Host-Parasite Interaction in Horses: Mucosal Responses to Naturally Acquired Cyathostomin Infections and Anthelmintic Treatment

Ashley Elaine Steuer
University of Kentucky, ashley.steuer@uky.edu
Author ORCID Identifier: https://orcid.org/0000-0003-1063-5723
Digital Object Identifier: https://doi.org/10.13023/etd.2020.160

Recommended Citation
Steuer, Ashley Elaine, "Host-Parasite Interaction in Horses: Mucosal Responses to Naturally Acquired Cyathostomin Infections and Anthelmintic Treatment" (2020). Theses and Dissertations--Veterinary Science. 47.
https://uknowledge.uky.edu/gluck_etds/47

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Ashley Elaine Steuer, Student

Dr. Martin K. Nielsen, Major Professor

Dr. Daniel K. Howe, Director of Graduate Studies
Host-Parasite Interaction in Horses: Mucosal Responses to Naturally Acquired Cyathostomin Infections and Anthelmintic Treatment

________________________________________

DISSERTATION

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
Ashley Elaine Steuer
Lexington, Kentucky

Director: Dr. Martin K. Nielsen, Schlaikjer Professor of Equine Infectious Disease, Associate Professor of Veterinary Science
Lexington, Kentucky
2020

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https://orcid.org/0000-0003-1063-5723
ABSTRACT OF DISSERTATION

Host-Parasite Interaction in Horses: Mucosal Responses to Naturally Acquired Cyathostomin Infections and Anthelmintic Treatment.

Cyathostomins are ubiquitous parasites in equids. In rare cases, cyathostomins lead to a generalized typhlocolitis and death. In healthy horses, local reactions are noted to the mucosal larvae; however, the mechanisms and importance of these reactions have not been elucidated. It has been hypothesized that anthelmintics can alter these reactions. Currently, three drug classes are approved for use in horses against cyathostomins; while all products target the adults, only two products are labeled as larvicidal. Adulticidal therapy is implicated in triggering the typhlocolitis, however, current evidence is contradictory. There is also conjecture that the larvicidal drugs can increase the risk for adverse inflammatory reactions; however, there has been limited investigation into possible mechanisms. One component of the local mucosal response is goblet cell hyperplasia (GCH), but its exact role in cyathostomin infections is unknown. We hypothesize that GCH in horses plays an important role with expulsion of the worms following treatment.

Two studies were conducted to elucidate the local and systemic immune response following larvicidal treatment based on histology, immunohistochemistry, and gene expression. In the first study, ponies with naturally acquired cyathostomin infections were allocated into three groups: fenbendazole-treated (FBZ), moxidectin-treated (MOX), and untreated control. Whole blood was collected weekly and tissue samples from the large intestine were collected at the 2- and 5-weeks post treatment (WPT) necropsies. Samples were evaluated for proinflammatory, anti-inflammatory and local mucosal responses. There were few significant differences between the three groups; however, there were significant correlations between luminal worm burdens, GCH, and goblet cell gene expression (MUC2, r= -0.2358 and RELM-β, r= -0.2261). This study identified the potential for seasonal turnover and expulsion of worms, where GCH score was lower at 2 WPT than 5 WPT across all groups (p<0.001), and not associated with treatment.

The second study aimed at evaluating and comparing a larvicidal treatment with a non-larvicidal treatment belonging to the same anthelmintic class to classify the local and systemic immune response following larvicidal treatment based on histology, immunohistochemistry, and gene expression. Horses with naturally acquired cyathostomin infections were allocated into three groups: Ivermectin (IVM)/praziquantel-
treated, MOX/praziquantel-treated, and untreated control. Whole blood was collected weekly and tissue samples from the large intestine were collected at the 2 and 5 WPT necropsies. Samples were evaluated for proinflammatory, anti-inflammatory and local mucosal responses, as above. MOX treatment was associated with lower GCH than the control and IVM treatment groups, and the IVM treated group had lower GCH than the control horses (p<0.0376). There were statistically significant positive associations between mucosal worm burdens and goblet cell hyperplasia and associated gene expression (GCH scores p<0.001, MUC2 p<0.001, RELM- β <0.001). Higher GCH was associated with higher worm burdens across all groups and was higher at 2 WPT than 5 WPT.

Overall, these studies demonstrate that treatment with an anthelmintic, both larvicidal and non-larvicidal, in healthy horses does not significantly change the proinflammatory or anti-inflammatory response. In fact, it may decrease the local mucosal response in horses, due to the decrease in worm burdens. Cyathostomins induce a proinflammatory response within the host tissue, while the encysted larvae may also induce an anti-inflammatory response as well. These studies demonstrate that that GCH is implicated in worm expulsion. This could further impact what we know of infections and how we interpret the disease complex.

**KEYWORDS: Cyathostomin, Equine, Goblet Cell, Host-Parasite Interaction, Anthelmintic treatment, Mucus.**
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By
Ashley Elaine Steuer

Dr. Martin K. Nielsen
Director of Dissertation

Dr. Daniel K. Howe
Director of Graduate Studies

05/07/2020
Date
DEDICATION

To my wonderful family, and the individuals that have supported me throughout my educational career. To my mother, whom has supported my dreams throughout my lifetime. To my husband, whom has been by my side through it all with constant support. To my teachers and friends and their constant encouragement. It takes a village to support an individual, and I have had that through them all.
ACKNOWLEDGMENTS

There are several individuals that I would like to acknowledge for their guidance, assistance, and encouragement along the way. First, I would like to acknowledge Dr. Martin K. Nielsen, for all of his guidance, endless support, and tolerance of my never-ending questions and run-on sentences. A thank you to my committee, Dr. Alan Loynachan, Dr. Amanda Adams, and Dr. Mary Rossano for all of your guidance and support through these four years. To my fellow lab mates, including Dr. Jessica Scare Kenealy, Jennifer Bellaw, Jennifer Cain, and Jamie Norris, and the numerous wonderful undergraduate and LMU students who contributed significantly to my learning, projects, and endless need of computer help. To Day Barker, Craig Stewart, and Allen Page for all of their help with immunology and Kristen Scoggin for IHC guidance. A giant thank you.

To the UK Farm crew, especially Courtney, Chad, Mason, Kevin and Lauren. Without you, this work would not have happened. Your commitment to the welfare of the farm animals inspires me every day. To the UKVDL, especially Donnie Becker, Judy Tucker, Dr. Craig Carter, Dr. Lynne Cassone, Sarah Welsh and the entire necropsy crew. Thank you for all of the learning experiences and opportunities!

To the National Center for Veterinary Parasitology, especially, Dr. Kelly Allen, Dr. Mason Reichard, Dr. Meriam Saleh, and Dr. Susan little. Thank you for your support, guidance, and making this all possible. I would not be here without you!

To my Tennessee family, Aly Chapman, Heidi Wyrosdick, Dr. Craig Reinemeyer, Dr. John Schaefer, Dr. Richard Gerhold, Dr. Charles Faulkner, and Dr. Sharon Patton, who started me on this incredible journey and have been an endless wealth of knowledge and
encouragement. Thank you for allowing me to continuously “bug you” throughout these seven years.

To Zoetis, LLC, thank you for your support and the opportunities that it brought us with these studies and through the NCVP and my fellowship.

Finally, to my family, Teresa, Anthony, Justin, Josh, Courtney, Kevin Sr., Kevin Jr., and Caitlin. Thank you for your support throughout these years. I would not be here without you. My vet school friends and family: for putting up with my incessant parasitology rants and for getting me this far.

It really does take a village and thank you for making it possible!
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CHAPTER 1. LITERATURE REVIEW

1.1 Parasites of Equids

Domestic horses (*Equus ferus caballus*) are hosts to one of the most diverse parasite populations in the animal kingdom (Lichtenfels et al., 2008). Of the kingdom Animalia, 3 phyla parasitize horses: Arthropoda, Nematoda, and Platyhelminthes. This literature review will focus primarily on the Nematoda and one platyhelminth that infect the gastrointestinal tract of horses, to allow for greater focus. When discussing the following classifications, phyla, classes, orders, suborders, and genera will be discussed as presented in Georgis’ Parasitology for Veterinarians 10th edition (Bowman, 2014).

1.1.1 Anoplocephalids

In the phylum Platyhelminthes, lies the family Anoplocephalidae. Within this family, there are three species, which infect the gastrointestinal tract of horses, *Anoplocephaloides mamillana*, *Anoplocephala magna*, and *Anoplocephala perfoliata*. *A. mamillana* and *A. magna* reside in the small intestine and share similar characteristics in life cycle to *A. perfoliata*; however, they are only reported sporadically and are considered to not be clinically relevant (Nielsen, 2016a; Rehbein et al., 2013). Therefore, they will not be discussed further. *A. perfoliata* is considered unique among tapeworms, as it is found at the ileocecal junction and within the cecum of horses, as most occur within the small intestine of their definitive host. Horses obtain infection by ingesting oribatid pasture mites that contain the infective cysticeroid stage and accumulate these parasites throughout the grazing season (Denegri, 1993); therefore, the highest worm burdens are usually found in the in second half of the year in the northern hemisphere, after the grazing season (Tomczuk...
et al., 2015). Infections with *A. perfoliata* may lead to hyperemia, mucosal thickening, and necrotic ulcers (Nilsson et al., 1995; Pearson et al., 1993; Williamson et al., 1997) of the ileocecal junction and cecum. The inflammatory reactions to this parasite will be reviewed in 1.3.2.3. Finally, associations between *A. perfoliata* and ileocecal, cecolic, and cecocecal intussusception, rupture, ileal hypertrophy, and ileal impaction are also reported (Barclay et al., 1982; Beroza et al., 1986; Foerner et al., 1980; Owen et al., 1989; Proudman et al., 1998; Ryu et al., 2001).

1.1.2 Ascarids

In the phylum Nematoda, within the class Secerentea, there are several orders and suborders that infect the gastrointestinal tract of horses, including: Ascaridida, Spirurida, Oxyurida, Rhabditida, and Strongylida. They reside from the stomach, small intestine, and large intestine of the horse. Within the order Ascaridida, is *Parascaris* spp., including *Parascaris equorum* and *Parascaris univalens*, with the former being most well-known but the latter more common worldwide (Martin et al., 2018; Nielsen et al., 2014). Horses are infected by ingesting the larvated eggs in the environment. The larvae hatch in the small intestine, and then undergo hepatotracheal migration, where they will mature and molt in the lungs (Clayton and Duncan, 1979). When they arrive back to the small intestine, the later stages mature into adults and start reproducing sexually (H. M. Clayton and Duncan, 1979). The entire process takes approximately 12-16 weeks (H. M. Clayton and Duncan, 1979). *Parascaris* spp. are common parasites in the small intestines of foals, and can cause severe intestinal impaction and potentially lead to intestinal rupture and death (Cribb et al., 2006; Southwood et al., 1966; Tatz et al., 2012). Horses gain immunity to the parasite,
though the exact mechanism is unknown, and will tend to not have significant burdens above 1-2 years of age (Clayton and Duncan, 1979; Nielsen, 2016b).

1.1.3 Spirurids

_Draschia megastoma, Habronema microstoma, and Habronema muscae_ are within the spirurids. Horses become infected when flies, primarily Musca autumnalis, *Musca domestica*, and Stomoxys calcitrans, carrying the infected larvae land near the lips, open wounds, or conjunctiva of the horse (Pugh et al., 2014). When placed near the mouth, the infective larvae are ingested and migrate to the stomach to complete the lifecycle. In the stomach, the adults pass larvated eggs in the feces, which are ingested by fly larvae maggots, and then undergo maturation to the infective stage within the fly larvae as they molt and mature (Pugh et al., 2014). The parasitic adults live in the glandular portion of the stomach, with _D. megastoma_ inducing large fibrous nodules along the margo plicatus. However, it is the larvae that cause the most significant disease during aberrant migration in the cutaneous and conjunctival tissues (Pugh et al., 2014; Rebhun et al., 1981; Underwood, 1936). This disease, known commonly as summer sores or habronemiasis, causes severe ulceration and granulation of wounds and the conjunctiva of the horse (Pugh et al., 2014).

1.1.4 Oxyurids

Horses are also host to the oxyurid parasites _Oxyuris equi_ and Probstmayria vivipara, both of which live in the large intestine of the horse. Horses become infected with _O. equi_ by ingesting the larvated egg (Enigk, 1949; Hasslinger, 1990). The larvae hatch and mature within the cecum (CEC), ventral colon (VC) and dorsal colon (DC) (Enigk,
1949; Hasslinger, 1990; Wetzel, 1930a). *Oxyuris equi* gravid females will deposit eggs around the edge of the anus, leading to intense anal pruritus, tail rubbing, and potential behavioral issues (Enigk, 1949; Hasslinger, 1990; Reinemeyer and Nielsen, 2014). These females can be large, up to 15 cm, have a pointed tail, and are routinely observed by horse owners in the feces (Reinemeyer and Nielsen, 2014). *Probstmayria vivipara* passes larvae into the lumen of the large intestine and undergoes auto reinfection within the large intestine of the host. There are no clinical signs associated with this parasite, and while most horses likely harbor all life stages, it is missed routinely due to its small size (<3mm)(Slocombe, 1985; Tolliver et al., 1987).

1.1.5 Rhabditids

Within the order Rhabditida is a very unique group of parasites with unusual lifecycles. In horses, only *Strongyloides westeri* is known to infect the gastrointestinal tract. Adult females live in the small intestine of foals and reproduce parthenogenically. Larvated eggs are passed in the feces, and hatch and grow to the infective stage, where, they can either continue to grow and reproduce sexually in the environment or infect the host and continue the lifecycle. Foals are infected by ingesting mare’s milk, percutaneous penetration, or by ingestion from the environment. The parasite has been loosely associated with foal frenzy syndrome and dermatitis, as well as foal heat diarrhea (Dewes, 1989; Lyons et al., 1973; Netherwood et al., 1996).

1.1.6 Strongylids

Finally, the order Strongylida is the main target of current adult equine parasite control. Within the Strongylida, one Trichostrongyle, *Trichostrongylus axei* infects the
stomach, and is important due to it being potentially zoonotic; however, most human cases currently seem to originate in the middle east and southeast Asia (Phosuk et al., 2013; Sharifdini et al., 2017). Of the strongyles, there are two classifications within horses, the cyathostomins (Cyathostominae; small strongyles), which will be the main focus of this dissertation, and the large strongyles (Strongylinae). The parasitic adults live in the CEC, VC, and DC. In domestic horses, the large strongyles consist of 14 species in 5 genera: Bidentostomum, Craterostomum, Oesophagodontus, Strongylus, and Triodontophorus (Lichtenfels et al., 2008). Of these, the most discussed due to pathogenicity are the three Strongylus species. Strongylus vulgaris, S. edentatus, and S. equinus undergo extensive migration in the horse. The most concerning of the three is S. vulgaris, whose larvae migrate through the aorta and cranial mesenteric, renal, and celiac arteries (Drudge, 1979; Slocombe, 1985). This produces significant arteritis, which leads to hemodynamic turbulence, thrombi formation, non-strangulating infarction, and death (Drudge, 1979; Duncan and Pirie, 1975; Nielsen et al., 2016). This parasite was the main target of control starting in the 1950s (Lyons et al., 1999); however, it has become rare in the United States, due to intense deworming regimens (Herd, 1990).

1.2 Cyathostomins in Equids

Cyathostomins are a unique group of parasites consisting of 14 genera and over 50 species (Lichtenfels et al., 2008). Within the last 30 years, due to the decreased prevalence of S. vulgaris, cyathostomins have become the main target of most deworming programs (Herd, 1990). Currently, cyathostomins are the strongyles that are most likely to cause disease in horses in North America and have widespread resistance to 2 of 3 available drug classes (Abbott et al., n.d.; Bellaw et al., 2018; Eysker et al., 1989; Gokbulut and McKellar,
They are unique among strongylids, due to location within the host and life cycle.

1.2.1 Life Cycle

The life cycle of cyathostomins has several characteristics of a classic strongylid lifecycle; however, they undergo unique phases that are clinically important for the disease process. Adults are found in the lumen of the CEC, VC, and DC. They copulate as a pair and the female then passes eggs into the organ lumen. The eggs are then shed within the feces, where they larvate to the first larval stage (L1) within the egg, in as little as 12 hours (Ogbourne, 1972; Rupashinge and Ogbourne, 1978). The larvae, under favorable conditions of warm temperature and high humidity, will then hatch. The L1 larvae then develop and molt to the L2 larvae. Both of these stages are coprophagous and feed to gain nutrients and energy to grow into the infective L3. The infective L3 larvae have a sheath (the cuticle from the L2 stage) to protect them from harsh environmental conditions and can survive for several months on pasture. At this stage, they do not acquire nutrients, and actively move to locations where horses will ingest them.
Figure 1-1. The lifecycle of cyathostominae within the horse. A. Adults in the lumen of the large intestine mate, and the female deposits eggs into the ingesta. B. Eggs are passed in the feces. C. Eggs embryonate and larvate to the L1 stage. D. L1 hatches into the environment. E. L1 molts to the L2 stage. F. L2 molts to the L3 stage, which infects the horse. G. After the horse ingests the L3, it encysts, to be then known as the EL3. H. The EL3 develops and molts to the LL3/L4 stage within the tissue. The L4 excysts, and enters the lumen of the large intestine, where they molt to the adult stage (A) and continue the lifecycle. The period of time to go from B-F (egg to infective L3) is typically 14 days on pasture. To go from F to A, the prepatent period, is 6 weeks to 2 years (Ogbourne, 1975).

The favorable conditions for environmental cyathostomin stages (eggs and larvae) have been well studied and modelled. The optimum temperatures for eggs to hatch and grow to the infective stage has been observed in vitro. The optimum temperature range is 25-33°C, with highest infective stage larval yield at 28°C (Lucker, 1941; Mfitilodze and Hutchinson, 1987; Ogbourne, 1972; Rupashinge and Ogbourne, 1978). The lower limit for egg hatching has been observed to be between 7.5-10°C, with the upper limit at 38°C; no eggs have observed to hatch at <4°C, and they die quickly at 40°C (Lucker, 1941;
Mfitilodze and Hutchinson, 1987; Ogbourne, 1972; Rupashinge and Ogbourne, 1978). Studies have also shown that long term freezing reduces larval yield; however, unembryonated eggs withstand colder temperatures more so than embryonated eggs, and L1 and L2 larvae were the most susceptible (Lucker, 1941; Rupashinge and Ogbourne, 1978; Zahner et al., 1997). L3 larvae are less susceptible to cold and more susceptible to higher temperatures. L3s survive longer at -5 and 3°C than at 26 and 31°C (Lucker, 1941; Rupashinge and Ogbourne, 1978; Zahner et al., 1997). The most detrimental effect on growth and survival was not a single freeze, but repeated freeze/thaw cycles or long term freezing over several months (Lucker, 1941; Rupashinge and Ogbourne, 1978; Zahner et al., 1997).

Once ingested by the horse, the L3 larvae exsheath in the small intestine, and then continue their migration to the large intestine. The L3 larvae are then considered an early third stage larva (EL3), which penetrates the mucosa of the CEC, VC, and DC, where a cyst is formed from connective tissue around the larvae (Ogbourne, 1978). At this point, the larvae may undergo arrested development (hypobiosis), where they remain dormant from 2 weeks to 2 years or more. (Gibson, 1953; Ogbourne, 1975). At this stage, it is suggested that no growth and activity occurs. Hypobiosis has been related to environmental factors, such as larval uptake, host factors, such as their individual immune response and age, and even due to the presence of other parasites (Smith, 1976a, 1976b). It has been suggested that the luminal stages have a negative feedback that inhibits the larval stages from further development. Removal of the adult parasites then stimulates the development of the inhibited larvae (Smith, 1976a, 1976b). Trickle experimental infections carried out to mimic how a host would obtain the parasites, have been shown to induce greater arrested
development than large quantities of infective larvae administered at once, indicating a feedback mechanism between the larvae, host, and luminal stages (Love et al., 1999). Once the larvae resume development, they molt into the 4th larval stage, and then excyst into the lumen of the intestine. In the intestine, they will undergo one final molt to the adult stage (Ogbourne, 1978). Overall, the prepatent period, the time from ingestion to eggs being passed in the feces can occur in as little as 2-3 months, but can be as long as two to three years (Gibson, 1953; Ogbourne, 1975; Round, 1969; Smith, 1976a).

1.2.2 Cyathostomins and their nutrient requirement and acquirement

When discussing cyathostomins and all parasitic stages, little work has investigated the nutrient needs and uptake of the parasites. This is due to the difficulty of evaluating the parasitic stages, particularly in vitro (0). However, studies have been performed with the environmental stages of these parasites.

Environmental stages are thought to be bacteriophagic and coprophagic, due to larval growth habits, composition of the larvae’s intestinal cells, and inference from other strongyloid parasites with similar life cycles. It is also noted that the lipids in the intestinal cells of L3 larvae are used to assess viability and survivability, with lower concentrations indicating lower infectivity rates (Baker et al., 1939). Studies have noted that repeated exposure to differing environmental conditions and long-term storage severely impact survival and lipid concentrations as well (Baker et al., 1939; Giovannola, 1936; Medica and Sukhdeo, 1997).
We do not know what or if the mucosal larval stage feeds on, whether it be the host or host secretions, passively absorbing nutrients from the intestinal content, or not feeding at all. Some theorize that no additional nutrients are required while in the larval stage, while others hypothesize that they feed on blood, due to bright red color of some of the larvae and their prominent tooth within the buccal capsule, or that they acquire nutrients from local cells and secretions (Cuillé et al., 1913; Ogbourne, 1978). It is important to note that there are those larvae that are undergoing hypobiosis, which would lead to reduced nutrient requirements, whereas developing larvae which likely do have differing metabolic requirements. Due to the difficulty of maintaining these larva stages in vitro (0), much is not known and subject to speculation and hypotheses.

There are two schools of thought on the feeding behavior of adult cyathostomins. One group theorizes that adults feed on the host and ingest blood and other serous nutrients by attaching to the large intestinal wall (Le Roux, 1924; Wetzel, 1930b). The more popular opinion is that the adults are bacteriophagic/coprophagic and survive off of luminal contents (Ihle, 1922; Looss, 1901; Rai, 1961; Theiler, 1923). This is due to the location of the adults found at necropsy, where they have been described as attached to the mucosa, along the mucosa, or within the luminal contents from just after death to a few hours, depending on the timing of necropsy (Ihle, 1922; Le Roux, 1924; Rai, 1961; Wetzel, 1930b). It is important to note that our lab has not seen cyathostomin adults attached to the mucosa (personal experience). Again, due to the difficulty of maintaining the adults in vitro (0), little is known about their nutrient requirements.

The species distribution of encysted cyathostomins within the mucosa of the large intestine remains largely unknown due to a lack of methods for differentiation of these
larval stages. One work (Ogbourne, 1975) suggests that of the 50 species described in equids, only 4 can be morphologically identified at the fourth larval stage. The species reported to infect the domestic horse in North America are reported in Table 1-1. Different species prefer different organs (Bellaw et al., 2018; Matheison, 1964; Ogbourne, 1978, 1975; Reinemeyer et al., 1986); however, the species prevalence and relative abundance may be impacted by treatments (Bellaw et al., 2018).

**Table 1-1. Species of small strongyles that infect the domestic horse. Asterisks indicate that it can be found infecting horses in North America. Information taken from Lichtenfels, 2008.**

<table>
<thead>
<tr>
<th>Cyathostomum</th>
<th>Coronocyclus</th>
<th>Cylicostephanus</th>
<th>Skrjabinodentus</th>
<th>Cylicodontophorus</th>
<th>Cylicocyclus</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. tetracanthum*</td>
<td>C. coronatus*</td>
<td>C. calicatus*</td>
<td>S. tschoijoi</td>
<td>C. bicorona*</td>
<td>C. radiatus*</td>
</tr>
<tr>
<td>C. alveatum*</td>
<td>C. labiatus*</td>
<td>C. asymmetricus*</td>
<td></td>
<td>T. gobi</td>
<td>C. ashworthi*</td>
</tr>
<tr>
<td>C. catinatum*</td>
<td>C. labratus*</td>
<td>C. bidentatus*</td>
<td></td>
<td></td>
<td>C. auriculatus*</td>
</tr>
<tr>
<td>C. montgomeryi</td>
<td>C. sagittatus</td>
<td>C. goldi*</td>
<td></td>
<td></td>
<td>brevicapsulatus*</td>
</tr>
<tr>
<td>C. pateratum*</td>
<td>C. ulambajari</td>
<td>C. hybridus*</td>
<td></td>
<td></td>
<td>C. elongatus*</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>C. longibursatus*</td>
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<td>C. insignie*</td>
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<td>C. leptostomum*</td>
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<td></td>
<td>C. nassatus*</td>
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<td></td>
<td></td>
<td></td>
<td>C. ultrajectinus*</td>
</tr>
<tr>
<td>Poteriostomum</td>
<td>Parapoteriostomum</td>
<td>Hsiungia</td>
<td>Caballonema</td>
<td>Gyalcephalus</td>
<td>Petrovinema</td>
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<tr>
<td><em>P. imparidentatum</em></td>
<td><em>P. mettami</em></td>
<td><em>H. pekingensis</em></td>
<td><em>C. longicapsulatum</em></td>
<td><em>G. capitatus</em></td>
<td><em>P. skrjabini</em></td>
</tr>
<tr>
<td><em>P. ratzii</em></td>
<td><em>P. euproctus</em></td>
<td></td>
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<td><em>P. poculatum</em></td>
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<td><em>P. mongolica</em></td>
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</tbody>
</table>

Table 1-2. (continued)

1.2.3 Pathogenicity and pathophysiology

While early reports associated cyathostomins with disease, they were generally considered of little clinical importance, especially when compared to other parasites, like *S. vulgaris* (Cuillé et al., 1913). After the significant reduction of *S. vulgaris* in managed herds, attention was drawn to horses presented for a severe clinical disease that was linked to larval cyathostomins, and therefore the disease was named “larval cyathostominosis” (Herd, 1990; Love et al., 1999). This disease occurs when there is mass excystment of L4 larvae from the mucosa into the lumen of the large intestine (Davidson et al., 2002; Love et al., 1999; Murphy and Love, 1997; Ogbourne, 1978).

