reads per sample.

5.2.6.2 RNA-seq analysis and selection of genes of interest

Specific details pertaining to RNA-seq analysis can be found in the supplementary files in Appendix 2. Reads were aligned and annotated to the *Parascaris univalens* reference genome and transcriptome, respectively (Wang et al., 2017). Mapping statistics are shown in Appendix 2 in Supplementary Table 5.1. The RNA sequencing data from this study were deposited in the Gene Expression Omnibus (GEO, NCBI, NIH) database under study GSE129514.

Functional annotation of the differentially expressed genes based on gene ontology (biological process and molecular function) was performed using Pantherdb.org, a pathway analysis program (Mi et al., 2013; 2017).

Genes of interest were selected following initial RNA-seq analysis based on the comparison between all drug treated and all control worms. Selection criteria for candidate genes were as follows: significant differences ($\alpha=0.05$) between all drug treated worms and all control worms, the differences in expression must show an obvious pattern between groups, and the candidate genes must not be significantly different between the *in situ* and *in vitro* control groups nor between worm sexes (*i.e.,* differences did not occur due to *in vitro* maintenance nor worm sexes). Due to the higher number of candidate genes remaining, some selections were based on annotated gene function and those which may play a role in parasite drug metabolism/defense/drug efflux.

5.2.7   Part 3: Further investigation of genes of interest with qPCR

Part 3 of this study was used to further examine the expression patterns of the genes of interest on more worms harvested from a fourth necropsy and maintained *in vitro* under the same drug exposure conditions as in Part 2. A total of five males and five females were used for each drug treatment/control group.

5.2.7.1   RNA-isolation and cDNA preparation

RNA-isolation occurred as described in section 2.6.1. RNA concentrations were estimated using the Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA). Reverse transcription PCR was performed to obtain cDNA according to the manufacturer's instructions (SuperScript IV First-Strand cDNA Synthesis Reaction, Invitrogen, Carlsbad, CA, USA).

5.2.7.2   Primer design and validation

Presently, no validated endogenous controls for mRNA transcript levels exist for *Parascaris* spp. Housekeeping genes previously examined for stability in albendazole and ivermectin treated *H. contortus* (Lecová et al., 2015) were used, namely nuclear cap binding protein subunit two (*ncbp*) and RNA-polymerase RPABC1 large subunit (*ama*). The selection of these reference genes was based upon their stable expression (FPKM values) across all treated and control groups in Part 2 of this study. Actin was not used as a control because the mode of action of OBZ is to target tubulin, and it is possible that actin transcription would be affected by this treatment as well.

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Primers were designed using the online program NCBI Primer-BLAST, and manufactured by Invitrogen (Waltham, MA). Primer pairs were selected based on GC content and melting temperature. Primer sequences are listed in Supplementary Table 5.2 in Appendix 2.

Specificity of primers were tested by performing real-time qPCR (Agilent Mx3000P qPCR System, Santa Clara, CA) with PowerUP SYBR Green Master Mix (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's instructions. Cycle parameters can be found in Appendix 2 in the supplementary files under the material and methods section. A disassociation curve analysis was performed to ensure product specificity.

5.2.7.3   Quantitative real-time PCR

qPCR analyses were performed using the ViiA 7 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) with SYBR green detection using PowerUP SYBR Green Master Mix (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's instructions. Each sample/primer combination was performed in duplicate. Pooled samples were used as positive controls and no-template (negative) controls were performed for each primer pair. Disassociation curve analysis was used to check for non-specific amplification.

PCR products from all male drug treated and *in vitro* control worms were sequenced to confirm amplification specificity. Sanger sequencing was performed at the University of Kentucky Genomics Core Lab. Sequence alignments were performed using the NCBI nucleotide BLAST tool.

5.2.8   Statistical analyses

5.2.8.1   Part 1: Initial assessment of parasite responses to *in vitro* drug exposure

Mean viability per flask of worms at each timepoint was calculated using the following formula (Scare et al., 2018):

$$\% \, Viability = 100 - \left(\frac{(initial \; score - score \; at \; time \; 'X')}{initial \; score} \times 100\% \right)$$

Mean scores and 95% confidence intervals were determined using Microsoft Excel 2016 (Redmond, WA, USA). Further statistical analysis was carried out using SAS software (version 9.4, SAS Institute, Cary, North Carolina, USA). Statistical analysis could only be carried out on worms harvested from the first necropsy because worms from the second necropsy were snap frozen before they had died (details provided in Appendix 2 in the supplementary files). Two mixed model analyses with repeated measures over time were performed to examine the effects of worm stage (immature/adult) and sex (male/female), and the effects of each drug (IVM or OBZ) at different concentrations (0.1, 1, or 10 μg/mL) on worm viability. The details for the covariates examined and random effects can be found in Appendix 2 in the supplementary file materials and methods section.

A third mixed model analysis (without repeated measures) was performed to examine the effects of the different drug concentrations on the viability of each worm stage/sex, and to determine if changing the drug concentrations altered the viability of each stage/sex. The model details can be found in Appendix 2 in the supplementary file materials and methods section.

For all three analyses described above, covariates identified as significant (α=0.05) were further examined in a 'least squares means' analysis for a Tukey's pairwise comparison.

5.2.8.2 Part 2: RNA-sequencing analysis

Identification of differentially expressed genes was performed using a total of five one-way ANOVA analyses, using the Benjamini-Hochberg correction for false discovery rate (FDR P < 0.01). Analyses were performed using JMP software (JMP®, Version 13. SAS Institute Inc., Cary, NC, 1989-2019). The first four analyses ignored the influence of worm sexes. The first analysis considered all drug treated worms, regardless of drug used, versus all control worms, regardless of *in situ* or *in vitro.* The second analysis compared only the IVM-treated and IVM-control worms, and the third analysis compared only the OBZ-treated and OBZ-control worms. The fourth analysis compared all *in vitro*

controls to all *in situ* control worms. Finally, the fifth analysis ignored drug/control group and considered any differences between worm sexes.

5.2.8.3   Part 3: Further investigation of genes of interest with qPCR

Fluorescence readings for each cycle (ΔRn) of all genes of interest per sample were corrected for variation in PCR efficiency using LinReg PCR analysis software (Ramakers et al., 2003) and corresponding Cq values were obtained. Mean Cq values were calculated for duplicate samples using Microsoft Excel 2016 (Redmond, WA, USA). The geometric mean for Cqs of the three housekeeping genes was calculated for each sample, and subsequently used to calculate the negative ΔCq for each gene of interest per sample using the following formula:

$$-\Delta Cq = 0 - (mean\ of\ GOI\ Cq\ - geometric\ mean\ of\ HKG\ Cq)$$

Negative ΔCq values were checked for normal distribution and subsequently normalized using JMP 13 software (JMP®, Version 13. SAS Institute Inc., Cary, NC, 1989-2019). Further statistical analyses were performed in SAS software (version 9.4, SAS Institute, Cary, North Carolina, USA). Three mixed linear analyses were performed to analyze gene expression of each gene of interest in response to drug treatment, between worm sex, and between *in situ* and *in vitro* controls. Covariates identified as significant (α=0.05) were further examined in a 'least squares means' analysis for a Tukey's pairwise comparison. The first model was used to analyze the influence of worm sex and the *in situ* verses *in vitro* environment. The second model was for a broad comparison between all drug treated and all control worms. The third model was more specific and examined direct comparisons between IVM treated and IVM controls, OBZ treated and OBZ controls, and IVM treated and OBZ treated.

5.3   Results

5.3.1   Part 1: Initial assessment of parasite responses to *in vitro* drug exposure

A graphical representation of worm viability following *in vitro* drug exposure can be found in Figure 5.1 A and B. Overall, IVM had a more immediate effect on worm

viability than OBZ, however there was some variability between worms harvested from the two necropsies.

Neither worm stage nor sex had a significant influence on viability (p=0.1868). Between the two drug treatments, IVM (0.1 µg/mL) had significantly lower viability than the RPMI-1640 control worms at hours three and four. No other significant differences were found between groups for the other time points.

When examining the influence of drug concentrations on the viability of each worm stage/sex, there was a significant decrease (p<0.0001) in viability of immature worms compared to female worms in OBZ (1 µg/mL). For all other drug concentrations, there were no significant differences in viability between immature and adult worms nor between male and female adult worms. For immatures (p=1.000), males (p=1.000), and females (p=0.9979), there was no significant difference in viability between the RPMI-1640 (no drug control) and the DMSO 10% control, which indicates that the DMSO did not affect worm viability and any observed changes were due to the drug. This analysis was also used to determine if there were significant differences in viability between the different drug concentrations employed for each stage/sex group. These results and the corresponding p-values are outlined in Table 5.2. For immatures, males, and females, all IVM concentrations resulted in significantly less viability than the control worms, except for females exposed to IVM (1 µg/mL) which was not significantly different from the control worms. There were no significant differences between the three IVM concentrations examined for each stage/sex of worms. However, the effects of the three different OBZ concentrations on each stage/sex of worms varied, but in general the immature worms were more susceptible to increasing OBZ concentrations, while the male and female adult worms showed a lesser change in viability in response to the changing concentrations. For immatures, males, and females, none of the three OBZ concentrations differed significantly from the control group.

Table 5.3 shows the time points at which worm viability decreased to ≥25% (sublethal) for each drug concentration which was used to determine the timepoints/concentrations used for RNA-seq analysis in Part 2. The optimal concentration of 10 µg/mL was determined for OBZ because the decrease in viability was consistent between the worms harvested from the two necropsies. The optimal

concentration of 1 µg/mL was determined for IVM because it appeared to have a more consistent decrease in worm viability after one hour than the other two concentrations (Figure 5.1 A and B). Viability had decreased ≥25% after 12 hours for OBZ (10 µg/mL) and after 1 hours for IVM (1 µg/mL). These time points were extended by two hours for IVM and 12 hours for OBZ to ensure a representative decrease in viability prior to snap freezing of worms for RNA-seq analysis (Part 2), and the final determined time points were three and 24 hours, respectively.

5.3.2   Part 2: RNA-sequencing analysis

Figure 5.2 illustrates the viability of the worms collected from the third necropsy and subsequently used for RNA sequencing. The total number of significant genes (α=0.01) for each of the group comparisons can be found in Table 5.4. Because of the sample size (1 male and 1 female worm per group), the comparison of all drug treated worms (n=4) versus all control worms (n=6) was used to select the genes of interest. The selected genes and associated p-values between all drug treated and control worms are displayed in Table 5.4. The genes of interest and stable expression of the house keeping genes between the treated and control groups are illustrated in Figure 5.3. There were no significant differences for any of the genes between the *in situ* and *in vitro* controls (p=0.9999; Fig. 5.3). Although the genes of interest were significantly different between all drug treated and control worms, they were not identified as significantly different when the groups were further divided between drug class (*i.e.* no significant differences between the specific OBZ and OBZ-control worms nor between the IVM and IVM-control worms; Fig. 5.3).

For the gene ontology (GO) analysis, the entire list of genes from the annotated genome (Wang et al., 2017) was initially used. Of these, 6% did not have a listed gene product and 24% were considered annotated as a 'hypothetical protein,' and were not included in the GO analysis. The following comparisons were made: all drug treated versus all controls, IVM 1 µg/mL versus IVM controls, and OBZ 10 µg/mL versus OBZ controls. The significantly different genes corresponding to these comparisons are listed as an additional file (Supplementary Data). Predicted biological processes and molecular function pathways are presented in Figure 5.4. Overall, it appears that treatment with

IVM reduced the number of categories for genes involved with molecular functions and biological processes. However, the categories of genes following OBZ treatment were similar to the gene categories representing all of the genes of the control worms.

5.3.3 Part 3: Further investigation of genes of interest with qPCR

Although the RNA-Seq data suggested differences in transcription of the genes of interest, qPCR did not reveal any significant differences for these genes between the treatment and control worms (0.7329; Fig. 5.5), between worm sex (p=0.0600; Fig. 5.5), nor between the *in situ* and *in vitro* controls (p=0.3265; Fig. 5.5). No differences were observed between each drug treated and the corresponding control group (Fig. 5.5; IVM/IVM control, p=1.0000; OBZ/OBZ control, p=0.8950).

Sequence information for the PCR products can be found in Appendix 2 in Supplementary Table 5.1.

5.4 Discussion

This is the first study to examine the phenotypic and transcriptomic responses in anthelmintic-naïve *Parascaris* spp. following *in vitro* drug exposure. The RNA-sequencing analysis revealed a number of gene transcripts that were significantly different between the treated and control groups (Table 5.4). The selected genes exhibit functions in other organisms related to drug detoxification, coordinating muscle contraction, regulation of membrane potential, and microtubule polymerization. The lack of significant differences in expression levels of the genes of interest between the *in situ* and *in vitro* controls indicate that these differences were not due to the worms being under *in vitro* conditions, but represent changes due to drug exposure (Figure 5.3A).

The predicted biological functions and metabolic pathways illustrate the variety of processes (Fig. 5.4) that are presumably affected by anthelmintic exposure. At this time, it is unknown how the differentially expressed genes are influenced by *in vitro* drug exposure. We also cannot conclude if they would elicit the same response *in vivo,* or if they have a role in anthelmintic resistance mechanisms. Therefore, these genes should be considered in future investigations. The proportion of significant genes related to various

biological process and molecular functions differ between the IVM treated/control and OBZ treated/control comparisons. For the IVM comparison, the genes related to biological processes are only related to metabolic and cellular processes while those related to molecular function are defined as catalytic activity, binding, and molecular function regulation (Fig. 5.4). This is interesting given IVMs paralytic mode of action. The target receptors of IVM (GluCl and GABA channels) are highly expressed on motor and sensory neurons, which regulate nematode locomotion, feeding behavior, and mediate sensory inputs (Wolstenholme, 2012). The predicted functions appear to be in agreement with the IVM mode of action, such as the disruption of feeding behavior may be related to changes in metabolic processes, and mediation of sensory inputs likely has some effect on cellular processes, catalytic activity, and regulation of molecular function. The BZ mode of action is primarily to disrupt energy metabolism and cell structure (Lacey, 1988). The pathway analysis resulted in a variety of genes, where those related to biological processes were primarily involved with localization, biological regulation, metabolic and developmental processes. The molecular function was dominated by genes related to binding and catalytic activity. The disruption of cellular structure is likely involved in localization, biological regulation, binding, and development processes. Likewise, it is not illogical to equate the disruption of energy metabolism with metabolic processes and catalytic activity. Overall, it can be speculated that the predicted gene ontology pathways have some relevance to the drug mode of action, and there are potential pathways involved with drug responses to be explored.

The selected genes of interest described herein have not been previously examined in any ascarid parasite following *in vitro* drug exposure. Interestingly, traditionally researched genes, such as p-glycoproteins (Janssen et al., 2013b, 2015; Chelladurai and Brewer, 2019) and the multi-drug resistant protein (Kotze et al., 2014), were not observed to be differentially expressed between drug exposed and control worms.

We identified a significant increase in *cyp4504c1* in drug treated worms over control worms. Xenobiotic metabolism occurs via three phases. The first being oxidation, hydrolysis, or reduction of the drug, the second is a conjugation reaction of the substrate, and finally active transport of the conjugate through the membranes (Cvilink et al., 2009).

The cytochrome P450 (CYP450) enzyme family is a major contributor for phase one and often catalyzes monooxygenation of the drug substrate (Cvilink et al., 2009). Drug metabolism is necessary for bioactivation of the drug within the target organism, but it can also result in undesirable effects, such as detoxification (Feyereisen, 1999; Cvilink et al., 2009). The CYP450s are a highly complex albeit evolutionarily conserved gene family. While they are organized into families and subfamilies based on sequence similarities, the diversity of chemical reactions possible by a single enzyme prevent the exact function of a specific enzyme from being derived from the sequence (Feyereisen, 1999). Elucidation of function is commonly theorized by the use of CYP450 inducers, such as phenobarbital, CYP450 inhibitors, such as carbon monoxide or piperonyl butoxide, or by the presence of possible metabolites (Feyereisen, 1999, Cvilink et al., 2009). CYP450s are a well-known driver for insecticide resistance (Feyereisen, 1999; ffrench-Constant et al., 2004; Li et al., 2007, Shi et al., 2015, 2016). They have also been shown to be up-regulated during starvation or stress and hypothesized to promote survival in *Caenorhabditis elegans* (McElwee et al., 2004), *Drosophila* variants (Dorszuk et al., 2012), and the cockroach, *Blaberus discoidalis* (Bradfield et al., 1991). Initially, CYP450s were presumably absent from nematodes (Barrett, 1998), however this is proven otherwise as 23 CYP subfamilies (Menzel et al., 2001) with over 80 genes have been identified in *C. elegans* (Menzel et al., 2001; Lindblom and Dodd, 2006), and 68 have been confirmed in *H. contortus* (Laing et al., 2015), some of which have been associated with multi-drug resistance (Yilmaz et al., 2017). Several studies have reported the presumed activity of CYP450s in *Haemonchus contortus* following xenobiotic exposure *in vitro* (Kotze, 1997, 1999, 2000; Kotze et al., 2006, Alvinerie et al., 2001), and larval stages consistently exhibited increased activity over adults (Kotze, 1997, 1999, 2000). It is suggested that the difference between larval and adult stages may be due to the lack of molecular oxygen available in the host's intestine where the adult stages reside. Only one study has compared the enzyme activity between macrocyclic lactone susceptible and resistant strains, but there was no evidence for elevated CYP450 activity associated with resistance status (Kotze, 2000). The role of CYPs in drug metabolism and the potential involvement in AR has not yet been studied in *Ascaris* or *Parascaris* species. The RNA-sequencing analysis performed in this study revealed a significant

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increase in expression of *cyp4504c1*. Given the evidence for drug metabolism by CYP450s in other organisms, the we hypothesize that this elevation occurred as a direct detoxification defense mechanism. This particular *cyp450* gene (*4c1*) has only been described once in the literature, and it was elevated in *B. discoidalis* following starvation and suggested to be elevated as a stress response (Bradfield et al., 1991). Therefore, it is possible that the increased *cyp450* expression may also be induced by a stress response elicited by the toxic anthelmintics. Finally, one must consider that the monooxygenase activity of CYP450 requires molecular oxygen to be present, and adult *Parascaris* spp. reside in the horse's intestine where oxygen availability must be limited, and extrapolations for the CYP450 activity *in vivo* cannot be made at this time.