The primary clinical signs associated with larval cyathostominosis are profuse watery diarrhea, weight loss, and emaciation (Love et al., 1999). Disease is characterized by a severe, sudden onset diarrhea, which then leads to dehydration, emaciation, and resulting in potential death in two to three weeks after clinical signs became apparent (Love et al., 1999; Peregrine et al., 2006). The diarrhea and emaciation are generally accompanied by a protein-losing enteropathy, hypoproteinemia, and edema (Love et al., 1999).
In Europe, a distinct seasonality and age distribution have been previously noted amongst larval cyathostominosis cases. Most cases are observed in late winter and early spring in horses 2-5 years of age (Reid et al., 1995). Whereas, in North America, cases occurred through October to May, with the majority occurring in October through December in horses with a median age of 1 year (Peregrine et al., 2006). Cyathostomins have also been implicated in disease in horses with recurrent diarrhea and weight loss, edema, and pyrexia without diarrhea in the fall (Mair, 1994) and other studies have implicated cyathostomins in horses with weight loss of several months duration prior to the onset of diarrhea (Mirck, 1977; Murphy et al., 1997; Reilly et al., 1993). Experimental infections with cyathostomin larvae have exhibited minor effects from change in fecal consistency and decreased intestinal motility to reduced weight gain and diarrhea for up to 52 weeks post infection (Love et al., 1999; Murphy and Love, 1997; Scháňková et al., 2014).

As far as diagnosis of clinical disease, there are no pathognomonic findings. Complete blood counts (CBC) and chemistry may reveal a neutrophilia and hypoalbuminemia, which fit with clinical signs mentioned above (Mair, 1994; Murphy et al., 1997). Other findings, that have been associated, are anemia, eosinophilia, and an increase in serum alkaline phosphatase (Murphy et al., 1997).

Cyathostomins have been implicated in several different types of colic, including: cecocecal intussusception, non-strangulating infarctions, and nonspecific medical colic (Lyons et al., 1994; Mair et al., 1999; Mair and Pearson, 1995; Uhlinger, 1990). This loose association with colic is reported for many diseases and is considered nonspecific in many cases, as colic arises from a broad range of etiologies. One possible mechanism behind
colic is decreased intestinal motility. This has been shown to occur in mixed cyathostomin infections; however, the exact mechanisms have not been identified at this time (Bueno et al., 1979).

At the mucosal level, tissues from horses with clinical disease and clinically healthy horses with cyathostomin infections are well described. In horses diagnosed with acute larval cyathostominosis, generalized inflammation and edema of the CEC and VC and DC occurs, with fibrin deposits, hemorrhagic foci and necrotic and granulomatous nodules throughout the mucosa. Large numbers of larvae are found both within the mucosa (20-50/cm²) and the contents, and there is enlargement of the mesenteric lymph nodes (Matheison, 1964; Tiunov, 1953; Velichkin, 1952). Histopathological examinations have revealed diffuse edema and disruption of the intestinal mucosa with a diffuse marked cellular response in the lamina propria and/or submucosa (Matheison, 1964; Tiunov, 1953; Velichkin, 1952). In a few studies, this reaction was more evident around encapsulated larvae (Giles et al., 1985; Matheison, 1964; Tiunov, 1953). The cellular response includes mixed populations of mononuclear cells, as mentioned above, eosinophils, and macrophages. Fibrous capsules were also found surrounding the larvae, as well as areas of hemorrhage with eosinophils. Dilated submucosal lymphatics and epithelial shedding were also noted. In one study, the inflammation in the large intestine was accompanied by inflammation and infiltration of inflammatory cells and edema into the lamina propria and submucosa of the jejunum and rectum (Church et al., 1986). Caution should be used when analyzing these findings, as these are locations that the larvae do not normally infect.

Clinically normal horses also exhibit pathology to the invasion of the parasites. Surrounding the larvae are fibroblasts that create a thickened layer of connective tissue,
encapsulating the larvae. Lymphocytes, plasma cells, macrophages, mast cells, neutrophils, and eosinophils surround the encapsulated larvae and occur infiltrate between the larvae and the basement membrane of the mucosa (Matheison, 1964; Steinbach et al., 2006; Tiunov, 1953). As the larvae grow, they increase in size, distort the normal tissue architecture, and induce goblet cell hyperplasia (GCH) and hypertrophy (Matheison, 1964). Larvae that encyst in the lamina propria have been reported to induce a primarily lymphocytic, plasmocytic reaction with a small fibrous capsule whereas larvae in the submucosa produce large fibrous and collagenous capsules with a plasmocytic, eosinophilic, histiocytic invasion (Matheison, 1964; Nielsen et al., 2015; Steinbach et al., 2006; Tiunov, 1953).

The current belief is that the disease causes a protein-losing enteropathy due to the mass excystment compromising the mucosal barrier and integrity (Love et al., 1999). Love et al. evaluated urinary excretion of chromium ethylenediaminetetraacetate to assess intestinal permeability. They found that the mucosal barrier was compromised following cyathostomin infections (Love et al., 1991). However, to assess a true protein-losing enteropathy, plasma proteins must be identified within the contents of the large intestine (Love et al., 1991). Studies have evaluated protein loss in later stage cyathostomin infections using radioisotope labeled protein markers; however, no differences were shown between infected ponies and controls (Love et al., 1991).

1.2.4 Treatment

Treatment options have been developed over the years to target several different species of parasites, and these are now known as “broad spectrum” anthelmintics. Over the past 100 years, 25 compounds in 6 drug classes were marketed (Lyons et al., 1999);
however, three drug classes with six compounds are currently available and labeled for use against cyathostomins in the United States: The benzimidazoles, pyrantel salts, and macrocyclic lactones (Branan et al., 2018; Nielsen et al., 2019). Each of these has a different mechanism of action, and differing levels of resistance reported in cyathostomins. They also target different stages of the parasite. There is also reports on how anthelmintics interact with the parasites and the host immune system. It has been documented that some adulticidal therapies (those strictly targeting adult & luminal cyathostomins) may increase the risk of larval cyathostominosis (Reid et al., 1995). Veterinarians and horse owners have raised concern that larvicidal activity may also increase the inflammatory reaction from dying larvae; however, this has not been evaluated in any studies. The following section will introduce the anthelmintic drug classes currently available in the United States. The inflammatory reactions that have been studied will be discussed in 1.3.2.1.1. Anthelmintic resistance will be mentioned; however, this is not a primary focus of this dissertation, aside from what effect a given anthelmintic may have on worm burdens and the immune response.

1.2.4.1 Benzimidazoles

Benzimidazoles (BZs) are well known parasiticides in the equine market and have been commercially available since the 1960s, making them the oldest drug class currently available in horses (Lyons et al., 1999; Nielsen et al., 2019). Benzimidazoles are labeled as broad spectrum anthelmintics, targeting large and small strongyles, ascarids, pinworms, and intestinal threadworms. Benzimidazoles inhibit the beta-tubulin formation within the nematodes. This leads to paralysis of the intestinal tract of the worms, leading to starvation and death, or a “slow-kill” (Lacey, 1988) These drugs have a wide margin of safety in
animals, as they are preferential to helminth and not mammalian microtubules (Lacey, 1988). The current compounds available in the US are fenbendazole and oxibendazole. Fenbendazole is sold in two dosing regimens: a single oral dose at 5 mg/kg and a five-day double dose at 10mg/kg. The main difference between these two treatment regimens is the ability of the five-day double dose to target the migrating and arrested larvae of the large strongyles, ascarids, and cyathostomins\(^\text{12}\). Anthelmintic resistance is reported to be widespread in cyathostomins to both the single and five-day double dose of fenbendazole and across all stages (Bellaw et al., 2018; Branan et al., 2018; Lyons et al., 2016; Reinemeyer et al., 2015).

### 1.2.4.2 Pyrantel Salts

Pyrantel salts, also known as pyrimidine compounds, are the second oldest drug class used in horses in the United States today. These were originally made available in the 1970s (Lyons et al., 1999). Pyrantel exists in two formulations for horses: Pyrantel pamoate and pyrantel tartrate. Pyrantel pamoate is a single oral dose suspension or paste, whereas pyrantel tartrate is a continuous daily pelleted feed. Pyrantel pamoate, at 6.6 mg/kg, is labeled as a broad spectrum product; however, it does not have larvicidal efficacy against encysted mucosal or migrating larvae\(^\text{3}\). Pyrantel tartrate, at 2.65 mg/kg daily has similar label claims as pyrantel pamoate; however, the label claim states that it is intended for the use of prevention of \textit{S. vulgaris} larval infections\(^\text{4}\).

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Pyrantel works by both depolarizing and causing excitatory effects on the muscle fibers of helminths (Reinemeyer, 2016). It is a nicotinic acetylcholine receptor agonist and acts on ionotropic receptors and not muscarinic receptors (Reinemeyer, 2016). It has a high affinity for nematode receptors, specifically. Resistance to these compounds is common in cyathostomins throughout the US (Branan et al., 2018; Reinemeyer, 2016).

1.2.4.3 Macrocyclic Lactones

Macrocyclic Lactones (MLs) were introduced in the 1980s as broad spectrum anthelmintics (Lyons et al., 1999). The first compound introduced was ivermectin, an avermectin class drug of MLs. Ivermectin induces flaccid paralysis and starvation by binding to the parasite’s glutamate gated chloride channels in the muscle and neural tissue, most specifically the somatic and pharyngeal muscles (Martin et al., 2016). Moxidectin was introduced in the 1990s and is a milbemycin ML. It too causes flaccid paralysis and starvation by the same mechanism as ivermectin (Martin et al., 2016). There are few key differences between ivermectin and moxidectin. Moxidectin is more lipophilic, which allows it to persist longer in the tissues and also allows it better access to the encysted stages of cyathostomins. Ivermectin, at 0.2 mg/kg single oral dose, is not labeled to target the encysted stages\(^5\); and the efficacies vary and are reported in Table 1-3; however, the single study reporting the high encysted larval efficacy is an apparent outlier. Moxidectin, on the other hand, at 0.4 mg/kg single oral dose, is labeled against the LL3 and L4s, EL3s in the US\(^6\). Moxidectin range of efficacies against the stages are reported in Table 1-3. One final note is that praziquantel, one of two compounds effective against equine tapeworms,

\(^5\) https://dailymed.nlm.nih.gov/dailymed/drugInfo.cfm?setid=8c8bf574-0b67-4248-a8f6-130e8e5c1ed3  
\(^6\) https://www.zoetisus.com/contact/pages/product_information/msds_pi/pi/Quest_Gel.pdf
the other being pyrantel; pamoate, is only available in combination with ivermectin or moxidectin.

**Table 1-3. Previously reported percent efficacies of moxidectin and ivermectin in horses with natural and experiential cyathostomin infections. Asterisk indicates that the percentages were calculated based on data presented, and not calculated by the authors. Dash indicates data not provided and blanks indicate that the anthelmintic was not evaluated in the study.**

<table>
<thead>
<tr>
<th>Study</th>
<th>Moxidectin</th>
<th>Ivermectin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EL3</td>
<td>LL3/L4s</td>
</tr>
<tr>
<td>(Klei et al., 1993)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Xiao et al., 1994)</td>
<td>0</td>
<td>62.6-79.1</td>
</tr>
<tr>
<td>(Love, 1995)</td>
<td>-</td>
<td>77%</td>
</tr>
<tr>
<td>(Monahan et al., 1996)*</td>
<td>34.0-52.6</td>
<td>16.7-50.0</td>
</tr>
<tr>
<td>(Eysker et al., 1997)</td>
<td>18.5</td>
<td>89.6</td>
</tr>
<tr>
<td>(Steinbach et al., 2006)*</td>
<td>96.7</td>
<td>100</td>
</tr>
<tr>
<td>(Reinemeyer et al., 2015)</td>
<td>63.6</td>
<td>85.2</td>
</tr>
<tr>
<td>(Bellaw et al., 2018)</td>
<td>71.8-73.8</td>
<td>0.9-74.6</td>
</tr>
</tbody>
</table>

1.2.5 Diagnosis

Diagnosing cyathostomin infection versus disease has led to much confusion and frustration for veterinary practitioners, horse owners, and even parasitologists. Disease is a clinical diagnosis, based on clinical signs, CBC/Chemistry and ultrasound, response to
treatment, and necropsy findings (Love et al., 1999; Peregrine et al., 2006). Routine
diagnostic testing for cyathostomins are as follows; however, they test for infection and
not disease.

1.2.5.1 Coprological Methods

The most common diagnostic tests involve using feces to identify infections. This
was first used in the early 1900s, after the discovery that many parasites pass stages in the
feces (Bass, 1909). This section will cover the most common fecal diagnostics for
cyathostomins below.

1.2.5.1.1 Fecal Flotation and Egg Counts

The primary fecal diagnostic method is using a fecal flotation. Parasite ova, larvae,
or cysts are floated based on a solution with greater specific gravity than the parasite
objects, but lower than miscellaneous fecal particulates. The primary principal is to
increase parasite yield and decrease background particulates. There are a few different
flotation methods, including passive, active, qualitative and quantitative.

Passive flotation involves allowing eggs to rise in the flotation media using just
gravity to separate the parasitic stages and feces. Active flotation involves enhanced G
force, usually with a centrifuge, to force the parasitic stages to the surface and particulates
to the bottom (Dryden et al., 2005).

Qualitative flotations are generally used in small animal practice. This involves
feces, usually a thumb sized amount, that is not accurately measured by neither weight nor
volume. The number of parasite ova, larvae, or oocysts, is generally not considered
important in small animal practice, as worm burden does not correlate with disease and
spread (Dryden et al., 2005). In large animals, quantitative fecal techniques, also known as fecal egg counts (FECs), are applied more commonly. With FECs, such as determined with McMaster or Mini-FLOTAC techniques, a known quantity of feces is used and added to a known quantity of flotation medium, and a subsample examined underneath the microscope (Ballweber et al., 2014; Cringoli et al., 2017a). This gives an estimation of the number of eggs per gram (EPG) being passed in the feces. EPG does not correlate with worm burden and disease. FECs are used for herd management and managing infection pressure and resistance (Nielsen et al., 2019). With regards to strongyles in horses, the eggs passed in the feces cannot be differentiated between genera or species by morphologic appearance, and therefore cannot be used to differentiate types of strongyle infections.

1.2.5.1.2 COPROCULTURE

Coprocultures are the next step for differentiating strongyle infections in horses. The principal is simple: allow the eggs to hatch and larvate to the infective stage, as they would on pasture. At this stage, the *Strongylus* spp., *Triodontophorus* spp., and a few of the strongylid and cyathostomin genera, and a few species, can be differentiated based on size and number of intestinal cells (Russell, 1948). This is useful in identifying large strongyle infections within a herd to develop management protocols (Bellaw and Nielsen, 2015; Kuzmina et al., 2012).

1.2.5.2 Enzyme-Linked Immunosorbent Assays (ELISA)

Enzyme-Linked Immunosorbent Assays (ELISAs) were first described in the 1970s (Engvall, 1971). The testing usually involves detecting and potentially quantifying antigen and antibodies (along with other peptides, proteins, and potentially hormones) (Engvall,
While this method is common in other host species for diagnosing parasite infection (i.e., heartworm in dogs (Zecca et al., 2018)), it is not routinely performed in horses in the US. In Europe and a few diagnostic laboratories in the US, an A. perfoliata antibody ELISA, which uses saliva and/or serum, is used to identify exposure and infection (Nielsen, 2016a). For small strongyles, a commercial test became available in the United Kingdom in 2019. While a previous serum antibody ELISA screened for infection of all parasitic stages for 11 species of cyathostomins (Mitchell et al., 2016), this platform is similar to Tzelos et al., and uses 5 cyathostomin antigens for the 5 most common species in horses (Tzelos et al., 2020). The current sensitivity is 98% and specificity is 81%; however, it does not differentiate between the luminal and encysted stages.

1.2.5.3 Polymerase Chain Reaction (PCR)

PCR is a diagnostic and research technique, where the general principle is that PCR takes small DNA segments and amplifies the target of interest (Mullis et al., 1992). They can be used for quantification (how much of a target is present) or qualitative testing (is the target present or not). In veterinary helminth parasitology, PCR has found limited use in commercialized diagnostic testing; however, various assays have been produced for use in research (Bredtmann et al., 2017; Davidson et al., 2005a; Hodgkinson et al., 2005; Manyazewal et al., 2018). In horses, few PCRs are available for diagnosing parasitic diseases, especially those of helminths (Andersen et al., 2013a). Currently, only one PCR is available in the United States for detection of Strongylus vulgaris in feces (Andersen et al., 2013a).

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7 https://www.austindavis.co.uk/small-redworm-blood-test
8 https://cdn.website-editor.net/88f85cd9154141f880badb10905b7906/files/uploaded/Austin%2520Davis%2520Small%2520Redworm%2520Guidelines%2520Final.pdf
al., 2013b). Others have tested PCR for cyathostomin infections and potential species differentiation; however, it is not commercially available due to the wide variation of species of cyathostomins (Bredtmann et al., 2017; Hodgkinson et al., 2005).

A combination PCR-ELISA has been developed for detection of strongyle eggs (Hodgkinson et al., 2005, 2003). A PCR–ELISA uses portions of PCR products and amplification to identify and quantify a target. In cyathostomins, this technique has been developed for the identification of cyathostomin fourth-stage larvae in horses with clinical cyathostominosis. This PCR-ELISA tested for 6 species and an overall marker for all cyathostomins. They found that disease was likely due to mixed infections in many cases (Hodgkinson et al., 2003). Hodgkinson et al. then expanded their PCR-ELISA for testing the species composition of strongyle eggs after treatment for 19 cyathostomin species to see their fluctuations after treatment; however, it does not quantify their burden (Hodgkinson et al., 2005).

Another PCR hybridization is the use of reverse line blot for identification of strongyle species (Cwiklinski et al., 2012; Ionita et al., 2010; Traversa et al., 2008; Van Doorn et al., 2010). This, similar to PCR-ELISA, allows for the detection of multiple species in one sample. It allows visualization of DNA bands on a gel via electrophoresis of the PCR products (Ionita et al., 2010; Traversa et al., 2008; Van Doorn et al., 2010).

1.2.5.4  *In vitro* cultivation of cyathostomins

Many studies have used culturing of cyathostomin larvae to the infective L3 stage for a variety of purposes, including antigen harvesting and anthelmintic testing (Hu et al., 2018; Kinsella et al., 2002; Payne et al., 2013; Peachey et al., 2016; Van Doorn et al.,
While these studies are useful for studying cyathostomins, the L3s are not the parasitic stages in horses, and few comparisons have been made between these and the parasitic stages, in terms of their in vitro behavior and antigenic responses. Few studies have attempted to culture the parasitic stages of cyathostomins with little success, as they do not survive well outside of the host (Brianti et al., 2009; Chapman et al., 1994). Additionally, adults do not survive well in RPMI 1640 medium for more than 24 hours (personal communications with Jennifer Bellaw and own observations). This is likely due to damage during harvesting, lack of proper nutrients, and temperature variability. The use of rodent models has been previously explored; however, they did not result in patent infections in rodents (De Blieck and Baudet, 1926; Klei and Chapman, 1999; Schoene, 1938; Scmid and Johannsen, 1937).

1.3 Equine Immune System

1.3.1 Overview of the Equine Immune System

The immune system is a complex barrier of cells, proteins, and signals that protects the body from foreign invaders. The body does this by responding in different mechanisms to pathogens (for example), such as bacteria, viruses, fungi, and parasites, generating inflammation. Through this process, the body must register “self” versus “non-self.” There are several ways to define the immune system and its response: innate and adapted, type 1 and type 2, proinflammatory and anti-inflammatory, etc.

Several organs and barriers are key to the immune response and protection of the host. Many of the circulating inflammatory cells are produced centrally, in the bone marrow or thymus, and occasionally lymph nodes and the spleen, and then migrate peripherally to lymph nodes, spleen, and mucosal associated tissues (mucosal associated
lymphoid tissues (MALT): this includes bronchus associated lymphoid tissue (BALT) and gut-associated lymphoid tissue (GALT)) to finish development and undergo activation. Cells will also circulate and migrate to the local tissues to react quickly to potential antigen. Epithelial cells of the skin and mucosa are also a barrier, both physically with clearance mechanisms and through secretions. These cells can also secrete inflammatory modulators in response to pathogens, recruiting inflammatory cells to respond (Felippe and Julia., 2016). This section will be broken down into innate vs adapted, cell types, type 1 and type 2, as well as an overview of cytokines and their potential role in horses.

1.3.1.1 Innate Immune Response

The innate immune system is considered a generalized, unspecific first response to pathogens (Sun et al., 2014). It involves unspecific cell-mediated immunity and barriers, including mucosal and skin barriers. All mucosal surfaces, and certain white blood cells, fall into the innate immune response including neutrophils, eosinophils, macrophages, dendritic cells, basophils, and mast cells (Sun et al., 2014). Certain cells induce inflammation and therefore recruitment of other inflammatory cells and upset the desirable conditions that the pathogen needs for survival (Sun et al., 2014).

1.3.1.2 Adaptive Immune Response

The adaptive immune response involves prior exposure to pathogens, to allow the immune system to recognize the pathogen from memory (Fagarasan et al., 2010). It allows for long term recognition of specific pathogens for quick attack. The innate immune response is still required for the adaptive immune response to mount an attack (Fagarasan
et al., 2010. The Adaptive immune response is further divided into cell-mediated and humoral responses (Fagarasan et al., 2010).

1.3.1.3 Type 1 Response

The immune response can further be classified into type 1 and type 2 responses. Type 1 immune response is typically thought of as acting against intracellular pathogens, such as bacteria, viruses, and protozoal infections. It’s comprised of both innate and adaptive immune responses, including interferon-gamma producing cells (such as CD4+ T helper cells), and cytotoxic lymphocytes (such as CD8+ T cells and natural killer cells). Cytokine producing cells produce inflammatory markers that can recruit and cause adaptation of B lymphocytes and other antibodies (Spellberg and Edwards, 2001). Type 1 responses typically allow chronic infection and fibrosis in helminth rodent models (Spellberg and Edwards, 2001).

1.3.1.4 Type 2 Response

The type 2 immune response is an important component in helminth infections. It is composed of portions of both the innate and adaptive responses. Type 2 immunity produces greater quantities of antibody than type one, and also suppresses phagocytosis, compared to a type one mediated response (Spellberg and Edwards, 2001). Except in the event of large pathogens, like parasites, type 2 immunity is typically involved with resolving cell-mediated inflammation (Spellberg and Edwards, 2001). Type 2 immunity usually causes a diminished worm burden in helminth infection models (Spellberg and Edwards, 2001).

1.3.1.5 Cells involved in the immune response
White blood cells are key components of the host pathogen response (Spellberg and Edwards, 2001). Entire textbooks and theses are dedicated to individual cell types and arms of their development. This section will give an overview of these cell types and function, with particular regard to parasitic disease, as these are used to evaluate the inflammatory response in subsequent chapters.

1.3.1.5.1 Neutrophils

Neutrophils are granulocytes that are a key component of the innate immune system. Cytokines, such as Interferon-γ (IFN-γ), direct neutrophils to the site of inflammation, which are released by epithelial cells, mast cells, and macrophages (Hellebrekers et al., 2018). Neutrophils primarily undergo phagocytosis and degranulation in response to bacteria and other, primarily intracellular, pathogens (Hellebrekers et al., 2018). In phagocytosis, they ingest microorganisms to remove them from the surrounding tissue, destroying the microorganisms in the process (Hellebrekers et al., 2018). During degranulation, the neutrophils release granules into the surrounding tissue that target the microorganisms, thereby leading to their death (Hellebrekers et al., 2018). Finally, neutrophils may also form neutrophil extracellular traps (NETs) (Caro et al., 2014). These traps form web fibers between the neutrophils, trapping extracellular bacteria and other pathogens (Caro et al., 2014). NETs may also serve as a physical barrier to prevent the infectious agent’s spread (Caro et al., 2014). In cyathostomin infections in horses, neutrophils are reported to surround the encysted larvae in the tissues (Matheison, 1964; M. K. Nielsen et al., 2015; Ogbourne, 1978; Steinbach et al., 2006; Tiunov, 1953). In larval cyathostominosis cases, neutrophilia is a potential component of the disease (Giles et al., 1985; Mair, 1994; Murphy and Love, 1997); however, neutrophil response, since it is part
of the innate immunity, is a part of all inflammatory responses and not just parasite infections.

1.3.1.5.2 EOSINOPHILS

Eosinophils are another key granulocyte of the innate immune system. These cells are implicated in both parasitic diseases and allergic reactions, and conditions such as asthma and anaphylaxis (Gleich, 2013). Eosinophils contain a number of important granules that target helminths during infections and eosinophil proliferation is mediated by IL-5, and can produce cytokines, such as Interferon-γ, Interleukins 4-6, and 10 (Adamko et al., 2013; Alam et al., 2013; Cadman et al., 2014; Wennerås et al., 2013).