We identified a significant increase in gene expression of *frmd4a* for drug-treated worms over control worms. Ikenouchi and Umeda (2009) reported the FRMD4A protein to regulate epithelial cell polarity. In this context, polarity refers to the apical-basal orientation of epithelial cells which is mediated by cell-to-cell interactions. FRMD4A facilitates the interaction of two complexes (Arf-6 and PAR-complex) at the primordial adherens junctions which subsequently facilitates the formation of the fused junctions forming 'belt-like' adherens structures. FRMD4A was also found to be a binding-partner for cytohesin-1 at the primordial adherens junctions (Ikenouchi and Umeda, 2009). The primary function of adherens junctions is to mediate cell-to-cell contact which is necessary for many processes including cellular organization, locomotion, and communication. Disruption of these junctions interrupts these processes (Meng and Takeichi, 2009). Adheren junction formation is dependent upon the presence of actin molecules, although less is known about the association of microtubules with adherens junctions. However, microtubules have been observed near them (Meng and Takeichi, 2009). Interestingly, Stehbens et al. (2006) noted that blocking microtubule polymerization toward adherens junctions will decrease the amount of E-cadherin, a necessary component of cellular junctions and proponent of organized cell-to-cell adhesion. Waterman-Storer et al. (2000) found that the assembly of filamentous actin (F-actin) is both directly and indirectly dependent upon the polymerization and organization of microtubles. Therefore, disruption of the polymerization process will negatively affect the formation of these junctions (Waterman-Storer et al., 2000; Meng and Takelichi,

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2009). The current study identified a significant increase in gene expression of *frmd4a* for drug-treated worms over control worms using RNA-seq analysis. Given the BZ mode of action is to disrupt microtubule formation (Lacey, 1988), it is possible that the increase in *frmd4a* was a direct result of microtubule depolymerization. This may cause the degradation of cell-to-cell contact by preventing the assembly of actin filaments and subsequently adherens junctions in turn disrupting epithelial cell polarity. FRMD4A is a direct regulator of polarity and may have been responding to this disturbance. Further studies are needed to confirm if there is a direct association between the BZ mode of action and *Parascaris* spp. epithelial cell polarity.

The gene *sup-9* was significantly increased among drug treated worms compared to control worms. In *C. elegans,* SUP-9 is known to encode a two-pore domain potassium channel (K2P) expressed in body-wall muscle, vulva, and intestinal cells (Perez de la Cruz et al., 2003). It also has sequence similarity to the mammalian TASK-1 and TASK-3 K2Ps (Perez de la Cruz et al., 2003). The TASK K2Ps are voltage and time dependent acid-sensitive channels. Although *sup-9* is somewhat orthologous to the TASK K2Ps, it is known that sequence similarity does not always predict functionality (Lesage and Lazdunski, 2000). Nevertheless, the TASK K2Ps primarily function to regulate cellular resting membrane potential through the passive transport of potassium cations, and in general K2Ps are physiologically associated with neurotransmitters and neuronal-muscular excitability (Lesage and Lazdunski, 2000). In C. elegans, SUP-9 is a proposed contributor of muscle contraction coordination, and forms a complex with SUP-10 and UNC-93 (Greenwald and Horvitz, 1980; Levin and Horvitz, 1993 Perez de la Cruz et al., 2003). Gain of function mutants were observed having uncoordinated, sluggish movements and decreased egg shedding. (Greenwald and Horvitz, 1980; Levin and Horvitz, 1993). Perez de la Cruz (2003) propose that these effects are due to hyperpolarization of the cellular membrane caused by an efflux of potassium ions. Hyperpolarization is also caused by an influx of chloride ions and is the primary mode of action of ML drugs, which act on GluCl and GABA receptors (Martin, 1997). Perez de la Cruz (2003) demonstrated that inducing chloride ion influx via GABA receptors enhances the phenotypic effects of the K2P gain of function mutants, but that the mechanisms act independently of each other. According to the RNA-seq analysis carried

out in the present study, the gene *sup-9* was significantly increased among drug treated worms compared to control worms. The relationship between GluCl receptors, the primary target of MLs, and SUP-9 in *Parascaris* spp. is unknown. However, given they have similar physiological effects due to hyperpolarization and SUP-9 functions to restore resting membrane potential, it is possible that ML treatment may affect the expression of *sup*-9, but no direct conclusions can be made at this time.

It is presumed that anthelmintic treatment induces the presence of free-radicals and oxidative stress in the parasite (James et al., 2009). This is evidenced by several reports of increased antioxidant defense mechanisms in anthelmintic resistant parasites, which has been summarized by James et al. (2009) and includes cambendazole resistant (Kawalek et al., 1984) and IVM resistant (Sotirchos et al., 2008) *H. contortus*. In the current study, RNA-seq analysis identified a significant increase in kelch-domain containing protein 10 (*klhdc-10*) in drug treated worms over control worms. In *Drosophila melanogaster,* the protein *slim* is orthologous to the mammalian *klhdc-10* (Sekine et al., 2012) and has been identified as an inducer of oxidative stress-induced cell death. An obvious hypothesis is that *Parascaris* spp. experienced oxidative stress in response to drug exposure, which caused increased expression of *klhdc-10*.

As described above, the differences in gene expression for the genes of interest identified by RNA-seq analysis were small (<2 fold), but significant. However, the qPCR analysis did not identify significant differences in gene expression. There are several possible reasons for the discrepancy between the RNA-seq and qPCR results. First, it is possible that the qPCR method employed was unable to detect such minute changes in gene expression. Second, the effect of variations in viability on transcriptomic expression is unknown. Next, the worms used were harvested from two different foals and the immune responses exhibited by the foals could initiate unknown changes at the transcriptomic level. While qPCR provides a relatively quick comparison of gene expression, there are several sources of variability that should be considered, as described by Bustin and Nolan (2004). Finally, it should be noted that even minute changes in RNA expression have been associated with important biological functions (Laurent et al., 2013). Therefore, while there was disagreement between the RNA-seq analysis and

qPCR results, the differences identified via RNA-seq and the gene of interest biological roles warrant future investigation.

In summary, this study was the first to perform *in vitro* drug exposure on anthelmintic-naïve *Parascaris* spp. and observe drug effects both phenotypically and transcriptionally. The *in vitro* drug exposure system described herein provides a reliable reference for future analyses directed at elucidating anthelmintic resistance mechanisms and identifying future drug targets. The wide-spread resistance status of *Parascaris* spp. warrants further investigation of these topics, and continuation of this work may provide information regarding parasite drug metabolism, identifying new drug targets, and/or preserving the efficacy of current anthelmintics against ascarids parasites and beyond.

Table 5.1 Number of *Parascaris* spp. worms used to observe responses to drug exposure *in vitro* for Part 1 of this study. Drugs were prepared in 10% DMSO. Worms were maintained in 200 mL RPMI-1640 media at 37˚C.

Drugs (µg/mL)	Necropsy 1			Necropsy 2			Total		
	Males	Females	Imm.	Males	Females	Imm.	Males	Females	Imm.
IVM (0.1)	2	1	2	2	2	0	4	3	2
IVM (1.0)	2	1	2	2	2	0	4	3	2
IVM (10.0)	2	1	2	2	2	0	4	3	2
OBZ (0.1)	2	1	2	0	0	0	2	1	2
OBZ (1.0)	2	1	2	0	0	0	2	1	2
OBZ (10)	2	1	2	2	2	0	4	3	2
OBZ (100)	0	0	0	2	2	0	2	2	0
RPMI-1640 & DMSO (10%)	4	2	4	2	2	0	6	4	4
RPMI-1640	6	3	6	4	4	0	10	7	6

Abbreviations: IVM, ivermectin; OBZ, oxibendazole; Imm, immature worms; RPMI-1640, Roswell Park Memorial Institute-1640 medium; DMSO, dimethyl sulfoxide

Table 5.2 Results of the statistical analysis evaluating the mean percent worm viability between drug treated and control worms, and between different concentrations of the same drug. Results are shown for each worm stage/sex.

Immature worms

OBZ Comparison 1			OBZ Comparison 2			IVM Comparison 1			IVM Comparison 2		
Control	Drug	Result	Drug 1	Drug 2	Result	Control	Drug	Result	Drug 1	Drug 2	Result
RPMI-1640	OBZ (0.1 µg/mL)	NSD	OBZ (0.1 µg/mL)	OBZ (1 µg/mL)	0.1 > 1 (p=0.0049)	RPMI-1640	IVM (0.1 µg/mL)	IVM < RPMI (p=0.0126)	IVM (0.1 µg/mL)	IVM (1 µg/mL)	NSD
RPMI-1640	OBZ (1 µg/mL)	NSD	OBZ (0.1 µg/mL)	OBZ (10 µg/mL)	0.1 > 10 (p=0.0493)	RPMI-1640	IVM (1 µg/mL)	IVM < RPMI (p=0.0258)	IVM (0.1 µg/mL)	IVM (10 µg/mL)	NSD
RPMI-1640	OBZ (10 µg/mL)	NSD	OBZ (1 µg/mL)	OBZ (10 µg/mL)	NSD	RPMI-1640	IVM (10 µg/mL)	IVM < RPMI (p=0.0501)	IVM (1 µg/mL)	IVM (10 µg/mL)	NSD

Male Worms

OBZ Comparison 1			OBZ Comparison 2			IVM Comparison 1			IVM Comparison 2		
Control	Drug 1	Result	Drug 1	Drug 2	Result	Control	Drug 1	Result	Drug 1	Drug 2	Result
RPMI-1640	OBZ (0.1 µg/mL)	NSD	OBZ (0.1 µg/mL)	OBZ (1 µg/mL)	NSD	RPMI-1640	IVM (0.1 µg/mL)	IVM < RPMI (p<0.0001)	IVM (0.1 µg/mL)	IVM (1 µg/mL)	NSD
RPMI-1640	OBZ (1 µg/mL)	NSD	OBZ (0.1 µg/mL)	OBZ (10 µg/mL)	NSD	RPMI-1640	IVM (1 µg/mL)	IVM < RPMI (p<0.0001)	IVM (0.1 µg/mL)	IVM (10 µg/mL)	NSD
RPMI-1640	OBZ (10 µg/mL)	NSD	OBZ (1 µg/mL)	OBZ (10 µg/mL)	NSD	RPMI-1640	IVM (10 µg/mL)	IVM < RPMI (p<0.0001)	IVM (1 µg/mL)	IVM (10 µg/mL)	NSD

Female Worms

OBZ Comparison 1			OBZ Comparison 2			IVM Comparison 1			IVM Comparison 2		
Control	Drug 1	Result	Drug 1	Drug 2	Result	Control	Drug 1	Result	Drug 1	Drug 2	Result
RPMI-1640	OBZ (0.1 µg/mL)	NSD	OBZ (0.1 µg/mL)	OBZ (1 µg/mL)	1 > 0.1 (p=0.0008)	RPMI-1640	IVM (0.1 µg/mL)	IVM < RPMI (p=0.0224)	IVM (0.1 µg/mL)	IVM (1 µg/mL)	NSD
RPMI-1640	OBZ (1 µg/mL)	NSD	OBZ (0.1 µg/mL)	OBZ (10 µg/mL)	NSD	RPMI-1640	IVM (1 µg/mL)	NSD	IVM (0.1 µg/mL)	IVM (10 µg/mL)	NSD
RPMI-1640	OBZ (10 µg/mL)	NSD	OBZ (1 µg/mL)	OBZ (10 µg/mL)	NSD	RPMI-1640	IVM (10 µg/mL)	IVM < RPMI (p=0.0082).	IVM (1 µg/mL)	IVM (10 µg/mL)	NSD

Abbreviations: NSD, no significant difference; OBZ, oxibendazole, IVM, ivermectin, RPMI-1640, Roswell Park Memorial Institute- 1640 medium

Table 5.3 Timepoints and drug concentrations when the mean viability *Parascaris* spp. adult worms decreased by ≥25%, but remained sub-lethal.

Drug (µg/mL)	Necropsy 1 Viability	Time (hours)	Necropsy 2 Viability (%)	Time (hours)	Hours of exposure used	Necropsy 3 Viability at time of freezing
OBZ (0.1)	36%	18	NA	NA	-	-
OBZ (1.0)	48%	6	NA	NA	-	-
OBZ (10.0)	50%	6	45%	12	24	22.22%
OBZ (100.0)	NA	NA	41%	24	-	-
IVM (0.1)	42.3%	1	100%	1	-	-
IVM (1.0)	40%	1	75%	1	3	75%
IVM (10.0)	30.4	1	40%	1	-	-

Abbreviations: OBZ, oxibendazole; IVM, ivermectin; NA, not applicable because worms were not maintained at this drug concentration for the corresponding necropsy.

Dashes indicate that the drug concentration was not used for molecular analysis.

Table 5.4 The top part of the table shows the number of significant transcripts (α=0.01) based on FPKM values obtained from part two of this study. The bottom part of the table shows the genes of interest selected for further investigation in part three of this study. The target genes were selected based on the comparison between all treated (n=4) and all control worms (n=6). Drug treated worms had significantly higher expression than control worms (α=0.01).

Comparison	Number of significant transcripts
In situ controls vs *in vitro* controls	115
All treated vs. all controls	88
All males vs. All females	5756
OBZ treated (10 µg/mL) vs. OBZ control	153
IVM treated (1 µg/mL) vs. IVM control	57
Selected target genes	**p-value***
Cytochrome P450 4C1 (*cyp4504c1*)	0.0008
Ferm domain containing protein 4a (*frmd4a*)	0.0006
Two-pore potassium channel protein (*sup-9*)	0.0004
Kelch domain containing protein 10 (*klhdc10*)	0.0008

Abbreviations: OBZ, oxibendazole; IVM, ivermectin

*All target genes had significantly higher expression in drug treated worms than control worms.
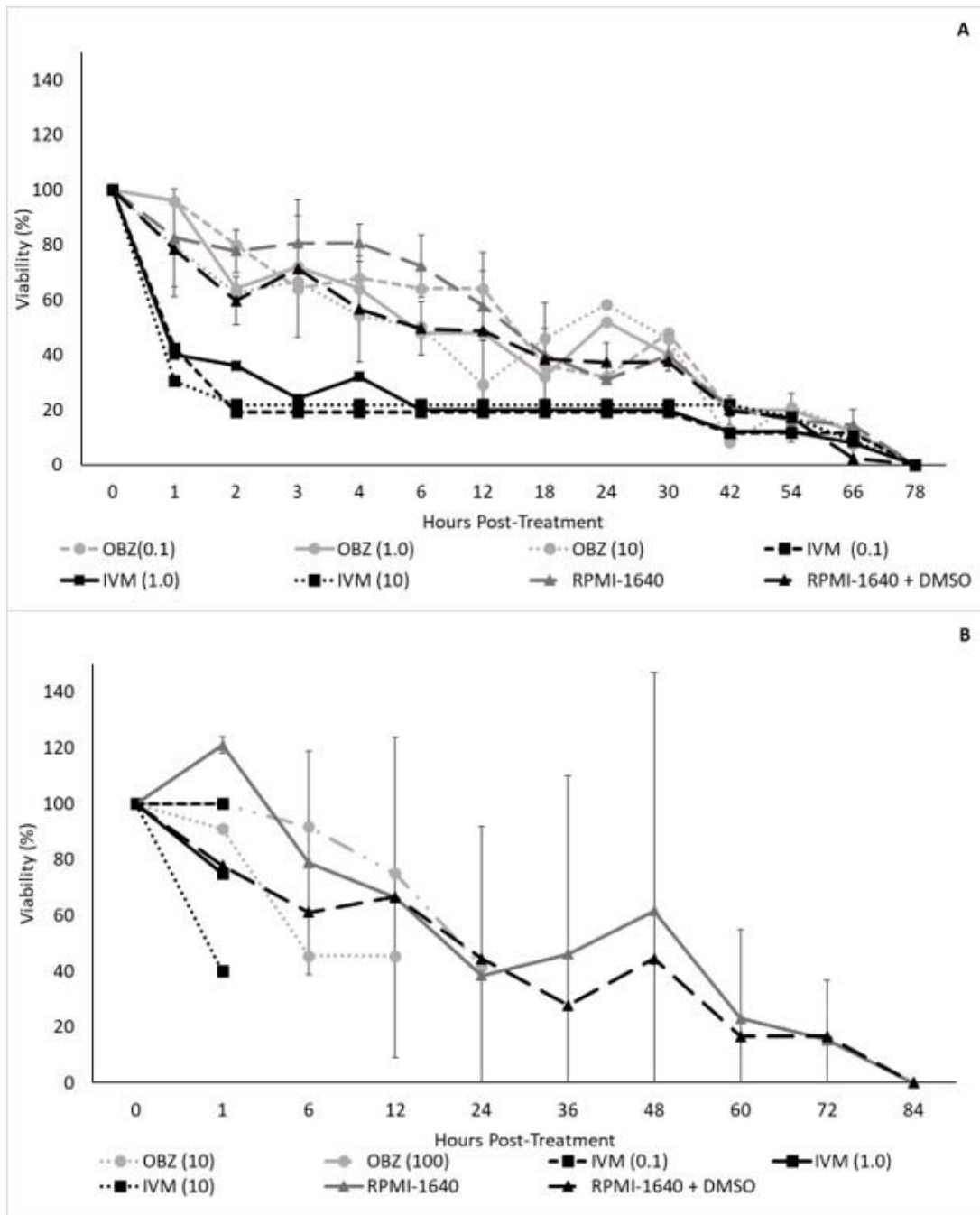
Figure 5.1 A graphical representation of mean worm viability following *in vitro* anthelmintic exposure. Both A and B are from Part 1 of this study in which initial observations about response to drug exposure were made. 'A' reflects worms obtained from the first necropsy and 'B' reflects worms obtained from the second necropsy. Control worms were maintained in RPMI 1640 medium only or with dimethyl sulfoxide (DMSO) which was used to prepare the anthelmintics. Error bars represent 95% confidence intervals ($\alpha=0.05$).
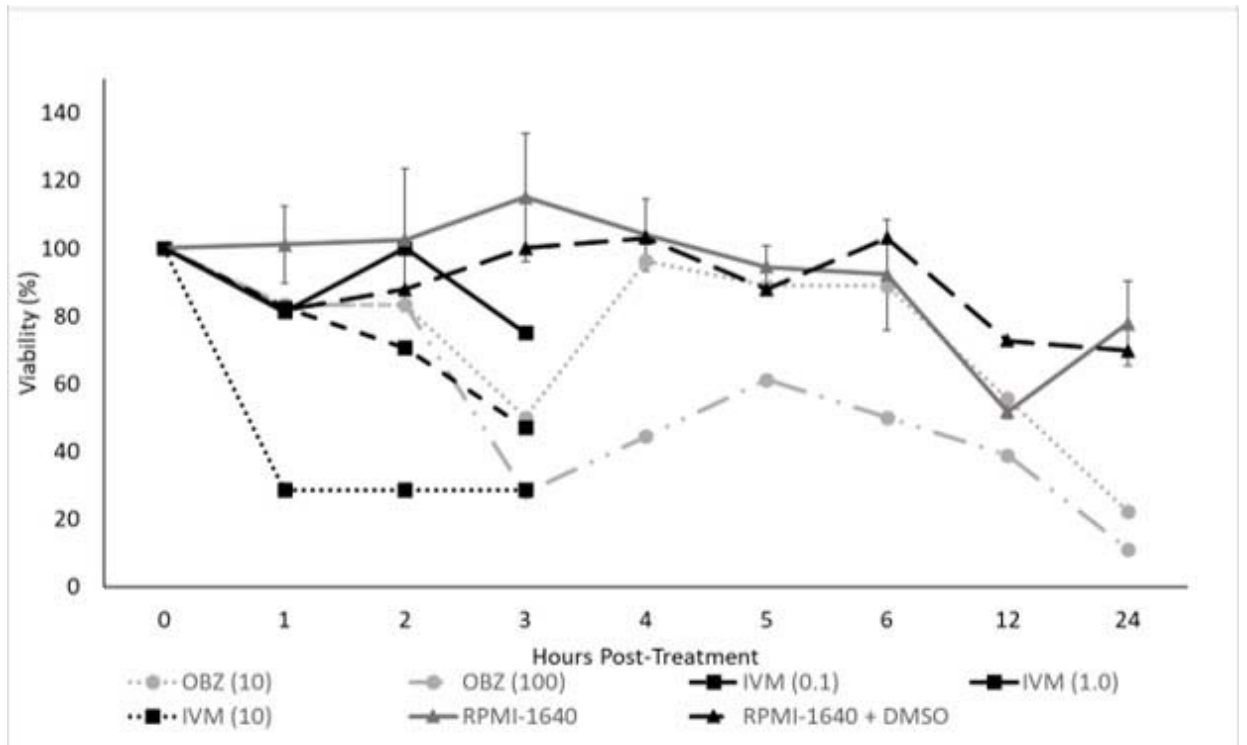
Figure 5.2 A graphical representation of mean worm viability of worms used in Part 2 of this study, where those exposed to oxibendazole (OBZ) at 10 µg/mL for 24 hours and ivermectin (IVM) at 1 µg/mL for three hours were used for RNA-sequencing analysis. Control worms were maintained in RPMI 1640 medium only or with dimethyl sulfoxide (DMSO) which was used to prepare the anthelmintics. Error bars represent 95% confidence intervals (α=0.05).

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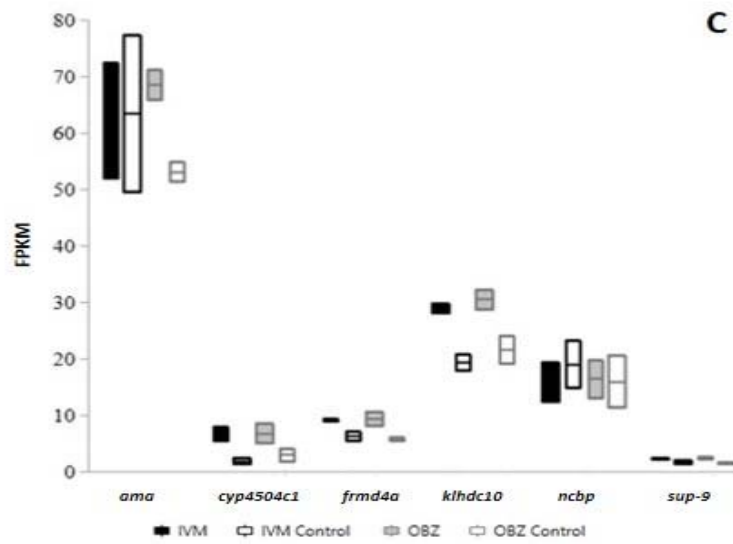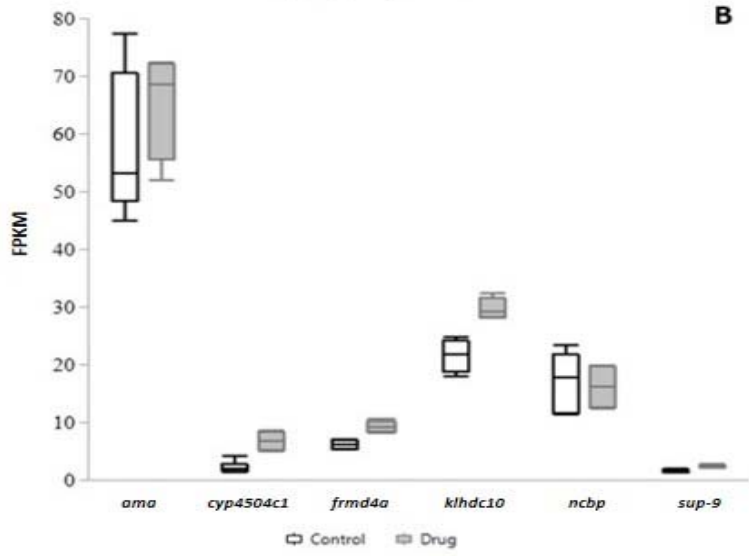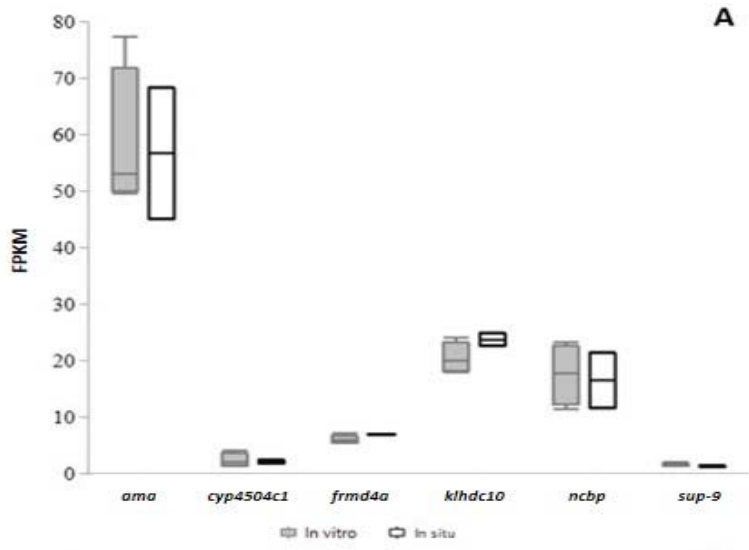
Figure 5.3 A graphical representation of select genes from the RNA-seq analysis performed in Part 2 of this study. The housekeeping genes are *ama* and *ncbp*. A) *in vitro* versus *in situ* controls, B) all control worms versus all drug treated worms, where all genes of interest had significantly higher expression in the drug treated worms ($\alpha=0.01$), C) ivermectin (IVM) treated (1 µg/mL), IVM control, oxibendazole (OBZ) treated (10 µg/mL), and OBZ control.

Figure 5.4 A graphical representation of the gene ontology pathway analysis for significantly different genes (SDGs) between groups. From left to right: all genes, all drug treated worms versus all control worms, ivermectin (IVM) treated (1 µg/mL) versus IVM control, oxibendazole (OBZ) treated (10 µg/mL) versus OBZ control. The top row reflects biological processes (BP) and the bottom row reflects molecular functions (MF).

A

B

C
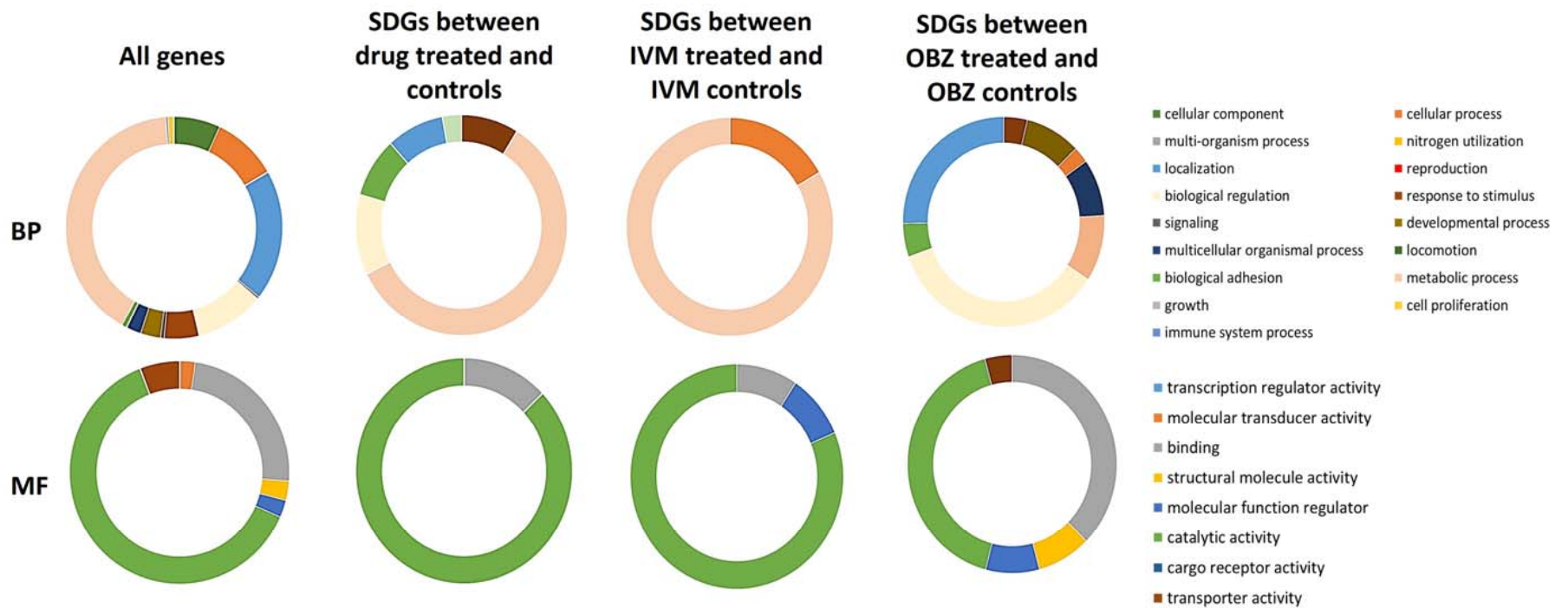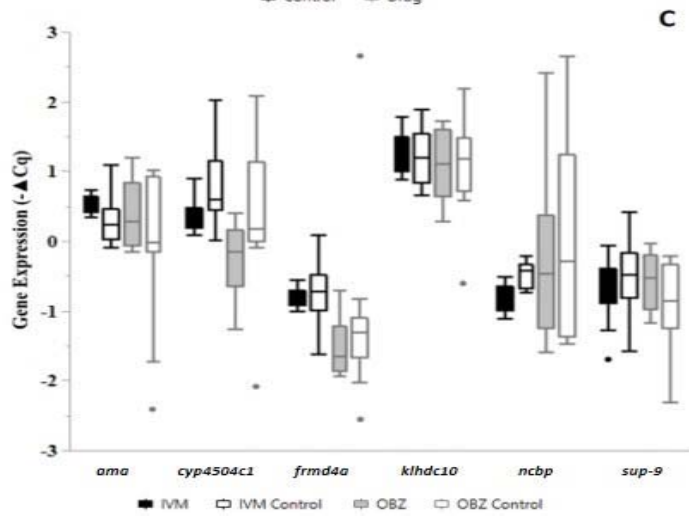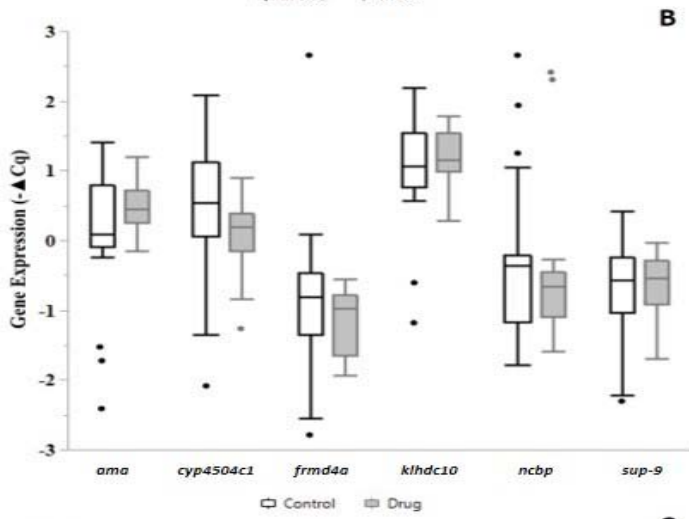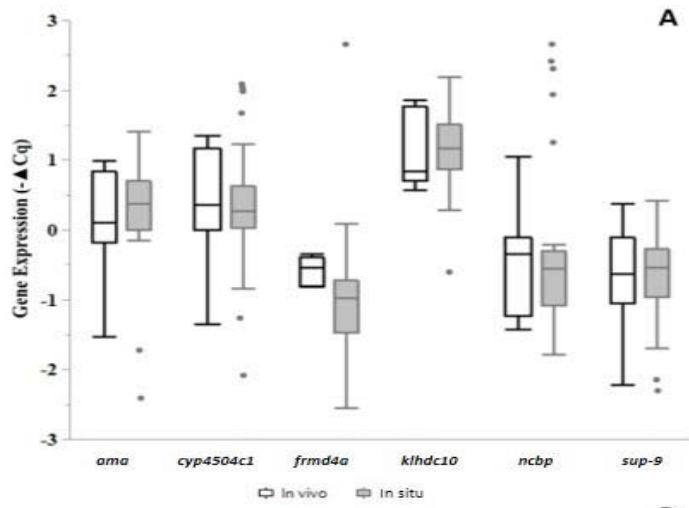
Figure 5.5 A graphical representation for gene expression analysis following quantitative real-time PCR (qPCR) from Part 3 of this study. A) *in vitro* versus *in situ* controls, B) all control worms versus all drug treated worms, C) ivermectin (IVM) treated (1 µg/mL), IVM control, oxibendazole (OBZ) treated (10 µg/mL), and OBZ control. No significant differences were identified for any qPCR analysis.

CHAPTER 6. DISCUSSION

6.1   Discussion introduction

Overall, the knowledge for combination treatment regimens and anthelmintic resistance mechanisms is lacking, particularly in equine parasites, and therefore these topics have been the focus of the current research. The discussion chapter is organized to first give a brief overview of the major findings described by the research presented herein. The subsequent sections offer detailed discussion of each chapter, including the major findings, explanations for the findings, future directions, and a brief conclusion. Finally, a generalized discussion regarding some of the significant questions in equine parasitology research is included.

6.2   Overview of the major findings

The first combination deworming study employing OBZ and PYR did not provide an additive effect beyond the first treatment. This is in contrast to findings and recommendations of ruminant parasites for reasons which will be discussed below. Regarding the second combination deworming study using OBZ and MOX, the results were somewhat expected given the full efficacy of MOX, and a longer-term study is needed to evaluate the impact on OBZ efficacy. The two studies provide a ground work for future field research and computer simulation studies. The evolving challenges from these studies speak to the complicated nature of cyathostomin biology and the sustainability of anthelmintic resistance. These topics are discussed further in the sections below.

Regarding the *in vitro* maintenance of adult *Parascaris* spp., it seems that this ascarid species has different energy/nutritional demands compared to other ascarid species, and as a result was not possible to maintain *in vitro* for more than 5-7 days. There are several possibilities for optimizing the *in vitro* maintenance protocol, and these methods will be discussed below. The gene expression analysis following *in vitro* drug exposure revealed a number of significantly differentially expressed genes, however, none of the commonly studied drug efflux genes (section 1.8.4) had increased expression

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in response to drug exposure. The selected genes of interest had functional applications that have yet to be explored in any ascarid parasite. The discussion will offer explanations for these differences, and how these findings will be useful in future studies.