While eosinophils appear to be a critical component of nematode infections, their role in worm expulsion has not been identified (Abraham et al., 2013; Allen and Sutherland, 2014; Klion and Nutman, 2004). In Nippostrongylus brasiliensis infections in mice, it has been found that a lack of eosinophils increases the susceptibility to infection, where greater numbers of larvae migrated to the lung, but did not contribute to their expulsion from the small intestine (Knott et al., 2007). In cyathostomin infections, eosinophils are reported in response to the larvae (Church et al., 1986; Giles et al., 1985; Matheison, 1964; Steinbach et al., 2006; Tiunov, 1953); however, eosinophilia is a less consistent finding in diseased animals (Murphy et al., 1997).

1.3.1.5.3 MACROPHAGES

Macrophages are another key component of the innate immune response. These phagocytic cells are found throughout the body and the tissues (Ruckerl et al., 2013). They specialize in removing cellular debris, and dead or dying cells, especially in chronic
inflammation, due to the death and clearance of neutrophils, and also eradicate microbes (Rückerl et al., 2013). After ingestion of cells, cellular debris, and portion of microbes, the macrophage can present antigens to helper T cells, initiating the adaptive immune response in hosts (Rückerl et al., 2013). Alternatively activated macrophages promote wound and tissue healing after infections and are key components of the Th2 and anti-inflammatory response (Rückerl et al., 2013).

1.3.1.5.4 DENDRITIC CELLS

Dendritic cells are antigen presenting cells that are specialized for the initiation of the adaptive immune response (Schraml and Reis e Sousa, 2015). Dendritic cells present antigen to T lymphocytes, forming a link between the innate and adaptive immune response by initiating and inducing T cell responses (Schraml and Reis e Sousa, 2015). They upregulate the expression of major histocompatibility complexes (MHCs) and secrete cytokines, such as Interleukin-12 to activate naïve T lymphocytes (Schraml and Reis e Sousa, 2015).

When dendritic cells are absent in several parasite models, a delayed and or compromised Th2 cell response occurs, indicating their crucial role in adaptive immunity (Ohnmacht et al., 2009; Reynolds et al., 2012)

1.3.1.5.5 BASOPHILS

Basophils are the least numerous granulocytes within the equine immune system, but they are the largest, accounting for <1% of peripheral white blood cells (Karasuyama et al., 2011). They contribute to acute and chronic allergic reactions and parasitic infections, especially in ectoparasitic infestations or parasites that ingest blood, as they
secret heparin, and anticlotting factor in the blood (Karasuyama et al., 2011). Basophils are also able to regulate T cells and their mechanisms and responses, and bind to immunoglobulin E, which is implicated in parasitic infections (Karasuyama et al., 2011). In some parasitic infections, basophils appear to be necessary for optimal worm expulsion (Reynolds et al., 2012).

1.3.1.5.6 MAST CELLS

Mast cells are another granulocyte, similar to basophils, that contain histamine and heparin (Mukai et al., 2018). They are also important in allergic and parasitic reactions, host defense, and immune tolerance (Mukai et al., 2018). When activated, mast cells release granules in response to immunoglobulin E or other pathogen recognition pathways (Mukai et al., 2018). When the mast cell degranulates, it releases important mediators, such as tumor necrosis factor-α, interleukin-4, and certain chemokines, such as eosinophil chemotactic factor, to attract other cell types (Mukai et al., 2018).

In *Heligosomoides polygyrus* infections in rodents, activated macrophages are an essential component in the hosts response to worm infection and expulsion. They also promote repairment of tissue damage created from the migration of *H. polygyrus* through the tissues (Reynolds et al., 2012).

1.3.1.5.7 B LYMPHOCYTES

B lymphocytes, or B cells, are a key component of humoral immunity of the adaptive immune system (Harris and Gause, 2011; Ludwig-Portugall and Layland, 2012). Their name arises because they initially develop in the bone marrow (Harris and Gause, 2011; Ludwig-Portugall and Layland, 2012). B cells secrete antibodies that bind to antigens
to target them for clearance and removal (Harris and Gause, 2011; Ludwig-Portugall and Layland, 2012). Depending on the type, they can also secrete cytokines and present antigens to other cell types (Harris and Gause, 2011; Ludwig-Portugall and Layland, 2012). On the cell surface, they express clusters of differentiation (CDs), during different phases of their life cycle (Casan et al., 2018). These can be used to identify them in the tissues. For example, we used a common marker, CD20 to identify B cells within the tissues of horses infected with cyathostomins (Steuer et al, 2018). CD20 concentrations increase until maturity (Casan et al., 2018). It’s exact function is not known, but it is critical for the immune response and suspected that it acts as a calcium channel in the membrane (Harris and Gause, 2011; Ludwig-Portugall and Layland, 2012). There are several types of B cells, including memory cells and plasma cells. Memory cells are designed to generate a rapid response to a pathogen that their activated parent B cell previously encountered (Harris and Gause, 2011; Ludwig-Portugall and Layland, 2012). Plasma cells are long-lived cells that secrete antibodies and immunoglobulins to mediate both B and T cells (Harris and Gause, 2011; Ludwig-Portugall and Layland, 2012).

1.3.1.5.8 T LYMPHOCYTES

T lymphocytes are a key factor of the cell-mediated adaptive immune response. They are so aptly named due to maturation and differentiation in the thymus of the host (Taylor et al., 2012). There are several types of T cells, including T helper cells, cytotoxic T cells, memory T cells, regulatory T cells, and innate T cells, such as Natural Killer T (NKT) cells and mucosal associated invariant T (MAIT) cells. T helper cells express CD4+ on their surface. They are activated when presented an antigen, and then secrete certain cytokines, depending on the type of helper cells. There are Th1, Th2, Th17, Th9, and Tfh
type helper cells within the body, named based on when they were identified and what they may excrete (Taylor et al., 2012). For example, Th1 cells are key in the inflammatory response in regard to intracellular microbes. They, in general, excrete cytokines such as Interferon-γ (Taylor et al., 2012). Th2 cells aid the B cells in production of antibodies and their differentiation. They secrete cytokines, such as Interleukin 4. Cytotoxic T cells, on the other hand, express CD8+ on their surfaces (Taylor et al., 2012). Their primary role is inducing cell apoptosis. They also excrete cytokines such as Interferon-γ and Interleukin-2. Memory cells play a similar role to memory B cells to aid in a quicker immune response to a pathogen. Regulatory T cells help maintain immunological tolerance and recognize self (Ludwig-Portugall and Layland, 2012). Finally, the innate cells, such as NKT cells and MAIT cells are associated with the bridging the adaptive and innate response. They can act as T helper cells and T cytotoxic cells.

Helminth infections, have been typically associated with a Th2 cell response (Taylor et al., 2012); however, while a Th2 is the primary response, it is an oversimplification to classify it as solely a Th2. Response, as several more types of cells and inflammatory markers are present in these more complex parasite interactions (Taylor et al., 2012). To aid in differentiation of the different T cell types and stages, CDs are used, as with B cells.

1.3.1.6 Immunoglobulins

There are a few different types of immunoglobulins (Ig), also known as antibodies, involved in the immune response. In general, Igs are produced by plasma cells, although a few of the aforementioned cell types may also produce small amounts (Baker et al., 2015; Woof and Mestecky, 2015). They are highly specific, with each of the subclasses and
isotypes performing a different function (Baker et al., 2015; Woof and Mestecky, 2015).

The types and general function are in Table 1-4.

**Table 1-4. Immunoglobulins produced in horses and their functions (Baker et al., 2015; Woof and Mestecky, 2015).**

<table>
<thead>
<tr>
<th>Immunoglobulin</th>
<th>Distribution</th>
<th>Produced by</th>
<th>Acts upon</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgA</td>
<td>Intravascular and secretions</td>
<td>Plasma Cells</td>
<td>Mucosal cells, enteric pathogens</td>
<td>Protection of mucus membranes</td>
</tr>
<tr>
<td>IgD</td>
<td>Lymphocyte surface</td>
<td>Immature B lymphocytes</td>
<td>B cells, Basophils, mast cells</td>
<td>Unknown</td>
</tr>
<tr>
<td>IgE</td>
<td>Basophil and mast cells</td>
<td>Plasma Cells</td>
<td>Eosinophils, Basophils, mast cells</td>
<td>Protection against parasites and allergies</td>
</tr>
<tr>
<td>IgG</td>
<td>Intra- and extra vascular</td>
<td>Plasma cells</td>
<td>Mast cells, antigens, initiating the complement pathway</td>
<td>Secondary response, innate, humoral</td>
</tr>
<tr>
<td>IgM</td>
<td>Primarily intravascular</td>
<td>Spleen; plasma cells</td>
<td>Antigens, activating the complement pathway</td>
<td>First response innate response, humoral</td>
</tr>
</tbody>
</table>
1.3.1.7 Cytokines and chemokines involved in the immune response

Cytokines and chemokines are essential for the host to defend itself from infections and to recognize self. These are messengers between the immune cells (Meyer et al., 2016). Table 1-5 highlights several important cytokines, including those in helminth infection and mucosal barrier function.

| Cytokine | Main producer(s): | Main Target(s) | Primary Function | Primary Classification | Researched in horses: | Role in helminth infections:
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>IL-1</td>
<td>Macrophages</td>
<td>Antigen Presenting Cells (APCs) T cells</td>
<td>Acute inflammation Pyrexia Stimulation of APCs and T cells</td>
<td>Proinflammatory</td>
<td>Yes (Nielsen et al., 2015)</td>
<td>Some (Anuradha et al., 2013)</td>
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<tr>
<td>IL-2</td>
<td>Activated Th1 Cells NK cells</td>
<td>B and Eosinophils T cells</td>
<td>Eosinophil recruitment and activation Proliferation of B and T cells</td>
<td>Proinflammatory</td>
<td>Yes (Davidson et al., 2002)</td>
<td>Yes (Turner et al., 2013)</td>
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<tr>
<td>IL-3</td>
<td>Activated T cells Eosinophils Mast cells</td>
<td>Basophils Eosinophils Hematopoietic progenitor cells Mast cells</td>
<td>Decrease Th1 response Eosinophil recruitment and activation Increase IgE Increase Th2 response</td>
<td>Proinflammatory</td>
<td>Yes (Janda et al., 2015)</td>
<td>Yes (Svetić et al., 1993)</td>
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<tr>
<td>IL-4</td>
<td>Activated Th2 cells Basophils Eosinophils Mast cells</td>
<td>B cells Eosinophils Mast Cells</td>
<td>B cell proliferation Mast cell growth Eosinophil recruitment and activation Increased mucin production and goblet cells</td>
<td>Proinflammatory</td>
<td>Yes (Davidson et al., 2002; Lanz et al., 2013)</td>
<td>Yes (Davidson et al., 2002; Lanz et al., 2013)</td>
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<tr>
<td>IL-5</td>
<td>Eosinophils Mast Cells Th2 Cells</td>
<td>Eosinophils</td>
<td>Eosinophil growth</td>
<td>Proinflammatory</td>
<td>Yes (Davidson et al., 2002)</td>
<td>Yes (Allen and Sutherland, 2002)</td>
</tr>
<tr>
<td>Cytokine</td>
<td>Main producer(s):</td>
<td>Main Target(s)</td>
<td>Primary Function</td>
<td>Primary Classification</td>
<td>Researched in horses:</td>
<td>Role in helminth infections:</td>
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</tr>
<tr>
<td>IL-6</td>
<td>Activated Th2 cells</td>
<td>B cells, T cells</td>
<td>Acute phase response, B cell proliferation, Increased IgE, T cell growth, Thrombopoiesis</td>
<td>Proinflammatory</td>
<td>Yes (Nielsen et al., 2015)</td>
<td>Yes (Nielsen et al., 2015; Licona-Limón et al., 2013)</td>
</tr>
<tr>
<td>IL-7</td>
<td>Bone Marrow, Thymus</td>
<td>Hematopoietic cells, T and B cell</td>
<td>Lymphopoiesis</td>
<td>Proinflammatory</td>
<td>Yes (Cook et al., 2008)</td>
<td>Yes (Wolowczuk et al., 1999)</td>
</tr>
<tr>
<td>IL-8</td>
<td>Macrophages, Somatic cells</td>
<td>Neutrophils, T cells</td>
<td>Chemotaxis</td>
<td>Pro-inflammatory</td>
<td>(Perkins et al., 2008)</td>
<td>Yes (Singh et al., 2013)</td>
</tr>
<tr>
<td>IL-9</td>
<td>Eosinophils, Th2 cells</td>
<td>Hematopoietic cells, Eosinophils</td>
<td>Lymphopoiesis, IgE production, Increase goblet cell growth and mucin production</td>
<td>Pro-inflammatory</td>
<td>Yes (Dewachi et al., 2006)</td>
<td>Yes (Licona-Limón et al., 2017)</td>
</tr>
<tr>
<td>IL-10</td>
<td>CD8+ B and T cells, Macrophages, Activated Th2 cells</td>
<td>B Cells, Mast cells, T cells</td>
<td>Antibody production, B cell proliferation, Decrease mast cell growth, Inhibits cytokine production</td>
<td>Anti-inflammatory*</td>
<td>Yes (Davidson et al., 2005a)</td>
<td>Yes (Licona-Limón et al., 2013)</td>
</tr>
<tr>
<td>IL-12</td>
<td>B Cells, Dendritic Cells, Macrophages, Monocytes, Th1 cells</td>
<td>B Cells, NK cells, T cells</td>
<td>Increased cell-mediated functions, Decrease Th2 response, Decreases IgE production, Proliferation of NK cells</td>
<td>Proinflammatory</td>
<td>Yes (Duran et al., 2013)</td>
<td>Yes (Setiawan et al., 2007)</td>
</tr>
<tr>
<td>IL-13</td>
<td>Basophils, Eosinophils, NK cells, Th2 cells</td>
<td>B cells, Eosinophils, Mast Cells</td>
<td>Same as IL-4, Increase goblet cells and mucin production</td>
<td>Proinflammatory</td>
<td>Yes (Lawson et al., 2019)</td>
<td>Yes (Lawson et al., 2019)</td>
</tr>
<tr>
<td>IL-17(a)</td>
<td>Activated CD4+ CD8+ memory cells</td>
<td>Neutrophils</td>
<td>Neutrophil recruitment, Activation of epithelial,</td>
<td>Pro-inflammatory</td>
<td>Yes (Page et al., 2017)</td>
<td>Yes (Serafini and Di</td>
</tr>
<tr>
<td>Cytokine</td>
<td>Main producer(s):</td>
<td>Main Target(s)</td>
<td>Primary Function</td>
<td>Primary Classification</td>
<td>Researched in horses:</td>
<td>Role in helminth infections:</td>
</tr>
<tr>
<td>----------</td>
<td>-------------------</td>
<td>----------------</td>
<td>-----------------</td>
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</tr>
<tr>
<td>IL-18</td>
<td>Macrophages, Th1 cells</td>
<td>NK cells, T cells</td>
<td>Induces IFN production by T cells and NK cells</td>
<td>Proinflammatory</td>
<td>Yes (O’Donovan et al., 2004)</td>
<td>Yes (Helmby and Grencis, 2002)</td>
</tr>
<tr>
<td>IL-25</td>
<td>Granulocytes, Mast Cells, Th2 cells, Tuft cells</td>
<td>Eosinophils, Goblet Cells, Somatic cells,</td>
<td>Increases cytokine production</td>
<td>Proinflammatory</td>
<td>No</td>
<td>Yes (Saenz et al., 2010)</td>
</tr>
<tr>
<td>IL-33</td>
<td>Epithelial Cells, Myeloid cells</td>
<td>Basophils, Dendritic Cells, Mast cells</td>
<td>Promotes accumulation, proliferation, and activation in the tissues, Increases cytokine production</td>
<td>Proinflammatory</td>
<td>No</td>
<td>Yes (Saenz et al., 2010)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Activated Th1 and NK cells</td>
<td>APCs, Macrophages, Neutrophils, Somatic cells</td>
<td>Antiviral effects, Decreases eosinophil counts, IgE and Th2 response, Induces antigen presentation, Promotes cell-mediated immunity</td>
<td>Proinflammatory</td>
<td>Yes (Davidson et al., 2005a)</td>
<td>Yes (Davidson et al., 2005a)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Macrophages, Mast cells, NK cells, Th1 cells</td>
<td>APCs, NK cells</td>
<td>Cell death, Inflammation</td>
<td>Proinflammatory</td>
<td>Yes (Davidson et al., 2005a)</td>
<td>Yes (Davidson et al., 2005a)</td>
</tr>
</tbody>
</table>

Table 1-6. (continued)
1.3.1.8 Other markers of inflammation

There are several other markers that can be used to assess inflammation in horses; veterinarians use these blood markers in routine screenings for signs of inflammation, and they are presented in Table 1-7.

**Table 1-7. Blood markers used routinely by veterinarians for diagnosis of inflammation in horses. Information sourced from (Stockham and Scott, 2008).**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Produced by</th>
<th>Function</th>
<th>State in inflammation</th>
<th>Status in cyathostomin infections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron</td>
<td>Uptake from diet</td>
<td>Transport oxygen in red blood cells</td>
<td>Decreased</td>
<td>Subtly associated (Andersen et al., 2014)</td>
</tr>
<tr>
<td>Serum Amyloid A (SAA)</td>
<td>Liver</td>
<td>Acute phase protein produced in response to cytokines. A potent inflammatory modulator</td>
<td>Increased</td>
<td>Not Associated (Andersen et al., 2014)</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>Liver</td>
<td>Increased inflammatory reaction</td>
<td>Increased</td>
<td>Subtly associated (Andersen et al., 2014)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>Liver</td>
<td>Increased in acute inflammatory disease</td>
<td>Increased</td>
<td>Subtly associated (Nielsen et al., 2013; Steinbach et al., 2006)</td>
</tr>
</tbody>
</table>
1.3.2 Equine Immune System in Parasitic Disease

Very little work has been done to date evaluating the equine immune system and parasitic disease. This section summarizes what we know of cyathostomins and other parasite infections in horses.

1.3.2.1 Responses to cyathostomins in vivo

For ease of breakdown, this section is further divided into the local and systemic inflammatory responses in cyathostomin infections. Given the concern that anthelmintic treatment may incite disease, many of the studies focus on these dynamic processes following treatment.

1.3.2.1.1 LOCAL

Few studies have evaluated the local response and other processes to cyathostomins within the tissues. In vitro work with these larval stages and adults has failed to yield high numbers of worms and attempts to keep these alive have generally not been successful as described in (Brianti et al., 2009). Therefore, at this time, the only option for studying these aspects of the lifecycle are through terminal studies.

In 1999, Klei and Chapman reviewed all literature concerning cyathostomins and the equine immune response. They reported the difficulties of establishing cyathostomin infections in rodent or other models. They also focused on the evaluation of age-related immunity. Older horses were reported to have lower FECs and worm burdens than younger horses, illustrating that a more developed immune system, as seen in older horses, decreases worm burdens and FEC output (Klei and Chapman, 1999). This was supported by Monahan et al., who described that older horses, compared to younger horses, had lower
mucosal larval burdens, specifically significantly lower EL3 burdens and slightly lower LL3/L4s burdens (Monahan et al., 1998).

Du Toit et al. (2007) evaluated the role of mast cells and mast cell proteinases in equine cyathostomin infections. They found that horses with encysted cyathostomin infections had significantly more tryptase activity in the VC and DC than uninfected horses. Also, the CEC of the horses expressed greater mast cell proteinase and had greater toluidine blue uptake than the other organs and therefore exhibited greater mast cell activity (du Toit et al., 2007). Pickles et al. (2010) also evaluated mast cells and their proteinases in the ceca of adult horses. They found that higher mast cell counts were associated with coinfection with *A. perfoliata*, but mast cell counts were not associated with proteinase activity. In this study, higher cyathostomin counts, both luminal and mucosal exhibited positive linear relationships with mast cell counts and their proteinases (Pickles et al., 2010). Collobert-Laugier et al. found increased numbers of eosinophils and mast cells in intestinal tissues with increased mucosal and luminal cyathostomin burdens. What they found overall, however, was that the cellular populations differed with age. In younger horses, there was a predominantly eosinophilic response, whereas older horses had a greater mast cell response in the tissues (Collobert-Laugier et al., 2002), suggesting an age related effect on the nature of the immune response.

When comparing larvicidal treatments, two studies have evaluated the mucosal response at 2 weeks post treatment (WPT) in cyathostomin infections. Steinbach et al., noted a mild increased inflammatory response to the five-day dose regimen of FBZ compared to moxidectin based on subjective histopathological findings (Steinbach et al., 2006). In 2015, Nielsen et al., semi-quantitatively evaluated the local histopathologic and
cytokine responses in horses treated with the larvicidal regimen of FBZ and MOX. While all responses were subtle, it appeared that MOX treated horses had a greater inflammatory response than the FBZ treated group, based on a greater cellular response and size of inflammatory nodules surrounding the larvae.

Few studies have evaluated the cytokine expression within the wall of the large intestine in horses with cyathostomin infection and disease. Davidson et al. (2005) quantitatively evaluated the presence of cytokines in horses infected with the encysted larval stages versus those that were not. They observed higher IL-4 expression with EL3 larvae in certain organs, such as the CEC compared to others; whereas other organs expressed higher IL-4 with later stage larvae comparatively (Davidson et al., 2005). TNF-α was difficult to quantify, as it was expressed at low concentrations within the tissues. IL-10 expression was significantly associated with EL3 and LL3/L4 larvae in the pelvic flexure of the colon. IFN-γ was not significantly associated with the larvae. They concluded that TNF-α was associated with intestinal pathology, whereas the other cytokines appeared to be involved with less pathology (Davidson et al., 2005). In horses with large intestinal disease, they did not detect the presence of TNF-α in horses with larval cyathostominosis (Davidson et al., 2002). They also found the larval burdens and gross mucosal pathology in larval cyathostominosis cases was most severe in the CEC and VC, where the greatest worm burdens occur (Davidson et al., 2002).

1.3.2.1.2 SYSTEMIC

In 2002, Dowdall et al. harvested and isolated antigen from encysted and luminal stages, to which they tested serum for IgG response to said antigens. They found that there
was a significant IgG response in horses infected with cyathostomins and none in uninfected controls; however, there was no association between the response and larval and adult cyathostomin burden. They hypothesized that later stages produce greater antigenic stimuli than early stages (Dowdall et al., 2002). Andersen et al. evaluated the acute phase proteins in the blood following cyathostomin and *S. vulgaris* infections in horses. This study reported possible hyperglobulinemia associated with parasitism in horses (Andersen et al., 2014).

Another study evaluated systemic circulation of acute phase inflammatory proteins and cytokines following treatment with moxidectin, pyrantel pamoate, or oxibendazole (Nielsen et al., 2013). These studies evaluated SAA, fibrinogen, and haptoglobin, as well as IL-1β, IL-4, IL-6 IL-10, IFN-γ and TNF-α. Minor increases in concentrations or expression occurred throughout the study. The only statistical differences between treatments occurred with fibrinogen, and, the only other factor contributing to statistically significant differences was time (Nielsen et al., 2013).

Another study evaluated the proinflammatory response following treatment and vaccinations in horses (Nielsen et al., 2015). While the data were not clear on the interactions, it was found that following vaccination and anthelmintic treatment, a less pronounced inflammatory response was produced (Nielsen et al., 2015). IL-10 was generally expressed in higher quantities in horses treated with anthelmintics versus the control group; however, IL-4 expression was highest in the untreated control group (Nielsen et al., 2015). The findings may have indicated a lower inflammatory response when anthelmintics were used in conjunction with vaccines; however, vaccine responses were still within normal parameters across the groups (Nielsen et al., 2015). Another study
evaluated the systemic response at two weeks post treatment in FBZ and MOX treated ponies and found that no systemic differences occurred between the FBZ and MOX treated groups and the horses in the untreated control group. There were no indications of a difference in systemic inflammatory responses to treatment (Nielsen et al., 2015).

Finally, systemic cytokine responses have been evaluated in older and middle aged horses (20-33 and 5-15 years old, respectively) following anthelmintic treatment with either pyrantel pamoate or MOX (Adams et al., 2015). They found that older horses had higher FECs and a more pronounced proinflammatory response to treatment than middle aged horses. This was not attributed to the worm burdens of the horses, but rather to the “inflamm-aging” phenomenon, where older horses have a greater inflammatory response than younger horses (Adams et al., 2015).

1.3.2.2 Cyathostomin responses in vitro

Few attempts have been made to harvest excretory/secretory (E/S) antigens from cyathostomins. For example, Paz-Silva et al. tested a liquid chromatography-based method for isolating proteins from E/S products of infective L3 larvae; however, they were not further classified or analyzed (Paz-Silva et al., 2011).

Another interesting study evaluated the stimulation of cyathostomin antigens on peripheral blood mononuclear cells (PBMCs) from healthy horses and horses with recurrent airway obstruction (RAO) (Lanz et al., 2013). They examined several cytokines, including IL-4, IL-10, IL-13, and INF-γ. They found that there were significant differences in IL-4 and Il-10 expression in those with RAO and those without RAO when exposed to the antigen (Lanz et al., 2013).
1.3.2.3 Immune reactions to other equine large intestinal parasites

The immune reaction to *Anoplocephala perfoliata* in the large intestine has also been evaluated. One study evaluated the effects of tapeworm antigen in vitro and the tissue pathology and gene expression in vivo in horses (Lawson et al., 2019). They described leucocyte and eosinophil infiltration and epithelial hyperplasia within the tissue surrounding the ileocecal valve where the parasites were attached (Lawson et al., 2019). They found a significant increase in IL-13 expression in these tissue samples, and a trend toward (but insignificant) an increase in IL-4 production. IL-10 and IFN-γ expression was lower in samples collected near the ileocecal valve (Lawson et al., 2019). When exposed to the antigen in vitro, there was an increase in expression of IL-2, IL-4, IL-5, IL-13, and IL-17 (Lawson et al., 2019).