6.3 Combination deworming studies

6.3.1 Major findings

The lack of sustainable control was the primary finding of the OBZ-PYR combination deworming study. Several of the combination deworming studies involving ruminant parasites (section 1.9.4) found it to be a beneficial alternative treatment regimen, when employed under appropriate conditions. However, modelling studies revealed lowered efficacies of combination products in populations where resistance was highly prevalent (section 1.9.4), and this is in agreement with the current findings. Nevertheless, some countries market combination dewormers for small ruminant and ruminant parasites, and they are used off label in other areas of the world. Likewise, combination products for horses are also marketed, but with a significant lack of evidence. The results described herein (sections 2.3 and 3.3) do not support the use of combination treatment against double-drug resistant cyathostomins, when drug resistance is well established and both drugs have low efficacies.

6.3.2 Explanations for findings

There are several variables which may contribute to the current findings. First is the suspected sustainability of anthelmintic resistance in cyathostomin parasites. Two long-term studies have described the sustainability of BZ resistance in two historic cyathostomin populations, known as *Population B* (Lyons et al., 2007) and *Population* S (Lyons et al., 2001). *Population* B cyathostomins originated from a farm in central Kentucky with known resistance to phenothiazine, thiabendazole, piperazine, and pyrantel pamoate (Drudge and Elam, 1961; Drudge and Lyons, 1965; Drudge et al., 1988, 1990). In 1966, *Population B* cyathostomins were established on a pasture at the University of Kentucky maintained in a herd of mixed light horses. This cyathostomin population was the subject of numerous efficacy studies, were resistance to variety of BZ

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compounds, including thiabendazole, was reported, but albendazole and OBZ remained efficacious (Drudge et al., 1977, 1979, 1984, 1991; Tolliver et al., 1993). A subset of this population (maintained in a selection of horses from this herd, but moved to a new pasture) was left unexposed to anthelmintics for 22 years (1970-2001) before a final critical test of various anthelmintic compounds. Despite the lack of selection pressure for over two decades, the resistance status of thiabendazole and FBZ was unchanged (Lyons et al., 2007). *Population S* cyathostomins were established at the University of Kentucky in 1974 and maintained in a herd of Shetland ponies. Over an 18 year period, resistance to cambendazole (Drudge et al., 1983) and OBZ (Drudge et al., 1985b) developed as a result of continuous anthelmintic treatments. Subsequently, administration of BZ type drugs ceased, and only PYR drugs were used for the next seven years (1992-1999) to induce PYR resistance. Despite the removal of selection pressure with BZ drugs for seven years, resistance to OBZ was maintained (Lyons et al., 2001). The *Population* S cyathostomins are still maintained at the University of Kentucky Main Chance farm in a herd of ponies, and exhibit substantial resistance to OBZ and PYR. This population was used in both combination deworming studies described herein (Chapters 2 and 3).

The two studies described above support the sustainability of BZ resistance in cyathostomins, even when the selection-pressure of drug treatment is removed. The sustainability factor also brings into question the 'fitness loss' theory proposed by Prichard (1990), as described in section 1.6. This theory is heavily relied upon in many combination deworming studies (section 1.9.4), but due to the lack of concrete and consistent knowledge of 'resistance genes,' the actual association of these with a fitness loss is unknown. Given the sustainability of resistance in cyathostomin parasites, it seems that this theory may not apply as they appear to pass on the genetics of resistance in a sustainable manner evidenced by the two long term studies described above (Lyons et al., 2001, 2007). Secondly, the initial efficacy levels of OBZ and pyrantel were 66.7% and 63.3%, respectively. As proposed by Learmount et al. (2012) and Leathwick (2012), the benefits of combination deworming are the greatest, when the resistance frequency to both drugs are low. Given the low starting efficacies of both actives used herein, one can presume the resistance alleles for both drugs are prevalent throughout *Population S* which may have negated the benefits of combination therapy. The portion of the population in

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refugia is also attributed as a major factor of combination therapy success, as modeled by Leathwick et al. (2012) and Leathwick (2013). However, the benefits of refugia to increase drug efficacy or slow the rate of resistance have not been evaluated for cyathostomin parasites, and their complicated biology affects the impact of refugia. All ponies in this study were treated, leaving the environmental stages and encysted larval stages as the only source of refugia. It is unlikely that the impact of the environmental stages as a source of refugia was observed within the timeframe of this study (one year). Because the adult cyathostomin lifespan is presumed to be one year (Ogbourne, 1975; Reinemeyer et al., 1986; Leathwick et al., 2019) and newly ingested stages undergo a period of arrestment, it is unlikely that the newly ingested stages would be in the lumen and have contributed to egg shedding. However, because neither OBZ nor pyrantel exhibit larvicidal efficacy, the encysted stages may have served as a source of refugia upon being recruited to the lumen. Removal of luminal stages by anthelmintic treatment is known to trigger recruitment of the arrested stages. Given treatment occurred every 8 weeks, it is likely that some of the arrested stages were recruited after each treatment. We can assume that the recruited population consisted of some fully susceptible individuals, single drug resistant worms, and double drug resistant worms. This is evidenced by the initial additive effect (76%) followed by the consistent albeit lower efficacies (40-43%) of the second, third, and fourth combination treatments. The first combination treatment was more efficacious because it removed the susceptible and single-drug resistant worms, leaving only the worms resistant to both OBZ and PYR. However, because treatment triggered recruitment of arrested stages bring a mixture of susceptible/resistant worms, this explains why there was low, but continued efficacy for the subsequent treatments. This multi-drug resistant population was continually targeted, but failed to be totally removed, preventing the efficacy from increasing. It is possible that if this study was allowed to continue for another year, we may have seen another additive effect in efficacy as new adult worms would have repopulated the lumen, and the susceptible and single-drug resistant worms would have again been removed. However, a break in treatments would likely be necessary otherwise the multidrug resistant worms would remain and again dominate the luminal burden, which prevent an additive effect. Next, it is also possible that interactions occurred between the two drug classes used. Currently,

there are no studies examining potential PK and PD interactions of anthelmintics occurring within the horse or in equine parasites. Little is known about the specific drug targets in equine parasites, but they are presumed to be the same as those modeled in *C. elegans* and other organisms. It is possible that the one drug may inhibit the receptor or effector effects of the other drug, or perhaps their interaction may trigger or enhance non-specific resistance mechanisms. These speculations of drug interactions show the limited knowledge available and support the need for future research in this area. The fact that these multi-drug resistant worms were already present in the population prior to this study makes one doubt that their resistance genetics are associated with a fitness loss, and it appears that they are capable of surviving and passing on the multi-drug resistant alleles to future generations. At this time, we conclude that combination deworming using OBZ and pyrantel is not a sustainable approach against OBZ-PYR resistant cyathostomins.

These results led us to our next study surrounding the question of combining an efficacious drug, or a drug naïve to the parasite population (MOX), with another anthelmintic (OBZ). Again, the rules of a refugia population and limited resistance present within the population still apply for achieving the maximum benefits of combination therapy. However, Leathwick (2012) did conclude that even when one of the anthelmintics offer only 50% efficacy, it can still offer some benefit to slowing the development of resistance to the new drug. This is because there is still a 50% chance that the older drug will be able to remove worms which become resistant to the new drug. Unfortunately, no new drug classes have been developed for horses in the last 40 years, and all of the current drugs have confirmed or emerging resistance among cyathostomin populations worldwide. Therefore, the ability to combine a new drug into a combination product is problematic. However, the *Population S* cyathostomins were naïve to ML drugs, providing a unique opportunity to use MOX as a novel anthelmintic. Therefore, the second combination deworming study targeted the *Population S* cyathostomins with MOX and OBZ, where the starting efficacy of OBZ was again low, at 46.7%. Moxidectin achieves some larvicidal efficacy against the arrested (EL_3) stage that is most recently reported as 73.8% (Bellaw et al., 2018), however the efficacy in a naïve population is unknown. In this study, anytime MOX was used, alone or in combination, it awarded

100% reduction in FECs, which was not surprising given the naïve ML status of the population. Although this study was carried out for two years, it only provided enough time to administer two combination treatments by the time FECs were high enough for an efficacy evaluation (10 horses with an EPG >100). In both cases, this took at least 40 weeks. This extended period between treatments was likely due to the perfect efficacy (100%) for removing the adult worms and the larvicidal efficacy of MOX, albeit incomplete. The total removal of adult worms removes the opportunity for any immediate egg shedding, and the ERPs were near or within historic reports of 16-22 weeks (DiPietro et al., 1997). Despite the high efficacy of MOX, the OBZ efficacies before and after combination treatment were not significantly different from each other. This finding may be due to the sustainability of BZ resistance, as evidenced by *Population B* (Lyons et al., 2007) and *Population S* (Lyons et al., 2001) cyathostomin populations. Therefore, it would likely be decades before MOX would completely eliminate the BZ resistance alleles from the population. The current timeframe of the study did not allow us to determine if OBZ could be beneficial to delaying the resistance development to MOX. Emerging resistance to the MLs is reported (section 1.7.1) and it is not unlikely that with routine ML exposure, *Population S* would also develop resistance, but ML resistance has yet to be reported on any managed horse farm. Given the observed sustainability of BZ resistance, it can be presumed that the last observed BZ efficacy within this population would continue to decrease as BZ exposure continued.

6.3.3 Comparison to other studies

As mentioned in section 1.9.4, only four studies have observed the effects of combination deworming in horses, all of which utilized only a combination drug treatment. Two of these studies will not be discussed here because either it did not report treatment efficacies (Lyons et al., 2016) or was focused on *Parascaris* spp. rather than strongyles (Wilkes et al., 2017). Of the remaining studies, one used a triple combination that achieved 99% efficacy where two of the three actives were <85% efficacious (Rolfe and Dawson, 1994), and the other used a combination of OBZ and pyrantel, and achieved 90-99% efficacy where one of the single actives used was not efficacious on a given farm (Kaplan et al., 2014). Both of these studies only observed one combination treatment, and

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the results are in agreement with efficacy achieved by the first OBZ-pyrantel combination treatment of the current study presented in Chapter 2. This is not surprising given that a resistant cyathostomin population would consist of fully susceptible individuals, some harboring resistance to each single active, and possibly some individuals with multi-drug resistance. The use of the combination would remove all of the susceptible and single active resistant worms providing an increase in efficacy compared to when each drug was used alone. However, it is unknown how sustainable this treatment would have been on a given farm. This depends on the initial resistance allele frequencies (*i.e.* initial single-active efficacies), farm management practices, refugia, modes of action, and potential drug interactions. Kaplan et al. (2014) used the same drug combination as described herein. Five of the eleven farms observed by Kaplan et al. (2014) had initial efficacies ≥90% for both single actives, and subsequently all of these farms achieved ≥99.3% efficacy for the combination treatment. Even the farms with the lowest single active efficacies had a combination efficacy ≥93.7%. The mean initial starting efficacies (OBZ, 89.9%; pyrantel, 87.1%) in Kaplan et al. (2014) were much higher than those in the long-term OBZ-pyrantel combination study (Chapter 2; OBZ, 66.7; pyrantel, 63.3%). Despite the differences in starting efficacies, both studies achieved an additive effect, as evidenced by the implementation of the additive effect formula, where Kaplan et al. (2014) achieved 97.2%, and the current study achieved 76.6%. The starting efficacies directly reflect the high and low combination efficacies observed, respectively. Because of the higher single-active efficacies in the former, it is possible that, in this scenario, subsequent combination treatments would have continued to achieve a high efficacy. However, given the likely presence of multi-drug resistant parasites, it is also possible that efficacy would have decreased and selected for multi-drug resistant worms. Given what we know about the sustainability of BZ resistance in cyathostomin populations, the latter outcome seems more likely. Finally, it must be considered that the Kaplan et al. (2014) study was actually conducted in 2004, and efficacy levels may have been higher than present day. If this was the case, then the combination therapy proposed may no longer be useful. Overall, more long-term studies are needed to evaluate the effects of combination deworming against cyathostomins where single-active efficacies are ≥85%.

Regarding the MOX-OBZ combination deworming study, this is the only study of equine cyathostomins using a 'new' anthelmintic (MOX), and therefore there are no other equine studies to which these results can be compared. There are some studies in ruminants evaluating combination therapies when one of the drugs is 100% efficacious and the other is considered ineffective (section 1.9.4). Overall, these studies found combination treatment to be beneficial. However, the majority of these studies employed drugs with mixed routes of administration and slow-release drugs. Currently, MOX is the only drug in horses considered to have extended persistence due to its water-soluble and lipophilic nature allowing it to quickly absorb into the horse's fat and retain its maximum concentration (Pérez et al., 1999; Gokbulut et al., 2010a). This characteristic is responsible for the extended ERP of MOX compared to other equine anthelmintics. This, coupled with the larvicidal efficacy, explains the prolonged efficacy observed in the current study and why it took 40 weeks for egg counts to rise to an acceptable level for reevaluation. There are a variety of administration routes for anthelmintics in cattle (intravenously, intramuscular, *per os*, intraruminal, subcutaneous). In horses, however, anthelmintics are only available for oral use. The PKs of pour-on anthelmintics (subcutaneous route) have been investigated in horses, and resulted in lower maximum plasma concentrations and shorter persistence when compared to oral IVM (Gokbulut et al., 2010b; 2016). Interestingly, the intramuscular administration of IVM resulted in prolonged retention of the maximum plasma concentration and prolonged availability than when IVM was given orally (Perez de la Cruz et al., 2003). Unfortunately, IVM is not labeled for horses via intramuscular administration due to a history of infections and reactions at the injection site (McKellar and Benchaouli, 1996). Therefore, it is obvious that the route of administration affects drug bioavailability and efficacy. The multiple routes of administration available in cattle provide more opportunities for combination deworming. A combination of IVM and albendazole when given intravenously resulted in an efficacy of 91.9%, but when IVM was given subcutaneously and albendazole intraruminally, the combined efficacy decreased to 70.8%. Additionally, the extended release capabilities should also be considered. The combination of doramectin (28-day) with albendazole resulted in significantly higher efficacy for up to a month after treatment than the 100-day slow release eprinomectin given alone. As illustrated herein,

there are many opportunities for combining anthelmintics in cattle and results are largely affected by the route of administration. Currently, no studies have examined combination deworming combined with alternative administration routes in horses. Given the extended release properties of MOX, and the longer persistence of IVM when given intravenously, it would be interesting to examine their effects when provided in combination with other drugs. However, the risks associated with injections may dampen the market and successes that have been observed in cattle.

Despite the success in the small ruminant industry, recommendation of combination deworming practices for equine parasites should not be extrapolated from the small ruminant GIN studies for several reasons. First, this is complicated by the known variation in drug receptor composition and receptor sensitivity between parasite species (as described in sections 1.5.2 and 1.5.3). The other equine combination deworming studies described herein have examined only a single combination treatment. While these initially have provided an additive effect, the OBZ-pyrantel combination study presented here (Chapter 2) where multiple treatments were given proved it not to be sustainable under the described conditions. Secondly, we must consider the differences in biology of the parasites. The clinically important GINs of small ruminants are able to undergo hypobiosis, where the larvae arrest development within the host, usually at the L_4 stage (Zajac, 2006). Generally, the period of hypobiosis is observed when environmental conditions are unfavorable for larval development and it would be unproductive for luminal stages to proliferate. Development of the hypobiotic stages resumes upon arrival of the next grazing season (Zajac, 2006). For cyathostomins, the hypobiosis phenomenon is different in that the larval stage undergoing hypobiosis is the EL_3, and they can remain arrested for at least two years (Smith 1976a,b). Although the combination deworming of cyathostomins is largely to target the luminal stages and not the hypobiotic stages, the lengthy arrested development presumably plays a role in the development and sustainability of resistance. A third reason is the lack of implementation and evidence for refugia benefits. As mentioned several times previously, refugia are important components for GIN management of small ruminants, and have been supported by several modelling and field studies. This management practice may aid in delaying the development of resistance in small ruminant GIN, even when resistance to one of the

actives is high. Refugia are easier to implement on small ruminant farms than on equine farms as all of the animals are generally owned and/or managed by a single individual, and selected treatments based on FEC and/or clinical symptoms is a common practice. However, refugia maintenance is not a common practice on most managed horse farms. Horse owners tend to either synchronize deworming treatments in an 'all or none' approach, or tend to manage their personal horses on their own terms, leaving little to no benefit of refugia (Robert et al., 2015; Nielsen et al., 2018a; Scare et al., 2018a). Therefore, implementation of combination deworming under the appropriate management conditions to maximize the combination deworming benefits will be a challenge for the equine industry. Overall, given the presence of resistance to the BZ and PYR drug class on managed horse farms world-wide, combination therapy using these two compounds is not recommended. While the MOX and OBZ combination treatment provided perfect FEC reduction and suppressed egg counts for several months, currently it is unknown if the same effects would be observed on managed horse farms where the ERP following ML treatment is shortened. Therefore, this should be evaluated before recommendations are made.

6.3.4 Future directions

Because of the limited timeframe allotted for the MOX/OBZ combination deworming study, it would be of primary interest to extend this study. It is difficult to know exactly how long of a study would be needed to determine the benefits and consequences of this combination. A five year study would allow for five generations of cyathostomins to be observed, given the proposed one year adult lifespan and the observed 40 week period necessary for egg counts to reach appropriate levels for evaluation. However, the known two year (or longer) arrestment period would complicate this as these worms would harbor BZ resistant alleles. This would drastically reduce the implications for observing the effects of MOX treatment on BZ resistance, and one could argue that an even longer study would be necessary. However, given the expense for maintaining a herd of horses (even miniature ones) it is challenging to secure funding for long term studies. Nevertheless, it would also be interesting to carry out a long-term study to observe the rate of resistance development to MOX, but again this would be

plagued by financial maintenance. Therefore, computer modelling studies implementing different variables and projecting over several decades would be of use. Currently, a model for cyathostomins has been developed employing the dynamics of the free-living/environmental stages (Leathwick et al., 2015b) and parasitic stages (Leathwick et al., 2019). Because the timeframes implemented in both of these studies were too short to observe long term effects on the population genetics, the details (horse age, seasonal/weather data, management practices, initial egg shedding levels, initial drug efficacies, anthelmintics used, treatment frequencies, etc) can be combined with the infection dynamics of the model. These parameters can be employed to predict the benefits and risks of combination therapy, and investigate the importance of refugia over several decades. These results would be useful to guide combination deworming recommendations to equine producers, and used as a baseline for future field studies. Another area of interest would be to observe genomic and transcriptomic differences between this double-drug resistant isolate and an anthelmintic naïve or susceptible isolate. However, such a study would be plagued by several challenges, such as the 50+ species of cyathostomins, the lack of pure isolates, and the genetic variation between isolates.

6.3.5 Conclusions

Overall, these studies emphasize the importance of not making equine parasite control regimens based off of findings in other species. The cyathostomin biology, particularly the prolonged period of larval arrestment, and the known sustainability of anthelmintic resistance introduce challenges that must be considered. The full effect of these factors, the influence of refugia, and the fitness loss theory remain largely unknown for cyathostomin parasites. It is clear that more long-term studies are needed to elucidate the influences on anthelmintic resistance and regimens for overcoming resistance. Based on these results, we conclude that combination deworming should not be implemented for double-drug resistant cyathostomins where initial efficacies are low, and combination therapy involving a new anthelmintic must be further evaluated on the longer-term under the conditions of managed horse farms.

6.4   *In vitro* maintenance of *Parascaris* spp. intestinal stages

6.4.1   Major findings

The primary conclusions obtained from the evaluation of the *in vitro* maintenance requirements of *Parascaris* spp. was that this ascarid species appears to have different nutritional demands and energy requirements than *Ascaris suum*. It was also interesting that implementation of a CO_2 incubator did not improve longevity or viability. This study also established an objective motility based viability assessment, and determined that worm body muscle tone was a better indicator of worm death rather than a lack of observed movement. It also appears that assessing worm viability over time may be a better observatory measure as opposed to worms being dead/alive.

6.4.2   Explanations for findings

In the current study, the addition of glucose as a nutrient did not improve worm viability. The concentration employed was 5mM. It is possible that this concentration was too low and thus no benefit was observed. Still, the constant replenishment of fresh medium and glucose nutrient should have offered some initial increase in viability, but it is possible that the 12 hour assessment interval was too infrequent to observe a short-lived improvement. Another possibility is that the worms are unable to metabolize glucose in its current form and perhaps an alternative, such as pyruvate, would be more suitable. However, the benefits of glucose as a nutrient cannot be ruled out, but rather it is plausible that glucose alone was not enough considering that RPMI-1640 medium, which contains glucose, offered significantly better longevity and viability than any other media/nutrient combination. RPMI-1640 contains glucose (11 mM) as a nutrient along with an array of inorganic salts, vitamins, glutathione (an antioxidant), and sodium bicarbonate (a pH regulator). While the current study did not explore the impact of these additional components on *Parascaris* spp. longevity and viability, it is evident that some or all of them play an important role in maintaining these parasites *in vitro*. Next, the implementation of a CO_2 incubator also did not affect *Parascaris* spp. viability. Given that only the saline-based media were tested in the CO_2 incubator it is possible that the influence of the gas was not evident due to the lack of sodium-bicarbonate in the media.

The use of a CO_2 incubator is common practice in cell culture, because the media employed contain sodium bicarbonate as a pH regulator, which requires CO_2. The pH levels of the saline-based media employed herein were adjusted using hydrochloric acid rather than continually maintained with sodium bicarbonate. Therefore, the full effects of a CO_2 incubator were not evaluated, and this was a limitation of the study. This study also identified significant differences in longevity and viability between the immature and adult intestinal stages. We observed the immature worms to frequently have more rapid and constant movements than the adult worms (unpublished observations), but this was short lived as the immatures appeared to decline in viability more quickly than the adults. Therefore, it is plausible to speculate that the immature stages and their growing status have higher metabolism and increased energy/nutritional requirements than the adults. Finally, this study identified the degree of motility to be a useful indicator of worm viability. We often observed that even when worms were not moving that they were still alive, which was based on movement stimulated by gentle prodding or by the presence of muscle tone. Therefore, worms exhibiting a lack of movement at the time of observation should not simply be assumed dead, as was done in several other studies (section 4.1). Given the constant peristalsis of the small intestine (the infection site of adult *Parascaris* spp.), we presume that worm motility is very important to prevent worm expulsion by the host. Therefore, we can speculate that observed worm motility may reflect viability both *in vitro* and *in vivo*.

6.4.3   Comparison to other studies

As described in section 4.1, several studies have maintained adult stages of *Ascaris suum in vitro*. Most of these studies used a relatively simple saline-based medium, where glucose was intermittently provided as a nutrient. McCoy et al. (2015) kept adult *A. suum* alive in *Ascaris* Ringer's Solution for 8 days without nutrient. However, when this medium was employed in the current study, *Parascaris* spp. lived a maximum of 60 hours (2.5 days) and an average of only 42 hours. A similar saline-based solution, artificial perienteric fluid, was capable of supporting adult *A. suum* for five days (Brownlee et al., 1997), whereas *Parascaris* spp. declined to <10% viability by 72 hours, and lived a maximum of 96 hours. Using RPMI-1640 medium, Islam et al. (2004) kept *A.*

suum alive for 2 weeks. However, *Parascaris* spp. was only capable of surviving for a maximum 168 hours (7 days). These stark differences in *in vitro* survivability between ascarid species may be due to several factors. First, we must acknowledge that the aforementioned studies did not evaluate worm viability in an objective manner, and most considered them only as dead or alive. From the current study, it is clear that the worm 'quality of life' must be considered during evaluations because worms with decreased motility are likely decreasing in viability and research quality. Therefore, while these studies report extended longevity, the results should be interpreted with caution as worms that lived the longest may have been barely viable. Secondly, it seems obvious that *Parascaris* spp. has more nutrient demands than *A. suum*. Because RPMI-1640 was the most successful medium, it appears that *Parascaris* spp. may benefit from the additional components included in this medium, however, direct conclusions cannot be made at this time. It is also possible that *Parascaris* spp. has a higher metabolism than *A. suum* and perhaps the nutrient concentrations employed were not enough or were not provided in the appropriate metabolite form for absorption and utilization. Another point for consideration is the effect of the immune system. As described in section 1.2.2, horses generally gain full immunity to *Parascaris* spp. infections by one year of age, and this is dependent on age rather than exposure. Pigs also gain substantial immunity to *A. suum* (Taffs, 1964), but this is driven primarily by exposure (Urban and Tromba, 1982, 1984; Urban et al., 1988; Eriksen et al., 1992), and it is not uncommon to find *A. suum* in mature pigs (Eriksen et al., 1992; Mejer and Roepstorff, 2006; Katakam et al., 2016). The intense immune response exhibited by foals may compromise the ability for *Parascaris* spp. to survive *in vitro* before they are even removed from the foal. Foals passively receive protective antibodies from their mares (Burk et al., 2016), and we can speculate that worms are immediately under constant attack by the immune system. It is possible that parasites surviving to adulthood may already be damaged by the immune system, or perhaps exhibiting mechanisms to evade the host immune response. Overall, it is likely that the immune response may hinder the survivability of adult worms *in vitro.* We attempted to control for this by harvesting worms from younger foals (4.5-5 months of age), but currently the extent of the immune system effects are unknown.

6.4.4 Future directions

The *in vitro* maintenance of *Parascaris* spp. intestinal stages provides vast opportunities to continue studying drug response mechanisms, comparisons in phenotypic/genotypic responses between resistant and susceptible isolates, immunological assays, collection and analysis of excretory/secretory products, and implementation of RNA*i* techniques. The parameters described herein may also prove beneficial in maintaining other ascarid species *in vitro*, such as *Toxocara canis*, *T. cati*, *Ascaridia galli*, and *Ascaris lumbricoides.* However, continued optimization of *Parascaris* spp. *in vitro* maintenance is warranted. There are several possible ways to explore the metabolic requirements of *Parascaris* spp. *in vitro*. One suggestion has been to decrease the maintenance temperature (normally at 37˚C) in order to slow worm metabolism (Dr. Richard Martin, *personal communication*). However, it is unknown what degree in temperature change would be optimal, or if a decrease would harm other physiological process, such as gene regulation and expression.

Several studies also employed the use of glucose as a nutrient supplement, but none of the studies actually measured if this improved worm longevity or viability. While the addition of glucose to a saline based media did significantly improve longevity in the current study, it did not affect worm viability. Some preliminary work using an over-the-counter medical grade glucometer to measure the concentration of glucose in the medium before and after a 12-hour period suggested that the worms were not consuming the glucose (Scare, unpublished data). However, the results were variable and it is unknown how accurate the glucometer is at reading glucose levels in a saline based medium. It would be interesting for studies to further explore the consumption of glucose and other energy metabolites (*i.e.* pyruvate) of various ascarid species. While the exact beneficial components of the RPMI-1640 media are unknown, one could easily test each individual component of the formulation and differentiate which are necessary for survival. However, perhaps it would be more time-worthy to determine what additional nutrients/components might further enhance the RPMI-1640 effects, such as the addition of fetal bovine serum. Immediately after harvest from necropsy, *Parascaris* spp. adult worms have been observed *in vitro* excreting/defecating what appears to be intestinal

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content which they ingested from the lumen of the horse's intestines (Scare, unpublished observations), and therefore it might be possible to collect these products and compare their components to horse small-intestinal digesta. This may provide some clues to what nutrients and compounds the worms are utilizing or discarding. The study described in Chapter 4, did not evaluate *Parascaris* spp. viability when maintained in RPMI-1640 and within a CO_2 incubator due to a limited number of worms. As described in 4.1, studies report ascarid maintenance with and without a CO_2 incubator, but none of these studied have assessed if it is a necessary component for *in vitro* maintenance. Most of these studies also used saline-based media, where the target pH was obtained using an acid, therefore it is unclear what benefits the investigators were hoping for by using the CO_2 incubator. Because of the sodium bicarbonate component of RPMI-1640 and its reliance on CO_2 to regulate pH, it would be of primary interest to observe worm viability/longevity when maintained in RPMI-1640 and a CO_2 incubator. Finally, it is unknown how the length of *in vitro* maintenance and observed worm viability affects/reflects physiologic processes, such as regulation of gene expression. This could be determined by observing the expression of some housekeeping genes in worms maintained *in vitro* for different lengths of time and in worms at different levels of viability. This would provide insight to how reliably motility represents viability. Another interesting comparison would be between *in vitro* maintained worms and worms harvested, but kept *in situ* and thus minimizing the impact of removal from the host and handling. This would provide insight to what physiological processes are disrupted by *in vitro* maintenance, and to what extent.

6.4.5 Conclusions

Overall, maintaining parasitic nematodes *in vitro* is challenging and currently no study has reported successful lifecycle replication of an intestinal helminth *in vitro*. This practice is plagued by many challenges, such as the unknown nutritional requirements, cellular communications between parasites and between the host and parasites, immunological effects, environmental/gaseous requirements. Additionally, it is unknown how these parameters might differ between developmental stages and/or sex. The current study provided the first *in vitro* assessment of nutritional and environmental requirements

for any ascarid parasite. It is apparent that despite sharing a phylogenetic superfamily (Ascaroidea), *Ascaris suum* and *Parascaris* spp. may have different nutritional and energy requirements. Therefore, direct extrapolations from one ascarid species to another should be made with caution. The results of this study provide groundwork for future *in vitro* studies, although direct extrapolations for *in vivo* representation require further investigation.

6.5   *In vitro* drug exposure of *Parascaris* spp.

6.5.1   Major findings

This study established an *in vitro* anthelmintic exposure protocol for adult *Parascaris* spp. Anthelmintics were prepared at varying concentrations to observe worm viability over time. An immediate finding was that worms responded more quickly to IVM treatment, regardless of concentration, than to OBZ treatment and there appeared to be some variation in viability following OBZ treatment.

6.5.2   Explanations for findings

The current findings provide a baseline for future drug exposure trials, as *Paracaris* spp. response to drug exposure had never been examined. The initial drug concentrations used to observe drug exposure (0.1, 1.0, 10, 100 μg/mL) were selected based on previous studies employing *in vitro* drug exposure of *A. suum* L_4 stage larvae (Hu et al., 2013) and adult *Parascaris* spp. (Janssen et al., 2013b), but these studies did not evaluate worm viability over time nor did these studies provide an explanation or reference for choosing these drug concentrations. This factor is further complicated by the fact that it is unknown how much active drug is reaching the parasite target site when inside the horse. Therefore, it was unknown how *Parascaris* spp. would respond. While it appears that the concentrations employed provided toxic effects on the worms, we must consider the possibility that perhaps these concentrations were either too low or too high to be therapeutically relevant. Additionally, the length of exposure may have been too long or too short. It is also unknown how well the viability scores reflect the toxic drug effects. The current study selected exposure length based on a sub-lethal decrease in viability, but presumably the worms begin responding to the drug immediately upon

exposure, whether this is phenotypically evident or not. The more immediate effect observed following IVM treatment than OBZ reflects the drugs' mode of action. As described in section 1.5, IVM causes immediate paralysis of the worm, whereas OBZ serves to slowly disrupt microtubule polymerization and energy metabolism. The latter occurs more slowly. Therefore, the *in vitro* findings are in agreement with the expected mode of action.

Furthermore, there are several variables that must be considered before direct interpretation of the drug effects can be made. First, it is unknown how an active host immune response may affect the worms before or after drug exposure, and worm age may also have an effect. Worms that are hindered by the immune response would likely be more affected by the drugs, however it also might be possible that attack by the immune system may prime worm defense mechanisms and they may already be exhibiting some protective responses before drug exposure begins. Thus they may have a 'head start' in protection. Worm age is another complicating factor. As described in section 4.3 immature (L_4) worms had a significantly shorter lifespan and lower viability under *in vitro* conditions (without any drug exposure) than adult males and females, and may have a higher metabolism than adults. It is unknown if the metabolism would increase the worm's ability to metabolize, detoxify, or pump out drugs, but differences in drug uptake based on life stage has been described in cattle GIN (El-Abdellati et al., 2011; AlGusbi et al., 2014). However, significant differences in worm viability between immature stages and adults upon drug exposure were only found under one drug concentration (OBZ 1μg/mL), and therefore no overall conclusions regarding drug metabolism of different stages can be made at this time. Finally, the horse's metabolism of the drug would influence the concentration and possibly the final substrate reaching the worm. Currently, these influences are unknown and cannot be reproduced *in vitro*.

6.5.3   Future directions

One of the most obvious next steps would be to optimize the *in vitro* drug exposure protocol by testing more drug concentrations. In the current protocol (section 5.2), media was changed every 12 hours to remove excretory/secretory products. Therefore, fresh drugs and media were provided every 12 hours. This was because the

length of drug exposure to observe viability changes was unknown, particularly between drugs with differing mode of actions. However, repeated drug exposure at full strength likely does not reflect *in vivo* conditions when a horse is treated with an anthelmintic. Typically, a single treatment is given and presumably the concentration decreases over time as it gets metabolized by the horse, however the persistence varies based on the drug used. Therefore, it would be interesting to compare the results of one drug exposure (media changes every 12 hours, but no fresh drug added) to the current method. It would also be interesting to observe changes when drug was added in decreasing concentrations every 12 hours. Another study would be to explore differences in phenotypic responses between various ascarid species following the same drug exposure protocol, and parasites of the same species, but with different anthelmintic susceptible/resistant status. In conjunction with this, the differences in responses between immature and adult stages could also be explored. Perhaps one of the most interesting studies would be apply the amount of active drug reaching the target parasites *in vivo* when administered to the horse. Some studies have investigated the drug concentrations reaching the gastrointestinal tract and parasites of ruminants (Lifschitz et al., 2017), but less is known for horses and their parasites. Some work has investigated the systemic anthelmintic concentrations and fecal excretion in horses (Pérez et al., 2001, 2010; Gokbulut et al., 2010a,b, 2016), and this would provide a starting point for estimating applicable *in vitro* drug concentrations. Future equine-based studies could mimic the work described by Lifschitz et al. (2017) to obtain more accurate estimations of drug concentrations within host intestinal tissues and various parasite tissues. Sublethal drug exposure is a known contributor to resistance. Therefore, some parallels may be made between *in vitro* and *in vivo* drug treatment studies that would lead to determining the optimum lethal drug concentrations reaching the parasite and then how much must be administered to the horse to achieve this goal. As previously mentioned, the host immune responses may affect worm responses to *in vitro* drug exposure. Because it is known that immunity to *Parascaris* spp. infections is driven by age rather than exposure, experimental infections of foals of different ages and immune status could be performed. Upon necropsy, worms would be harvested and maintained *in vitro* with and without drug exposure as described in section 4.2 to evaluate the effects on only worm viability (without drug exposure) and

to investigate if the immune response hinders or primes worm defense mechanisms upon drug exposure. Additionally, characterization of the host immune response could be performed using blood parameters and samples of the small intestinal wall for histopathology and differential expression of cytokines.

6.5.4 Conclusion

Overall, the *in vitro* drug trial of *Parascaris* spp. provided an important starting point for future anthelmintic-based research, and has applications for other parasite species as well. However, nonetheless, results must be interpreted with caution as there are several factors contributing to *in vivo* drug exposure that currently cannot be controlled for.

6.6 Gene expression analysis of adult *Parascaris* spp. following *in vitro* drug exposure

6.6.1 Major findings

This was the first study to investigate the effects of *in vitro* drug exposure on the whole transcriptome of adult *Parascaris* spp. worms. The predictive pathway analysis showed clear differences in the functionality of differentially expressed genes when worms were exposed to either IVM or OBZ. Upon closer examination of the genes between the drug treated and control group, four of the top significantly different genes also had functional implications, however the fold change difference was <2. Based on their annotated function, it appears that these genes may have been upregulated by the worms in direct response to drug exposure, and provide a basis for future investigations regarding their role in resistance and drug response mechanisms. Furthermore, the more commonly researched genes associated with resistance did not have a significant increase in expression.