1.4 Overview of Helminths and the immune system

1.4.1 Goblet Cells and Mucosal Barriers

Goblet cells are an integral part of the mucosal barrier of organs such as lungs, stomach, and intestines. They are specialized cells within the epithelium that secrete several products, including proteins and mucins, including mucus. Goblet cell hyperplasia is noted in relation to parasite infections, including cyathostomins (Hasnain et al., 2010; Ogbourne, 1978; Reynolds et al., 2012; Rodrigues et al., 2018).

Fallon et al., 2006 evaluated the role of IL-25 (IL-17E), a cytokine that drives the Th2 response, compared to IL-17A, which is generally more involved with the Th1 response. They elucidated that IL-25 knockout mice failed to eradicate infections with *N. brasiliensis*, a strongyle of rodents that is used extensively for hookworm models in
humans (Fallon et al., 2006). This study also noted a decreased Th2 cytokine production, specifically with IL-13 and IL-5 being produced at a fivefold lower level than in the wild type mice; however, IL-4 was elevated at later timepoints. In this study, however, there was no delay in GCH production. Once IL-25 was administered to these mice, a rapid expulsion of the worms occurred; however, it was neither B nor T cell dependent. They suggested that IL-25 may have another impact as to why GCH may have occurred without the presence of worm expulsion, such as a lack of effect on intestinal contraction and motility (Fallon et al., 2006).

In another study, IL-22 deficient mice infected with *N. brasiliensis* had delayed worm expulsion, despite adequate Th2 cytokine response (Turner et al., 2013). This was replicated with *T. muris*, where they also noted decreased GCH and delayed worm expulsion in IL-22 deficient mice. This indicated its significant role of the mucosal epithelial barrier in nematode infections in rodents (Turner et al., 2013).

Resistin-like molecule β (RELM-β) has been evaluated in nematode infections in murine hosts. It is a product of goblet and epithelial cells and is implicated in gastrointestinal immunity (Hogan et al., 2006). They found that RELM-β was a key component of maintaining the mucosal integrity in inflammatory processes (Hogan et al., 2006), and that it was essential in nematode clearance (Artis et al., 2004). In a Th2 response, RELM-β increased and helped induce a protective mucosal immunity, whereas an IFN-γ and Th1 response decreased RELM-β expression and prolonged nematode infections (Artis et al., 2004). Finally, IL-13 drove RELM-β expression in the intestines, and RELM-β inhibited chemotaxic functions of nematodes in vitro (Artis et al., 2004). Another goblet cell product is MUC2, a protein rich mucin excreted within the intestines,
has been found to be upregulated in *H. polygyrus* infections, and directly involved in worm expulsion in *T. muris* and *N. brasiliensis* (Hasnain et al., 2013a, 2013b; Reynolds et al., 2012).

### 1.4.2 Other nematode models

*Heligosomoides polygyrus* is a rodent model parasite that is used routinely to simulate small intestinal strongylid infections (Reynolds et al., 2012). They have a similar lifecycle to cyathostomins where they migrate and encyst into the mucosa of the small intestine (Reynolds et al., 2012) and have been successfully cultured in vitro to harvest their E/S products (Reynolds et al., 2012). A combined T and B cell response was reported to be necessary to adequately expel the worms in vivo (Reynolds et al., 2012). Another study evaluated multiple infections of mice with *H. polygyrus* and found that a compromised B cell function was not critical in expulsion of primary infections or subsequent infections (Harris and Gause, 2011). *Heligosomoides polygyrus* also induces a strong Th2 type response. *In vitro* studies have demonstrated an increase expression of IL-4, IL-5, IL-9, and IL-10 from spleen and lamina propria cultured cells and IL-3, IL-4, IL-5, and IL-9 from mesenteric lymph nodes and Peyer’s patches of the small intestine *in vivo* (Finney et al., 2007; Reynolds et al., 2012; Setiawan et al., 2007; Svetić et al., 1993). Studies have also found that IL-4 and, to a lesser extent IL-13, are the most critical for *H. polygyrus* expulsion in mice (Reynolds et al., 2012).

In another rodent study with *N. brasiliensis* infections, IL-5, IL-13, IL-25, and IL-33 were produced by epithelial cells in the lumen of the small intestine and excreted as an alarm during damage to the tissue (Reynolds et al., 2012).
1.4.3 Immune Modulation

Parasites produce excretory and secretory products, which are usually pooled together as E/S antigens. These have been shown in several studies to modulate the host parasite interaction, using many different mechanisms and proteins. This has been comprehensively laid out by Hewitson et al. (2009) and provided in a brief summary in Table 1-8. Briefly, there are many types of immunomodulation used by cestodes, trematodes, and nematodes to allow them to parasitize the host (Hewitson et al., 2009). These allow for cell inhibition or proliferation and recruitment and/or decrease in inflammatory response (Hewitson et al., 2009).

<table>
<thead>
<tr>
<th>E/S Product type</th>
<th>Helminth identified in:</th>
<th>Target(s)</th>
<th>Function(s)</th>
<th>Pro/anti-inflammatory</th>
</tr>
</thead>
</table>
| Alpha-1 and omega-1 | Trematode (Schistosomes) | Basrophils | • Degranulation  
• IL-4 production  
• Induction of Th2 response | Proinflammatory |
| Glycoproteins | Trematodes  
Nematodes | Varies. Can include DCs | • Induce Th2 response  
• Granuloma formation  
• Induce IL-10 production | Proinflammatory (in general) |
| Cytokine homologues | Trematodes  
Cestodes  
Nematodes | Varies. | • Induce pro-inflammatory response  
• IL-4  
• Generation of Treg cells | Proinflammatory |
| C type lectins and galectins | Nematodes (filariids, ascarids, strongylids) | Varies (T cells) | • Immune evasion  
• Eosinophil chemoattractant | N/A |
| Protease inhibitors (Cystatins, serpins) | Nematodes | Varies | • Reduced antigen presentation  
• Reduced T cell priming  
• Increase IL-10 production | Anti-inflammatory |

Table 1-8. Types of E/S products. Produced by helminths, as presented by Hewison et al., 2009. None of these have been evaluated in cyathostomin infections.
Table 1-9. (continued)

1.5 Aims and hypotheses

- **H₁**: Equids that receive anthelmintic treatment will have a lower local and systemic inflammatory responses than equids that do not

- **H₂**: MLs will have a greater inflammatory response than BZs due to their higher efficacy and removal of larger encysted and luminal cyathostomin burdens

- **H₃**: Larvicidal treatment will incite a greater inflammatory response from encysted and luminal worm die-off than non-larvicidal treatment

- **H₄**: A greater cyathostomin burden, both mucosal and luminal, will decrease the proinflammatory response

- **H₅**: Encysted cyathostomins will be able to potentiate an anti-inflammatory response
• H₆: Local inflammatory responses to treatment will appear in systemic circulation as “spillover”

The aims of the following studies are to evaluate the local and systemic immunologic responses in naturally acquired cyathostomin infections across extended timepoints (2 and 5 weeks post treatment), specifically after receiving anthelmintic treatment. Another goal is to characterize the inflammatory response and role of goblet cells in cyathostomin infections and following anthelmintic treatment. Finally, a third aim is comparing the inflammatory response to benzimidazoles versus macrocyclic lactones, and larvicidal versus non-larvicidal effects at extended timepoints.
CHAPTER 2. EVALUATION OF THE MUCOSAL INFLAMMATORY RESPONSES TO EQUINE CYATHOSTOMINS IN RESPONSE TO TREATMENT

This research was originally published in Veterinary Immunology and Immunopathology 199, 1-7.

2.1 Introduction

Equine cyathostomin parasites are omnipresent in grazing horses across the world, and infection is virtually inevitable. This group of parasites consists of 14 genera and 50 species, of which 8 genera and 40 species are described infecting horses (Lichtenfels et al., 2008). The life cycle is unique among strongylids, as cyathostomin larvae are known to undergo arrested development at the early third larval stage (EL3) (Eysker and Mirck, 1984), where hundreds of thousands of larvae can accumulate over time, presumably under the influence of a host response to the invading larvae (Chapman et al., 2003). Furthermore, EL3 counts have been reported to be significantly higher during seasons characterized by weather conditions that are unfavorable for parasite transmission on pasture (Chapman et al., 2003; Eysker et al., 1990; Ogbourne, 1975). Larvae eventually mature into late third (LL3) and fourth (L4) stages before they leave their cysts and make their way back to the intestinal lumen. This process has been associated with a pronounced inflammatory reaction, and when large numbers of larvae emerge synchronously, it can cause a severe typhlocolitis known as larval cyathostominosis (Love et al., 1999). While the disease complex is well described, the local inflammatory and immunologic mechanisms are poorly understood.

Of the current marketed equine anthelmintics, only fenbendazole (10 mg/kg or 7.5 mg/kg) administered orally once daily for five consecutive days and moxidectin gel (0.4
mg/kg) administered once orally are labeled for use against encysted cyathostomins. Of the two treatments, cyathostomins have documented resistance to the adulticidal dose of fenbendazole, as well as the other benzimidazoles (Kaplan et al., 2004; Lester et al., 2013; Peregrine et al., 2014) and the larvicidal dose regimen (Bellaw et al., 2018; Reinemeyer et al., 2015).

When considering the inflammatory response to treatment, one study suggested a significantly reduced local inflammatory response in ponies treated with moxidectin compared to the five-day fenbendazole regimen (Steinbach et al., 2006). We recently evaluated local and systemic inflammatory markers in response to moxidectin and the larvicidal regimen of fenbendazole and found no systemic responses to any of the two treatments (Nielsen et al., 2015). Locally, subtle inflammatory reactions were associated with moxidectin treatment but not fenbendazole treatment; however, this was largely contributed to the reduced larvicidal efficacy of fenbendazole (M. K. Nielsen et al., 2015).

Recently, we completed another treatment trial comparing the larvicidal effects of the five-day fenbendazole treatment regimen (n=12) with moxidectin (n=12) and an untreated control group (n=12). In addition, to comparing the larvicidal efficacy of these two anthelmintic formulations, we also evaluated two different time intervals between treatment and necropsy; 2 weeks and 5 weeks. Anthelmintic efficacy results from this study are presented elsewhere (Bellaw et al., 2018). Overall, mucosal worm burdens were substantially higher than in the previous study (Reinemeyer et al., 2015), but the 2-week post treatment larvicidal efficacies were very similar with reduced larvicidal efficacy of the fenbendazole regimen (50.4%), whereas moxidectin efficacy (73.8%) was within historically reported ranges (Bellaw et al., 2018).
The aims with the present study were to collect further histopathological information on local inflammatory responses to anthelmintic therapy and perform the first comparison of histopathological reactions observed at the two different time intervals post anthelmintic treatment.

2.2 Materials and Methods

2.2.1 Study Design

The University of Kentucky’s Institutional Animal Care and Use Committee approved this study, protocol number 2015–2092. The study was carried out between September and October of 2015. Thirty-six ponies ranging from two to four years of age with naturally acquired cyathostomin infection were kept on pasture throughout the study. Ponies were blocked into groups of three based off age and decreasing magnitude fecal egg counts (performed with mini-FLOTAC, detection limit of 5 eggs per gram Barda et al., 2013). One pony from each block was randomly allocated into one of three treatment groups: fenbendazole treated, and moxidectin treated, and untreated control groups. Twelve ponies were allocated into each of the 3 treatment groups. All study personnel were blinded to group allocation and treatments throughout the study.

On Day-1, each pony was weighed on a certified livestock scale and individual doses of larvicidal anthelmintic were prepared based off weight and group assignment. Ponies assigned to the fenbendazole treatment group received 10 mg/kg fenbendazole (Panacur PowerPak, Merck Animal Health, Madison, NJ, USA) for five days, Days 0–4. Ponies assigned to the moxidectin treatment group received 400 μg/kg of moxidectin (Quest, Zoetis, Kalamazoo, MI) orally once on Day 4. On Days 18 and 19 (2 weeks post treatment), three ponies randomly selected from each group were necropsied and tissues
samples taken. Three complete replicates were necropsied each day to ensure that equal numbers of ponies from each treatment group were necropsied simultaneously. The rest of the 18 ponies were kept on pasture until Days 35 and 36 (5 weeks post treatment), when they were euthanized, and tissue samples were taken.

2.2.2 Histology

Tissue samples of approximately 2.5cm were collected from grossly normal appearing middle portions of the cecum, ventral colon and dorsal colon. The samples were stored in 10% formalin for 24h then transferred to 70% ethyl alcohol until use for histopathology purposes. These tissue samples were routinely processed and stained with Harris hematoxylin and eosin. All samples were analyzed by light microscopy and immunohistochemistry as previously described (Nielsen et al., 2015). In summary, larvae were evaluated based on size and morphological characteristics to determine larval stage (L3 or L4). Lesions associated with 3rd and 4th stage cyathostomin larvae were counted and histologically assessed for location (colonic glands, mucosal epithelium, lamina propria, or submucosa), size, inflammatory cell composition (neutrophils, eosinophils, macrophages, and lymphocytes), and severity of inflammation and fibrosis (0=none; 1=1–5 cell layers, mild inflammation or fibrosis; 2=6–15 cell layers, moderate inflammation or fibrosis; or 3= >15 cell layers, severe inflammation or fibrosis). Additionally, tissue sections were analyzed for the presence or absence of goblet cell hyperplasia, mucosal ulceration, and submucosal granulomas.
2.2.3 Immunohistochemistry

Immunohistochemistry was utilized to characterize the mononuclear leukocyte population. Tissue sections were immunohistochemically stained (Dako, EnVision+Dual Link System-HRP) for Cluster of differentiation 3 (CD3) (Dako polyclonal rabbit antihuman CD3, Code A0452), CD20 (Thermo Scientific rabbit polyclonal antibody, Catalog #RB-9013), and MAC387 (Dako monoclonal mouse anti-human Myeloid/Histiocyte antigen, clone 387) to quantify T cells, B cells, and activated macrophages, respectively. The number of immunohistochemically stained cells were semi quantitatively graded (0=none; 1=1–5 cell layers; 2=6–15 cell layers; or 3= >15 cell layers) for the radius of each parasite associated inflammatory focus.

2.2.4 Statistical analyses

All statistical analyses were carried out using SAS University Edition (SAS Institute, Cary, NC, USA). Generalized mixed linear models were developed to analyze the effects of treatment groups and weeks post treatment on the histopathological findings. Inflammatory nodule radius size and total mucosal length were the only continuous variables, while all others were labeled as categorical variables. Horse ID was kept as a random effect. Models were generated to analyze cell counts, larval counts, goblet cell hyperplasia, mucosal ulcerations, and inflammatory nodules with and without parasites. L3s, L4s, and degenerative larvae were evaluated in the cecum, ventral colon, and dorsal colon in respect to their locations in the submucosa, lamina propria, and mucosa. For all, ‘organ’ and ‘location’ were covariates. Total length of the organ mucosa of all samples of all groups was analyzed for possible bias as well. Influence of all measured parameters for
each analysis was evaluated using traditional backward and forward elimination of variables. All variables with p-values<0.25 were kept in the model. The interaction between ‘groups’ and ‘weeks post treatment’ was also evaluated in each analysis. When variables were significant, a ‘least square means’ for a Tukey’s pairwise comparison, odds ratio, and estimate were all performed, and interpretation of results occurred at the significance level $\alpha=0.05$.

2.3 Results

2.3.1 Worm Burdens

Bellaw et al., 2018 published the mean worm burden of these ponies Table 2-1. Postmortem mean luminal adult worm and larval counts, as well as the mean encysted larval counts from the three groups. Data taken From Bellaw et al. 2017. Briefly, all luminal worm burdens increased from 2 to 5 weeks post treatment, with the exception of a decrease of luminal L4s in the control group. All encysted stages decreased from 2 to 5 weeks post treatment across all groups. The average larval counts per 50mm of tissue evaluated is included as Table 6-1.

Table 2-1. Postmortem mean luminal adult worm and larval counts, as well as the mean encysted larval counts from the three groups. Data taken From Bellaw et al. 2017.

<table>
<thead>
<tr>
<th>Group</th>
<th>Weeks Post Treatment</th>
<th>Luminal Adult</th>
<th>Luminal L4</th>
<th>EL3</th>
<th>LL3/L4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fenbendazole</td>
<td>2</td>
<td>10,750</td>
<td>4,608</td>
<td>77,436</td>
<td>32,226</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>62,873</td>
<td>20,136</td>
<td>31,333</td>
<td>8,000</td>
</tr>
<tr>
<td>Moxidectin</td>
<td>2</td>
<td>1,150</td>
<td>1,371</td>
<td>40,900</td>
<td>28,000</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>17,240</td>
<td>12,016</td>
<td>18,200</td>
<td>9,200</td>
</tr>
<tr>
<td>Control</td>
<td>2</td>
<td>147,869</td>
<td>78,991</td>
<td>156,000</td>
<td>110,200</td>
</tr>
</tbody>
</table>
2.3.2 Macrophages

Total macrophage score differences between groups were significant (p=0.05), with moxidectin treatment group having a higher mean macrophage score than the fenbendazole treatment group (p<0.0185), in which scores only ranged between 0 and 2 (Figure 2-1a). Degenerative larvae were associated with a higher macrophage score than L3s and L4s, and L4s were associated with a higher score than the L3 stages (p<0.05) (Figure 2-2). Significantly more activated macrophages (MAC387-positive) were found in the control untreated group than the fenbendazole and moxidectin groups (p<0.0104, and p<0.0004), in which scores ranged from 0 to 2, and the untreated control group’s scores ranged from 0 to 3 (Figure 2-3a). There were significantly more activated macrophages associated with inflammatory nodules in the ventral colon than the dorsal colon and lower numbers in the lamina propria than the submucosa (p<0.0004). The average macrophage scores and activated macrophage scores at the two time points per group is presented in Supplemental Figure 2-1.
Figure 2-1. Box-whisker plots representing inflammatory cell scores (0 = none; 1 = 1-5 cell layers, mild inflammation or fibrosis; 2 = 6-15 cell layers, moderate inflammation or fibrosis; or 3 = >15 cell layers, severe inflammation or fibrosis) and lesion size (mm) in the three treatment groups. Upper and lower bounds of the box represent the upper and lower quartile, respectively. The diamond represents the mean of the observations and the whiskers represent the minimum and maximum observations per group. Asterisks indicate a statistically significant difference between the groups (p<0.05). a) macrophage scores, b) lymphocyte scores, c) neutrophil scores, d) eosinophil scores, e) fibrous connective tissue scores, f) inflammatory nodule size radius, g) granuloma nodule size radius, h) goblet cell hyperplasia scores.
Figure 2-2. Histopathology sections of representative findings. The size bar in all sections except d is equal to 100μm. a) Dorsal colon with goblet cell hyperplasia score of 0 (100x, H&E). Moxidectin group 5 weeks post treatment. b) Ventral colon with goblet cell score of 2 (100X, H&E). Control group 5 weeks post treatment. c) Dorsal colon with L3 larva in the submucosa surrounded by a large population of inflammatory cells and fibrous connective tissue (100X, H&E). Moxidectin group 2 weeks post treatment. d) L3 larva in the glands of the cecum surrounded by a thin layer of inflammatory cells and fibrous connective tissue (400X, H&E scale bar is 40μm). Control group 2 weeks post treatment. e) Cecum with degenerative L4 larva in the submucosa (200X, H&E), surrounded by degenerative eosinophils, fibrous connective tissue, and inflammatory cells. Fenbendazole group 2 weeks post treatment. f) Intact L4 larva in the mucosa of the cecum, control group 2 weeks post treatment (100X, H&E). g) Intact L4 larva in the submucosa of the cecum (40X, H&E). h) Ventral colon with CD20-positive B lymphocytes (100X), representing a lymphocyte score of 3. Moxidectin group 2 weeks post treatment. The arrow identifies a large aggregate of B lymphocytes.

Figure 2-3. Box-whisker plots of the immunohistochemically (IHC) stained cell scores per treatment group. Upper and lower bounds of the box represent the upper and lower quartile, respectively. The diamond represents the mean of the observations and the whiskers represent the minimum and maximum observations per group. Asterisks indicate a statistically significant difference between the groups (p<0.05). a) MAC387-positive activated macrophage scores. b) CD20-positive B lymphocyte scores. c) CD3-positive T lymphocyte scores.

2.3.3 Lymphocytes

Total lymphocyte population did not differ between treatment groups (Figure 2-1b). In the CD20-positive B lymphocytes, scores ranged from 0 to 3 in all treatment groups and there were no statistical differences between the groups (Figure 2-3b). Significantly more B lymphocytes were in inflammatory nodules in the mucosa than in the lamina propria and
submucosa (p<0.05) (Figure 2-2). CD3-positive T lymphocytes were elevated in the moxidectin group when compared to the control group (Figure 2-3c). T cell scores were also elevated in the mucosa when compared to the submucosa (Table 2-3). The lymphocyte scores, B lymphocyte scores, and T lymphocyte scores at the two time points per group are presented in Supplemental Figure 2-1.

Table 2-3. P values of response variables tested in the mixed linear models and the input variables given in the analysis. Dashed lines indicate variables not included in the model.

<table>
<thead>
<tr>
<th>Response variable</th>
<th>Group</th>
<th>Weeks Post Treatment</th>
<th>Sample</th>
<th>Location</th>
<th>Larval Stage</th>
<th>Macrophages</th>
<th>Neutrophils</th>
<th>Eosinophils</th>
<th>Lymphocytes</th>
<th>Inflammatory Nodule Size</th>
<th>Fibrous Connective Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eosinophils</td>
<td>&gt;0.25</td>
<td>0.0497</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&lt;0.0001</td>
<td>0.0062</td>
<td>&gt;0.25</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&gt;0.25</td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>&gt;0.25</td>
<td>0.1895</td>
<td>&gt;0.25</td>
<td>0.1103</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&lt;0.0001</td>
<td>&gt;0.25</td>
<td>&lt;0.0001</td>
<td>0.016</td>
<td>&gt;0.25</td>
</tr>
<tr>
<td>Macrophages</td>
<td>0.05</td>
<td>0.1929</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&lt;0.0001</td>
<td>-</td>
<td>0.0362</td>
<td>0.0528</td>
<td>&lt;0.0001</td>
<td>0.0003</td>
<td>&gt;0.25</td>
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<tr>
<td>Lymphocytes</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>0.047</td>
<td>0.0047</td>
<td>&gt;0.25</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.1271</td>
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<tr>
<td>Larval Stages</td>
<td>0.104</td>
<td>&gt;0.25</td>
<td>0.0019</td>
<td>&lt;0.0001</td>
<td>-</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>0.0393</td>
<td>&gt;0.25</td>
<td>0.0356</td>
<td>0.0357</td>
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<tr>
<td>Fibrous Connective Tissue</td>
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<td>0.0014</td>
<td>0.1058</td>
<td>0.0013</td>
<td>0.1006</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td></td>
</tr>
<tr>
<td>B Lymphocytes</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>0.0002</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
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<td>&gt;0.25</td>
<td></td>
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<tr>
<td>T Lymphocytes</td>
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<td>&gt;0.25</td>
<td>0.0769</td>
<td>0.0219</td>
<td>0.1403</td>
<td>&gt;0.25</td>
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<td></td>
</tr>
<tr>
<td>Activated Macrophages</td>
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<td>&gt;0.25</td>
<td>0.0409</td>
<td>0.0004</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
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</tr>
<tr>
<td>Goblet Cell Hyperplasia</td>
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<td>0.0047</td>
<td>0.0032</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Inflammatory Nodule Size</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>0.0672</td>
<td>0.0003</td>
<td>0.027</td>
<td>0.0793</td>
<td>0.0001</td>
<td>0.0003</td>
<td>&gt;0.25</td>
</tr>
<tr>
<td>Radii</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td></td>
</tr>
<tr>
<td>L3s</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td></td>
</tr>
<tr>
<td>**L4s</td>
<td>&gt;0.25</td>
<td>0.0009</td>
<td>0.0047</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>***&lt;0.02</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Degenerative Larvae</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td></td>
</tr>
<tr>
<td>Mucosal Ulcerations</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td></td>
</tr>
<tr>
<td>Submucosal Granulomas</td>
<td>0.122</td>
<td>0.1131</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>0.0028</td>
<td>0.0396</td>
<td>&gt;0.25</td>
<td>&gt;0.25</td>
<td>0.1108</td>
<td>-</td>
<td>0.1708</td>
</tr>
</tbody>
</table>

* L4s in the submucosa
** L4s in the mucosa
*** Difference values between the larval stages in the model

2.3.4 Neutrophils

Neutrophils had no significant difference among groups, weeks post treatment, organ (cecum, dorsal colon, and ventral colon), location or with larval stages (Figure 2-1c).

The neutrophil scores at the two time points per group are presented in Supplemental Fig. 2.1.
2.3.5 Eosinophils

Eosinophil scores were elevated at 2 weeks post treatment versus 5 weeks post treatment and were elevated across all groups (Table 2-3); however, no difference was seen between treatment groups (Figure 2-1d). Eosinophil scores were also higher in the presence of degenerative larvae than both L3 and L4 larvae and higher scores were associated with L4s than L3s (p<0.05). The eosinophil scores at the two time points per group are presented in Supplemental Figure 2-1. The average eosinophil scores per organ per time point per group are presented in Supplemental Table 2-2.