6.6.2 Explanations for findings

The RNA-seq analysis revealed a number of differentially expressed genes between all drug treated worms and all control worms. Comparisons between individual drug treatments (IVM and OBZ) and the corresponding controls (IVM control and OBZ control) were not used for further analysis due to the small sample sizes, and this was a

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limitation of the study. The small fold changes in gene expression levels may have occurred for a number of reasons; Laurent et al. (2013) discusses the biological importance of many genes with minute fold changes. Currently, it is unknown how the death of the host and *in vitro* conditions affect transcriptomic processes, as parasites may begin responding immediately to the death of their host. Although these worms were harvested within a few hours of euthanasia, it is possible that the lack of peristalsis and decrease in host body temperature may trigger a response in the parasites. Additionally, we don't know how the *in vitro* conditions affect physiological process, such as transcriptomic processes. It is possible that the minute fold change differences between drug treated and control worms were because overall transcriptomic processes were deteriorating from *in vitro* conditions. Finally, the effects of the host immune response must always be considered. It is possible that protective mechanisms of the worm are shared between responses to immune system attack and drug treatments. At this time, *Parascaris* spp. responses to host immune responses remain unknown and further research on this speculation is needed.

This was the first study to examine an ascarid population that was completely naïve to anthelmintics, and using susceptible worms reduces the genetic variability associated with resistant isolates. As described in section 1.8, parasites are opportunistic organisms and have the capability to quickly develop mutations when under selection pressure, such as anthelmintics which leads to varying drug response mechanisms between species and isolates of the same species (James et al., 2009; Gilleard, 2013). Therefore, using a susceptible isolate may reduce the variability associated with selection pressure. However, the absence of drug selection may not accurately reflect the resistance scenarios on managed horse farms. Furthermore, as described in section 1.5, the drug receptor subunits often vary between parasite species, making cross-species extrapolations challenging.

The genes of interest were selected based on having significantly increased expression and functional application. The functionalities of these genes and proposed explanations for their increased expression are provided in detail in section 5.4, and thus will not be repeated here. Furthermore, the RNA-seq analysis described herein failed to

identify commonly implicated resistance genes, such as the Pgps and multidrug resistance genes. As described in section 1.8.4, the Pgps have been of significant focus in several resistance studies. While increased expression of Pgps has been associated with resistance in *H. contortus* and *C. elegans*, findings within other parasite species are inconsistent and contradictory. The lack of increased expression of Pgps in the drug treated groups may have occurred for three reasons. First, this may be because the parasite population used has not been subject to any anthelmintic selection pressure and perhaps development of increased expression occurs over time. Secondly, it must be noted that *C. elegans* and *H. contortus* are within the same phylogenetic clade (Clade III along with other strongylid species), however *Parascaris* spp. belongs to Clade V, and perhaps the phylogenetic distance promotes *Parascaris* spp. to implement other drug response mechanisms. Third, a study by Janssen et al. (2013b; described in 1.8.4) did not identify an increase in expression of Pgp genes between *Parascaris* spp. isolates that were presumably susceptible or resistant, nor when *Parascaris* spp. adults were exposed to IVM *in vitro*. Results described in the current study (Chapter 5) were in agreement with Janssen et al. (2013b). Additionally, Janssen et al. (2013b) identified three missense mutations in a *P. equorum* isolate that was presumably ML resistant. However, it must also be noted that the majority of specimens used by Janssen et al. (2013b) did not have a resistance status confirmed by a FECRT, but rather farms reported a lack of decreased egg shedding after treatment. Although the current study failed to identify increased expression of common resistance genes, such as Pgps, it does not mean that these genes are not involved in resistance mechanisms. Populations that have undergone routine anthelmintic treatment and thus selection pressure may have selected for overexpression of these genes. As suggested by Janssen et al. (2013b), the mechanisms of resistance may be related to mutations rather than expression levels. This was outside of the limitations of the current study as the population of worms used had not been under anthelmintic selection pressure, but should be observed in future studies. Overall, it is interesting that *Parascaris* spp. so far do not appear to increase Pgp expression in response to drug exposure, and there could be alternative mechanisms at work.

Finally, we must consider the limitations of the annotated *Parascaris* spp. transcriptome (Wang et al., 2017). In its current form, 30% of the listed gene IDs did not

have an associated gene name. This missing information may have crucial functions related to resistance and drug response mechanisms, and further supports the need for future transcriptomic work in the *Parascaris* species.

6.6.3   Future directions

The most logical next step would be to repeat this study on more worms, perhaps of different isolates, to validate the findings. It would be interesting to select worms from multiple populations with known resistance and susceptibility. Several studies of other parasite species have reported variations in findings between different isolates with the same anthelmintic sensitivity status, but the level of variation in *Parascaris* spp. isolates is unknown. As described in section 1.8.1, piperonyl butoxide is a known CYP450 inhibitor. Exposing *Parascaris* spp. to drugs with and without the inhibitor may provide more evidence for the functional role of this gene as a drug response mechanism. A similar approach could be taken for Pgps, using the inhibitor verapamil. A more molecular approach would be to further investigate the selected genes of interest using the *C. elegans* model. First, differential expression of these genes could be evaluated in a drug exposure trial to observe if this nematode responds in a similar way to *Parascaris* spp., both transcriptionally and phenotypically. Secondly, mutant strains of *C. elegans* could be used to determine if the *Parascaris* spp. gene rescues the phenotype. For example, given that the CYP450 enzymes are important drug detoxification mechanisms, a *cyp450* knockout strain of *C. elegans* could be used to observe if a lack of this gene increases the drug toxic effects. Then, the *C. elegans* knockout could be transformed with the *Parascaris* spp. *cyp450* gene to observe if it exhibits a rescue phenotype. As discussed later in section 6.8, despite the flexibility of the *C. elegans* model, one must keep in mind the associated challenges associated with applying this free-living nematode as a parasitic model.

Aside from investigating gene function, the several influences exhibited by the host on the parasite likely introduce unknown sources of variability. Therefore, it would be of interest to determine how the *in vitro* conditions affect various physiological processes compared to *in vivo* conditions, and how the host immune system affects transcriptomic processes. Regarding the host's drug metabolism on the worm responses,

it would be interesting to compare the transcripts of worms treated *in vivo* and *in vitro*. An obvious challenge would be obtaining worms from *in vivo* treatment before they died and RNA degraded. Alternatively (or additionally), worms could be obtained from drug resistant isolates and used for both the *in vivo* and *in vitro* models. In this way, some *in vivo* worms would likely be surviving at the time of necropsy. Furthermore, an expansion of this would be to compare *in vivo* and *in vitro* worms from resistant and susceptible isolates. As described previously, the effect of immune responses on worm drug responses is unknown, and this could be further evaluated using experimental infections of foals with different ages. The differences could be explored both phenotypically and transcriptomically. Unfortunately, the aforementioned studies require the sacrifice of young horses which makes it challenging to obtain specimens, and of course is never an ideal scenario. Finally, considering all of these influences, a common theme has been that results obtained from lab isolates are rarely also found in field isolates (Blackhall et al., 1998; Beech et al., 2011; Kotze et al., 2014).

6.6.4   Conclusions

This study provides the first whole-transcriptome analysis of adult *Parascaris* spp. worms in response to *in vitro* drug exposure. Based on functionality, four of the top six significantly different genes show potential involvement as drug response mechanisms. These genes have not been previously reported in any ascarids species in response to drug exposure and warrant further investigation.

6.7   Differences of cyathostomin and *Parascaris* spp. infections

One of the primary questions following the research described herein is if there are any cross extrapolations which can be made between the cyathostomin and *Parascaris* spp. study findings. It is possible that the cyathostomin family and the *Parascaris* genus may share similar resistance and drug response mechanisms, but there are several biological reasons that strongly support why direct extrapolations between cyathostomins and *Parascaris* spp. should not be made.

First, the phylogenetic distance between equine cyathostomins and *Parascaris* spp. must be considered. Cyathostomins belong to Clade V, with all other strongylids, while

Parascaris spp. are within Clade III (Geary and Thompson, 2001; Hashmi et al., 2001; Gilleard, 2004). While there is a large amount of evolutionary conservation of nematode genes, there is a great amount of variation in the biology between different clades and the associated genetic variation is largely unknown (Gilleard, 2004). Therefore, the differences in cyathostomin and *Parascaris* spp. dynamics discussed below are likely driven by years of ecological adaptions, all of which should be carefully considered before generalizations are made.

There are several differences between the dynamics of cyathostomin and *Parascaris* spp. infections. First, as previously described, cyathostomins can infect horses of any age, while *Parascaris* spp. is primarily found in foals (<1 year of age). Perhaps what is most interesting is that the immunity obtained for *Parascaris* spp. is age driven rather than exposure driven. Clayton and Duncan (1979b) established experimental infections in foals at either 2-4 weeks of age or 6-12 months of age. Both age groups had foals raised under conditions of natural exposure (*i.e.* on pasture) or in a worm-free environment (*i.e.* concrete stall). The younger foals, regardless of rearing environment, established higher infections than the older foals, and there were no significant differences between the older foals reared under natural conditions or worm-free conditions. Overall, the younger foals had a higher rate of establishment and higher FECs than the older foals (Clayton and Duncan, 1979b). In contrast, the immune responses to cyathostomin infections, albeit incomplete, appear to be exposure driven which tends to be a function of age. The immune response to cyathostomin infections is most commonly observed by the number of encysted and arrested larvae, as immune responses are evidenced to play a primary role in the arrestment process (Poynter, 1969; Love et al., 1999; Klei, 2000). Interestingly, studies report foals and parasite naïve horses having a shorter cyathostomin prepatent period (Smith 1976a,b), no evidence of larvae in arrested development (Reinemeyer et al., 1988; Chapman et al., 2002, 2003; Nielsen and Lyons, 2017), and high adult worm burdens (Monahan et al., 1998; Chapman et al., 2002; 2003). These findings are in contrast to those of mature horses. As the horse matures, the cyathostomin exposure and subsequent immune responses increase. This results in a longer prepatent period (Smith, 1978) and increased numbers of encysted and arrested larvae (Monahan, 1998; Chapman et al., 2002, 2003). Therefore, it appears that immunity

to *Parascaris* spp. infections is more innate in nature, while the incomplete immunity to cyathostomins is driven by the acquired immune response. Currently, we can only speculate how the immune responses might influence anthelmintic resistance. In general, the *Parascaris* spp. immune response limits the patent infection to only a few months during the foal's life, and this may limit the spread of resistant alleles in a given population. However, the life cycle and prolific nature permits multiple generations of *Parascaris* spp. to pass through a single foal. This, coupled with the hardiness of the eggs in the environment, may support the quick spread of resistant alleles through the parasite population, given the resistance is not associated with a fitness loss. However, historically it has been hypothesized that the high number of eggs in the environment would support a continuous refugia population, and this was recently supported by a 40-year model simulation (Leathwick et al., 2017), but this is largely dependent upon the absence of resistance alleles in the refugia population. Finally, it is also unknown if *Parascaris* spp. exhibits protection mechanisms from the horse's immune response that may also contribute to protection mechanisms from anthelmintics. Regarding cyathostomins, they can infect horses of all ages which greatly increases the opportunity for resistance to develop. However, it is presumed that the immune response causes the encysted stages to arrest, and the arrested stages can persist for at least two years. This, coupled with the one year life span of adult worms, slows down the lifecycle and passing of resistant alleles through subsequent generations. However, it should also be considered that the arrestment phase allows resistant alleles to persist in refugia (when non-larvicidal drugs are used), and may contribute to the gene pool for many subsequent generations. Overall, for both cyathostomins and *Parascaris* spp., there are several factors that must be considered for resistance, and more studies are needed to clarify the influence of the immune responses.

A second interesting point is that *Parascaris* spp. infections do not appear to be affected by seasonality (Fabiani et al., 2016), while cyathostomin infections are (Ogbourne, 1975; Eysker et al., 1990; Leathwick et al., 2015b, 2019; Nielsen and Lyons, 2017). This includes cyathostomin larval development in the environment, arrestment, the excystment of arrested larvae, the number of adults in the lumen, and egg shedding. The environmental stages consist of the non-embryonated egg, the embryonated egg, and

the L_1, L_2, and L_3 stage larva. These stages are affected differently by varying environmental conditions and this has been reviewed by Nielsen et al. (2007). In summary, the infective L_3 has the most resistance to conditions of frost and excessive dryness, and some resistance to freeze/thaw cycles however, they are very susceptible to heat (temperatures ranging from 30-38˚C). Interestingly, the other stages are more resistant to high temperatures, but less resistant to the other environmental factors than the L_3 stage. The hardiness of the L_3 stage is attributed to the double-layered cuticle that surrounds the larva and is a characteristic unique to this stage. Some evidence also suggests that the cuticle may even offer protection in times of desiccation, if temperatures remain below the heat threshold (Nielsen et al., 2007). Overall, the ideal conditions for development are warm temperatures (25-33˚C) and humidity of at least 20%. Given these environmental constraints, the climatic region must be considered when focusing anthelmintic treatments around the grazing period (*i.e.* when larval development and infection pressures have peaked; Nielsen et al., 2017). On the contrary, the only environmental stage of *Parascaris* spp. is the egg. These eggs, within which the infective larva develops, has a reputation for being hardy as it is surrounded by a thick proteinaceous coat. They are found to remain viable under extreme temperature and chemical conditions. Parascaris *spp.* eggs have been reported viable up to 59˚C (Koudela and Bodeček; 2006; Rakhshandehroo et al., 2015) and some viability (77.3%) when frozen at -20˚C for 168 hours (Koudela and Bodeček, 2006). The eggs are also somewhat resistant to various chemicals, such as bleach and potassium dichromate, a reagent known to be highly toxic and corrosive (Rakhshandehroo et al., 2015). It is assumed that the eggs can survive on a pasture of years, or even decades, but no long-term studies have evaluated this claim. Overall, seasonal conditions more drastically affect the development of cyathostomins than *Parascaris* spp. This may be due to the seemingly delicate cyathostomin L_1 and L_2.

In mature horses with previous cyathostomin exposure, the number of arrested larvae (EL_3) increases during periods when environmental conditions are unfavorable for larval development, such as excessive heat and desiccation, or extreme cold (Ogbourne, 1975; Eysker et al., 1990; Chapman et al., 2003; Scháňková et al., 2014). Likewise, larvae tend to resume development and excyst as more favorable conditions arise

(Ogbourne, 1975; Eysker, 1990; Love et al., 1999; Chapman et al., 2003), and the maximum number of adults are found in the lumen during periods of moist, mild weather conditions (Ogbourne, 1975, 1976; Reinemeyer et al., 1986; Chapman et al., 2003). It should be noted that the fluctuations in the number of arrested larvae reflect the infection pressure as the seasons affect the development of the environmental stages. However, more studies are needed before direct conclusions can be made. Egg shedding is also affected by seasonality, however fluctuations may be less obvious depending on the climatic region. One study observed equine strongyle egg shedding on horses in the United Kingdom for one year and found distinct changes in egg shedding following seasonal changes (Wood et al., 2012). A recent study observed strongyle egg shedding in horses over one year in Kentucky (USA), and while fluctuations directly associated with seasons was not observed, the egg shedding was increased in the month of September (nearing the end of the grazing season), but was decreased in the month of May (at the very beginning of the grazing season; Steuer et al., *in preparation)*. These results are likely due to regional climatic differences, where more northern climates (like the UK) have more distinct seasons than milder, southern regions of the United States (Nielsen et al., 2007). Regarding cyathostomin resistance, seasonality and climatic differences likely affect the rate of resistance development. In temperate regions the infection pressure is more consistent resulting in a continual intake of cyathostomins which would increase allele turnover and possibly cause a faster developmental rate of resistance. In contrast, regions where seasonal conditions hinder larval development and infection pressure decreases during certain times of the year may regularly disrupt allele turnover. Therefore, climatic regions should be considered when deciding when to administer anthelmintic treatment. Currently, in the United States, it is recommended to deworm horses around the grazing season to control for cyathostomins (Nielsen et al., 2016). Deworming during the grazing season reduces egg shedding and thus the infection pressure, however, worms surviving treatment (resistant worms) would then be the only worms contributing to egg shedding and subsequent generations, thus contributing to a faster rate of resistance. This has recently been modeled by Nielsen et al. (2019) and described in section 1.9.2. Regarding animal health and decreasing the immediate risk for disease, deworming during the grazing season seems plausible. However, regarding the

development of resistance, it may be more beneficial to deworm during seasons not ideal for larval development (hot/dry), that way eggs shed by resistant worms would be unlikely to contribute to the infection pressure. In any case, managing parasites on a short and long term basis is a delicate balance.

A third major point of difference is the larval development and adult lifespan of *Parascaris* spp. and cyathostomins. The maturation of *Parascaris* spp. *in vivo* is well documented, but cyathostomins undergo a period of arrested development that is poorly understood and may continue for at least two years. Regardless, the transcriptional changes that may occur between developmental stages for cyathostomins and *Parascaris* spp. is unknown. One similarity, however, is that it appears some anthelmintics affect the larval stages of both species. DiPietro et al. (1987) observed IVM to remove immature intestinal stages of *Parascaris equorum*, and later also reported IVM efficacy against the migrating stages in the liver and lungs (DiPietro et al., 1988). MOX and FBZ (double dose, five days) are labeled to exhibit some efficacy against encysted cyathostomins. The incomplete efficacy is likely due to insufficient bioavailability of the drug at the target site. Encysted stages that are exposed to sub-lethal levels may be triggered to exhibit detoxification mechanisms and evade future drug treatments. The adult lifespan is also an important factor for resistance. The life expectancy of the *Parascaris* adult is thought to be around 270 days (Mozgovoy, 1953 in Morand, 1996). Because of the arrested stage, the exact adult lifespan for cyathostomins is unknown, but it is presumed to be around one year. This is evidenced by seasonal fluctuations in the number of adult worms in the lumen (Ogbourne, 1975, 1976; Reinemeyer et al., 1986; Chapman et al., 2003), where more adults are found in the lumen when environmental conditions favor larval development on pasture (i.e. mildly warm temperatures, humidity), but begin to decrease when conditions are less favorable (i.e. excessive/prolonged heat and dryness).

Next, the stages affected by anthelmintic treatments are an important point for consideration. For migrating stages of *Parascaris* spp., IVM initially exhibited 100% efficacy against migrating larval stages (DiPietro et al., 1988) and was 98.2% efficacious against immature stages in the intestine (Austin et al., 1991), however the high levels of ML resistance (Table 1.2) make this a less than ideal treatment option today. The only

other larvicidal treatment is FBZ (FBZ (10 mg/kg for 5 days) which historically exhibited near perfect efficacy against migrating larvae in the lungs and was 99.8% effective against immature intestinal stages (Vandermyde et al., 1987). However, no recent studies have evaluated larvicidal efficacy for either drug class against *Parascaris* spp. larval stages. For encysted cyathostomins, only MOX (0.4 mg/kg) and FBZ (10 mg/kg for 5 days) are labeled for larvicidal efficacy. In North America, MOX is only labeled for efficacy against the LL_3/L_4 stages, and not the EL_3 stage. A recent study reported a decrease in larvicidal efficacy for the FBZ treatment, whereas MOX was 73.8% and 74.6% efficacious against EL_3 and LL_3/L_4 stages, respectively, which was within the range of original label claims of 50-100% (Reinemeyer et al., 2015; Bellaw et al., 2018). However, the ERP of MOX was 4 weeks, which is a drastic decrease compared to the original 16-22 weeks. The shortened ERP is speculated to be due to luminal L_4 stages surviving treatment and quickly maturing to egg laying adults (Lyons et al., 2009, 2010; Rossano et al., 2010; Lyons and Tolliver, 2013; Reinemeyer et al., 2015; Bellaw et al., 2018). Regardless, removing the larval stages eliminates it as a source of refugia and delays the opportunity for egg shedding into the environmental refugium as well. The lack of 100% efficacy of the ML drug class and FBZ 5-day regimen against larval stages of both *Parascaris* spp. and cyathostomins, has likely contributed largely to their development of anthelmintic resistance. Every treatment, a portion of larvae survive and it is unknown if they have simply not received a high enough drug concentration, and/or if perhaps they have superior detoxification mechanisms. Regarding the adult stages, all three drug classes marketed for equine parasites in the United States were initially effective against adult stages of cyathostomins and *Parascaris* spp. (section 1.5), however the increasing levels of resistance (Tables 1.1 and 1.2), complicate treatment regimens. Currently it appears that the cyathostomins are still affected by the drugs for which *Parascaris* spp. is resistant (ML class), whereas *Parascaris* spp. is still susceptible to the drugs for which cyathostomins are heavily resistant (BZ). Some reports of resistance to the PYR drug class exist for both.

As described in section 1.8, there is evidence for some shared non-specific resistance mechanisms between *Parascaris* spp. and cyathostomins. For example, Pgps have been associated with resistance or reduced drug sensitivity in both cyathostomins

and *Parascaris* spp. Two putative Pgp domains have been identified across several cyathostomin species (Drögemüller et al., 2004c), and Peachey et al. (2017) identified increased expression of Pgp-9 in cyathostomin populations subjected to routine ML exposure (Peachey et al., 2017). Pgp-11 and Pgp-16 have been characterized in *Parascaris equorum,* and mutations within Pgp-11 are associated with resistance (Janssen et al., 2013b). While the influence of Pgps are shared between cyathostomins and *Parascaris* spp. the currently identified Pgps are different, and it appears that mechanisms are as well (expression vs. sequence mutations). Additionally, mutations in Pgp-11 of *D. immitis* have also been identified and associated with decreased IVM susceptibility (Bourguinat et al., 2011a). This is particularly interesting as *D. immitis* (a filarial nematode) and *Parascaris* spp. are within the same phylogenetic clade (Clade III). Furthermore, cyathostomins harbor substantial BZ resistance (section 1.7.1), as do several small ruminant strongylid species (section 1.7.2). As described in section 1.8.2, mechanisms for resistance are most understood for the BZ drug class and commonly attributed to three polymorphisms in the β-tubulin gene. These associations are mostly based on findings in the small ruminant GIN (section 1.8.2). While F200Y appears to be the most common SNP in BZ resistant small ruminant GIN populations, the F167Y mutation is more commonly reported in resistant cyathostomins populations. In contrast, these polymorphisms do not appear to associate with resistance in *Ascaris* species (section 1.8.2).

Overall, the dynamics of cyathostomin and *Parascaris* spp. infections, such as life cycle, host immune responses, and seasonality, influence treatment regimens and the development of anthelmintic resistance. The phylogenetic distance between cyathostomins and *Parascaris* spp. is illustrated by the aforementioned biological differences, and is further evidenced by the variation in anthelmintic resistance mechanisms. Therefore, it does not appear useful or wise to make extrapolations between *Parascaris* spp. and cyathostomins.

6.8   Challenges with using C. elegans as a parasitic model

Given the heavy use of the free living, soil-dwelling nematode, *C. elegans,* as a model organism for parasitic nematodes, it is relevant to discuss the challenges associated

with its implementation. Perhaps the primary challenge of parasite research is the host dependency, and currently no life cycles of equine parasites can be replicated *in vitro*. Leading experts attempt to overcome these challenges by using the model organism *C. elegans*. Every gene in this nematode is characterized, and numerous mutant strains are available for study. Additionally, it can be maintained *in vitro* as pure isolates and has a fast replication rate. However, there are several points to consider before using *C. elegans* as a parasitic model and/or extrapolating results from studies to a particular parasite species (Geary and Thompson, 2001; Hashmi et al., 2001; Gilleard, 2004). First, one should consider the phylogenetic relationships involved. *Caenorhabditis elegans* belongs to the Clade V nematodes, as do all strongylids, however filariid and ascarid species belong to Clade III (Geary and Thompson, 2001; Hashmi et al., 2001; Gilleard, 2004). This relationship results in several differences that should be considered. For example, *C. elegans* resides in the environment as a free-living nematode, while parasites may live within the lumen of various host organs, and/or perhaps migrate within tissues or systems. Functionally, *C. elegans* operates in an aerobic environment where as most parasites are within an anaerobic environment. The genetic differences between a parasitic and non-parasitic nematode are unknown (*i.e.* the 'parasite' genes have not been identified) (Gilleard, 2004; Geary and Thompson, 2001). Further, the genetic variability between similar and dissimilar parasites also remains unknown (Geary and Thompson, 2001). Therefore, the application of *C. elegans* largely depends on the characteristic to be studied. Despite these challenges, this model organism has proven beneficial for anthelmintic screening assays (Geary and Thompson, 2001; Hashmi et al., 2001), and has some applications for studying parasite gene regulation and function (Gilleard, 2004; Geary and Thompson, 2001; Hashmi et al., 2001). Exploring gene regulation in *C. elegans* is a common and relatively simple technique as this nematode can be easily transformed with DNA, and expression patterns can be observed using reporter genes. However, the promoter and regulatory regions of a given gene of interest should be considered as it may not be the same in the parasite species (Gilleard, 2004). For gene function analysis, there is a plethora of *C. elegans* mutant strains available for which the gene of interest can be inserted and observed for functional restoration (Gilleard, 2004; Geary and Thompson, 2001). However, it must be considered that these results only

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provide evidence for gene function, but do not provide proof. The gene may not function identically in *C. elegans* as it does in the parasite species, and other genes with a shared function of the deletion may partially restore the phenotype. Another challenge is that some knockouts are lethal. In this case, other technologies such as RNA*i* or overexpression may be useful. The former requires a high-level of sequence homology, while the latter requires a clear hypothesis for the gene function (Gilleard, 2004). Overall, it is clear that *C. elegans* can be a useful model for studying parasites, but there are many considerations that must be taken into account and results should be interpreted with caution.

6.9   Lack of anthelmintic resistance in Strongylus vulgaris

Finally, the last point of discussion will be on the lack of resistance reports for stongylids, such as *S. vulgaris*, when it has developed so broadly for the cyathostomins. Although *S. vulgaris* was not a focus of the research described herein, the implication for discussion is still warranted due to the high pathogenicity of this parasite and need for preventing the development of resistance. There are four primary hypotheses which may contribute to the lack of resistance in this parasite. First, it must be considered that the cyathostomins consist of over 50 species. While it is unknown which species, or if all of them, harbor resistance genes, the species diversity gives the cyathostomins a much larger variety of genes which may associate with resistance. Secondly, the prepatent periods of cyathostomins and *S. vulgaris* differ greatly. *S. vulgaris* takes at least 6 months from the time of initial infection until the adult worms are sexually mature and females begin laying eggs. The prepatent period for cyathostomins is much more complicated due to the arrested stages. In foals, the prepatent period has been documented as early as 5 weeks, but in young horses (4-5 years old) it may be 12-15 weeks (Smith, 1976b), while in older horses (9-10 years old) it has been observed at 17-18 weeks (Smith, 1978). Nevertheless, the prepatent period of *S. vulgaris* is longer than the cyathostomins, and likely contributes to a slower rate of resistance development as it would take longer to cycle the resistant alleles through to subsequent generations. It is also unknown how cyathostomins are recruited from arrested development to continue development within the lumen, but it is presumed to occur as a trickle effect. This method of recruitment

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would likely contribute to a faster rate of resistance spreading through the population. Next, the mechanisms of drug uptake should be considered. The *Strongylus* species are known blood feeders, and if the drug used has a high systemic concentration, then *S. vulgaris* would receive direct exposure. The feeding action of cyathostomins is unknown, but they are found free floating in the lumen of the intestine rather than attached to the mucosa. This suggests that they do not take a blood meal, but perhaps ingest intestinal content. Therefore, they may take up drug through oral ingestion during feeding, and/or perhaps the drugs are absorbed transcuticularly. If this is the case, then either method may result in indirect and/or lower drug bioavailability for cyathostomins than *S. vulgaris*, and inadequate drug bioavailability is a known driver for resistance. Finally, the arrested development stage of cyathostomins is complex and largely unknown, but likely has some role in the development of resistance as the larvicidal drugs (MOX single dose 0.4 mg/kg or FBZ at 10 mg/kg for 5 days) are not 100% efficacious, allowing a portion of surviving worms harboring resistance to propagate. Perhaps those that survive have developed pertinent mechanisms. It is also unknown if there is a time lapse between when the cyathostomin L_4 stage excysts and when metabolic/feeding processes begin in the lumen. If there is a period where the L_4 is present in the lumen, but not feeding, then the amount of drug reaching the target receptor may be too low to cause lethal effects. For *S. vulgaris*, IVM treatment affects nearly all stages, including larval stages within the CMA, except for the L_5 residing in the CMA. Most L_5s are reportedly observed retaining the L_4 cuticle before migrating to the large intestine. Presumably, this extra layer provides additional protection from anthelmintic treatment. One might assume that this would drive resistance, but if the extra cuticle provides complete protection, then possibly no resistance mechanisms are necessary.

6.10   Overall conclusion

The anthelmintic resistance crisis is not a new problem, yet there is limited knowledge regarding resistance mechanisms and alternative treatment regimens. Some research in livestock supports alternative treatment and management regimens (*i.e* refugia management, combination deworming, etc.), but few field studies have been performed for equine parasites. Currently, the use of combination deworming where drug resistance

is prevalent within a cyathostomin population only selected for multi-drug resistance parasites and was not a sustainable control option. Some experts suggest using combination therapies with a new anthelmintic with perfect efficacy, but the lack of drug development prevents this opportunity on most managed horse farms. However, with a unique ML naïve cyathostomin population, we investigated the use of a ML in combination with OBZ. While the ML drug was capable of perfect efficacy as evaluated by FECs and delaying egg counts for several months, the long-term effects on OBZ efficacy are still unknown. The consequences of this combination on managed horse farms where the ML ERP is shortened must be evaluated before recommendations are made. Overall, it appears that cyathostomin populations with a history of drug resistance will be challenging parasites to overcome. Regarding resistance mechanisms, very few studies have focused on equine parasites, and most research has focused on results obtained from other parasite phyla, such as generalized resistance mechanisms and changes in subunit composition. However, it is likely that resistance to a single drug is due to numerous mechanisms. Identifying drug response mechanisms of the whole worm, as presented herein, has provided the opportunity for identifying a detoxification mechanism shared among numerous phyla (plants, insects, nematodes etc). It also identified potentially novel genes which may be involved with compensating for the drug's toxic effects, however no firm conclusions can be made at this time. It is clear that there are vast opportunities for research in equine parasites going forward, and swift rate of resistance development demands knowledge for preventing resistance from developing to future drugs.

APPENDICES

APPENDIX 1. SUPPLEMENTARY TABLE 1

Supplementary Table 1. Mean treatment efficacies at 2 weeks-post treatment calculated using the FECRT. Predicted efficacies were calculated using the additive effect formula.
95% Confidence intervals are included in parenthesis.
FECRT = [(pre-treatment EPG) – (post-treatment EPG)/ pre-treatment EPG] x 100%

Treatment	FECR (%)
2015 Oxibendazole	66.7 (54.62 – 78.78)
2015 Pyrantel pamoate	63.3 (51.32 – 75.28)
2015 Predicted combination treatment	80.73 (72.29 – 89.17)
1st Combination treatment	76.64 (64.82 – 88.46)
2nd Combination treatment	42.56 (28.09 – 57.03)
3rd Combination treatment	41.59 (25.7 – 57.48)
4th Combination treatment	40.67 (27.21 – 54.13)
2016 Oxibendazole	42.29 (32.84 – 54.27)
2016 Pyrantel pamoate	42.7 (28.27 – 57.12)
2016 Predicted combination treatment	68.68 (57.54 – 79.82)

APPENDIX 2. SUPPLEMENTARY INFORMATION FOR CHAPTER 5

Supplementary Table 1. The top portion of the table presents the mapping statistics for individual each *Parascaris* spp. sample to the *Parascaris univalens* reference genome (Wang et al., 2017) generated in Part 1 of this study. The second portion of the table presents the results of the sequence alignments for the PCR products generated in Part 2 of this study compared to the predicted sequence.

Mapping statistics	
Sample	Uniquely Mapped Reads (%)
In vivo control, Female	72.73
In vivo control, Male	76.65
Ivermectin (1 µg/mL), Female	76.06
Ivermectin (1 µg/mL), Male	68.03
Ivermectin control, Female	71.94
Ivermectin control, Male	62.65
Oxibendazole (10 µg/mL), Female	77.67
Oxibendazole (10 µg/mL), Male	73.94
Oxibendazole control, Female	43.02
Oxibendazole control, Male	71.80

PCR product sequence alignment to predicted sequence (% identity)						
Sample	Target gene					
	ncbp	*ama*	*cytp450*	*frmd4a*	*sup-9*	*klhdc-10*
IVM Male	100	100	98.54	98.4	NSA	100
IVM-C Male	99	100	100	98.93	98	100
OBZ Male	98	100	97.84	98.91	94.83	100
OBZ-C Male	100	99.28	97.96	99.45	92.06	NSA

Abbreviations: IVM, ivermectin treated; IVM-C, ivermectin control; OBZ, oxibendazole treated; OBZ-C, oxibendazole control; NSA, no significant alignment

Supplementary Table 2. Primer sequences for housekeeping genes and genes of interest. Primers were designed using NCBI Primer Blast.

Gene	Primer Name	Sequence (5'-3')	Amplicon Size (bp)
Nuclear cap binding protein subunit 2 (*ncbp*)*	Pgr002_g120F Pgr002_g120R	ATCAGCATACGAAATGGACG TTCAGAAAGCAAATTGGGAC	206
RNA polymerase RPABC1 large subunit (*ama*)*	PgR065_g009F PgR065_g009R	CCATGACTTTACTTCCGTCA AAGAAGGTTATCATCTCGGC	185
Cytochrome P450 4C1 (*cytp450*)	PgR071_g005F PgR071_g005R	TCCTATCTTCATCCCGTGTA CCTTTCACCAGTTCCGTATT	188
FERM domain containing protein 4a (*frmd4a*)	PgR045_g021F PgR045_g021R	CAGACAGTGAACTCCAGAAA CCAACATAGCCTCAGAGTTT	231
Two pore potassium channel protein (*sup-9*)	PgB01_g100F PgB01_g100R	GGCCAGACTATTAGCAAAGG AACAAATGAACGCCAAAAGG	94
Kelch domain containing protein 10 (*klhdc-10*)	PgR401_g001F PgR401_g001R	ACAACGGAGTTCTTTACCAA CTCCAAAGATGAGGTTCAGG	133

Asterisks indicate housekeeping genes.

Supplementary Table 3. Concentrations and RIN scores for samples used in RNA-seq analysis analyzed by the Agilent Bioanalyzer (Agilent, Santa Clara, CA, USA) at the University of Kentucky Genomics Core Lab.