2.3.6 Goblet Cells

Goblet cell hyperplasia scores were elevated at 5 weeks post treatment compared to 2 weeks post treatment across all groups (Figure 2-1h) and were also elevated in the ventral colon in comparison with the dorsal colon (Table 2-3); however, no statistical difference was noted between the ventral colon and the cecum. The goblet cell hyperplasia scores at the two time points per group are presented in Supplemental Figure 2-1. The average goblet cell hyperplasia scores per organ per time point per group are presented in Supplemental Table 2-2.

2.3.7 Larvae

When multiple stages of larvae were present in a sample, no statistical significance was apparent between the larval stages present. The number of intact L4s in the mucosa varied significantly by weeks post treatment, with greater numbers of L4s being present at two weeks versus five weeks (Table 2-3). The dorsal colon contained fewer L4s than the cecum and ventral colon (Table 2-3). Larvae were surrounded by variably sized
inflammatory nodules ranging from 0.01 to 1.13mm, with no statistically different radii between groups (Figure 2-1g, Figure 2-2, and Table 2-3). The fibrous connective tissue scores (FCT) were significantly elevated in the submucosa when compared to the lamina propria (p<0.0003). Fibrous connective tissue scores were also significantly lower at 5 weeks post treatment than 2 weeks post treatment (Table 2-3). Mucosal ulcerations were noted only in the control group at 2 weeks post treatment. Submucosal granulomas ranged in size from 0.1 to 1.65mm and there were no statistical differences in the number of submucosal granulomas between the groups (Figure 2-1f). The larval counts at the two time points per group are presented in Supplemental Figure 2-1.

2.4 Discussion

This study is the first to characterize the local inflammatory response at both 2- and 5-weeks post treatment, as well as further characterize the local inflammatory response to the parasites themselves and to the treatments. While there were subtle differences between treatment groups, this study determined several novel findings among the treatment and control groups not previously reported.

In previous studies, goblet cell hyperplasia had been noted (Nielsen et al., 2015; Steinbach et al., 2006); however, it had not been found to be statistically significant between weeks post treatment, organ, or negatively associated with encysted worm burden. In the present study, goblet cell hyperplasia varied by weeks post treatment and the organ it was located in. Scores were elevated at five weeks post treatment when compared to two weeks post treatment, and scores were higher in the ventral colon than the cecum and dorsal colon. As these observations were made amongst all treatment groups, including the control group, it is unlikely that this is treatment related and it may instead be related to
seasonality, infection pressure, or stage of infection. It may also be hypothesized that the significantly lower larval counts and luminal worm counts observed at five weeks post treatment (Bellaw et al., 2018) may be due to an increased goblet cell reaction and resulting in worm expulsion; however, more work is needed to understand the role of goblet cell hyperplasia in luminal as well as encysted cyathostomin infections. In other species, such as rodents, interleukins (IL) 4 and 13 serve a large role in worm expulsion and are the primary drivers of goblet cell hyperplasia induction from intestinal epithelial cells (Fallon et al., 2006). In rodents experimentally infected with *Nippostrongylus brasiliensis* and *Heligosomoides polygyrus*, members of the order Strongylida (where *H. polygyrus* possesses a life cycle similar to cyathostomins, in that the larvae encyst in the mucosa of the small intestine), it was found that these goblet cells upregulated Resistin-Like Molecule beta (RELM-β) in response to infection, after migration of the worms to the small intestine. It was also noted that RELM-β knockout mice had prolonged infection times, suggesting a role of RELM-β in the expulsion of adult *N. brasiliensis* and *H. polygyrus* infections, but not in the larval encysted forms of *H. polygyrus* (Herbert et al., 2009). In another study, upregulation of Mucin 5AC (MUC5AC) in cecal goblet cells during *Trichuris muris* infection was found necessary for worm expulsion (Hasnain et al., 2011). Overall, it has been shown in other species that the regulation of goblet cell hyperplasia is mediated by IL-4 and IL-13 and leads to the upregulation of important components for worm expulsion. To date, limited work has been done with IL-4 and IL-10 in equine cyathostomin infections (Davidson et al., 2005b; M. K. Nielsen et al., 2015) and the connection between these cytokines and goblet cell hyperplasia has not been established. Currently, no work has been
done with cyathostomin parasites in regard to IL-13 and MUC5AC/RELM-β, and this remains a path for future investigation.

The overall macrophage scores of the fenbendazole group were significantly lower than those of the moxidectin group, and the mean scores of the activated macrophage a significant difference between the control and the two treatment groups in the mean activated macrophage scores, suggesting a difference in the inflammatory response between the two treatment groups (Figure 2-3a). It could be inferred that the lower macrophage scores in the fenbendazole group could be due to the lack of efficacy reported by Bellaw et al., (2018). While there were no overall differences in the total lymphocyte population (Figure 2-1b), the treatment groups had significantly more T lymphocytes than the untreated control group (Figure 2-3c). Anthelmintic treatment may upregulate the immune response in regard to the T lymphocyte pathway; however, future studies are needed to evaluate this potential role of immunoregulation. In total, these inflammatory cell findings differ somewhat from two previous studies, where inflammatory responses were only noted to one of the two treatments evaluated; moxidectin (Nielsen et al., 2015) or fenbendazole (Steinbach et al., 2006).

The finding of intact L4s in the mucosa varied by weeks post treatment, with a large number being present at two weeks compared to the five week time point (Table 2-3); this is in accordance with mucosal digest data generated in the same study (Table 2-1; Bellaw et al., 2018). The cecum and ventral colon contained more L3s and L4s than the dorsal colon, which is also in agreement with previously reported counts determined with a mucosal digest method (Bellaw et al., 2018; Reinemeyer and Herd, 1986). The larvae (L3 and L4) were also more likely to be encysted in the mucosa, specifically in the lamina
propria, than in the submucosa. Steinbach et al. (2006) also reported that the majority of all larvae were more likely to encyst in the lamina propria and mucosa; however, these authors also indicated that the majority (67–90%) of L4 larvae encysted in the submucosa in all treatment groups. Taken together, these two studies suggest that larval populations may prefer to encyst in the mucosa over the submucosa. It is also hypothesized that the L3s may not have persisted long enough to stimulate a larger host response around the individual larvae in the form of fibrous connective tissue or increased inflammatory cells (Table 2-3) or that they may be less immunogenic. Mucosal ulcerations were only found in the control group which differs from Steinbach et al. (2006), where ulcerations were found only in fenbendazole treated groups.

In summary, there were subtle differences in the inflammatory response between treatment groups. However, both treatment groups had elevated T cells in comparison to the control group, and the fenbendazole group had significantly lower macrophage scores than the control group. It is also worth noting that some lesions, such as mucosal ulcerations, were solely noted in the untreated control group. Furthermore, an increase in goblet cell hyperplasia scores was observed between the two time points, coinciding with decreased encysted larval counts and further investigation can shed more light on these processes.

2.5 Conflicts of Interest

The authors declare no conflicts of interest.

2.6 Acknowledgements

This study was funded by Zoetis LLC. The authors would also like to thank Eric Roemmelle for statistical support.
CHAPTER 3. CYTOKINE AND GOBLET CELL GENE EXPRESSION IN EQUINE CYATHOSTOMIN INFECTION AND LARVICIDAL ANTHELMINTIC THERAPY

THE FOLLOWING CHAPTER WAS PUBLISHED IN Parasite Immunology. 2020. 00:E12709
LICENSE NUMBER FOR REUSE IN DISSERTATION: 4793690425223

Abstract

Aims: The role of the immune response to cyathostomin infections in horses remains unknown. Intestinal goblet cell hyperplasia has previously been noted as a component in cyathostomin infection; however, the function is unclear. The goal of this study was to evaluate the local and systemic gene expression to cyathostomin infections following larvicidal treatment and explore their relation to goblet cells. Methods and results: 36 ponies with naturally acquired cyathostomin infections were randomly allocated into 3 groups: fenbendazole-treated (10mg/kg PO 5 days), moxidectin-treated (0.4mg/kg PO once), and untreated control. Whole blood from all horses was collected weekly, and tissue samples from the large intestine collected during necropsy at 2- and 5-weeks post treatment (WPT). Gene expression of interleukin (IL)-4, IL-5, IL-6, IL-10, IL-13, IL-17A, IL-22, IFN-γ, Resistin-like Molecule beta (RELM-β), Mucin 2 (MUC2), and Tumor Necrosis Factor (TNF)-α was measured using qRT-PCR. There were statistically significant linear correlations between luminal worm burdens and MUC2 (r= -0.2358) and RELM-β (r= -0.2261). Conclusion: This suggests an active role of immune system post treatment in parasite expulsion, specifically in goblet cells, and that the organs respond differently to treatment and the larvae themselves. This may have implications in the disease process and treatment.
3.1 Keywords

Cyathostomin; goblet cell; inflammation; worm expulsion; larvicidal; equine; anthelmintic

3.2 Introduction

Domestic horses are hosts to a plethora of parasites, including cyathostomins, a group of parasites composed of 8 genera and 40 species infecting domestic horses alone (Lichtenfels et al., 2008). Cyathostomins are pervasive parasites of horses, and their lifecycle is unique among strongylids. After ingestion of the infective 3rd larval stage (L3), the Early 3rd larval stages (EL3) encyst in the mucosa of the dorsal and ventral colons (DC and VC, respectively), as well as cecum (CEC), where they can undergo hypobiosis for up to 2 years (Ogbourne, 1975). The larvae proceed to develop into Late 3rd larval stage (LL3s) and mucosal 4th larval stage (L4s). All mucosal stages are collectively referred to as developing larvae (Eysker and Mirck, 1984). The L4s then emerge into the intestinal lumen to molt to the adult stages and complete the lifecycle. In rare cases, the emergence of the L4s from the mucosa to the lumen of the large intestine causes larval cyathostominosis, a life-threatening disease. The mass emergence of larvae leads to an acute or chronic generalized typhlocolitis and protein-losing enteropathy as well as secondary bacterial infections (Love et al., 1999). This disease is reported to be fatal in 50% of cases and reported risk factors include recent treatment with a non-larvicidal anthelmintic, horse age (2-5 years of age), and season (such as autumn or winter) (Reid et al., 1995).

Considerable inflammation is associated with the encysted EL3s, LL3s, and L4s, as well as profound, diffuse inflammatory reactions associated with the process of
excystment of the L4s into the lumen (Love et al., 1999). Few studies describe the disease process and evaluate the mechanisms and inflammatory reactions associated with the progression of parasitic stages, as well as from these parasites in response to both larvicidal and nonlarvicidal treatment. Davidson et al. evaluated the local tissue gene expression levels of Interleukin (IL)-4, IL-5, IL-10, Interferon (IFN)-γ, in large intestinal tissues with and without encysted larvae present (Davidson et al., 2005). Tissues with encysted cyathostomin larvae present expressed higher levels of IL-4, IL-5, and IL-10 than those without, whereas expression of IFN-γ did not differ between the two groups; however, the study did not evaluate other cytokines or genes involved in the Th1 and Th2 immune responses (Davidson et al., 2005). Only one study thus far has evaluated cytokine gene expression in intestinal tissues in horses with and without large intestinal disease, including three larval cyathostominosis cases (Davidson et al., 2002). This study found Tumor Necrosis Factor (TNF)-α was expressed in horses with inflammatory bowel disease, and less likely to be expressed in cases of larval cyathostominosis and those without parasites; however, there were no significant differences between the groups with and without large intestinal disease, including those with encysted cyathostominis present (Davidson et al., 2002). The study did not quantify the gene expression of the cytokines present, and further investigation is warranted to evaluate quantitative differences that may occur in parasitic disease and at various stages of cyathostomin infection, as well as following anthelmintic treatment.

Currently, there are two anthelmintic products labeled for larvicidal treatment of encysted cyathostomin larvae: fenbendazole (10 mg/kg or 7.5 mg/kg orally for 5 consecutive days) and moxidectin (0.4 mg/kg orally once). Studies have described the
inflammatory response to these two larvicidal treatments both locally and systemically ((Nielsen et al., 2015; Steinbach et al., 2006; Steuer et al., 2018). While these studies evaluated the inflammatory response to the larvae and treatment, their scope of testing of the inflammatory response (especially in regard to the local cytokine and local gene expression) was limited. Based on histopathological evaluation of local tissue reaction, Steinbach et al. concluded that there was substantially more inflammation in the fenbendazole-treated group compared to the moxidectin group (Steinbach et al., 2006). In comparison, we have reported only subtle differences in local pro-inflammatory cytokine gene expression between groups of horses treated with the same anthelmintics ((M. K. Nielsen et al., 2015). Most recently, we evaluated the histopathologic effects of both moxidectin and five-day fenbendazole-treatment over two post treatment time intervals, and noted inflammatory reactions both at 2 and 5 weeks post-treatment (WPT) (Steuer et al., 2018). One of the primary novel findings was the correlation of goblet cell hyperplasia with decreased mucosal larval and luminal worm counts at 5 WPT (compared to 2 WPT) across all groups, including the untreated controls (Steuer et al., 2018). This suggests a process of worm expulsion from the mucosa occurring, regardless of anthelmintic treatment. Goblet cells are an integral part of the epithelial barrier of organs and produce mucin products. Goblet cell hyperplasia has been observed in concordance with worm expulsion in other animal species, and the process involves IL-4, IL-13, and upregulation of Resistin-Like Molecule (RELM)-β and Mucin-2 (MUC2) ), which help increase the mucosal barrier and allow for worms to be expelled (Fallon et al., 2006; Herbert et al., 2009). Other cytokines, such as IL-22, have been described as mediating goblet cell hyperplasia and inducing higher mucin production, which also aids in worm expulsion
(Hasnain et al., 2010; Turner et al., 2013). None of these mechanisms have been studied in relation to cyathostomin infection in horses.

The aim of this study was to evaluate gene expression of selected cytokines and goblet cell-associated components following anthelmintic treatment with larvicidal doses of fenbendazole and moxidectin in ponies naturally infected with cyathostomin parasites. This study evaluated two post treatment time intervals (2 and 5 weeks) with emphasis on the role of goblet cells and their potential involvement with worm expulsion. An additional aim was to further evaluate the local and systemic pro- and anti-inflammatory gene expression to the larvae and to treatment.

3.3 Materials and methods

3.3.1 Study design

This study was conducted under the approval of the University of Kentucky’s Institutional Animal Care and Use Committee, protocol 2015-2092. Power calculation was performed with G*Power analysis, version 3.1.5 (Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany). For comparison at two time points, a minimum detectable effect size of 0.65, with $\alpha=0.05$ and $\beta=0.80$ occurred with a group size of six. For overall determination of treatment efficacy using a group of 12 yielded a minimum detectable effect size of 0.54, with $\alpha=0.05$ and $\beta=0.80$. Briefly, thirty-six mix-breed ponies, ranging from 2-4 years of age with naturally acquired cyathostomin infection and allocated into three treatment groups: fenbendazole treated (10 mg/kg orally for 5 consecutive days (Panacur PowerPak, Merck Animal Health, Madison, NJ, USA)), moxidectin treated (0.4 mg/kg once orally (Quest, Zoetis, Kalamazoo, MI, USA)), and untreated control. Allocation into blocks was established on decreasing magnitude of fecal egg counts.
completed with Mini-FLOTAC (Cringoli et al., 2017b), detection limit of 5 eggs per gram) and age. From each block, ponies were randomly allotted into each of the three treatment groups, allowing for 12 ponies into each treatment group. Ponies were kept on pasture from September through October 2015 and were euthanized at 2 and 5 WPT. All study personnel were blinded to treatment information and group allocation throughout the study.

Ponies were weighed on a certified livestock scale and individual doses of larvicidal anthelmintic were prepared based on treatment group assignment and weight on Day -1. Ponies receiving fenbendazole were treated once daily on Days 0-4, and the ponies receiving moxidectin treatment were treated once on Day 4. At two weeks post treatment (Days 18 and 19) six ponies from each treatment group (three each day) were randomly selected for euthanasia and necropsy. The remaining 18 ponies remained on pasture and were then euthanized and necropsied at 5 WPT (Days 35 and 36). Worm burdens and enumerations, anthelmintic efficacy and evaluation of histopathologic reaction data have previously been reported from this study (Bellaw et al., 2018; Steuer et al., 2018).

3.3.2 Sample collection

Weekly blood samples, starting at Day -7, were collected from all ponies until euthanasia and placed into Tempus blood RNA tubes (Applied Biosystems, Foster City, CA, USA) per the manufacturer’s instructions. The filled tubes were vigorously shaken for 15-30 seconds and incubated at room temperature for 24 hours. The blood samples were then placed at -20°C until further processing.

As part of the necropsy procedure, tissue samples measuring approximately 10x10mm were collected at necropsy from the CEC, VC, and DC. Tissue samples were
placed directly into RNALater (Life technologies, Grand Island, NY, USA), and left at room temperature for 24 hours. Then, all samples were refrigerated at 4°C until further processing.

3.3.3 Cytokine and gene expression

3.3.3.1 Blood RNA extraction and reverse transcription

Total RNA was isolated from whole blood tempus tube samples as described by Page et al. Briefly, Samples were placed into 50mL tubes and washed with 3mL 1xPBS and centrifuged at 300g for 10 minutes. 600µl of Viral Lysis buffer (Invitrogen, Carlsbad, CA, USA) were added and to each sample and vortexed to suspend the pellet. RNA was extracted with iPrep Total RNA Kit (Invitrogen) and the iPrep Purification Instrument (Invitrogen). Samples were stored at -20°C until further use (Page et al., 2017).

Reverse transcription of the samples was carried out similarly to Nielsen et al. 2015. Briefly, RNA was quantified with a Biotek Epoch Biophotometer (Biotek, Winooski, VT, USA). Reverse transcription reactions were carried out in an Applied Biosystems Veriti 96 well Thermal Cycler (Applied Biosystems, Foster City, CA, USA), using Promega reagents (Promega, Madison, WI, USA). The cDNA was then diluted with an equivalent volume of RNase-free water (80µL; Qiagen, Hilder, Germany). Samples were stored at -20°C until further use.

3.3.3.2 Tissue RNA extraction and reverse transcription

One hundred mg of the intestinal mucosa from the intestinal tissue samples were used for RNA extraction. Samples were processed similarly to Liu et al., with the following
difference: Trizol (Invitrogen) was used in this process instead of RNA-STAT60 (Liu et al., 2012). Reverse transcription was carried out as in 2.3.1, with the following exception: 41.5µl of tissue RNA was used for all tissue reactions to improve potentially low RNA yields due to protein interference in spectrophotometer readings. cDNA samples were stored at -20°C until further use.

3.3.3.3 Quantitative real time polymerase chain reaction

Gene expression was evaluated through quantitative RT-PCR assay (Breathnach et al., 2006; M. K. Nielsen et al., 2015) for all blood and tissue samples. Equine specific TaqMan intron spanning primers and probes (Thermo-Fisher Scientific, Carlsbad, CA, USA) were used for this study. β-glucuronidase (β-GUS, Ec03470630_m1), IL-4 (Ec03468790_m1), IL-5 (Ec03468691_m1), IL-6 (Ec03468678_m1), IL-10 (Ec03468647_m1), IL-13 (Ec03470543_m1), IL-17A (Ec03470096_m1), IFN-γ (Ec03468606_m1), and TNF-α (Ec03467871_m1) were commercially available through Thermo-Fisher Scientific and used in this study. Custom TaqMan® gene expression assays were developed through Thermo-Fisher Scientific for IL-22 (AR9HJZX), MUC 2 (ARRWFPW), and RELM-β (AP47XR4). β-GUS was used as a housekeeping gene (Breathnach et al., 2006).

Each reaction contained 5µl of Sensimix II probe kit master-mix (Bioline, London, UK), 0.5µl of 20x TaqMan assay for primers and probes of interest (assays-by-design, Applied Biosystems), and 4.5µl of diluted cDNA. Duplicate wells were prepared for each sample and gene. Applied Biosystems 7900HT Fast-RT-PCR system was used to assess gene expression. Incubation was the same as Nielsen et al 2015. LinRegPCR was used to adjust qRT-PCR efficiencies (Ramakers et al., 2003). qRT-PCR data were normalized to
the housekeeping gene (β-GUS), and analyzed using the 2-ΔΔCT method (Livak and Schmittgen, 2001).

3.3.4 Statistical analysis

Statistical analyses were carried out using SAS 9.4 (SAS Institute, Cary, NC, USA). Mixed linear models were constructed for evaluating the effect of treatment group and days post treatment on gene expression. The ‘mixed procedure’ was used and ‘horse ID’ kept as a random effect. ‘Group’, ‘day’, and ‘organ’ were kept as class variables. Models were built to analyze each cytokine, and organ was kept as a covariate. The traditional forward and backward elimination of variables was used to evaluate the influence of all parameters. Variables with P-values of 0.2 or below remained in the model and the interaction between group and day was evaluated in all analyses (Kee and Koval, 1997). Log transformation of variables was used, when appropriate, to achieve normal distribution. A ‘least square means’ analysis was used for a Tukey’s pairwise comparison. A significance level of α=0.05 was used for data interpretation. Figures and linear pairwise correlations were conducted through JMP Pro 14 (SAS Institute, Cary, NC, USA), and a Pearson correlation coefficient obtained. For the correlations, luminal and mucosal worm counts and stages, goblet cell hyperplasia scores and associated gene expression, and all inflammatory cytokine expression were used as variables.

3.4 Results

3.4.1 Goblet cell-associated, and anti-inflammatory cytokine gene expression

3.4.1.1 Gene expression in the intestinal tissue
Differences were noted among the groups and timepoints for gene expression of IL-22, MUC2, IL-10, and RELM-β (Figure 3-1). MUC2 gene expression was greater at 2 WPT compared to the 5 WPT time point in all groups (Figure 3-2, p=0.0011), and greater in the control group compared to the two treatment groups (p=0.0337, 0.0367) at 2 WPT. In the moxidectin group, MUC2 was also elevated in at 2 WPT compared to 5 WPT (Figure 3-2, p=0.0033), which was noted neither in the fenbendazole treated nor control groups. The anti-inflammatory IL-10 gene expression was elevated at 2 WPT compared to 5 WPT (Figure 3-1, p <0.0001). Tissue RELM-β gene expression was elevated at 2 WPT compared to 5 WPT across groups (Figure 3-2, p=0.0361).

Differential gene expression of these genes occurred not only between treatment groups and timepoints, but between the three intestinal organs as well. IL-10 gene expression of the DC in the fenbendazole group was elevated compared to all organs and time periods, whereas the control CEC expressed higher levels of IL-10 than the CEC of both treatment groups (p<0.0001 and 0.0009, respectively). Additionally, the CEC and DC of the fenbendazole group expressed higher levels of IL-10 than the CEC and DC in the moxidectin group (p<0.05). Independent of treatment group, the CEC and DC expressed higher levels of IL-10 than the VC (p=0.0095 and 0.0033, respectively), and gene expression of IL-13 was higher in the DC compared to both the CEC and VC (p<0.0032). IL-22 gene expression was also elevated in the DC compared to the VC (p=0.0181), and MUC2 gene expression was lower in the CEC compared to the DC and VC (Figure 3-3, p=0.0337 and 0.0367, respectively). Finally, RELM-β gene expression was elevated in the DC compared to CEC and VC (Figure 3, p=0.0074 and 0.0182, respectively).
Figure 3-1. Box-whisker plots of relative quantities of the expression of interleukins IL-10, IL-13, and IL-22 in intestinal tissues at two and five weeks post treatment. Upper and lower bounds of the boxes represent the upper and lower quartiles, respectively. Letters indicate significant differences between groups (p<0.05).
Figure 3-2. Box-whisker plots of relative quantities of the expression of goblet cell-associated genes MUC2 (a) and RELM-β (b) in large intestinal tissues at two and five weeks post treatment in the three different treatment groups (Control, Fenbendazole, and Moxidectin). Upper and lower bounds of the boxes represent the upper and lower quartiles, respectively. Letters indicate significant differences between groups (p<0.05).
3.4.1.2 Gene expression in the systemic circulation

There were no differences in the circulating IL-10 and IL-13 gene expression between the treatment groups and timepoints \( p>0.05 \), Figure 3-2). IL-22, MUC2, and RELM-β gene expression in circulation were elevated at prior to 3 WPT, where then relative gene expression decreased across all groups, regardless of treatment (Figure 6-3, \( p<0.0421 \)).

3.4.2 Pro-inflammatory cytokine gene expression

3.4.2.1 Gene expression in the intestinal tissue

At 2 WPT, IL-4 gene expression of the control group was elevated compared to the fenbendazole group (Figure 3-5, \( p=0.0027 \)). IL-5 gene expression followed similar
patterns, with the control group exhibiting higher gene expression than both treatment groups (Figure 5, p<0.0237). Overall, there was higher gene expression of TNF-α in the fenbendazole treated group than the moxidectin treated group (Figure 3-4, p<0.0352). At 2 WPT, the fenbendazole treated group also expressed higher levels of IFN-γ when compared to the moxidectin treated group (Figure 4, p=0.0387). Regardless of treatment group, IL-5, IL-6, and IFN-γ gene expression were elevated at 2 WPT compared to 5 WPT (Figure 3-4, Figure 3-5, p=0.0113 and 0.0042, respectively). In the CEC, the control group exhibited higher IFN-γ gene expression at 2 WPT compared to both treatment groups (p<0.0078).

Regardless of time, the control CEC IL-4 gene expression was elevated compared to all other organs of all groups (p<0.001). IL-5 gene expression followed similar patterns with higher gene expression in the control CEC at 2 WPT compared to all other organs and time points (p<0.0445). At 2 WPT, IL-5 gene expression was elevated in the fenbendazole DC compared to moxidectin DC, and overall, IL-5 gene expression was higher in the control DC compared to moxidectin DC (p<0.0389). Finally, in the VC, TNF-α was expressed in higher levels in the fenbendazole-treated group compared to both the control and moxidectin-treated groups (p<0.0073).
Figure 3-4. Box-whisker plots of the relative quantities of the pro-inflammatory cytokines IFN-γ (a) and TNF-α (b) in the intestinal tissues at two and five weeks post treatment in the three treatment groups (Control, Fenbendazole, and Moxidectin). Upper and lower bounds of the boxes represent the upper and lower quartiles, respectively. Letters indicate significant differences between groups (p<0.05).
Figure 3-5. Box-whisker plots of the relative quantities of the pro-inflammatory cytokines IL-4, IL-5, and IL-6 in the intestinal tissues at two and five weeks post treatment in the three treatment groups (Control, Fenbendazole, and Moxidectin). Upper and lower bounds of the boxes represent the upper and lower quartiles, respectively. Letters indicate significant differences between groups (p<0.05).

3.4.2.2 Gene expression in systemic circulation

There were no differences in the circulating IL-10 and IL-13 gene expression between the treatment groups and timepoints (p>0.05, Figure 6-4). IL-22, MUC2, and RELM-β gene expression in circulation were elevated at prior to 3 WPT, where then relative gene expression decreased across all groups, regardless of treatment (Figure 6-3, p<0.0421).

3.4.3 Correlation of gene expression, goblet cell hyperplasia, and worm burdens

3.4.3.1 Correlations in the intestinal tissue
There was negative correlation between worm burdens of the large intestinal organs and IL-4, and negative correlations between worm burdens and IL-22 (Table 3-1, p<0.05). Interestingly, goblet cell hyperplasia had a negative correlation with IFN-γ and IL-10. Luminal worm counts also negatively correlated to MUC2 and RELM-β (Table 3-1, p<0.05). Some of the evaluated genes also exhibited linear relationships with other genes, (Table 6-4). IL-22, MUC2 and RELM-β were strongly correlated with each other in tissue (Table 6-4, p<0.05).
Table 3-1. Pairwise Pearson product-moment correlations to assess linear relationships of gene expression within the tissues and worm burdens and goblet cell scores. The r values are presented here. Those in BOLD are statistically significant with p<0.05.

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3.4.3.2 Correlations in systemic circulation

IL-13 and IL-6 positively correlated to both luminal and encysted worm counts, whereas IL-22, MUC2, and RELM-β only had positive correlation to encysted worm burdems (Table 3-2, p<0.05). Many of the cytokines exhibited linear correlations in the blood as well, including MUC2 and RELM-β (Table 6-3).
Table 3-2. Pairwise Pearson product-moment correlations to assess linear relationships of gene expression within the blood and total worm counts and GCH scores. The r values are presented here. Those in BOLD are statistically significant with p<0.05. Abbreviations for table above: EL3, early third larval stage; LL3, late third larval stage; L4, fourth larval stage; GCH, Goblet Cell Hyperplasia average score; IL, Interleukin; MUC2, Mucin 2, RELM- β, Resistin-Like Molecule Beta; TNF-α, Tumor Necrosis Factor- α.

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3.5 Discussion

This is the first study to evaluate reactions to equine cyathostomin infection with a specific focus on goblet cells and their function, and the first to evaluate gene expression of IL-13, IL-17A, IL-22, RELM-β, and MUC2 following larvicidal treatment. The study has provided new insight into host-parasite interactions with cyathostomin infections and suggested mechanisms involved with spontaneous worm expulsion.

The study population was uniquely suited for this study. These ponies were born on the research farm and had been kept together on the same pastures since then, and they had not received anthelmintic treatments since the year before. The age range (2–4 years) was particularly appropriate for this study, as this is when encysted cyathostomin burdens are reported to be at their highest (Melanie R Chapman et al., 2003). Furthermore, larval cyathostominosis is reported to occur at a higher frequency in horses younger than 5 years of age (Reid et al., 1995). Finally, it is important to study these aspects in naturally infected horses as no experimental infection protocol can appropriately mimic daily ingestion of infective cyathostomin larvae and their species composition. Concerning the role of goblet cells and their function, it is interesting to note the higher gene expression of MUC2 and RELM-β in the tissues across all groups at the 2 WPT time interval compared to 5 WPT, while IL-13 exhibited the opposite effects. In our previous histopathological study, we reported an increase in goblet cell hyperplasia at 5 WPT compared to 2 WPT, which suggests the increase in IL-13 reported herein could be associated with this process, as reported in other species (Steuer et al., 2018; Turner et al., 2013). This and the decreased gene expression of MUC2 and RELM-β coincided with the previously reported decreased
mucosal worm burdens at 5 WPT (Hasnain et al., 2013b). While typically associated with worm expulsion and goblet cell proliferation, the higher gene expression of IL-10 reported herein coincided with the previously reported decreased goblet cell scores (summarized in Supplemental Figure 3-5) and higher counts of encysted larvae in the mucosal tissues (Bellaw et al., 2018; Steuer et al., 2018). This suggests that IL-10 negatively correlates with goblet cell hyperplasia in these horses; however, it is likely not a linear relationship (Table 1). There may also be a direct correlation with IL-22, IL-10, and the goblet cell products MUC2 and RELM-β (Supplemental Table 3-2). This suggests a potential negative feedback loop on goblet cell hyperplasia with IL-13 and positive feedback loops with IL-22 and IL-10. Here, IL-22 and IL-10 may result in a decrease in goblet cells (Table 3-1), and IL-13 may be associated with an increase in cyathostomin infections; however, this needs further investigation to unravel the exact role that cyathostomins play in the potential immune modulation. It would also be interesting to further investigate the course of events between 2 and 5 WPT to follow the progression of expression of these genes to better understand the exact mechanisms occurring in the tissues. In rodent models infected with *Trichuris muris*, IL-22 is known to mediate goblet cell hyperplasia and induce worm expulsion, as with other worm models in rodents and worm expulsion (Inclan-Rico and Siracusa, 2018; Turner et al., 2013). The decrease in gene expression of IL-22, MUC2 and RELM-β at 2 WPT in whole blood corresponded with our observations in the mucosal tissue samples, indicating a tissue spill-over of cells expressing these cytokines and genes into the blood circulation, as previously suggested (Nielsen et al., 2015).

In regard to the pro-inflammatory cytokines expressed in the intestinal tissues, we generally found the same pattern as the other cytokines and genes associated with goblet cell hyperplasia.
cell hyperplasia, with higher gene expression at 2 WPT compared to the 5 WPT time point. However, there were additional differences associated with anthelmintic treatment. In contrast to fenbendazole, moxidectin-treatment appeared to suppress IL-5 and TNF-α at 2 WPT, when compared to the control. The lack of a similar fenbendazole effect could be due to the reduced anthelmintic efficacy for this treatment in the study, and thus a reflection of the worm burden rather than treatment (Bellaw et al., 2018). This is supported by the elevated gene expression of IFN-γ and TNF-α in the control CEC and VC, respectively. Overall, gene expression of IL-5 at 2 WPT was greater in the control group than the two treatment groups, and greater in the fenbendazole-treated group compared to the moxidectin group, suggesting that moxidectin’s higher larvicidal and overall treatment efficacy caused suppression of IL-5 by decreasing worm burdens. Thus, encysted larval burdens may play a significant role in IL-5 production within these mucosal tissues. Nematode mediation of IL-5 has previously been reported in rodent models infected with *N. brasiliensis* (Fallon et al., 2006).

Finally, this study demonstrated that the intestinal organs had significantly different levels of gene expressions. This may reflect differences in resident worm burdens between these organs, or it could be due to different immunological reactions at the organ level. Further unravelling these mechanisms remains a future area of research, as this has not been previously reported in studies and is a new area of interest in understanding not only infections, but the disease process as well (Davidson et al., 2005, 2002). There was a general trend for the DC to express higher levels of cytokines and goblet cell hyperplasia-associated genes, which are generally associated with the lower cyathostomin worm burdens found in the DC. In our previous work, we found that the VC had more goblet cell
hyperplasia and higher mucosal larval counts, which is in agreement with the greater MUC2 gene expression observed in the VC and reported herein (Bellaw et al., 2018; Steuer et al., 2018).

It is important to consider the efficacy of both moxidectin and fenbendazole in this study, which were 70.8% and 74.6% for encysted LL3s/L4s at 2 WPT and 73.8% and 50.4% for encysted EL3s, respectively (Bellaw et al., 2018). These larvicidal efficacies were not statistically different from each other, and neither exhibited a 100% larval reduction. However, they both removed similar proportions of encysted larvae, but utilized different modes of action. Despite this, there were limited differences between treatment groups, including the untreated control. Nonetheless, it remains challenging to separate effects associated with the drugs themselves from changes in larval numbers present within the large intestine. The remaining larvae within the mucosa still maintain a potentially significant antigenic potential, and while we compare against an untreated control group, these remaining larvae may serve as a source of confounding in interpretation of the data. However, as documented by our laboratory, none of the currently available anthelmintic products labelled for larvicidal therapy can be expected to completely eliminate the encysted burden, so this study is evaluating a realistic real-life scenario (Bellaw et al., 2018; Reinemeyer et al., 2015). The data reported herein suggest that larvicidal treatment itself may not have an effect long term (2-5 weeks); however, more work is needed evaluating responses closer to the time of treatment allowing a better assessment of effects associated with the resultant acute worm die-off.

In conclusion, this is the first study evaluating gene expression with particular focus on goblet cells in equids naturally infected with cyathostomins during a five-week time
period post larvicidal treatment. Herein, we have provided evidence of interaction between parasites, anthelmintic treatment, and the equine immune system. This study illustrated that goblet cells likely play an important role in the host response to mucosal cyathostomin burden and appears involved with the worm expulsion observed between the 2 and 5 WPT time intervals. Furthermore, we have illustrated that the CEC, VC, and DC respond differently to these stimuli and these aspects represent compelling avenues of future research aiming at improving our understanding of host-parasite interactions with cyathostomin parasites.

3.6 Acknowledgments

The authors would like to thank Zoetis, LLC for financial support of this research.

3.7 Disclosures

All authors report no conflicts of interest.

3.8 Author Contribution Statement

AES, AAA, and MKN all participated in the generation of the concept of the project and participated in obtaining funding. AES, VDB, and JCS all participated in development of protocol and data collection. ALL AUTHORS participated in analyses of the data and interpretation. AES and MKN wrote the manuscript, with detailed contributions and feedback from AAA, VDB, and JCS.
4.1 Introduction

Cyathostomins are inevitable parasites in horses with infection rates reaching between 90-100% of grazing horse herds (Chapman et al., 2003; Monahan et al., 1998; Reinemeyer et al., 1986; Verkaaik, 2016). The collective cyathostomins includes 8 genera of 40 species infecting domestic horses (Lichtenfels et al., 2008). The life cycle is considered unique and complex among strongylid parasites of animals, where the encysted larvae undergo hypobiosis at the early third larval stage for up to two to three years (EL3) (Eysker and Mirck, 1984; Smith, 1976a), then proceed through development to the late third stage and fourth stage larvae (LL3/L4, respectively). Hundreds of thousands of larvae can accumulate over time, and this accumulation is believed to be driven by the host reaction to the parasites (Chapman et al., 2003). Larval accumulation appears to be associated with seasons, with notably higher mucosal counts occurring after the grazing season, and when pasture conditions will be unfavorable for parasite development and maturation (Leathwick et al., 2016; Nielsen et al., 2018b, 2007).

Since the 1970s, the processes of encystment and excystment have been associated with a local inflammatory reaction surrounding each individual larva (Ogbourne, 1978). A pronounced typhlocolitis reaction and disease has been associated with mass excystment of the L4s from the tissue, a disease complex known as larval cyathostominosis (Love et al., 1999). While the disease complex is well described, the local and systemic inflammatory and immunological mechanisms of disease and infection are still poorly understood.
There are currently three drug classes available for use against the cyathostomins: benzimidazoles, pyrantel salts, and the macrocyclic lactones. Cyathostomins have widespread resistance to the benzimidazoles and pyrantel salts; however, there is very little evidence of resistance to the macrocyclic lactones (MLs) at this time (Kaplan et al., 2004; Lester et al., 2013; Peregrine et al., 2014). All MLs are broad spectrum anthelmintics; however, only moxidectin gel administered at 0.4 mg/kg once orally targets the encysted stages, specifically it is only labeled in the United States for the LL3/L4s. Ivermectin at the labeled 0.2 mg/kg once orally dose is not labeled to target the encysted stages, and most studies support this finding even with elevated doses (Klei et al., 1993).

When evaluating the local histopathological inflammatory response to treatment, studies have compared the only two larvicidal regimens available, which are in different drug classes: moxidectin as dosed above and fenbendazole (Nielsen et al., 2015; Steinbach et al., 2006; Steuer et al., 2018). However, few significant differences were actually noted between the studies, and the earlier studies had contradictory findings. One study suggested that there was a significantly reduced local inflammatory response in ponies treated with moxidectin compared to the larvicidal fenbendazole protocol (Steinbach et al., 2006). Inversely, In 2015, Nielsen et al. elucidated subtle decreased inflammatory reactions in the fenbendazole treated group; however, this was suggested to be due to the decreased efficacy of fenbendazole and therefore decreased efficacy of the treatment and lower inflammatory reaction to dying larvae (Nielsen et al., 2015). In this same study, no differences in systemic inflammatory responses were noted (Nielsen et al., 2015). In 2018, we completed a similar study, with results reported in chapters 2 and 3 of this dissertation. Briefly, we noted that there were no significant differences in the overall local and systemic
inflammatory response to these to treatments that had similar larvicidal efficacies. The most significant finding was the relationship between the worm burdens, and goblet cell hyperplasia (GCH) and the related genes, thus potentially implicating GCH in worm turnover, which has been described in other host-parasite interactions (Hasnain et al., 2013a, 2010; Herbert et al., 2009).

Studies have not evaluated the local inflammatory response to the solely adulticidal ivermectin, even though adulticidal therapy is implicated in larval cyathostominosis (Reid et al., 1995). Previous studies that have evaluated the inflammatory reaction to ivermectin compared it to oxibendazole, moxidectin and a few other products. These studies noted no differences between the treatment groups; however, these studies noted intestinal erythema, edema and ulceration to worm burdens themselves (Love, 1995; Monahan et al., 1998, 1996). This is consistent with our previous findings (Steuer et al., 2018). The aims with the present study were to collect further histopathological information on local inflammatory responses to anthelmintic therapy and perform the first comparison of histopathological reactions observed at the two different time intervals post anthelmintic treatment between larvicidal and nonlarvicidal ML treatment in adult horses.

4.2 Materials and Methods

4.2.1 Study design

This study was performed under the University of Kentucky’s Institutional Animal Care and Use Committee, protocol number 2018-3134. Power calculations for the group sizes have previously been reported (Bellaw et al., 2018). Thirty-six horses with naturally acquired cyathostomin infections, aged 2-5 years old, were kept on pasture from August-
October 2019. To allocate into groups, horses within a birth year were grouped by sex and then ranked by decreasing magnitude of egg counts (Cringoli et al., 2017). Then horses were blocked into groups of three and within each block randomly allocated into each group. Finally, the horses were then randomly allocated into one of the two time intervals. Each group contained a total of 12 horses, with 6 horses allocated to each of the two time intervals. All study personnel were blinded to group allocations and treatments throughout the study.

On day -10, each horse was weighed on a certified livestock scale, and feces taken for group allocation, as defined above. Horses in the moxidectin group received 0.4mg/kg orally (Quest Plus, Zoetis Inc., Parsippany, NJ, USA), and horses in the ivermectin group received 0.2mg/kg ivermectin orally (Zimectrin Gold, Boehringer Ingelheim, Duluth, GA, USA) on Day 0. The horses in the untreated control group did not receive any treatment.

Six horses from each group were euthanized on days 14 and 15 (2 weeks post treatment). Three horses from each group were necropsied each day to ensure equal numbers of horses from each group were necropsied simultaneously. The remaining 18 horses were kept on pasture until they were euthanized in the same manner as above, on days 35 and 36 (5 weeks post treatment).

4.2.2 Mucosal worm enumeration

This method was previously reported (Bellaw et al., 2018). Briefly, 5 % mucosal sections by weight were taken from each of the large intestinal organs. The mucosa and submucosa were scraped off the surface and placed within 1:100 mixture of hydrochloric acid to water, with 10mg pepsin added per liter. The tissue was then digested at 37°C for 2
hours. A 2% subsample was taken, washed, and stained with Lugol’s iodine solution and evaluated for EL3 and LL3 larvae under a dissection scope. Larvae totals were estimated by taking the number of larvae found and multiplying by a factor of 1000.

4.2.3 Tissue sample harvesting

At necropsy, tissue samples, approximately 2x2cm, were taken along a taenial band, approximately 6cm apart, from grossly normal cecum (CEC), dorsal colon (DC), and ventral colon (VC). Samples were placed in 10% buffered neutral formalin for 24h and then stored in 70% ethanol at 4°C until further processing.

4.2.4 Histology

All tissue samples were routinely processed and stained with Harris hematoxylin and eosin. All samples were analyzed by light microscopy as previously described (Steuer et al., 2018), and approximately 50mm in length of mucosa was examined. Briefly, larvae were evaluated based off of size and morphologic characteristics to determine stage (L3 or L4). Larvae were counted, and lesions surrounding the larvae, were evaluated for location (colonic glands, mucosal epithelium, lamina propria, and submucosa), size, inflammatory cell composition (neutrophils, eosinophils, macrophages, and lymphocytes) and severity of inflammation and fibrosis. Scores were given based off of the following: 0=none, 1=1-5 cell layers, 2= 6-15 cell layers, and 3=>15 cell layers. Tissue sections were also evaluated and scored for the presence of goblet cell hyperplasia, mucosal ulcerations, and submucosal granulomas as previously described (Steuer et al., 2018).
4.2.5 Immunohistochemistry

Immunohistochemistry (IHC) was used to characterize and semi-quantify the products of the goblet cells. Tissue sections were immunohistochemically stained (Leica, BOND-MAX Stainer, Leica Biosystems, Buffalo Grove, IN, USA) for Mucin 2 (antibody PA5-79702, Invitrogen, Carlsbad, CA, USA), Mucin5AC (antibody PA5-83504, Invitrogen, Carlsbad, CA, USA), and Resistin-Like Molecule Beta (antibody PA5-61896, Invitrogen, Carlsbad, CA, USA). The number of immunohistochemically stained cells were counted per 10 intact glands within a sample. Along with IHC staining, samples were stained with PAS/Alcian Blue (Richard-AllenTM Scientific Alcian Blue/PAS Special Stain Kit, Thermo ScientificTM, Waltham, MA, USA) per manufacturer’s instructions for evaluation and enumeration of mucins within the goblet cells.

4.2.6 Statistical Analyses

Statistical analyses were carried out using SAS 9.4 (SAS Institute, Cary, NC, USA). Mixed linear models were constructed for evaluating the effect of treatment group and days post treatment on histopathological findings. The ‘glimmix procedure’ was used and ‘horse ID,’ ‘Age,’ and ‘sex’ kept as a random effects. ‘Group’, ‘day’, and ‘organ’ were kept as class variables. The traditional forward and backward elimination of variables was used to evaluate the influence of all parameters. Variables with P-values of 0.2 or below remained in the model and the interaction between group and day was evaluated in all analyses (Lee and Koval, 1997). A ‘least square means’ analysis was used for a Tukey’s pairwise comparison for categorical variables when p<0.05. A significance level of α=0.05 was used.
for data interpretation. Figures were developed in JMP Pro 14 (SAS Institute, Cary, NC, USA).

4.3 Results

4.3.1 Worm burden

Mucosal worm burden and anthelmintic efficacy is described in Table 4-1. There were no significant differences in mucosal larval burden between the groups. Significantly lower EL3 and LL3 counts were observed in the VC compared to the CEC and DC; however, the VC had higher encysted larval burden (p<0.001, Figure 4-1). Mucosal worm burdens were highest at 2 WPT. This was supported within the histological evaluation of the larval stages as well (p>0.05).

<table>
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**Total Mucosal Counts**

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<td>3000-101000</td>
<td>42.18</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>32500</td>
<td>8000-62000</td>
<td>16.3</td>
</tr>
<tr>
<td>Ivermectin</td>
<td>2</td>
<td>98833</td>
<td>18000-297000</td>
<td>-32.4</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>62667</td>
<td>34000-147000</td>
<td>-61.4</td>
</tr>
<tr>
<td>Control</td>
<td>2</td>
<td>7466</td>
<td>7000-184000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>38833</td>
<td>10000-71000</td>
<td></td>
</tr>
</tbody>
</table>

Table 4-2. (continued)
4.3.2 Inflammatory Cells

There were no significant differences between treatment groups, organs, or timepoints in the present study (Figure 4-2). Neutrophil scores were associated with larval stage; L4s were associated with higher scores than LL3s and EL3s (p=0.0040).
Figure 4-2. Inflammatory scores presented by groups and weeks post treatment. There were no significant differences noted between the groups or time intervals. Upper and lower bounds of the boxes represent the upper and lower quartiles, respectively.

4.3.3 Goblet Cells

Across all groups, GCH was elevated at 2 compared to 5 WPT (p=0.0029, Figure 4-3). GCH scores were lowest in moxidectin treated versus the ivermectin treated and untreated controls (p<0.0001, Figure 4-3). Scores were positively associated with worm burden, and highest in the VC compared to the DC and CEC, with the DC higher than the CEC (p=0.0495). Alcian blue and PAS stained counts were positively associated with GCH, and significantly followed similar trends with organs, WPT, and groups (p=0.0252),
and the ratio between the stained cell counts positively associated with higher GCH scores (p=0.0043).

MUC2 counts were highest in the VC, followed by the CEC and then the DC (p<0.0367, Figure 4-4). MUC2 counts were also negatively associated with total organ encysted larval counts (p<0.0001). MUC5AC was positively associated with GCH and negatively associated with total encysted larval counts (p=0.0066). In the tissues, MUC5AC expression was highest in the DC, compared to CEC and VC (p=0.0317, Figure 4-4). RELM-β followed similar trends to MUC2 and MUC5AC; however, it was lowest in the CEC (p=0.0032, Figure 4-4).

**Figure 4-3.** Goblet Cell Hyperplasia scores by group and weeks post treatment. Different letters denote significant differences between timepoints. Upper and lower bounds of the boxes represent the upper and lower quartiles, respectively.
4.4 Discussion

This study is the first to evaluate ivermectin in regard to the local histopathological findings following treatment at two time points. Overall, there were no significant differences in the pro-inflammatory cells present; however, we did note decreased GCH in horses after receiving treatment, with lowest GCH scores occurring in the moxidectin treated horses compared to ivermectin. This does indicate a difference in the two treatments, with a stronger host response when more encysted larvae remain.

The most interesting finding of the present study was the association between GCH and worm burdens. Higher GCH scores at 2 WPT were associated with the higher larval burdens, and this is supported by studies in other species (Hasnain et al., 2010; Turner et
al., 2013); however, this contradicts our previous findings, where mucosal worm counts were inversely correlated with GCH scores (Steuer et al., 2018). Our present study more accurately depicts a role in worm expulsion occurring over time. Findings in our previous study led to the hypothesis that worm turnover was occurring regardless of treatment (Steuer et al., 2018). This current study supported this hypothesis, as we observed the same trend in mucosal counts and GCH was associated with these decreasing worm burdens. At present, we are unable to evaluate the role of luminal counts in this process, but GCH directly correlated with luminal worm burdens in the previous study (Steuer et al., 2018). It is speculated that this is the case here as well. This is directly supported by the fact that there were significant differences between the treatment groups in regard to GCH, with the moxidectin group, which had the lowest worm burdens, having lower scores than the ivermectin group, which, in turn had lower scores than the control group. This signifies that with less worms, there is less of a mucosal inflammatory reaction to worms and decreased worm expulsion; however, luminal counts need to be assessed to support this claim.

This is the first study to identify MUC5AC in the intestinal tissue of horses. It was reported in low numbers of stained goblet cells and associated with GCH and worm counts. This indicates a potential active component in worm expulsion, which has been previously reported only in other species (Hasnain et al., 2011, 2010; Ma et al., 2018; Rousseau et al., 2011). This is neither a common, nor typical mucus produced within the intestines, and is only previously reported in the airways of horses; however, it is also found in the gastric glands of the horses, which was used as a positive control in this study. MUC2 and RELM-β stained cells followed similar patterns to MUC5AC, and this indicates their critical role.
in worm expulsion, as depicted in other host and parasite species (Chen et al., 2018; Hasnain et al., 2013a, 2010; Herbert et al., 2009; Ma et al., 2018). This was previously reported in chapter 3 of this dissertation, where MUC2 gene expression significantly correlated with GCH and worm burdens.

One further component was evaluating acidic versus neutral mucins. This study aimed at evaluating neutral pH mucins versus acidic pH mucins, to see if there was a difference in the types of mucins potentially involved. The presence of both acidic (PAS stained mucins) and neutral mucins (Alcian blue stained mucins) increased with GCH and worm counts; however, it was interesting to note is that, with higher GCH and worm burdens, the ratio changed as well, with an increase in acidic mucins being produced. This may suggest that in higher worm burdens are associated with a more acidic environment. This may be the host response to incite turnover of the worms into the lumen and out of the host.