Sample ID	Concentration	Average	Average RIN

	(ng/μl)	
In vivo Male	432	9.75
In vivo Female	1105	9.75
OBZ (10) Female	459	9.8
OBZ Control Female	583	9.75
IVM (1) Female	440.5	9.8
IVM Control Female	531.5	10
OBZ (10) Male	328.5	9.5
OBZ Control Male	366	9.35
IVM (1) Male	487	9.35
IVM Control Male	574.5	9.45

Abbreviations: IVM, ivermectin; OBZ, oxibendazole; RIN, RNA integrity number

Materials and Methods Supplementary Information

2.2.1 Collection of Parascaris spp.

Collection of live worm specimens at necropsy occurred as previously described (Scare et al., 2018). Briefly, all worms were milked out of the small intestine onto a mesh sieve, rinsed with room temperature (RT) tap water, and placed in a container of RT RPMI-1640. The container was placed in a water bath maintained at 37˚C for transport to the laboratory. Worms were classified as adult or L4, and adult worms were further characterized by sex as described by Scare et al. (2018).

2.5 Part 1: Initial assessment of parasite responses to in vitro drug exposure

Viability assessments on worms harvested from the first necropsy were performed every hour for the first six hours, and then every once every six hours until all worms had died (78 hours). For the second necropsy, viability assessments were performed at one, six, and twelve hours post-treatment, then at 12-hour intervals until all worms had died (84 hours). Because the number of worms to be collected for part two of this study was unknown (*i.e.,* there was a risk of no worms present), worms from the second necropsy were snap frozen once a sub-lethal decrease in viability (≥25%) was observed. This included the IVM worms at 1 hour, OBZ (10μg/mL) at 12 hours and OBZ (100μg/mL) at

24 hours, along with the corresponding control worms. The remaining control worms were allowed to continue the observation period.

2.6.2 RNA-seq analysis

Adaptor trimming and quality control were performed using TrimGalore Version 0.4.4 (Babraham Bioinformatics) and reads were subsequently aligned to the *Parascaris univalens* reference genome (Wang et al., 2017) using STAR Version 2.5b (Dobin et al., 2013). Reads were annotated to the *Parascaris* reference transcriptome (Wang et al., 2017) using Cufflinks (Release 2.2.1) (Trapnell et al., 2012). The parasite sources used to develop the genome and transcriptome by Wang et al. (2017) were obtained from the same drug naïve *Parascaris* spp. population used in the current study (section 2.2). Read counts were normalized as fragments per kilobase of exon per million mapped reads (FPKM) and differential gene expression analysis was performed on normalized read counts.

Selection criteria for candidate genes were as follows: significant differences ($\alpha=0.05$) between all drug treated worms and all control worms, the differences in expression must show an obvious pattern between groups, and the candidate genes must not be significantly different between the *in situ* and *in vitro* control groups nor between worm sexes (*i.e.,* differences did not occur due to *in vitro* maintenance nor worm sexes). Due to the higher number of candidate genes remaining, some selections were based on annotated gene function and those which may play a role in parasite drug metabolism/defense/drug efflux.

2.7.2 Primer design and validation

Specificity of primers were tested by performing real-time qPCR (Agilent Mx3000P qPCR System, Santa Clara, CA) with PowerUP SYBR Green Master Mix (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's instructions. Cycle parameters can be found in the supplementary files. The total reaction volume was 10 μl and prepared according to the manufacturer's instructions with forward and reverse primers at a concentration of 400 nM each and the cDNA sample at approximately 10 ng. This was performed on a pooled-sample of cDNA from all drug treated and control worms. The cycle parameters were as follows: activation step at 50°C for two minutes followed by the

Dual-Lock DNA polymerase at 95˚C for two minutes, then 40 cycles of denaturation at 95˚C for 15 seconds and anneal/extension at 60˚C for one minute. Lastly, a dissociation step was performed to determine gene-specific amplification evident by a single peak in the melting curve. The dissociation step was 60˚C to 95˚C at a ramp rate of 0.15˚C/second.

2.8 Statistical analyses

2.8.1 Part 1: Initial assessment of parasite responses to in vitro drug exposure

Two mixed model analyses with repeated measures over time were performed where 'percent viability' was the response variable. The first analysis examined the differences between worm stage (adult or immature) and between adult males and females. The covariates examined were 'time', 'stage/sex', and the interaction term of 'time'*'stage/sex'. The variable 'drug' was kept as a random effect. The second analysis examined the effects of drugs (IVM or OBZ) at each concentration (0.1, 1, or 10 μg/mL), and RPMI-1640 and DMSO (10%) controls for all worms over time. The covariates examined were 'time', 'drug/concentration', and the interaction term of 'time'*'drug/concentration'. The variable 'stage/sex' was kept as a random effect.

A third mixed model analysis (without repeated measures) was performed to produce two sets of results. First, to examine differences between worm stage/sex for the different drug concentrations and control groups, and secondly to determine if changing the drug concentrations altered viability within each stage/sex. The response variable was 'percent viability', and the covariates examined were 'drug/concentration', 'stage/sex', and the interaction term of 'drug/concentration'*'stage/sex'. The variable 'time' was kept as the random effect.

2.8.3 Part 3: Further investigation of genes of interest with qPCR

Further statistical analyses were performed in SAS software (version 9.4, SAS Institute, Cary, North Carolina, USA). Mixed linear analyses were performed to analyze gene expression of each GOI in response to drug treatment, and any variability between worm sex and between *in situ* and *in vitro* controls. The forward construction and backward elimination approach was employed in all models and only covariates where p<0.2 remained in the model. Covariates identified as significant (α=0.05) were further examined in a 'least squares means' analysis for a Tukey's pairwise comparison. For all

models, the response variable was 'normalized –ΔCq.' The first model was used to analyze the influence of worm sex and the *in situ* verses *in vitro* environment. The covariates examined were 'sex' and 'environment', whereas 'drug', 'gene' 'PCR plate' and 'sample replicate' were kept as random effects. The second model was for a broad comparison between all drug treated and all control worms. The covariates examined were 'drug or control', 'gene', 'sex', 'environment' and the interaction term of 'drug or control'*'gene'. The third model was more specific and examined direct comparisons between IVM treated and IVM controls, OBZ treated and OBZ controls, and IVM treated and OBZ treated. The covariates examined were 'drug/control type', 'gene', 'sex' and the interaction term of 'drug/control type'*'gene.'

Sequences for genes of interest for products from all male worms from section 3.3

ncbp

>Predicted Sequence

ATCAGCATACGAAATGGACGCGTAAATTAAATTAGCAAGTAGAAGCAGGC GGATATGTGGGTTGAGGATTTATGATTGGCACTGCCCGGCAGCTTCCTGAGG CCTCATCTGGTTATGTGCTTTTACGTTCAGTTATATGCTACTTGAATATGCTTT ATGGAAAAGCGGTCAAACACGTTGAAGAGCGTCCCAATTTGCTTTCTGAA

>NCBP Male IVM 1µg/mL

AGGGCGGATATGTGGGTTGAGGATTTATGATTGGCACTGCCCGGCAGCTTCC TGAGGCCTCATCTGGTTATGTGCTTTTACGTTCAGTTATATGCTACTTGAATAT GCTTTATGGAAAAGCGGTCAAACACGTTGAAGAGCGTCCCAATTTGCTTTCTG AAA

>NCBP Male IVM Control

AGCAGGCGGATATGTAGGGTTGAGGATTTATGATTGGCACTGCCCGGCAGCT TCCTGAGGCCTCATCTGGTTATGTGCTTTTACGTTCAGTTATATGCTACTTGAA TATGCTTTATGGAAAAGCGGTCAAACACGTTGAAGAGCGTCCCAATTTTGCTT T

CTGAAA

>NCBP Male OBZ 10µg/mL

TCCAGCAGGCGGATATGTGGGTTGAGGATTTATGATTGGCACTGCCCTACAG
CTTCCTGAGGCCTCATCTGGTTATATGTTTTTACGTTCAGTTATATGCTACTTG
AATATGCTTTATGGAAAAGCGGTCAAACACGTTGAAGAGCGTCCCAATTTGC
TTTCTGAAA

>NCBP OBZ Control

ACCAGCAGGCGGATATGTGGGTTGAGGATTTATGATTGGCACTGCCCGGCAG
CTTCCTGAGGCCTCATCTGGTTATGTGCTTTTACGTTCAGTTATATGCTACTTG
AATATGCTTTATGGAAAAGCGGTCAAACACGTTGAAGAGCGTCCCAATTTGC
TTTCTGAAA

ama

>Predicted Sequence

CCATGACTTTACTTCCGTCAGGAACGACTATAAAAATGAATTACCTTAGCA
AGTGGAGCTAAGCAGTTGGTGGTGCAAGATGCATTCGAGATGACATGGTTGT
TAGCAGCATCATATTTATCTTCGTTGACGCCCATAACGAACATTGGTGCATCC
GCAGATGGCGCCGAGATGATAACCTTCTT

>AMA Male IVM 1µg/mL

CGCTGGAAGCTAAGCAGTTGGTGGTGCAAGATGCATTCGAGATGACATGGTT
GTTAGCAGCATCATATTTATCTTCGTTG
ACGCCCATAACGAACATTGGTGCATCCGCAGATGGCGCCGAGATGATAACCT
TCTTCACGTT

>AMA Male IVM Control

AGGTGGGAGCTAAGCAGTTGGTGGTGCAAGATGCATTCGAGATGACATGGTT
GTTAGCAGCATCATATTTATCTTCGTTGACGCCCATAACGAACATTGGTGCAT
CCGCAGATGGCGCCGAGATGATAACCTTCTTAAAGCCCA

>AMA Male OBZ 10 µg/mL

AGCAGGAGCTAAGCAGTTGGTGGTGCAAGATGCATTCGAGATGACATGGTTG
TTAGCAGCATCATATTTATCTTCGTTGACGCCCATAACGAACATTGGTGCATC
CGCAGATGGCGCCGAGATGATAACCTTCTTCATGT

>AMA Male OBZ Control

AGGATAGCAGGTGGAGCTAAGCAGTTGGTGGTGCAAGATGCATTCGAGATGA

CATGGTTGTTAGCAGCATCATATTTATCTTCGTTGACGCCCATAACGAACATT

GGTGCATCCGCAGATGGCGCCGAGATGATAACCTTCTTGGTCTA

Cytp450

>Predicted Sequence

TCCTATCTTCATCCCGTGTAGACCACTTTCATGGTCTCATCAGAAAACTCTC

CCCTTGAACCATCTCACGGCATATATTTAAGAAGGCGCGCAGAATTTCGCCTA

CCTGTTTCAATTAATAAAATTGTATTTCTTTTCAACGAACACCTTCAGCCAAT

ACTCGAGAGCAATACGGAACTGGTGAAAGG

>CYTP450 Male IVM 1 µg/mL

TAGCCCCTTGACCATCTCACGGCATATATTTAAGAAGGCGCGCAGAATTTCGC

CTGCCTGTTTCAATTAATAA

AATTGTATTTCTTTTCAACGAACACCTTCAGCCAATACTCGAGAGCAATACGG

AACTGGTGAAAGGACTAATA

>CYTP450 IVM Male Control

CTCCCCTTGAACCATCTCACGGCATATATTTAAGAAGGCGCGCAGAATTTCGC

CTACCTGTTTCAATTAATAAAATTGTA

TTTCTTTTCAACGAACACCTTCAGCCAATACTCGAGAGCAATACGGAACTGGT

GAAAGGATT

>CYTP450 Male OBZ 10µg/mL

CACTACCCTTGACCATCTCACGGCATATATTTAAGAAGGCGCGCAGAATTTCG

CCTGCCTGTTTCAATTAATAAAATTGT

ATTTCTTTTCAACGAACACCTTCAGCCAATACTCGAGAGCAATACGGAACTG

GTGAAAGGACGTGT

>CYTP450 Male OBZ Control

AGTAAACTCTCCCCTTGACCATCTCACGGCATATATTTAAGAAGGCGCGCAG

AATTTCGCCTGCCTGTTTCAATTAATAA

AATTGTATTTCTTTTCAACGAACACCTTCAGCCAATACTCGAGAGCAATACGG

AACTGGTGAAAGGA

Klhdc10

>Predicted Sequence

ACAACGGAGTTCTTTACCAAGTTGGTGGTACCACAGGACACATATATAACA
TGGAGGTGCGGAGTTTGACACCCGTTCGGACTGAGACGAACAAACCGTGTGC
AAGCAATTTCCCTGAACCTCATCTTTGGAG

>klhdc10 Male IVM 1µg/mL Male

CGACCGGCTTACTGGAGGTGCGGAGTTTGACACCCGTTCGGACTGAGACGAA
CAAACCGTGTGCAAGCAATTTCCCTGAACCTCATCTTTGGAGGATGGCCTCGT
TATGCTCTATGGAAAAGCGCCGTAACGACGTTGTGATTATACCCC
TTTGGTTTCT GAAAAATGCATT

>klhdc10 Male IVM Control

TGCGCAGGTGCGGAGTTTGACACCCGTTCGGACTGAGACGAACAAACCGTGT
GCAAGCAATTTCCCTGAACCTCATCTTTGGAGGACTAAATCGGTAATCCTAGA
TGGAGCTAGATGATGTGCTGATTAGGGAATTTTAATTTGAAGA
GGTCCT

>klhdc10 Male OBZ 10 µg/mL

CCCTGCTCTCTTACTGGAGGTGCGGAGTTTGACACCCGTTCGGACTGAGACGA
ACAAACCGTGTGCAAGCAATTTCCCTGAACCTCATCTTTGGAGGAGCGAAAT
TGGTGGATCCATATATAAAAGCTAAAGACGTTGAAAGTTCGTGGCG
GTTGTTTTCTGAAAGGACTTTC

>klhdc10 Male OBZ Control (No significant alignment)

TGGAGGGAGGAAGTTTCTTCAGCCCTTTTTCTCTCTGACTGGACTGTCTGTTC
GTGAGCCATTCCCTATCACGTTCTTCTCGATATTGTTTAAAAAGCTTTTTTATT
ATATTTGAGCAATATATATTTTTGTGGTAGTGAGC

Sup-9

>Predicted sequence

GGCCAGACTATTAGCAAAGGCGCTTTCATCAGGCAGCTCTAAGAAATTAGA
GAATCGGGCATCACTCGCCGAATCCTTTTGGCGTTCATTTGTT

>sup-9 Male IVM 1µg/mL

No sequence product generated

>sup-9 Male IVM Control

GGGGGGCAGCCTCCTAGAATTAGAGATCGGGCATCACTCGCCGAATCCTTTT
GGCGTTCATTTGTTAGAGGGCCGCTTCTCTAGTTTATATGCTGTGGCTATATCT
ACACGAAGGCGCATCACAAATGGCGTACAGATGATTAGCATTAT

>sup-9 Male OBZ 10 µg/mL

GTCGGGCCGCCTCTAGGAATTAGAGATCGGGCATCACTCGCCGAATCCTTTTG
GCGTTCATTTGTTCGAGGGCCATTTCTTTTTTTTTTTGTTGCAGATACGAAATT
GTTATGCCCTACACAAGAGCGCTATCAACGCTTGATGTATACCCCTTTTTTTC
TGAAA

>sup-9 Male OBZ Control

GGGGGCGGCTCTAGAATTAGAGATCGGGCATCACTCGCCGAATCCTTTTGGC
GTTCATTTGTTAATGAGCCCCAACTTCTTGTGCTTCCCTTTGCAGATATAAAC
ATTGAATATGCCAAATAGAAAGCGCGATGACGACCTGAAACGTATA
CCCTTTT TCTCAGAAAA

Frmd4a

>Predicted sequence

CAGACAGTGAACTCCAGAAATTGATGAAGATTTTAAGGAAGAAATCCAGA
CGGGAATCTGTTGAGATAAGGACGGGAAAATCTTGGCAAATTATACGTTCGG
GAAACATCCGTTCCATATGTGAAACTGCAGATTACTTCAACGAGCAGTAACA
AATCATATTTATTCTGAGTATTCTTTGCAGTCATAGCCGAGAATGATAACTTC
GTTCAAACTCTGAGGCTATGTTGG

> frmd4a Male IVM 1 µg/mL

TCGGAGCAATCCAGACGGGATCTGTTGAGATAAGGACGGGAAAATCTTGGCA
AATTATACGTTCGGGAAACATCCGTTCCATATGTGAAATTGCAGATTACTTCA
ACGAGCAGTAACAAATCATATTTATTCTGAGTATTCTTTGCAGTCATAGCCGA
GAATGATAACTTCGTTCAAACTCTGAGGCTATGTTGG

>frmd4a Male IVM Control

CCAGAACGGGAATCTGTTGAGATAAGGACGGGAAAATCTTGGCAAATTATAC

GTTCGGGAAACATCCGTTCCATATGTGAAATTGCAGATTACTTCAACGAGCA

GTAACAAATCATATTTATTCTGAGTATTCTTTGCAGTCATAGCCGAG

AATGATAAC TTCGTTCAAACTCTGAGGCTATGTTGGA

>frmd4a Male OBZ 10 µg/mL

AGAACGGGCAATCTGTTGAGATAAGGACGGGAAAATCTTGGCAAATTATACG

TTCGGGAAACATCCGTTCCATATGTGAAATTGCAGATTACTTCAACGAGCAGT

AACAAATCATATTTATTCTGAGTATTCTTTGCAGTCATAGCCGAGAA

TGATAACTTCGTTCAAACTCTGAGGCTATGTTGG

>frmd4a Male OBZ Control

TCCGACGGGAATCTGTTGAGATAAGGACGGGAAAATCTTGGCAAATTATACG

TTCGGGAAACATCCGTTCCATATGTGAAATTGCAGATTACTTCAACGAGCAGT

AACAAATCATATTTATTCTGAGTATTCTTTGCAGTCATAGCCGAGA

ATGATAACTTCGTTCAAACTCTGAGGCTATGTTGG

APPENDIX 3.LIST OF SIGNIFICANTLY DIFFERENT GENES

Due to the length of the list, please see the separately included excel sheet.

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PEER-REVIEWED PUBLICATIONS

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PEER-REVIEWED ABSTRACTS AND PRESENTATIONS

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