In regards to encysted larval counts, efficacies were similar to those that were previously reported (Bellaw et al., 2018; Eysker et al., 1997, 1992; Love, 1995; Xiao et al., 1994). The overall larval counts were not significantly different between the treatment groups, which may help explain the lack of significant differences between treatment groups in regard to the inflammatory response. This is consistent with previous studies as well (Nielsen et al., 2015; Steinbach et al., 2006; Steuer et al., 2018). Inflammatory cell populations were not significantly different among groups. This is again consistent with previous reports (Nielsen et al., 2015; Steinbach et al., 2006; Steuer et al., 2018). The focal inflammatory reaction surrounding the encysted larvae did not appear to be significantly impacted by treatment, which is what we have previously reported (Steuer et al., 2018).
However, as developing and later stage larvae had greater inflammatory cell populations, this indicates the potential for immune modulation by the parasites, or parasites undergoing immune modulation. In fact, the inflammatory reaction, regardless of treatment, is most likely driven by the host-parasite interaction and larval stages, and this requires further investigations.

Finally, this study once again demonstrated that the three large intestinal organs may have a different response to worm burdens, as noted in Chapters 2 and 3 of this dissertation. This raises the question into why we see differences in worm burdens and in the inflammatory responses between the organs, and what this may indicate in intestinal disease. In general, during cyathostomin infections, the large intestine is regarded as a whole; however, this study, along with our previous study, suggests that the VC may have less of a proinflammatory reaction pattern compared to the DC, and the CEC may have high turnover of larvae overall, as demonstrated here and previously (Steuer et al., 2018). It is possible that identifying the exact mechanisms behind this may shed light on managing worm burdens, diagnosis, and treatment.

In conclusion, this study found no significant differences in inflammatory cell populations following larvicidal versus nonlarvicidal treatment and untreated horses; however, GCH and its role in response to treatment is necessary to consider in future studies. Further investigations into the role of goblet cell hyperplasia and worm turnover need to be considered in the future to understand the reactions to anthelmintic treatment.
5.1 Introduction

Horses are host to cyathostomins, a dynamic groups of parasites, with over 8 genera and 40 species in domesticated horses alone (Lichtenfels et al., 2008). The somewhat complex direct lifecycle involves up to several hundred thousand larvae infecting the host’s cecum (CEC), ventral colon (VC), and dorsal colon (DC). Larvae penetrate as early third stage larvae (EL3s) and encyst, where they may undergo hypobiosis for up to 2 years and/or proceed through development to the late third and fourth stage encysted larvae (LL3s/L4s, respectively) (Eysker and Mirck, 1984; Ogbourne, 1975). This hypobiosis is believed to be driven by the host response to the parasites; however, the mechanism is poorly understood (Chapman et al., 2003). Larger accumulations are associated with age and seasons, with noticeably higher counts occurring in horses 2-5 years of age and the highest counts occurring after the grazing season (Ogbourne, 1975). There are noted inflammatory responses surrounding each individual larvae; however, disease is characterized when large numbers of L4 excyst *en masse*, producing a generalized typhlocolitis, a condition also known as larval cyathostominosis (Love et al., 1999). Few studies have evaluated the mechanisms behind the immunological and inflammatory processes in diseased as well as clinically normal horses. Only one study thus far has evaluated cytokine gene expression in the large intestinal tissues of horses with and without large intestinal disease, including three cases with larval cyathostominosis (Davidson et al., 2002). They found that Tumor Necrosis Factor (TNF)-α was expressed in horses with inflammatory bowel disease; however, there were no significant differences occurring between the groups with and
without large intestinal disease, including those with encysted cyathostomins present (Davidson et al., 2002). Another study evaluated local tissue gene expression levels of Interleukin (IL)-4, IL-5, IL-10, Interferon (IFN)-γ, in large intestinal tissues with and without encysted larvae present (Davidson et al., 2005). Tissues with encysted cyathostomins present expressed higher levels of IL-4, IL-5, and IL-10 than those without, whereas expression of IFN-γ did not differ between the two groups (Davidson et al., 2005).

When evaluating the local inflammatory response to treatment, studies have targeted comparing the only two larvicidal regimens available: moxidectin at 0.4mg/kg once orally as dosed above and fenbendazole at 10 mg/kg orally for five days (Nielsen et al., 2015; Steinbach et al., 2006; Steuer et al., 2018). These studies were based on evaluating the differences in responses to two different drug classes; however, few significant differences were noted between the studies, and the earlier studies had contradictory findings. One study suggested that there was a significantly reduced local inflammatory response in ponies treated with moxidectin compared to the larvicidal fenbendazole protocol based on qualitative histopathological findings (Steinbach et al., 2006). Inversely, we have evaluated the local and systemic inflammatory response to moxidectin and fenbendazole in two different studies. In 2015, we found subtle decreased inflammatory reactions in the fenbendazole treated ponies compared to moxidectin; however, this was attributed to the decreased efficacy of fenbendazole, and not to actual differences between the two anthelmintics themselves (Nielsen et al., 2015). In 2018, we completed the study reported in Chapters 2 and 3 of this dissertation. Briefly, we noted that there were no significant differences in the overall local and systemic inflammatory response to these treatments that had similar efficacies. The most significant finding was
the relationship between the worm burdens, and goblet cell hyperplasia (GCH) and the related genes. This potentially implicated GCH in worm turnover, similar to what has been described in other host-parasite interactions (Hasnain et al., 2013a, 2010; Herbert et al., 2009).

Of the three currently available drug classes, only the macrocyclic lactones (MLs) do not have widespread resistance reported in cyathostomin populations (Kaplan et al., 2004; Lester et al., 2013; Peregrine et al., 2014). While both MLs are labeled as broad spectrum, only moxidectin gel administered at 0.4 mg/kg once orally targets the encysted stages. Specifically, in the United States it is labeled for the LL3/L4s, whereas in the rest of the world it has a label claim for all stages. Ivermectin at the labeled 0.2 mg/kg once orally dose is not labeled to target the encysted stages, and most studies support this finding even with elevated doses (Klei et al., 1993); however, few studies have demonstrated some efficacy against the encysted stages (Eysker et al., 1992; Love, 1995).

Studies have not thoroughly evaluated the local inflammatory response to solely adulticidal ivermectin, even though adulticidal therapy is implicated in the disease process behind larval cyathostominosis (Reid et al., 1995). Previous studies that have evaluated the inflammatory reaction to ivermectin and compared to oxibendazole, moxidectin and other products noted intestinal erythema, edema and ulceration to worm burdens, but no differences between treatments (Love, 1995; Monahan et al., 1998, 1996). These studies have not quantified the inflammatory reaction, nor evaluated cytokine and gene expression within the tissue following treatment.

The aims with the present study were to collect further local and systemic information on inflammatory responses of selected cytokines and goblet cell-associated
components following anthelmintic treatment with a single dose of moxidectin and ivermectin in horses naturally infected with cyathostomin parasites to compare the effects of a larvicidal versus nonlarvicidal product within the same drug class. This study evaluated two post treatment time intervals (2 and 5 weeks) with emphasis on the role of goblet cells and their potential involvement with worm expulsion. An additional aim was to further evaluate the local and systemic pro- and anti-inflammatory gene expression related to the larvae and to treatment.

5.2 Materials and methods

5.2.1 Study design

As stated in 4.2.1, this study was performed under the University of Kentucky’s Institutional Animal Care and Use Committee, protocol number 2018-3134. Power calculations for group sizes have previously been reported (Bellaw et al., 2018). Thirty-six horses with naturally acquired cyathostomin infections, aged 2-5 years old, were kept on pasture from August-October 2019. To allocate into groups, horses within a birth year were grouped by sex and then ranked by decreasing magnitude of egg counts (Cringoli et al., 2017). Then horses were blocked into groups of three and randomly allocated into each group. Finally, the horses were then allocated into one of the two time intervals. Each group contained a total of 12 horses, with 6 horses allocated to each of the two time intervals. All study personnel were blinded to group allocations and treatments throughout the study.

On day -10, each horse was weighed on a certified livestock scale, and feces taken for group allocation, as defined above. Horses in the moxidectin group received 0.4mg/kg orally (Quest Plus, Zoetis Inc., Parsippany, NJ, USA), and horses in the ivermectin group
received 0.2mg/kg ivermectin orally (Zimectrin Gold, Boehringer Ingelheim, Duluth, GA, USA) on Day 0. The horses in the untreated control did not receive any treatment.

Six horses from each group were euthanized on days 14 and 15 (2 weeks post treatment). Three horses from each group were necropsied each day to ensure equal numbers of horses from each group were necropsied simultaneously. The rest of the 18 horses were kept on pasture until they were euthanized in the same manner as above, on days 35 and 36 (5 weeks post treatment).

5.2.2 Sample collection

Weekly blood samples, starting at Day -10, were collected from all ponies until euthanasia and placed into TempusTM blood RNA tubes (Applied BiosystemsTM, Foster City, CA, USA) per the manufacturer’s instructions. The filled tubes were vigorously shaken for 15-30 seconds and incubated at room temperature for 24 hours. The blood samples were then placed at -20°C until further processing.

As part of the necropsy procedure, tissue samples were taken with a 6mm biopsy punch the CEC, VC, and DC, weighing 20-30mg. Tissue samples were placed directly into RNALater (Life technologies, Grand Island, NY, USA), and left at room temperature for 24 hours and then placed at 4°C until further processing.

5.2.3 Cytokine gene expression

5.2.3.1 RNA Isolation and reverse transcription

Total RNA was isolated from whole blood Tempus tube samples as described by Page et al., 2017. Briefly, Samples were placed into 50mL tubes and washed
with 3mL 1xPBS and centrifuged at 300g and 4°C for 10 minutes. 600µl of Viral Lysis buffer (Invitrogen, Carlsbad, CA, USA) was added and to each sample and vortexed to suspend the pellet. RNA was extracted with Kingfisher Flex (Thermo Scientific, Waltham, MA, USA) instrument and MagMax CORE Nucleic Acid Purification Kit (Applied BiosystemsTM, Foster City, CA, USA). Samples were stored at -80°C until further use.

Tissue was processed similarly to the blood. Approximately 25mg of tissue were lysed with 10µl proteinase K and 90µl proteinase K buffer from the MagMax CORE Nucleic Acid Purification kit for 2h at 55°C. The sample was then added to the 96 well plate and processed according to manufacturer’s tissue extraction instructions, with using the Kingfisher Flex tissue RNA program.

Reverse transcription of the samples was carried out similarly to Nielsen et al. 2015. Briefly, RNA was quantified with a Biotek Epoch Biophotometer (Biotek, Winooski, VT, USA). Reverse transcription reactions were carried out in an Applied Biosystems Veriti 96 well Thermal Cycler (Applied BiosystemsTM, Foster City, CA, USA), using 6µl Maxima Reverse transcriptase (Thermo ScientificTM, Waltham, MA, USA), 4µl RNA, and10µl nuclease free water. The cDNA was then diluted with 120µl RNase-free water (Qiagen, Hilder, Germany). Samples were stored at -20°C until further use.

5.2.3.2 Quantitative Real Time Polymerase Chain Reaction

Gene expression was evaluated through quantitative RT-PCR assay for all blood and tissue samples (Breathnach et al., 2006; Nielsen et al., 2015). Equine specific TaqMan intron spanning primers and probes (Thermo-Fisher ScientificTM, Waltham, MA, USA) were used for this study. β-glucuronidase (β-GUS, Ec03470630_m1), IL-4
(Ec03468790_m1), IL-5 (Ec03468691_m1), IL-6 (Ec03468678_m1), IL-10 (Ec03468647_m1), IL-13 (Ec03470543_m1), IL-17A (Ec03470096_m1), IFN-γ (Ec03468606_m1), and TNF-α (Ec03467871_m1) were commercially available through Thermo-Fisher Scientific and used in this study. Custom TaqMan® gene expression assays were developed through Thermo-Fisher Scientific for IL-22 (AR9HJZX), MUC 2 (ARRWFPW), and RELM-β (AP47XR4). β-GUS was used as a housekeeping gene.

Each reaction contained 5µl of SensiFAST® Lo-Rox master-mix (Bioline, London, UK), 0.5µl of 20x TaqMan assay for primers and probes of interest (assays-by-design, Applied Biosystems), and 4.5µl of cDNA. Duplicate wells were prepared for each sample and gene. QuantStudio 7 Flex Real-Time PCR System (Applied BiosystemsTM, Foster City, CA, USA) was used to assess gene expression. LinRegPCR was used to adjust qRT-PCR efficiencies (Ramakers et al., 2003). qRT-PCR data were normalized to the housekeeping gene (β-GUS), and analyzed using the 2-ΔΔCT method (Breathnach et al., 2006; Livak and Schmittgen, 2001).

5.2.4 Statistical Analyses

Statistical analyses were carried out using SAS 9.4 (SAS Institute, Cary, NC, USA). Mixed linear models were constructed for evaluating the effect of treatment group and days post treatment on gene expression. The ‘glimmix procedure’ was used and ‘horse ID’ kept as a random effect. ‘Group’, ‘day’, and ‘organ’ were kept as class variables. Models were built to analyze each cytokine, with repeated measures taken into account for systemic expression. The traditional forward and backward elimination of variables was used to evaluate the influence of all parameters. Variables with $P$-values of 0.2 or below remained in the model and the interaction between group and day was evaluated in all analyses (Lee
and Koval, 1997). A ‘least square means’ analysis was used for a Tukey’s pairwise comparison of categorical variables with \( p<0.05 \). A significance level of \( \alpha=0.05 \) was used for data interpretation. Figures and linear pairwise correlations were conducted through JMP Pro 14 (SAS Institute, Cary, NC, USA), and a Pearson correlation coefficient obtained. For the correlations, goblet cell hyperplasia associated gene expression, and all inflammatory cytokine expression were used as variables.

5.3 Results

5.3.1 Goblet cell-associated and anti-inflammatory cytokine gene expression

5.3.1.1 Gene expression in the intestinal tissue

There were no significant differences in the goblet cell-associated and anti-inflammatory gene expressions between the treatment groups or among the 2 timepoints within the intestinal tissue (\( p>0.05 \), Figure 5-1). IL-13 was elevated in the DC compared to the CEC; whereas MUC2 expression was highest in the cecum (\( p=0.0246 \)); however, there were no other significant differences of genes within the organs (Figure 5-2). Finally, IL-22 was positively associated with the number EL3 larvae (\( p=0.0418 \)). Correlations among cytokines in the tissue are presented in Table 6-5. Correlation between the genes expressed in the intestinal tissues. Bold indicates significant correlations.
Figure 5.1. Relative expression of goblet cell associated gene expression and anti-inflammatory gene expression within the tissue at 2 and 5 weeks post treatment. Upper and lower bounds of the boxes represent the upper and lower quartiles, respectively.
5.3.1.2 Gene expression in the systemic circulation

In systemic circulation, there were no significant differences of goblet cell and anti-inflammatory associated genes between the treatment groups (p>0.05). All genes followed similar trends in systemic circulation, with 4 WPT as a significant timepoint among all genes (p=0.0494). Regarding goblet cell gene expression over time, significant differences were noted among the timepoints (p=0.0020, Figure 5-3). IL-10 expression was low at 2, 3, and 5 WPT compared to treatment and 4 WPT (p<0.001). IL-13 increased slightly to 3 WPT, dropped significantly at 4 WPT, and then increased dramatically at 5
WPT. IL-22 has similar trends to IL-13; however, it was significantly decreased at 2 and 4 WPT (p<0.001). MUC2 was highest at 2 WPT, lowest at 4 WPT, with a rebound at 5 WPT (p<0.0001), whereas RELM- β was lowest at 2 WPT, highest at 4 WPT, with a significant drop at 5 WPT (p=0.0494). Correlations between genes are listed in Table 6-6.

Figure 5-3. Relative quantitites of goblet cell associated gene expression in systemic circulation over time. Different letters denote significant differences between timepoints. Upper and lower bounds of the boxes represent the upper and lower quartiles, respectively.
5.3.2 Pro-inflammatory cytokine gene expression

5.3.2.1 Gene expression in the intestinal tissue

IFN-γ, had the highest expression in ivermectin treated horses compared to moxidectin and the controls, and with the lowest expression in the moxidectin group (p<0.001). It was also negatively associated with encysted larval burdens (p<0.001) and expression was highest at 5 WPT compared to 2 WPT (p<0.001). Highest expression was in the CEC, followed by VC, with the lowest in the DC (p=0.001). TNF-α expression was decreased in the two treatment groups, with moxidectin expressing the lowest level of TNF-α (p<0.001). Expression was highest in the VC compared to the cecum and DC, with lowest expression in the DC (p<0.001). Comparing 2 versus 5 WPT, TNF-α expression decreased in the moxidectin group, remained similar in the ivermectin group, and increased in the control group (p<0.001). Correlations between proinflammatory genes within the tissues are listed in Table 6-6.

Other pro-inflammatory (IL-4, IL-5, IL-6) cytokine expression was negatively associated with encysted larval burdens (p=0.0013). Expression of IL-5 was highest in the cecum, followed by DC and then VC (p=0.0077). IL-17A, on the other hand, was highest in the DC, compared to the CEC and VC (p=0.0340) and positively associated with MUC2 (p=0.0294).
Figure 5.4. Relative quantities of expression of pro-inflammatory cytokine genes within the tissues. Different letters denote significant differences between organs. Upper and lower bounds of the boxes represent the upper and lower quartiles, respectively. Abbreviations are cecum (CEC), ventral colon (VC), and dorsal colon (DC).

5.3.2.2 Gene expression in systemic circulation

There were no significant differences of proinflammatory cytokine gene expression in systemic circulation between groups (p>0.05). Proinflammatory cytokine gene expression exhibited significant associations with other genes. IL-4 gene expression in
systemic circulation was positively associated with IL-5, IFN-γ, but negatively associated with IL-6, IL-10, MUC2, RELM-β, TNF-α, and encysted larval counts (p=0.0172). Expression of IL-4, and IL-5 cytokines was highest at 4 WPT, and lowest at 0 and 1 WPT post treatment (p=0.010). Across all groups, IL-6, IL-10, MUC2, RELM-β, TNF-α, expression was lowest at 4 WPT, and then increased in expression at 5 WPT (p=0.0477, Figure 5-5). IL-6 gene expression was highest at treatment and 5 WPT, and lowest at 4 WPT (p=0.0321, Figure 5-5).

Interestingly, IFN-γ was negatively associated with EL3 counts (p=0.0167). IFN-γ gene expression increased from treatment to 3 WPT, then dropped at 4 and 5 WPT, and TNF-α followed a similar pattern (p=0.0224, Figure 5-5). Correlations between the proinflammatory cytokine gene expression levels in systemic circulation are listed in Table 6-6.
Figure 5-5. Relative gene expression of proinflammatory cytokines from treatment to 5 WPT. Different letters denote significant differences between timepoints. Upper and lower bounds of the boxes represent the upper and lower quartiles, respectively.
5.4 Discussion

This is the first study to evaluate the local tissue gene expression to ivermectin treatment. There was significant difference in the proinflammatory response between the two treatment groups and between the untreated control horses. Within the tissues, both treatment groups had considerably lower pro-inflammatory response, specifically with regards to IFN-γ and TNF-α, compared to the untreated control group; however, moxidectin treatment was associated with decreased expression when compared to ivermectin treated horses. This may not be a direct effect of the treatment itself, but more likely due to the removal of the luminal worms and some of the encysted stages within the tissues, because we see the significant difference between the treatment groups and the untreated control group.

Previously, we reported the decreased encysted larval burdens and GCH at the 5 WPT (Chapter 4). While it appears that a similar trend was followed in GCH gene expression in this current study, it was not statistically significant within the tissues; however, the expression of these GCH related genes within the systemic circulation was significantly different among the timepoints. Also, at 4 WPT, there were several significant spikes and drops in cytokine expression. For example, IL-4 and IL-10 significantly increased, whereas MUC2 and IL-22 significantly decreased. It is possible that a significant event occurred within the tissues, whether it be parasite maturation and excystment into the lumen, further worm expulsion from the lumen, but this remains unknown. It may also suggest that GCH decreased briefly within the tissues, and therefore there may have been a decrease in worm burden or worm expulsion. This will be evaluated in more depth when the luminal counts of cyathostomins become available. The GCH markers and the
relationship they exhibit with each other, GCH, and worm burdens is similar to what we previously reported (Chapter 2); however, this study reports a significant pro-inflammatory response to the worms present in the tissues, which was previously reported by other studies (Davidson et al., 2005b, 2002).

Finally, we further describe the differences between the large intestinal organs, which we previously reported in Chapters 2, 3, and 4. The organs clearly produce a different response to encysted larvae and treatment. For example, the DC appears to express higher pro-inflammatory cytokine levels (such as IL-6), which may lead to the lower mucosal larval burdens observed; whereas, the CEC had a greater GCH response, but not pro-inflammatory response, and may indicate higher turnover of larvae in this organ. However, this requires further investigation into the mechanisms behind the infection.

In regard to systemic circulation, IL-22 and MUC2, and RELM-ß may be potential markers for epithelial cell turnover, goblet cells, and worm burden, and they appear to be from spillover from the intestines, as previously described; however, as with all other markers, there are other potential factors that may influence these in systemic circulation, such as coinfections with bacteria, other inflammatory insults or diseases that may increase goblet cell turnover within the host (Nielsen et al., 2015; Steuer et al., 2018).

In conclusion, this is the first study to evaluate the local tissue gene expression to ivermectin treatment. While we noted that ivermectin treated horses had a few increased proinflammatory markers, this was not likely due to the compound itself, but that higher worm burdens (specifically mucosal encysted larval burden) elicit a greater pro-
inflammatory response. GCH and its related genes also appear to be associated with worm burdens and remain an area of interest and further exploration.
CHAPTER 6. DISCUSSION

6.1 Introduction to the discussion

The studies presented herein form a small bridge over the current knowledge gaps concerning the host parasite interaction between horses and cyathostomins, and the effects of anthelmintic treatment. The discussion is presented with an overview of major findings, differences between the treatment classes, larvicidal versus nonlarvicidal treatment, the differences between timepoints, systemic responses, and goblet cells and their potential role.

6.2 Overview of major findings

The work presented herein reports the first studies to evaluate local and systemic inflammatory and goblet cell responses to cyathostomin infections and anthelmintic treatment at 2 and 5 weeks post treatment (WPT). The unique design in the studies allows for a comparison across all four chapters. Age range, time of year, group size, blinding, allocation, and treatment protocol, as well as necropsy techniques were kept equivalent to allow comparisons between the two studies; however, the first study used a population of ponies and the second study used horses, which may account for some differences.

Overall, reducing the number of encysted larvae and/or adult worms reduced the proinflammatory response in the horses and ponies, as presented in Chapters 2-5. FBZ may lead to a slightly increased proinflammatory cytokine response compared to the macrocyclic lactones (MLs), specifically moxidectin (MOX); however, MOX had higher inflammatory cell populations (see section 6.3). Whereas the nonlarvicidal ivermectin (IVM) led to a greater proinflammatory response than MOX (see section 6.4), but there was no difference in the inflammatory cell populations between the MLs.
Another unique aspect of these studies is the two post treatment necropsy timepoints. This is the first time that IVM, MOX, and FBZ have been evaluated at an interval greater than 2 WPT. This was originally performed to capture the egg reappearance period of the anthelmintics and to study changes in the worm populations (Bellaw et al., 2018). However, this allowed us to evaluate the local immunological reaction and study what may happen with the phenomenon of larval excystment, adult worm expulsion, and general parasite turnover. We found that, even without anthelmintic treatment administered, there was a significant decrease in mucosal worm burdens from 2 to 5 WPT, whereas luminal worm burdens increased from 2 to 5 WPT in the first study. This will be discussed in greater length in 6.5.

Another aspect of this study is the novel comparison of the CEC, VC, and DC in both studies. We found that the organs produced significantly different inflammatory and immunologic responses, which correlated with their different worm burdens, which will be discussed further in section 6.6.

Finally, the investigation of Goblet cells and their potential role in cyathostomin infections and horses was a main component of this dissertation. Goblet cell hyperplasia (GCH) has been reported with infections and disease since 1978 (Ogbourne, 1978); however, the exact role remains unclear. GCH and mucins were positively associated with treatments and encysted and luminal cyathostomin burdens in both studies and IVM treated horses had higher GCH responses, which will be discussed further in 6.7.
6.3 Benzimidazole versus macrocyclic lactone treatments and their immunologic effects

The limited differences in the inflammatory cell response described in the current work was attributed to the encysted and luminal worm burden present within the host, rather than the parasites that were removed and the potential reaction that may cause. The inflammatory cell populations primarily surround the larvae remaining in the intestinal wall and is associated with the larvae rather than focal areas without larvae present or a diffuse response (Chapters 2 and 3). This is also supported by the lack of differences between our treatment groups, and the control group, in general, expressing a greater inflammatory response. In both studies, IVM, MOX and FBZ exhibit different larvicidal efficacy; however, some larvae remain, regardless of treatment (Bellaw et al., 2018). At maximum, 74.6% of larvae were removed from the tissues, with values ranging from 0-74.8% at the 2 and 5 WPT timepoints (Bellaw et al., 2018, Chapter 3). Therefore, a large proportion of the worms were left behind in all groups, allowing for continued stimulation of the immune system in all horses. However, if removal were to cause a greater response with dying and decaying larvae, there would be greater expression of proinflammatory cytokines in those with the highest percentage of removal, which was not found (Chapters 3 and 5). While the low level of efficacy is due to drug resistance of the parasites to FBZ (Bellaw et al., 2018), the exact mechanism for MOX is not currently known; however, the variable efficacy is well described (Table 1-3). The groups with higher larval counts had a higher proinflammatory response than those that do not (Chapters 2-4).

The inflammatory cell populations and reactions reported herein contradict the findings by Steinbach et al. and Nielsen et al. Briefly, Steinbach et al. noted a greater
inflammatory cell response in the FBZ treated group compared to the MOX group; whereas Nielsen et al. described a subtle response to MOX (Nielsen et al., 2015; Steinbach et al., 2006). It was suggested that this was due to the reduced FBZ larvicidal efficacy, which contrasts Steinbach et al. (2006), who reported adequate efficacy of FBZ, although the exact efficacy was not reported. Our study observed increased macrophage and T cell counts following MOX treatment compared to the FBZ treated group; however, overall the two products had similar larvicidal efficacies, which contradicts the suggestion that the lack of inflammatory response to FBZ could be due to reduced efficacy. When taking into account the mechanisms of action of the anthelmintics, FBZ and MOX act different upon the parasites, which was discussed in 1.2.4. In other hosts, such as in as dogs with *Dirofilaria immitis* infections, the use of MLs inhibits E/S secretions of the microfilaria (L1 larvae) and therefore allows the host immune response to detect and remove the larvae (Vatta et al., 2014; Zahner et al., 1997). In comparison, FBZ kills the parasites, and then allows for removal by the host immune system. This allows for different antigenic stimulation and reaction to the parasites within the host tissues.

6.4 Larvicidal versus non-larvicidal treatments and the immunologic effects

The larvicidal efficacies of moxidectin and ivermectin were within the ranges previously reported (Bellaw et al., 2018; Eysker et al., 1997, 1992; Love, 1995; Monahan et al., 1998, 1996; Xiao et al., 1994). The hypothesis that more larvae decrease the inflammatory response is rejected by Chapters 4 and 5, comparing IVM and MOX. Ivermectin treated horses had an increased proinflammatory response compared to MOX. GCH related genes had no significant differences within the tissues between the groups and timepoints. IFN-γ expression was highest in IVM treated horses, lowest in MOX,
suggesting that a reduction in parasite burden results in a lower proinflammatory response. Proinflammatory cytokine expression significantly associated with encysted larval burdens. It is also reported that ivermectin (or nonlarvicidal treatments) is implicated in disease (Reid et al., 1995), which is not true of other strongylid parasites and host systems. This indicates a unique case within cyathostomins and how the parasites response to treatment and interact with the host immune system.

6.5 The differences between the timepoints: larvae and worm turnover revisited

Due to the longer follow-up times post treatment, these studies (Chapters 2 and 4) detected an interesting phenomenon within the encysted burdens, where they were highest at 2 WPT and lowest at 5 WPT across all groups. Originally, this was attributed to a lab error; however, given the repeatability, it appears that another influence may be occurring. One potential factor is seasonality and worm turnover. Once per or twice per year, the adult worms will die and be replaced by the immature worms. This developed from the notion that at the adults live for 270-470 days within the host, and that L4s were more numerous in the spring than summer, and again in the fall (Gibson, 1953; Matheison, 1964; Ogbourne, 1975; Smith, 1976a). While the previous authors believed that this occurs in the spring before the grazing season, this turnover may actually occur in the fall, after the grazing season, which is supported by these data, that they found high numbers of L4s in the fall, and that larval cyathostominosis occurs in the late fall/winter-early spring (Gibson, 1953; Ogbourne, 1975; Peregrine et al., 2006; Reid et al., 1995; Schäfer et al., 2019; Smith, 1976a). Treatment inducing development of the encysted worms is also supported by the luminal counts from Chapter 2 where luminal counts, especially luminal L4s in the treatment groups increased from 2 to 5 WPT; and encysted larval counts in Chapter 2 and
Chapter 4 which decreased from 2 to 5 WPT (Gibson, 1953; Matheison, 1964; Ogbourne, 1975; Smith, 1976a). However, since this was also noted in the treatment group, this may also indicate the seasonality of the parasite as well.

The limited differences in the inflammatory cell response described in the current work were thought to be from the encysted and luminal worm burden present within the host, rather than the parasites that were removed and the potential reaction that may cause. Higher eosinophil, macrophage, and other inflammatory cell scores were associated with later stage and degenerative larvae and all of these inflammatory cell scores were higher at 2 WPT across all groups in Chapter 2, and neutrophils in Chapter 3.

6.6 Organs and tissue responses: are all large intestinal compartments created equal

The control group had the highest response within the tissues. The best explanation for this phenomenon is that higher numbers of cyathostomins, both encysted and luminal, increase the inflammatory response, which was opposite of what we hypothesized. The findings that there is a different response between the organs also makes logical sense.

Organs responded differently in both studies, histologically and in cytokine expression. Overall, it appears that a higher proinflammatory response occurs in the DC, and the CEC has a higher goblet cell reaction (Chapters 3 and 5). This is consistent with lower worm burdens in the DC, followed by CEC, then VC, reported in this study. And may attribute to the DC’s lower encysted and luminal worm burdens, or the different species composition of the DC may contribute to a greater inflammatory response; however, this is consistent with previous studies (Chapman et al., 2003; Davidson et al., 2002; Hogan et al., 2006; Maizels et al., 2012; Reinemeyer et al., 1986). Why the organs have different worm species compositions, worm burdens, and mucosal reactions remains
an area of interest and pursuit when trying to understand the infection dynamics and possible disease processes.

Finally, we know that horses develop a level of immunity to cyathostomins, as represented by older (>4 years) horses having decreased egg counts compared to young horses and foals (1-3 years of age, and <1, respectively) (Klei and Chapman, 1999). Younger horses have higher encysted larval counts and differing responses in tissues, including in the DC, than older horses (Collobert-Laugier et al., 2002), illustrating a difference that occurs within this tissues of horses, and that the immunological and inflammatory response changes over time (as described in 1.3.2.1.1). The proinflammatory response likely triggers worm expulsion from the tissues, creating an inhospitable environment for most worm populations and these are areas of future exploration.

6.7 Systemic responses

Overall, the studies reported herein support the hypothesis of “spillover” previously presented (Nielsen et al., 2015), where the systemic cytokine and gene expression and differences between these may result from what is occurring in the large intestine. We measured goblet cell genes in systemic circulation that correlated positively with the tissue expression of these genes (Chapters 3 and 5). Systemically, while there were differences among timepoints and these correlated with tissue expression of all genes, many of these were not statistically significant (Chapters 3 and 5). This is likely due to the overall complexity of cytokines and their functions, and many crossovers with the immune system and its signaling. What is of note is those involved with the mucosal response, such as MUC2, RELM-β, and IL-22 directly correlated with the tissue expression, though at lower
levels, and were identified in systemic circulation, indicating potential markers for monitoring goblet cell function within horses.

6.8 Goblet cells and their role

Goblet cell hyperplasia positively correlated with the luminal counts. This is interesting, as it may have implicated the role of GCH in larval excystment into the lumen of the large intestines and/or expulsion of the worms from the host. In other host models, GCH, along with increased mucin and product production allow for decreasing the interaction between the epithelium and parasites, and along with increased contractility, allow expulsion from the host (Hasnain et al., 2013a; Murgia et al., 2017; Rousseau et al., 2011). The parasites in these rodent models investigated do not have an encysted stage within the large intestine, as cyathostomins do. The triggering of excystment, and the potential involvement of goblet cells and their mucins to aid this process is therefore unknown. These reactions remain an area of future interest.

In this study, we observed a potential spillover of gene expression from the large intestinal tissues, including those relating to GCH. In other species it was found that IL-22, GCH, MUC2 (among other mucins), and RELM-β expression were directly related to worm burden and worm expulsion, with higher expression and concentrations associated with greater worm burden and worm expulsion (Chen et al., 2012; Hansson, 2012; Hasnain et al., 2010; Maizels et al., 2012). IL-10 was inversely correlated with worm expulsion in studies as well (Hasnain et al., 2013b). We made similar findings, where GCH and related genes appeared to be related to worm turnover; however, higher GCH scores were associated with lower mucosal scores, but higher luminal counts. This may suggest that the luminal counts have a greater and more direct effect than the mucosal counts on GCH, or
vice versa. However, IL-10 and IL-22 may directly be correlated with mucosal worm burdens, as illustrated in Chapter 3. This may indicate a role in the encysted worms modulating the host immune system.

These studies found that a greater GCH response occurred with the untreated controls compared to the treatments and was lowest in the MOX group. This suggests that greater encysted burdens may be implicated in GCH and worm turnover; however, this is the opposite of the previous study (Chapter 2). This suggests that the encysted larval stages do not have a role in GCH, or vice versa; however, it remains a possibility that GCH may play a crucial role in larval excystment and luminal worm turnover may or vice versa. It is unsure what relationships luminal counts will have with GCH in this study; however, it is likely that luminal counts will be significantly associated with GCH, which is supported by other host parasite models (Hasnain et al., 2010; Turner et al., 2013). Without luminal counts, it is hard to determine the role of GCH; however, it is likely that the excystment into or expulsion out of the lumen of the large intestine is influenced by GCH or vice versa in cyathostomin infections.

Goblet cells and their products are key components of the host inflammatory response, as illustrated here and in other host/parasite interactions (Hasnain et al., 2010; Herbert et al., 2009; Mishra et al., 2007; Rodrigues et al., 2018). The goblet cells directly relate to luminal and encysted cyathostomin burdens in horses; however, other coinfections, such as with bacteria, may play a significant role. Treatment does not directly modulate GCH in horses; however, treatment may modulate GCH by modulating worm burdens.
6.9 Concurrent parasitic infections

One other component of this study to discuss is concurrent *A. perfoliata* infections in these horses. There were no tapeworms found in the treated horses; however, the control horses had *A. perfoliata* burdens in both studies (unpublished data); however, they were not correlated with any of the inflammatory responses in these horses. The histopathological samples were not taken near the ileocecal valve in these horses, where the primary *A. perfoliata* burdens reside. However, it is possible that these burdens would have an influence as seen in another study where epithelial proliferation, GCH, and cytokine responses were higher in *A. perfoliata* infections (Lawson et al., 2019). However, these studies did not account for cyathostomin infections and samples were collected directly at the site of *A. perfoliata* attachment and did not compare to those tissues within the same host (Lawson et al., 2019). Therefore, it is unlikely that *A. perfoliata* coinfections contributed significantly to the findings of our studies.

6.10 In vivo versus in vitro

This work is a first step toward identifying the effect of anthelmintic treatment on the inflammatory response and the role of goblet cells in cyathostomin infection. Further evaluations would require *in vitro* cultivation of large intestinal mucosa (or an alternative tissue representation), exposure of other equivalent cell lines (as in (Lawson et al., 2019) inoculated with cyathostomins, or different study approaches *in vivo*. As previously described in 0, there is a lack of success culturing the parasitic stages of cyathostomins. Furthermore, immune and cell population responses can change rapidly, within minutes to hours of insult, making it challenging to identify appropriate timepoints for *in vivo* studies.
In place of the two- and five-week timepoints used in the current studies, an alternative would be more immediate timepoints, such as one, two, and seven days post treatment. However, this may introduce the ethical challenges concerning the amount of horses needing to be euthanized to achieve statistical significance. Another option would be cannulation of horses for repeated biopsies; while the stomach, ileum, and CEC have been readily cannulated (Austbø and Volden, 2006; Meyer and Klingeberg-Kraus, 2002; Peloso et al., 1994; Williams et al., 2018); the colons, due to location, size, and risk of infection and peritonitis pose a unique set of concerns. The large intestinal cannulation of horses also poses an ethical potential with the increased risk of infection, disease, and rupture. In one study with long term dual cecal and ileal cannulation, 63% of subjects were euthanized due to intestinal herniation (Williams et al., 2018). Cannulations would also not allow for large subsampling, or sampling of multiple known areas. Overall, cannulation would be a potential option; however, there are great ethical, financial, and study design considerations beforehand.

One facet to consider is in vitro studies, as well as using other host models. As mentioned, being able to culture the parasitic stages of cyathostomins is not currently possible in vitro (Brianti et al., 2009; Klei and Chapman, 1999). As far as using other host models, cyathostomins have not been established in other hosts, including immunosuppressed rodent models (Klei and Chapman, 1999). Using other host/parasite models, there are no other strongyloid parasites or other laboratory animal models that include a hypobiotic developmental phase in the large intestine; making exact correlations difficult at this time.
6.11 Overall conclusion

Healthy horses with large encysted and luminal burdens that receive a treatment do not have a greater proinflammatory response than horses that do not receive treatment. Larvicidal treatments have a lower proinflammatory response than non-larvicidal treatment in healthy horses. The greater the encysted cyathostomin burden within a horse, the greater proinflammatory response they will have. In horses, goblet cells have an association with cyathostomin infections. Systemically, it appears that spillover of goblet cell related genes occurs and may be a future area of monitoring the goblet cell reaction in horses.

- **H1**: Equids that receive anthelmintic treatment will have a lower local and systemic inflammatory responses than equids that do not- **Reject**; the untreated control horses had higher pro-inflammatory responses in the tissues and there was no difference in systemic circulation

- **H2**: MLs will have a greater inflammatory response than BZs due to their higher efficacy and removal of larger encysted and luminal cyathostomin burdens- **Reject**, efficacies and responses were similar

- **H3**: Larvicidal treatment will incite a greater inflammatory response from encysted and luminal worm die-off than non-larvicidal treatment- **Reject**, proinflammatory responses were highest in the controls, which had the greatest encysted and luminal worm burdens

- **H4**: A greater cyathostomin burden, both mucosal and luminal, will decrease the proinflammatory response- **Reject**; the opposite was reported in these studies
- H₅: Encysted cyathostomins will be able to potentiate an anti-inflammatory response- **Accept**; There seems to be an association between the anti-inflammatory markers and encysted burden

- H₆: Local inflammatory responses to treatment will appear in systemic circulation as “spillover”- **Accept**; There seems to be some spillover of genes related to GCH in systemic circulation
APPENDICES
Figure 6-1. Box-whisker plots representing inflammatory cell scores (0 = none; 1 = 1-5 cell layers, mild inflammation or fibrosis; 2 = 6-15 cell layers, moderate inflammation or fibrosis; or 3 = >15 cell layers, severe inflammation or fibrosis), larval numbers, and lesion size (mm) in the three treatment groups at 2 and 5 weeks post treatment. Upper and lower bounds of the box represent the upper and lower quartile, respectively. The diamond represents the mean of the observations and the whiskers represent the minimum and maximum observations per group. a) Fibrous Connective Tissue Scores, b) L3 larvae in lamina propria, c) L4 larvae in in the mucosa, d) total mucosal length, e) goblet cell hyperplasia scores, f) B lymphocyte scores (IHC), g) T lymphocyte scores (IHC), h) macrophage scores (IHC), i) neutrophil scores, j) macrophage scores, k) eosinophil scores, l) lymphocyte scores. The fenbendazole group contains 6 animal at both 2 and 5 weeks post treatment, the Moxidectin group contains 7 individuals at 2 weeks post treatment and 5 animals at 5 weeks post treatment, and the control group contains 5 animals at 2 weeks post treatment and 7 animals at 5 weeks post treatment.
Table 6-1. Average mean larval counts per 50mm. Ventral Colon is abbreviated VC and Dorsal Colon is abbreviated DC.

<table>
<thead>
<tr>
<th>Group</th>
<th>Weeks Post Treatment</th>
<th>Organ</th>
<th>Intact L3 in lamina propria</th>
<th>Degenerative L3 in lamina propria</th>
<th>Intact L3 in submucosa</th>
<th>Degenerative L3 in submucosa</th>
<th>Intact L3 in glands</th>
<th>Degenerative L4 in mucosa</th>
<th>Intact L4 in submucosa</th>
<th>Degenerative L4 in submucosa</th>
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<td>1.48</td>
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<td></td>
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<td>VC</td>
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<td></td>
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<td></td>
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<td>VC</td>
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Table 6-2. Supplemental Table 2. Average histopathology scores for statistically significant variables Ventral Colon is abbreviated VC and Dorsal Colon is abbreviated DC.

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<tr>
<th>Weeks Post Treatment</th>
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<th>Eosinophil Scores</th>
<th>Goblet Cell Scores</th>
<th>Fibrous Connective Tissue Scores</th>
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<td>-</td>
</tr>
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<td>5</td>
<td>Cecum</td>
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Figure 6-2. Box-whisker plots of the natural log of the relative quantities (RQ) of mRNA expression of selected cytokines in the blood over time, as shown by treatment groups and study week the blood was drawn (-1-5). Upper and lower bounds of the boxes represent the upper and lower quartiles, respectively. -1 is the pretreatment blood sample. 0 is treatment, followed by 1, 2, 3, 4, and 5 weeks post treatment of IL-10 and IL-13.
Figure 6-3. Box-whisker plots of the natural log of the relative quantities (RQ) of mRNA expression of goblet cell-associated genes in the blood over time, as shown by treatment groups and week of blood collection. Upper and lower bounds of the boxes represent the upper and lower quartiles, respectively. -1 is the pretreatment blood sample. 0 is treatment week, followed by 1, 2, 3, 4, and 5 weeks post treatment. WPT (weeks post treatment).
Figure 6-4. Box-whisker plots of the natural log of the relative quantities (RQ) of circulating mRNA for pro-inflammatory cytokines in the blood over time, as shown by treatment groups and week of blood collection. Upper and lower bounds of the boxes represent the upper and lower quartiles, respectively. -1 is the pretreatment blood sample. 0 is treatment week, followed by 1, 2, 3, 4, and 5 weeks post treatment. WPT (weeks post treatment).
Figure 6-5. Box-whisker plots of the natural log of the relative quantities (RQ) of circulating mRNA for pro-inflammatory cytokines in the blood over time, as shown by treatment groups and week of blood collection. Upper and lower bounds of the boxes represent the upper and lower quartiles, respectively. -1 is the pretreatment blood sample. 0 is treatment week, followed by 1, 2, 3, 4, and 5 weeks post treatment. WPT (weeks post treatment) for TNF-α, IFN-γ, and IL-17A.
Table 6.3. Pairwise correlations between the different genes within the blood. The r values are presented to illustrate linear relationships. BOLD values indicate statistical significance.

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<th></th>
<th>IL-13</th>
<th>0.2349</th>
<th>IL-10</th>
<th>0.1103</th>
<th>MUC2</th>
<th>0.9879</th>
<th>RELM-β</th>
<th>0.1342</th>
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<tr>
<td>IL-13</td>
<td>0.2349</td>
<td>0.9861</td>
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<td>0.9879</td>
<td>0.1103</td>
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<td>0.1103</td>
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<td>0.1103</td>
<td>0.1103</td>
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<td>0.1103</td>
<td>0.1213</td>
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<td>0.2349</td>
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<td>0.2349</td>
<td>0.9861</td>
<td>0.2349</td>
<td>0.9861</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

r values are presented to illustrate linear relationships. BOLD values indicate statistical significance.
Table 6.4. Pairwise correlations between the different genes within the tissue. The \textit{r} values are presented to illustrate linear relationships. BOLD values indicate statistical significance.

\begin{tabular}{cccccccc}
\textbf{Gene} & \textbf{IL-10} & \textbf{IL-13} & \textbf{IL-22} & \textbf{IL-4} & \textbf{IL-5} & \textbf{IL-6} & \textbf{MUC2} & \textbf{RELM-β} & \textbf{TNF-α} \\
\hline
IL-10 & 0.7435 & 0.8626 & 0.9093 & 0.9887 & 0.8122 & 0.8883 & 0.9622 & 0.4179 & 0.3926 & 0.4026 & 0.1621 \\
IL-13 & 0.7435 & 0.8626 & 0.9093 & 0.9887 & 0.8122 & 0.8883 & 0.9622 & 0.4179 & 0.3926 & 0.4026 & 0.1621 \\
IL-16 & 0.751 & 0.755 & -0.0179 & & & & & & & & \\
MUC2 & 0.9417 & 0.2197 & & & & & & & & & \\
RELM-β & -0.1278 & & & & & & & & & & \\
TNF-α & & & & & & & & & & & \\
\end{tabular}
## Table 6-5. Correlation between the genes expressed in the intestinal tissues. Bold indicates significant correlations.

<table>
<thead>
<tr>
<th></th>
<th>IL-10</th>
<th>IL-13</th>
<th>IL-17A</th>
<th>IL-22</th>
<th>IL-4</th>
<th>IL-5</th>
<th>IL-6</th>
<th>MUC2</th>
<th>RELM-β</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>0.926</td>
<td>0.041</td>
<td><strong>0.945</strong></td>
<td>0.611</td>
<td><strong>0.986</strong></td>
<td><strong>0.989</strong></td>
<td>0.525</td>
<td>0.761</td>
<td>0.752</td>
<td>0.982</td>
</tr>
<tr>
<td>IL-10</td>
<td><strong>0.859</strong></td>
<td>0.941</td>
<td>0.877</td>
<td><strong>0.862</strong></td>
<td><strong>0.941</strong></td>
<td>0.761</td>
<td>0.898</td>
<td>0.902</td>
<td>0.935</td>
<td></td>
</tr>
<tr>
<td>IL-13</td>
<td>0.921</td>
<td>0.793</td>
<td>0.782</td>
<td>0.487</td>
<td>0.556</td>
<td>0.789</td>
<td>0.801</td>
<td>0.842</td>
<td></td>
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</tr>
<tr>
<td>IL-17A</td>
<td>0.904</td>
<td>0.945</td>
<td>0.955</td>
<td>0.889</td>
<td>0.923</td>
<td>0.930</td>
<td>0.946</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-22</td>
<td>0.606</td>
<td>0.691</td>
<td>0.861</td>
<td>0.999</td>
<td>0.992</td>
<td>0.784</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>IL-4</td>
<td>0.935</td>
<td>0.667</td>
<td>0.691</td>
<td>0.705</td>
<td>0.983</td>
<td></td>
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<tr>
<td>IL-5</td>
<td>0.631</td>
<td>0.788</td>
<td>0.784</td>
<td>0.962</td>
<td></td>
<td></td>
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<tr>
<td>IL-6</td>
<td>0.865</td>
<td>0.865</td>
<td>0.260</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>MUC2</td>
<td>0.998</td>
<td>0.881</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>RELM-β</td>
<td></td>
<td></td>
<td>0.882</td>
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<td></td>
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</tr>
</tbody>
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## Table 6-6. Correlation between genes expressed in systemic circulation. Bold indicates significant correlations.

<table>
<thead>
<tr>
<th></th>
<th>IL-10</th>
<th>IL-13</th>
<th>IL-17A</th>
<th>IL-22</th>
<th>IL-4</th>
<th>IL-5</th>
<th>IL-6</th>
<th>MUC2</th>
<th>RELM-β</th>
<th>TNF-α</th>
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<tbody>
<tr>
<td>IFN-γ</td>
<td>0.077</td>
<td><strong>0.115</strong></td>
<td>0.312</td>
<td>0.101</td>
<td>0.062</td>
<td><strong>0.815</strong></td>
<td><strong>0.368</strong></td>
<td>0.508</td>
<td>0.142</td>
<td>0.098</td>
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<tr>
<td>IL-10</td>
<td>-0.270</td>
<td>0.030</td>
<td><strong>-0.243</strong></td>
<td><strong>0.527</strong></td>
<td>0.003</td>
<td>-0.228</td>
<td>0.093</td>
<td><strong>0.348</strong></td>
<td><strong>-0.244</strong></td>
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</tr>
<tr>
<td>IL-13</td>
<td>0.068</td>
<td><strong>0.984</strong></td>
<td>-0.092</td>
<td><strong>0.105</strong></td>
<td>0.035</td>
<td>-0.195</td>
<td>0.034</td>
<td><strong>0.997</strong></td>
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<tr>
<td>IL-17A</td>
<td>-0.008</td>
<td>0.092</td>
<td><strong>0.230</strong></td>
<td>-0.060</td>
<td>-0.004</td>
<td>0.146</td>
<td>0.049</td>
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<tr>
<td>IL-22</td>
<td>-0.167</td>
<td><strong>0.124</strong></td>
<td>0.010</td>
<td>0.0357</td>
<td>0.137</td>
<td><strong>0.984</strong></td>
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<tr>
<td>IL-4</td>
<td>-0.007</td>
<td>-0.483</td>
<td><strong>-0.230</strong></td>
<td>-0.028</td>
<td>-0.112</td>
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<td>IL-5</td>
<td><strong>0.412</strong></td>
<td>0.613</td>
<td>0.036</td>
<td><strong>0.100</strong></td>
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<tr>
<td>IL-6</td>
<td>0.472</td>
<td>0.087</td>
<td>0.029</td>
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<tr>
<td>MUC2</td>
<td>0.444</td>
<td><strong>-0.149</strong></td>
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<tr>
<td>RELM-β</td>
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<td>0.075</td>
<td></td>
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VITA

Ashley E. Steuer

EDUCATION:
PhD Candidate in Veterinary Sciences, August 2016-Present
Maxwell H. Gluck Equine Research Center
University of Kentucky; Lexington, Kentucky 40546
Anticipated Graduation: May 2020

Doctor of Veterinary Medicine, May 2016
College of Veterinary Medicine
University of Tennessee; Knoxville, Tennessee 37916

Bachelor of Sciences in Animal Science and minor in Spanish, May 2012
Lyman Briggs College & College of Agriculture
Michigan State University; East Lansing, MI

HONORS AND AWARDS:
Young-Investigator Travel Grant (awarded by WAAVP)—2019
Young-Investigator Travel Grant (awarded annually by AAVP)—2017
Merck Award in Veterinary Parasitology—2012
Michigan State University Tower Guard Alumni -2008

PEER-REVIEWED PUBLICATIONS:

