2014

MODULATION OF VACCINE-INDUCED RESPONSES BY ANTHELMINTIC TREATMENT IN PONIES

Emily Rubinson
University of Kentucky, efr930@verizon.net

STUDENT AGREEMENT:

I represent that my thesis or dissertation and abstract are my original work. Proper attribution has been given to all outside sources. I understand that I am solely responsible for obtaining any needed copyright permissions. I have obtained needed written permission statement(s) from the owner(s) of each third-party copyrighted matter to be included in my work, allowing electronic distribution (if such use is not permitted by the fair use doctrine) which will be submitted to UKnowledge as Additional File.

I hereby grant to The University of Kentucky and its agents the irrevocable, non-exclusive, and royalty-free license to archive and make accessible my work in whole or in part in all forms of media, now or hereafter known. I agree that the document mentioned above may be made available immediately for worldwide access unless an embargo applies.

I retain all other ownership rights to the copyright of my work. I also retain the right to use in future works (such as articles or books) all or part of my work. I understand that I am free to register the copyright to my work.

REVIEW, APPROVAL AND ACCEPTANCE

The document mentioned above has been reviewed and accepted by the student's advisor, on behalf of the advisory committee, and by the Director of Graduate Studies (DGS), on behalf of the program; we verify that this is the final, approved version of the student's thesis including all changes required by the advisory committee. The undersigned agree to abide by the statements above.

Emily Rubinson, Student

Dr. Martin K. Nielsen, Major Professor

Dr. Joe Springer, Director of Graduate Studies

MODULATION OF VACCINE-INDUCED RESPONSES BY ANTHELMINTIC TREATMENT IN PONIES

THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the College of Medicine at the University of Kentucky

By

Emily Frances Rubinson
Lexington, Kentucky

Director: Dr. Martin K. Nielsen, Professor of Veterinary Sciences
Lexington, Kentucky

2014

ABSTRACT OF THESIS

MODULATION OF VACCINE-INDUCED RESPONSES BY ANTHELMINTIC TREATMENT IN PONIES

Vaccines and anthelmintics induce an inflammatory response in equids. Since they are commonly given concurrently, it is practical to study any interaction between them. This study evaluated whether IVM and PYR would modulate the acute phase inflammatory response, the systemic gene expression of pro-inflammatory cytokines, and vaccine-specific titers induced by WNV, EHV, and KLH vaccines. Naturally-infected, yearling ponies were sorted by gender, then fecal epgs. They were randomly assigned to three treatment groups: IVM, PYR, and control. All ponies received vaccinations intramuscularly on days 0 and 29. Whole blood, serum, and plasma samples were collected 1, 3, and 14 days post-vaccination. Samples were analyzed for inflammatory markers, cytokine, mRNA expression, and vaccine-specific IgG titers by ELISA. The acute-phase inflammatory marker data showed no statistical significance; they did show an increase in SAA, haptoglobin, and fibrinogen, and a decrease in iron after vaccination. The mRNA data showed that anthelmintics had a significant effect on interleukin mRNA levels, but not on TNF-α or IFN-γ levels. The ELISA assays showed no biologically significant reduction in IgG as compared to the control group. We conclude that deworming does not affect vaccine IgG titers; therefore, ceasing vaccinating and deworming concurrently is not necessary.

KEYWORDS: parasite, cytokine, anthelmintic, ELISA, vaccine

Emily Frances Rubinson

12/17/2014

MODULATION OF VACCINE-INDUCED RESPONSES BY ANTHELMINTIC TREATMENT IN PONIES

By

Emily Frances Rubinson

____Martin K. Nielsen_____
Director of Thesis

____Joe Springer_____
Director of Graduate Studies

___12/16/2014____
Date

Acknowledgements
The author would like to acknowledge the Greyson Jockey Club Research Foundation for their funding of this project; Dr. Stine Jacobsen at the University of Copenhagen (Denmark) for her lab's work on the inflammatory markers; Dr. Bettina Wagner at Cornell University for her lab's work on the West Nile Virus ELISA's; Alejandra Betancourt (Horohov lab, Univ. of Kentucky) for her work on the cytokine gene expression assays; Stephanie Reedy (Chambers lab, Univ. of Kentucky) for her work on the KLH and EHV vaccine ELISAs; Ms. Bonnie Decker at the University of Kentucky Veterinary Diagnostic Laboratory for performing the hematology; Chip Stamper and John Allen at Pin Oak Farm (Univ. of Kentucky) for all of their help with the ponies and for their upbeat attitudes; Jennifer Bellaw (Univ. of Kentucky) for her guidance while I learned the techniques used in the project; Holli Gravatte (Univ. of Kentucky) for her help with my adjustment to the laboratory; and Dr. Martin K. Nielsen for his veterinary assistance (giving vaccines and anthelmintics), allowing me to work in his lab, and for all of his advice throughout the project.

Table of Contents

List of tables

List of figures

1. Introduction

Intestinal invertebrate parasites of the horse, known as helminths, can alter the immune response both locally and systemically (Betancourt *et al*. 2014, Davidson *et al.* 2005). Drugs targeting these multicellular organisms are known as anthelmintics; they are commonly given to horses on the same day as routine vaccinations, such as for West Nile virus and equine herpes rhinopneumonitis virus. Both anthelmintics and vaccines are known to induce inflammation in horses (Steinbach *et al.* 2006, Andersen *et al.* 2012), but studies into the effect of deworming on the systemic inflammatory response induced by the vaccines has not been extensively studied.

2. Background

Helminths are parasites that can be characterized into three main phyla: Platyhelminthes (flukes and tapeworms) Nematoda (roundworms) and Acanthocephala (thorny-headed worms) (Castro 1996). This thesis concentrates on the equine intestinal nematode parasites known as the strongyles (order Strongylida), the largest order of parasitic helminths in horses. The adults of family Strongylidae are identified by a well-developed buccal capsule and a copulatory bursa (Lichtenfels 1975). The adults can range from 5 mm to 4.5 cm in length, with the males being smaller than the females (Reinemeyer *et al.* 2007, Taylor *et al.* 2013). The family Strongylidae is further divided into two subfamilies: the Strongylinae (large strongyles) and the Cyathostominae (small strongyles), differentiated using the shape of their buccal capsule. The Strongylinae have a funnel-shaped buccal capsule, while Cyathostominae have a cylindrical buccal capsule (Lichtenfels 2008). The focus of the study described herein is the family Cyathostominae.

2.1 Cyathostominae

The cyathostomins are a family consisting of 13 morphologically different genera that contain 51 species in total; 40 of these species are found in horses and ponies, while the other 11 are only found in other equids (Lichtenfels *et al.* 2008). They are microscopically similar to the large strongyles at their early stages (egg, first two larval stages), but can be differentiated from the other Strongylida by their internal morphology once they reach the third infective larval stage (**Figure 1**).

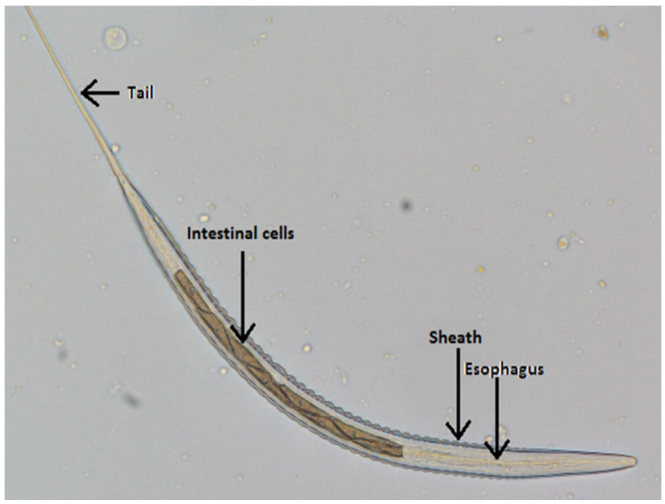

Figure 2.1*- A third infective larval stage of a cyathostomin; note the 8 intestinal cells, the sheath, the long tail and the filariform esophagus.*

2.2 Life cycle

Strongyle eggs are excreted from the horse via the feces, where they develop into the first three larval stages (L1, L2, and L3) in the environment (**Figure 2**). Once the third infective larval stage (L3) on the pasture is ingested by the horse, it penetrates into the mucosal layer of the large intestine, where it develops into the early third larval stage (EL3), late third larval stage (LL3) then the fourth larval stage (L4) and sometimes early fifth stage (L5) before exiting the mucosa and developing into reproductive adults. The exit of the cyathostomins into the intestinal lumen causes an inflammatory response,

exacerbated if the encysted stages (EL3, LL3 and L4) are induced to exit the mucosa by the administration of fenbendazole (see section 2.4). The entire process from L3 ingestion to finding eggs in the feces, also known as the prepatent period, ranges from six to eight weeks for the first maturing species, if not interrupted by arrestment of EL3s (Ihler 2010, Lyons 2011). Classification of the different stages is by morphology and location of the stage, which is most efficient if the horse is necropsied. In this thesis, we will be identifying the different strongyle species via microscopic observation of third-stage larvae (Appendix B). Identification of the different species of strongyle is necessary to determine the correct treatment regimen.

2.2.1. Effects of encysted L3 and L4 stages on the intestinal mucosa

The free-living L3 stage is able to survive on pasture for several months at a time; once inside the horse, the EL3 stage can arrest within the mucosal tissue of the cecum and ventral colon, emerging as early as one month and as late as three years after arrest. (Gibson 1953, Reinemeyer and Nielsen 2012). In northern temperate climates of the U.S., the LL3s encyst in the mucosa in the fall, develop into L4s within the cyst over the winter, and then emerge in the spring (Reinemeyer 1986). In most cases, these L4s emerge from the mucosa into the lumen a small fraction of the population at a time, causing diffuse necrotic swellings (Love *et al.* 1999).

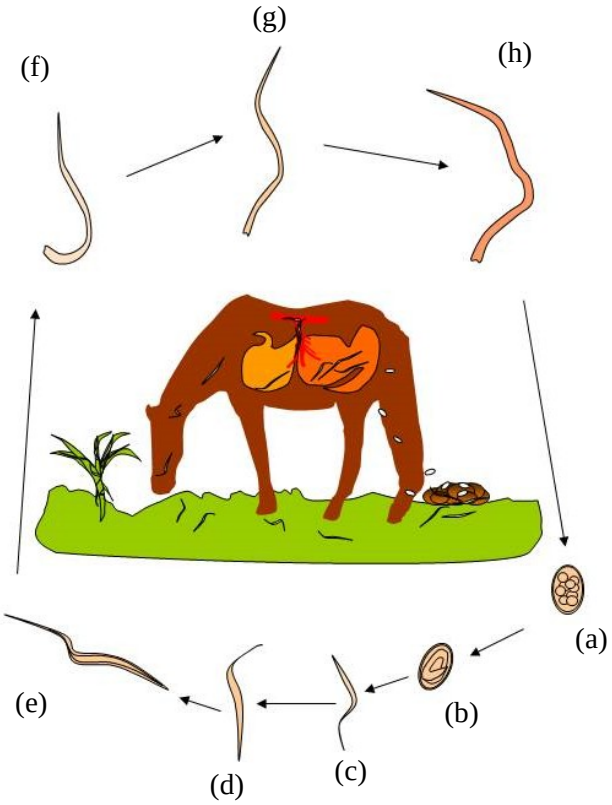

Figure 2.2*- Cyathostomin life cycle. Clockwise from (a) to (h): egg, larvated egg, L1, L2, L3, EL3, L4, adult.*

If large numbers of larvae emerge synchronously, this causes mucosal cell rupture accompanied by loss of fluid and proteins into the lumen, also known as protein-losing enteropathy (Lyons *et al.* 1999, Steinbach *et al.* 2006). This greater damage causes a massive inflammatory reaction in the mucosa, along with intestinal lesions. These effects, in turn, can cause chronic diarrhea (Love *et al.* 1999, Peregrine *et al.* 2005). This condition, called larval cyathostomiasis, can be severe enough to cause death of the horse (Love *et al.* 1999). Although some horses may present with symptoms in the clinic, most infected horses are asymptomatic.

Although a horse may not exhibit visible symptoms, an infection with cyathostomins can cause hematological changes; in highly parasitized horses, these changes can include low neutrophil counts, low serum albumin levels, anemia, and high β-globulin (Love *et al.* 1999). In addition, the horse will exhibit local cytokine release associated with Th2 (IL-4 and IL-10) and Th1 (IFN-γ and TNFα) responses (Davidson *et al.* 2005), with Th2 being the predominant response with non-filarial parasites. The Th1 response is more associated with the killing of intracellular pathogens and is involved with the vaccine response (Berger 2000).

2.3. Anthelmintic types

Anthelmintics are classified based on their subcellular targets in the parasite. Three different classes of anthelmintics are approved for use in equids to treat strongyles in the United States: macrocyclic lactones, tetrahydropyrimidines, and benzimidazoles (Holden-Dye and Walker 2007). We can test the efficacy of these compounds using the equation in section 3.2.

2.3.1. Macrocyclic lactones

Macrocyclic lactones (MLs) such as ivermectin (IVM) and moxidectin (MOX) are used to treat cyathostomin infections by paralyzing the body wall muscles of the worm, specifically causing flaccid paralysis (Holden-Dye and Walker 2007, Claerebout and Vercruysse 2012a). This paralysis is caused by the interaction of the drug with several ligand-gated ion channels including nicotinic acetylcholine (nACh) receptors, ACh-gated chloride channels, GABA-gated chloride channels, and histamine-gated chloride channels. Of these various channels, it is their strong interaction with glutamate-gated chloride channels that is the main method of action of the macrocyclic lactones (Holden-Dye and Walker 2007). The paralyzed worm is then passed out of the horse via normal peristalsis (Claerebout and Vercruysse 2012b). MLs are generally effective against strongyles, but ascarids currently are mostly resistant to this class of drugs (Peregrine *et al.* 2014). The current efficacy of a single dose of ivermectin (0.2 mg/kg IVM) against strongyles in some studies is between 89% and 100% (Canever *et al.* 2013, Lester *et al.* 2013, Relf *et al.* 2014, Stratford *et al.* 2014). However, there are recent reports of reduced activity of IVM and MOX against small strongyles in horses in several geographical sites (Demeulenaere *et al.* 1997, Costa *et al.* 1998, Traversa *et al.* 2009, Lyons *et al.* 2011). This is based on finding earlier returns of epgs after treatment compared initial activity when MLs were first marketed. Critical tests recently have shown that activity on adult small strongyles was still high but less on immature intestinal luminal stages; the result being that the life cycle is shortened (Lyons *et al.* 2009).

2.3.2. Tetrahydropyrimidines

Pyrantel pamoate (PYR) and pyrantel tartrate act as excitatory nACh receptor agonists in the muscles lining the body wall of the small strongyle (Holden-Dye and Walker 2007, Alverez *et al.* 2009). As an agonist, the drug binds competitively to the nACh receptor and over-activates it. As a result, pyrantel causes spastic paralysis (Holden-Dye and Walker 2007). As with the macrocyclic lactones, the paralyzed worm is then passed out of the horse via normal peristalsis (Claerebout and Vercruysse 2012b). Strongyles have a large range of resistance to pyrantel, with current single-dose (19 mg/kg) efficacy between 50% and 100% (Canever *et al.* 2013, Hsu and Martin 2013, Lester *et al.* 2013, Relf *et al.* 2014, Stratford *et al.* 2014).

2.3.3. Benzimidazoles

Benzimidazoles affect the microtubular cytoskeleton of the parasite by binding to β-tubulin. This causes impaired movement and impaired reproductive ability of the parasite (Holden-Dye and Walker 2007). Fenbendazole, the only benzimidazole approved for larvicidal use globally, needs to be given at higher doses to eliminate those cyathostomins which are encysted in the large intestinal mucosa (Claerebout and Vercruysse 2012c). Currently, fenbendazole has between 0% and 84% efficacy against luminal strongyles when given as a single dose (Canever *et al.* 2013, Lester *et al.* 2013, Relf *et al.* 2014, Stratford *et al.* 2014). Unfortunately, when fenbendazole is given to a horse in the larvicidal dose (10 mg/kg for 5 days), it triggers a local response by T lymphocytes, eosinophils and macrophages, which surround L3 and L4 cyathostomins in a thick granuloma. Within 4 to 6 days after fenbendazole treatment, the encysted larvae die, releasing nematode waste products, to which intestinal eosinophils and lymphocytes react, causing localized edema (Love *et al.* 1999). Within 2 weeks of treatment with the larvicidal dose, morphological changes to the large intestine, including ulceration and erosions into the mucosa, appear; these lesions can extend into the bowel wall (Steinbach *et al.* 2006).

2.4. Intestinal response to anthelmintic treatment in the horse

Though anthelmintics are a necessary part of parasite control in the equid, they can also trigger adverse reactions in the intestine as the adults are killed. These adverse reactions include weight loss, diarrhea, subcutaneous edema and fever, a condition known as larval cyathostomiasis (Love *et al.* 1999). In a study by Reid *et al.* (1995), they used several statistical methods (student's t test, chi-square test and multiple logistic regression analysis) to find the variables most likely to contribute to chronic diarrhea, which they classified as larval cyathostomiasis; the effect of time since last anthelmintic treatment (i.e. less than 2 weeks gave a higher "score" than anthelmintics given more than two weeks prior to admittance to an equine hospital) was a significant contributor ($P=0.02$) to the development of larval cyathostomiasis. The only other factors which were of equal or higher significance were age ($P=0.001$) and season of diagnosis ($P=0.02$) (Reid et al. 1995).

2.5. Anthelmintic resistance and herd management

The FDA defines anthelmintic resistance as the ability of a subset of parasites to survive treatment with a specific anthelmintic to which they were previously susceptible

(USFDA-CVM 2013a). This phenomenon is due to changes in their genome and that of their descendants. Resistance is determined by several factors, including the immune status of the host animal and treatment protocols (USFDA-CVM 2013a). With regard to detecting efficacy of the drug in a specific herd — and therefore the level of parasite resistance to the drug — a fecal egg count reduction test (FECRT) is performed. The test is performed on data taken from a group of animals and involves the calculation of the percent reduction in the number of parasite eggs in a fecal sample between Day 0 and Day 14 after treatment (USFDA-CVM 2013b). As recommended by Stratford *et al.* (2014), any FECR found to be less than 90% indicates resistance is developing, or has already developed, to the drug.

2.6. Immune modulation and inflammatory response to helminths

In humans, infecting parasites, such as *Strongyloides stercoralis*, induce inflammation by releasing pathogen-associated molecular patterns (PAMPS). The PAMPs regulate transcriptional activation of tumor necrosis factor (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6), all of which are pro-inflammatory cytokines. If chronically present, they can cause ulcerative colitis and inflammatory bowel disease (Berridge 2012, Romero-Cabello *et al.* 2012).

Infection of helminths into tissues causes the initiation of the type 2 helper T cell (Th2) inflammatory response; this response involves the recruitment and activation of basophils, eosinophils, and mast cells (shown in **Figure 3**) (Koyasu and Moro 2011). Th2 cells are one of three types of CD4+ helper T cells; the other categories are the type 1 helper T (Th1) and type 17 helper T (Th17) cells (Zhu *et al.* 2010). Anti-inflammatory cytokines, such as interleukin-25 and interleukin-33 (IL-25 and IL-33), are produced in response to helminth infection by epithelial cells in the intestine. Their role is to induce the blockage of helminth invasion of the mucosa until an adaptive response can be initiated. In addition, IL-4 is produced by basophils and mast cells when stimulated by the invading parasite and IL-6 is produced constitutively by natural helper cells, causing antibody production and iron sequestration (Koyasu and Moro 2011).

Levels of non-heme iron transported by ferritin in the blood are used as marker of inflammation by veterinary and human clinicians; when an inflammatory stress response is triggered by the secretion of IL-6, it, in turn, induces the upregulation of the hormone hepcidin. Hepcidin is a peptide that binds to an iron transport protein, ferroportin, in the intestinal mucosa. When hepcidin is upregulated, ferroportin is downregulated, causing iron withdrawal from the body and its subsequent sequestration, causing anemia (Johnson and Wessling-Resnick 2012). In addition, this sequestration of iron restricts the parasites' access to the element, which is important in the function of redox enzymes, such as NADH-fumerate reductase (NFR). This enzyme contains an iron-sulfur cluster as the catalytic center and is responsible for the conversion of fumarate to succinate in the helminth respiratory chain (Omura *et al.* 2001, Johnson and Wessling-Resnick 2012).

Helminth infection is known to block Th1 response activation, and instead induces the activation of the Th2 response in rodents and immunocompromised humans (Peón *et al.* 2013). The Th1 response is normally associated with infectious diseases because of its induction of IFN-gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) (**Figure 3**), which in turn stimulates phagocytosis and oxidative burst, as well as antigen

presentation to cytotoxic T cells (Spellberg and Edwards 2001, Davidson *et al.* 2005). In contrast, Th2 responses are associated with IL-4 and IL-10 and cause antibody production, B cell proliferation, and infiltration of eosinophils and basophils into the tissues; this action leads to a more "allergic" response (Spellberg and Edwards 2001, Davidson *et al.* 2005).

Davidson *et al.* (2005) published a study which explains the inflammatory response to cyathostomins with regard to cytokine levels in naturally-infected horses. Similar to aspects of the study described in this thesis, these cytokines were measured by reverse transcriptase-competitive PCR (RT-cPCR) using the method described by Guiguère and Prescott (1999). The reaction products were run on an agarose gel, and then measured using densitometry, from which standard curves were graphed. All values were normalized to β-actin levels and expressed as fold change in cytokine mRNA expression (Guiguère and Prescott 1999). However, unlike our study, Davidson *et al.* measured local inflammation by taking mucosal samples of 6 sites in the intestine and measuring cytokine and encysted larval burdens for each sample for all 17 horses. The study found that increased IL-10 and IL-4 levels, therefore Th2 responses, are linked to the presence of cyathostomins in the large intestinal wall, while IFN-γ was not associated with larval parasite burdens. TNF-α was only seen in those areas of the excised intestine which had signs of mass excystment and/or empty cysts (Davidson *et al.* 2005).

Helminths, such as *Schistosoma* spp. and *Trichinella* spp., have been found to modulate Th1 inflammatory responses in rodent and human subjects (Mangan *et al.* 2004, McKay 2009, Walsh *et al.* 2009, Harn *et al.* 2009, Osborne *et al.* 2014); prior to the current study, the modulation of inflammation by helminths in the horse has not been studied. Previous studies in mice noted that interleukin-10 (IL-10) was the main cytokine that contributed to protection against the Th2 response induced by infection (Mangan *et al.* 2004, McKay 2009). For example, Mangan *et al.* (2004) found that *Schistosoma mansoni* can induce the proliferation of B cells producing IL-10, which protects mice from severe anaphylaxis. In a recent study by Osborne *et al.* (2014), they examined whether *Trichinella spiralis* modulates the murine immune system by direct interaction or by perturbing the natural gut microbiota. They found that a Type 2 T cell response was induced and that the cytotoxic T cells specific to the virus given were not only decreased in number, but also reduced in their effector functionality (i.e. stimulating the caspase cascade leading to apoptosis of the target cell) when coinfected with the parasite (Harty *et al.* 2000). In addition, they found that the helminths directly regulate antiviral T cell responses, which could affect the efficacy of vaccines (Osborne *et al.* 2014).

Due to the helminths' ability to decrease inflammatory reactions in the intestine, live helminths and helminth-derived proteins, such as those from *Trichinella* spp. have been used therapeutically to decrease inflammation associated with inflammatory bowel diseases, such as Crohn's (Motomura *et al.* 2008, Chu *et al.* 2013, Sandborn *et al.* 2013, Hiemstra *et al.* 2014). Ironically, in addition to the helminths controlling the inflammatory response in the intestine, one of the anthelmintic drugs used in these studies, ivermectin, suppresses inflammation in rodent models by decreasing immunoglobulin G (IgG) and decreasing infiltration of neutrophils, lymphocytes and macrophages leading to a decrease in allergic inflammation, i.e. decreasing the Th2 response (Yan *et al.* 2011).

When the immune response is differentiated toward a Th2-type response during helminth infection, B cells begin to produce immunoglobulin E (IgE) and immunoglobulin G (IgG) in response to the release of IL-5 by eosinophils (Harris and Gause 2011).

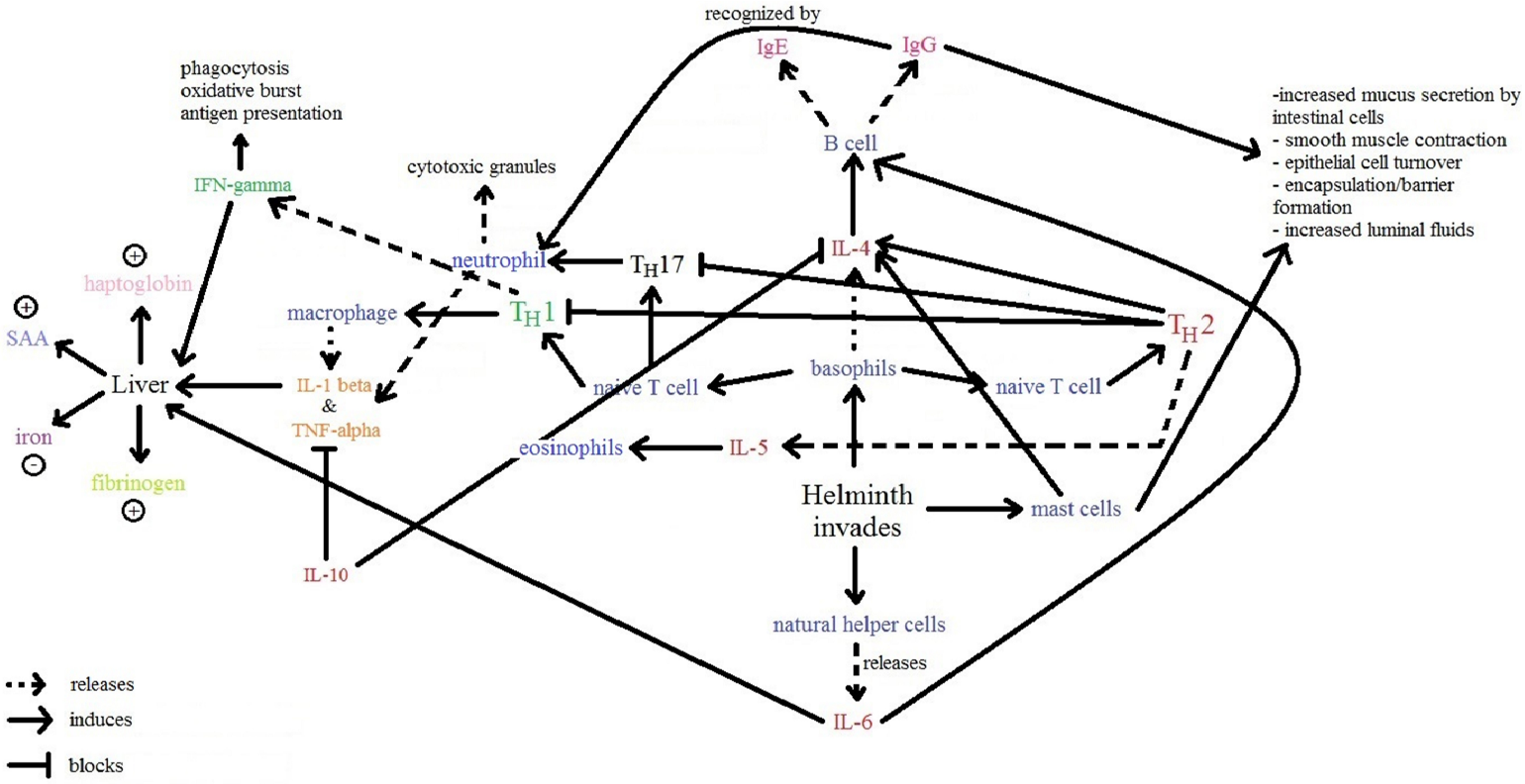

Figure 2.3*- The adaptive immune response to helminth infection with the pertinent cytokines and acute phase proteins. Colored labels are the acute inflammatory markers commonly used in the veterinary clinic; the positive signs mean that when inflammation is present, the systemic levels of these factors increase, while systemic iron decreases with inflammation. Cytokines involved in the T_H2 response are shown in red font; those which are involved in the T_H1 response are shown in green. Immune cells are shown in dark blue. Sources: Gause* et al. *2013, Koyasu and Moro 2011.*

2.6.1. Acute inflammatory response to vaccination

Acute-phase proteins (APPs) are serum proteins originating in the liver that can be used as a measure of levels of inflammation or infection (Eckersall 1995). The panel of APPs used in quantification of the inflammatory response in the clinic is haptoglobin, fibrinogen, and serum amyloid A (SAA). In addition to these three proteins, it is also common to test serum iron levels as a marker of inflammation.

In a study by Andersen *et al.* (2012), ten horses were given, intramuscularly, an immune stimulating complex (ISCOM) vaccine, specifically an inactivated influenza/tetanus toxoid vaccine, or an adjuvanted, live recombinant vector vaccine containing two influenza strains and tetanus toxoid. They found that serum SAA and fibrinogen levels increased within 24 hours of injection with both vaccines, with elevated inflammatory marker levels throughout the 96-hour study. As would be expected with the SAA and fibrinogen level increases, there was a decrease in serum iron levels for 48 hours post-vaccination with both vaccines. Overall, the ISCOM vaccine gave a higher inflammatory response than the vector vaccine. They concluded that vaccination is a viable inducer of mild inflammation in the horse for study of the acute phase response.

2.6.2. Cytokine expression in response to vaccination

In addition to monitoring the inflammatory markers mentioned above, inflammation can be monitored via leukocyte-produced cytokine levels and hematological properties. The cytokines associated with the inflammatory Th2 vaccine response in the horse are IL-4, IL-5, and IL-10, while IL-2, IFN-γ and TNF-α are released by Th1 cells after vaccination (Romagnani 1999). With the EHV vaccine we would expect to see an increase in blood levels of IL-4, IL-5 and IL-10, while the WNV vaccine would induce IFN-γ and/or TNF-α release. The cytokines measured in this study (IL-1β, IL-4, IL-10, TNF-α and IFN-γ) are released at the highest level between at about 10 and about 56 days after vaccination (Paillot *et al.* 2005, Kydd *et al.* 2006).

2.6.3. Hematological effects of vaccine and measuring vaccine antibody titers

Adjuvanted vaccines, such as the EHV and WNV vaccines in this study, or the ISCOM vaccine in the Andersen *et al.* study described above, cause increases in total WBC count, while protein vaccines have less of an effect on WBC levels (Andersen *et al.* 2012).

In addition to measuring cytokine levels and hematological features, the response of the host immune system to the vaccine can be directly measured via antibody titers, specifically different isotypes of IgG.

Four isotypes of IgG are commonly found in horse serum: IgG(a), IgG(b), IgG(c), and IgG(T). IgG(a) and IgG(b) act via complement activation and opsonization (i.e. they are cytophilic antibodies) and are associated with an acute inflammatory vaccine response, while IgG(c) and IgG(T) act via competing with IgG(a) and IgG(b) for antigens, with IgG(T) being associated with a more chronic inflammatory vaccine response. Production of IgG(a) and IgG(b) is induced by the release of IFN-γ by Th1 cells, IgG(c) is associated with respiratory burst by peripheral leukocytes, and IgG(T) release occurs with the end of active infection (Cunha e*t al.* 2006, Lewis *et al.* 2008). When vaccinated, IgG isotypes appear in the serum at different times post-inoculation: IgG(c) appears first, IgG(a) appears at approximately 1 week, IgG(b) at approximately 3 weeks, and IgG(T) appears only after acute infection resolves, between 4 and 16 weeks

post-inoculation (Cunha *et al.* 2006).

2.6.4. Modulation of inflammation by helminths and by anthelmintics

It is well established in large animals, both ruminant and horse, that vaccination causes a potent inflammatory response, but modulation of this inflammatory response by anthelmintics in horses is not as well defined (Killi 2008, Hepburn *et al.* 2009, Andersen *et al.* 2012).

A recent study on the subject of modulation of inflammation by anthelmintics was performed in calves (Schutz *et al.* 2012). They measured IL-4, IL-6, TNF-α, and IFN-γ levels in calves after treatment with fenbendazole at one of two time points: 2 weeks pre-vaccination or at same time as vaccination. Another group of calves was given no anthelmintic treatment but was vaccinated. When the calves were given the deworming at the same time as they were vaccinated, there was a suppression by treatment on Th2 cytokine levels, specifically IL-4 levels. In addition, there was a decrease in parasite burden. The decrease in Th1 response could influence the efficacy of the response against repeat viral infection, i.e. decreased vaccine efficacy (Schutz *et al.* 2012). If this is true in horses, then the vaccine efficacy should decrease with anthelmintic treatment.

The only previous study looking into the effect of removal of helminths on the vaccination response in equids was published in 2001 by Edmonds *et al.* The study was performed by dosing naturally-infected ponies with pyrantel pamoate (two doses, three weeks apart) or moxidectin (one dose). Twenty-four days after the first treatment of pyrantel or the dose of moxidectin, keyhole limpet hemocyanin (2 mg) was injected i.m. Pre-treatment, 2 weeks and 4 weeks post-treatment blood samples were taken in order to determine cytokine (IL-2, IL-4, IL-5, and IFN-γ) and vaccine-induced antibody (IgA, IgG(a), IgG(b), and IgG(T)) levels, and to determine the peripheral blood mononuclear cell (PBMC) proliferation in response to two different stimulants (KLH and pokeweed mitogen).The proliferation was measured as the mean count per minute for the stimulated versus the unstimulated cells. At 4 weeks, the ponies were euthanized, and necropsied in order to recover parasites (large and small strongyles, ascarids, and fly larvae) for characterization and enumeration. They found that the parasitism level did affect the KLH-induced cytokine response by PBMCs; ponies with fewer parasites had higher IL-4 and IFN-γ levels as compared to the non-vaccinated and heavily parasitized or the KLH-vaccinated and moderately-to-highly parasitized ponies. There was no statistical significance to the KLH-specific Ig levels, but the trend seemed to lean toward low parasite-burdened ponies having higher total Ig and IgA levels at both two and four weeks post-vaccination, while IgG(T) levels were higher for the less parasitized ponies at two weeks only (Edmonds *et al.* 2001). These results suggest that removal of helminths, as an effect of treatment, could increase the effectiveness of a vaccine when the horse is challenged.

2.6.5. Parasite dynamic changes with equine host age

It has been established that the proportions of cyathostomin stages change as the horse matures. As horses age, the production of pro-inflammatory cytokines such as IFN-γ and TNF-α increase in the peripheral blood (Adams *et al.* 2008, Hansen *et al.* 2013). One previous study performed by Katepalli *et al.* (2008) used radioactive thymidine incorporation to test the hypothesis that as horse telomeres shorten with PBMC division, there will be a decline in immune function. They confirmed this hypothesis by finding

that as telomeres shorten in PBMCs, the total serum IgG decreased and pro-inflammatory cytokine expression increased.

Prior to the study by Katepalli, Chapman *et al.* (2003) studied the prevalence and amount of cyathostomins in a range of ages of ponies. Previously untreated ponies between the ages of 4 months and 5 years were naturally infected and fecal egg counts were taken. At various times of the year, these ponies were necropsied and cyathostomins were enumerated and classified as EL3, developing third stages in the mucosa, early fourth stages still encysted in mucosa, luminal L4s or adults. The results showed that the group with the highest percentage of EL3s was the 2 year olds, the L4s tended to make up the highest percentage of cyathostomins in the 3 to 5 year-old pony lumens, and 1 year olds had the highest number of adults in their intestinal lumen. The percentage of DLs was, after considering the standard deviation, the same in all three age groups.

2.7 Knowledge gaps and how we fill them

Before this study, there were no studies which evaluated how the vaccination response could be affected by the use of anthelmintics; this is a concern because foals are commonly vaccinated and dewormed monthly for the first 12 months of life, with the vaccinations and deworming occurring on the same day. In addition, as Th1 responses are the selected for with most infections, and therefore vaccines, while deworming would cause a shift toward a Th2 response (Nielsen *et al.* 2013). It is the aim of this study to determine whether there is an interaction between the vaccine-induced inflammation and the anthelmintic-induced inflammation which could affect vaccine response, either increasing or decreasing that response.

3. Experimental methodology

3.1 Aims and hypotheses

The study's main hypothesis is that any inflammation caused by vaccination, and the vaccine efficacy, will be modulated by the administration of a concomitant anthelmintic treatment. To test this hypothesis, we analyzed acute phase proteins and cytokines in response to a combination of vaccinations, and, simultaneously, compared the impact of the two anthelmintics on the vaccine response using ELISAs, inflammatory markers, hematology and cytokine analyses.

Over 60 days in August and September 2013, a total of 23 female and intact male ponies of approximately 8 months of age were involved in the study. The treatment protocol included vaccination of all ponies with three separate vaccines: a protein vaccine without adjuvant (keyhole limpet hematocyanin, KLH; 10 mg/kg for first dose, 5 mg/kg for subsequent dose; Sigma, Saint Louis, MO), a live recombinant, adjuvanted vaccine (West Nile virus, WNV; Recombitek rWNV, Merial; 1 mL/dose), and an inactivated viral vaccine containing an Carbomer adjuvant (equine herpes rhinopneumonitis, EHV; Prodigy with Havlogen, monovalent, Merck Animal Health; 2 mL/dose). Before administration of vaccines and anthelmintics, the ponies were weighed using a platform scale (model #700, Tru-Test Inc., Mineral Wells, TX). All vaccines were given i.m, and each vaccine was given in one of three locations: the left side of the neck, the right side of the neck and the pectoral region. All ponies were dosed two times, four weeks apart with the vaccines. Concurrently, one cohort received, *per oral*, ivermectin (IVM; Eqvalan, Merial; 200 μg/kg, 8 ponies, Group A), a second received pyrantel pamoate (PYR; Strongid Paste, Pfizer Animal Health; 6.6 mg base/kg, 8 ponies, Group B), and the third cohort were kept untreated (Control, 7 ponies, Group C). At various time points after each vaccination and anthelmintic treatment, we took blood and fecal samples from all ponies. Blood was taken in order to analyze cytokine RNA levels (Tempus, Life Technologies), inflammatory marker levels (BD Vacutainer® for serum, BD Technologies, Durham, NC), to run vaccine titer analyses (BD Vacutainer® Plus Citrate Tubes, BD Technologies, Durham, NC), and to do hematological counts (BD Vacutainer® Plus K2 EDTA Tubes, BD Technologies, Durham, NC), such as white blood cell counts, hematocrit and leukocyte subtyping. Feces were collected in order to find the efficacy of the two anthelmintics in this cohort of ponies. The treatment and testing protocols are displayed in **Table 1**.

Table 3.1*-Experimental protocol by day number. "X" indicates the action taken that day.*

	Day													
	-4	0	1	3	4	14	21	29	30	32	35	42	50	59
Vaccines		x						x						
Anthelmintics		x						x						
Whole blood	x*	x	x	x	x	x		x	x	x		x		
Feces	x	x				x	x	x			x	x	x	x

* On this day, only serum was taken.

3.2 Egg counts

In order to quantify efficacy of the two anthelmintics in the horses, fecal egg counts (FECs) were performed. Feces were collected on the experimental days shown in

Table 1. The sampling at Day -4 was performed in order to determine groupings for dosing of the anthelmintics (see section 3.2 for more detail). Using the mini-FLOTAC method, we were able to find the egg count per gram with a sensitivity of 5 eggs per gram (epg) (Barda *et al.* 2013, Cringoli *et al.* 2010) (**Figure 4**; for further details, please see Appendix A). After all counts were performed for all time points, a fecal egg count reduction test (FECRT) was calculated against the pre-treatment counts for the strongyles using the equation

$$FECR = 100*(FEC_{Day\ 0} - FEC_{Day\ 14})/FEC_{Day\ 0}$$

to determine the efficacy of the anthelmintics in each individual in the treatment groups versus the ponies in the control group.

In addition to the FECs, the eggs were characterized based on morphology (i.e. strongyle eggs were differentiated from ascarid and tapeworm eggs) (Zajac and Conboy 2012).

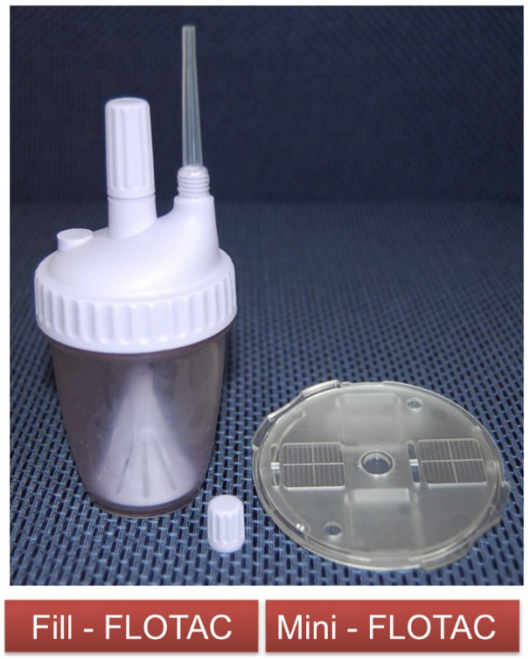

Figure 3.1. *Setup of mini-FLOTAC apparatus. Picture from Barda* et al. *(2013).*

3.3 Selection of groups

Grouping of ponies for treatment was based on the epg for Day -4 samples. The ponies were sorted first by gender and then ranked by descending eggs per gram, i.e. have equal numbers of low, medium, and high egg shedders in each group. After these parameters were satisfied, the ponies were allocated to each group (1st pony was put into group 1, the second was put into group 2, the third into group 3, etc.) The range of epg was between 35 and 1725 strongyles. The anthelmintic type and distribution were as follows (female, male): IVM (4,4); PYR (4,4) and Control (4,3).

3.4. Hematology

All hematological assays were performed by the University of Kentucky Veterinary Diagnostic Laboratory. The following hematology tests were measured on samples collected on the dates shown in **Table 1**: white blood cell (WBC) and red blood

cell (RBC) counts, percentage of leukocytes (neutrophils, lymphocytes, basophils, and monocytes), hematocrit, hemoglobin levels, and other RBC characteristics (mean corpuscular hemoglobin, mean corpuscular volume, and mean corpuscular concentration) using a Forcyte hematology analyzer (Oxford Science Inc., Oxford, CT).

3.5. Inflammatory markers

All inflammatory markers were measured using automated spectrophotometric methods in the laboratory of Dr. Stine Jacobsen (Univ. of Copenhagen). Serum amyloid A levels were measured using an immunoturbidometric assay (LZ SAA EIKEN, EIKEN Chemical Co., Ltd., Shanghai, China), haptoglobin levels were measured using a peroxidase assay (Phase Range Hp Assay, Tridelta Development, Ltd., Maynooth, Ireland), fibrinogen levels were measured using the Clauss method on an automated coagulometric analyzer, and iron levels were measured by colorimetric spectrophotometry (ADVIA 1650, Siemens Healthcare, Erlangen, Germany) (Andersen *et al.* 2014, Jacobsen *et al.* 2005a).

3.6. Cytokine expression

Blood was collected via venipuncture into Tempus tubes (Life Technologies, Grand Island, NY) on the days outlined in **Table 1**. The samples were analyzed by Alejandra Betancourt (Gluck Equine Research Center). RNA isolation was performed using an iPrep Purification Instrument (Life Technologies, Grand Island, NY) and the Purelink RNA isolation kit (Life Technologies, Grand Island, NY) as per the manufacturer's instructions. RNase-free water was added to 1 μg of isolated total RNA to a volume of 41.5 μL, and 38.5 μL of MasterMix (16 μL 5X AMV buffer, 16 μL $MgCl_2$, 4 μL dNTP, 1 μL RNasin and 0.5 μLL of AMV reverse transcriptase) was added to each sample. Reactions were incubated at 42°C for 15 minutes and 95°C for 5 minutes in a Veriti 96-well thermocycler (Applied Biosystems, Foster City, CA) to form cDNA. Intron-spanning equine cytokine-specific primer/probe sets were designed for analysis of cDNA of the five cytokines (IL-1β, IL-4, IL-10, TNF-α and IFN-γ) (Assays-by-Design, Applied Biosystems) using real-time PCR (ABI RT-PCR system, Applied Biosystems, Foster City, CA) as described in Breathnach *et al.* (2006).

Relative quantification of cytokine expression changes were calculated using the $2^{-\Delta\Delta C_T}$ method, as described by Livak and Schmittgen (2001). Briefly, the cycle threshold (C_T) values from the real-time PCR instrument are imported into a graphing program. The results are normalized to the endogenous control, β-glucuronidase (Betancourt *et al.* 2014). The results are given as the mean relative change in cytokine gene expression (i.e. gene amplification) in each sample relative to the initial number of cDNA molecules in the sample (i.e. RQ value), assumed as proxy for the cytokine levels (Livak and Schmittgen 2001).

3.8. Vaccine ELISAs

KLH and EHV vaccine ELISAs were performed by Stephanie Reedy (Gluck Equine Research Center), and WNV vaccine ELISAs were performed by Dr. Bettina Wagner (Cornell University) in order to measure antigen-specific serum antibody levels of whole IgG, IgG(a), IgG(b), and IgG(T). The 96-well microtiter plates were coated with

300 μg of KLH/well or with 50 μg of 5 μg/mL whole EHV-1 virus/well, and kept at 4°C overnight. The sera samples were diluted in order to find the optimal concentration for each IgG subtype. The control sera were 2-fold serially diluted to a final concentration of 7.81 EU/mL. A 1% polyvinyl alcohol (PVA) blocking solution, made in PBS, was diluted 1:1 with PBS and stored at room temperature until needed. The blocking solution stops non-specific binding to the plastic. The secondary antibody was peroxidase-conjugated AffiniPure Goat Anti-horse IgG (Jackson Immuno Research, West Grove, PA). When the plates were ready, they were washed three times with PBS/TWEEN-20 (PBS-T) and 200 μL of PVA blocking solution was added, and the plates incubated at RT for one hour. The plates were washed three more times with PBS-T before 100 μL of diluted serum samples or standard was added to the appropriate wells in duplicate, and the plates incubated for one hour at 37°C. The plates were washed three more times with PBS-T before 100 μL of secondary antibody was added and incubated for 1 hour at 37°C. The plates were washed, and 100 μL of SureBlue (KPL, Inc., Gaithersburg, MD) was added to the wells. Stop Solution (KPL, Inc., Gaithersburg, MD) was added after 1 minute, and the plate was read at 450 nm on a spectrophotometer (BioRad Benchmark Plus). The results were then calibrated against the standard curve to determine relative ELISA units (ELISA units/mL) (personal communication).

3.9. Statistical analyses

Mixed linear model analyses with repeated measures were performed using SAS (version 9.3, SAS Institute Inc., Cary, NC, USA) to determine interactions between cytokine expression, acute phase markers, vaccine IgGs, and egg counts (**Table 4**). The random effect was the FEC and the class variables were group and time-point; the rest of the variables were continuous. Interaction terms were calculated between group and time and when time was found significant, a least square means analysis was used for a Tukey's pairwise comparison of the time variable. A 5% significance level was used to determine those parameters most affected.

4. Results

4.1. Egg counts

The average fecal egg count reductions for each group are shown **Table 2**. A negative number in the FECR table means that there was an increase in the levels between Day 0 (treatment) and Day 14 in each month. Overall, we saw an average efficacy of 96.8% with ivermectin, <1% efficacy with pyrantel, and an average increase in egg counts of 13.2% in the control group.

Table 4.1*- The observed efficacies of the two anthelmintics in the study cohort.*

	Avg FECR-Aug (%)	**Avg FECR-Sept (%)**
IVM	100.00 ± 0.00	93.51 ± 17.59
PYR	53.51 ± 68.89	-55.43 ± 187.68
Control	-25.03 ± 114.39	-1.55 ± 58.31

4.2. Hematology

As the graphs (**Figure 5)** indicate, the treatment groups had a 4.28-12% decrease in total WBCs, a 2.47-3.25% increase in hematocrit, a 1.13-5.38% increase in neutrophils, and a 0.42-3.72% decrease in lymphocytes within the first 5 days after the first treatment. In contrast, the control group had a 15.2% decrease in total WBCs, a 2.51% increase in hematocrit, a 0.43% decrease in neutrophils, and a 1.00% decrease in lymphocytes within the first 5 days after the first treatment. The second treatment had similar effects to the first, except neutrophil percentages increased. In summary, there were negligible changes in all of the hematology over the eight weeks.

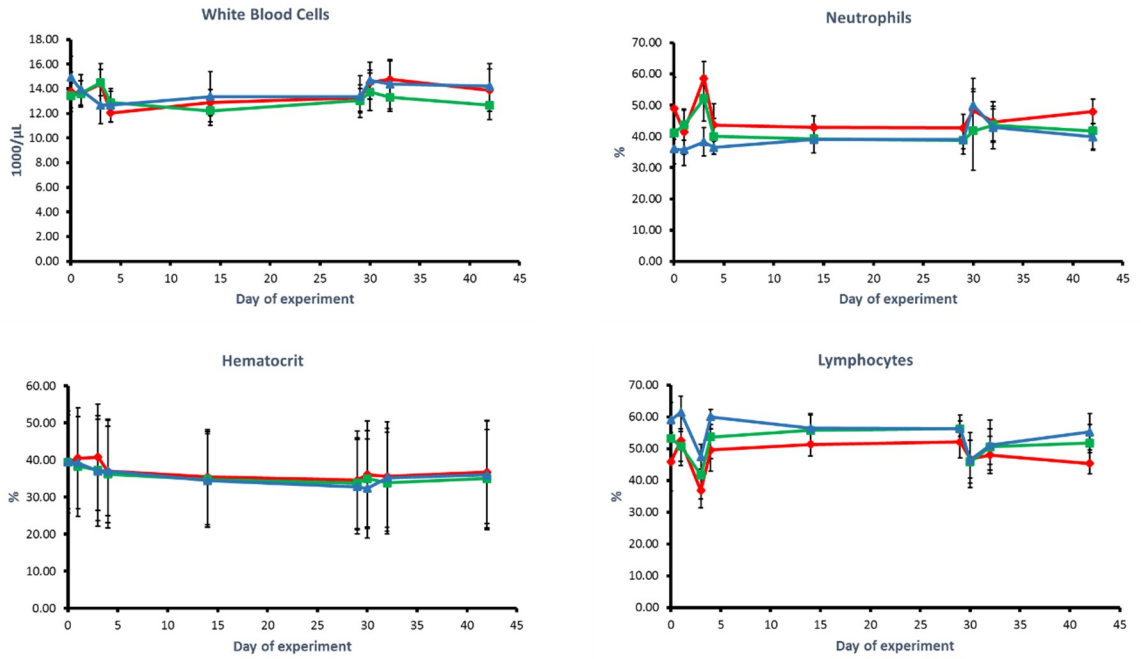

Figure 4.1*- Hematological analysis results. Red= ivermectin; green=pyrantel pamoate; blue=control. Error bars are the 95% confidence interval.*

4.3. Inflammatory markers

As the graphs (**Figure 6**) indicate, there is a 55.9-133% increase in haptoglobin, a 21-40.1% increase in fibrinogen, a 2- to 45-fold increase in SAA, and a 4.0-23.9% decrease in iron within the first 72 hours after the second treatment. There was a statistical difference in the marker levels between the control and treatment groups for fibrinogen, but not for SAA, haptoglobin or iron as determined by fixed effects analyses (**Table 3**).

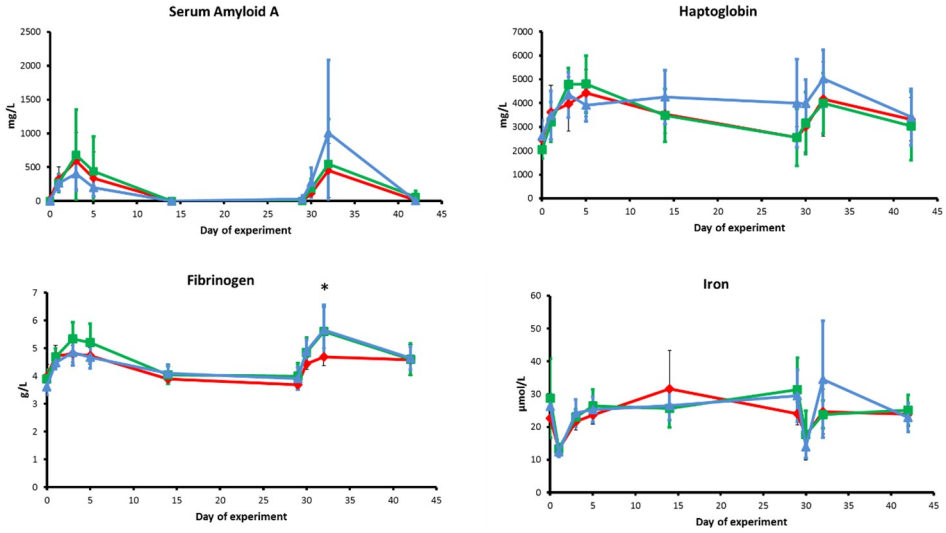

Figure 4.2*- Acute phase marker analysis results. Red= ivermectin; green=pyrantel pamoate; blue=control. Error bars are the 95% confidence intervals.*

4.4. Cytokine expression

With the use of mixed linear analyses and by looking at the graphs (**Figure 7**), there are no statistically significant effects of time on IL-1β, IL-4, IL-10, and TNF-α levels, but there is a significant statistical and biological decrease in IFN-γ mRNA levels over time, specifically between days 29 and 32. In addition, we see that anthelmintic treatment did have a statistically significant effect on the levels of IL-1β and IL-10, but not on IL-4, TNF-α or IFN-γ mRNA levels as determined by fixed effects analyses.

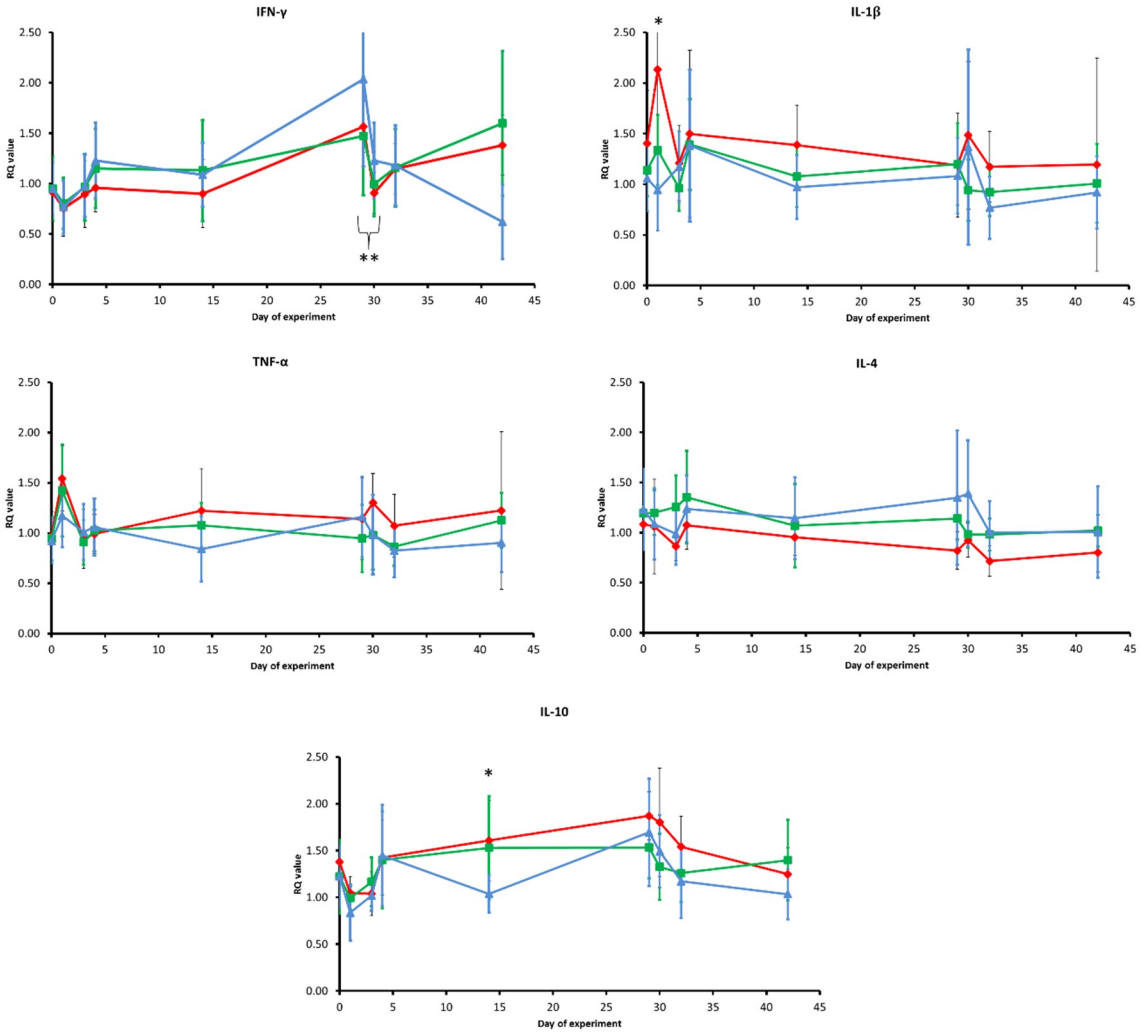

Figure 4.3*- Cytokine expression results. Red= ivermectin; green=pyrantel pamoate; blue=control. Error bars are the 95% confidence interval. Asterisks signify the timepoints at which the groups had significant differences. Double asterisks signify where there was a significant difference between timepoints.*

4.5. Vaccine ELISAs

As seen in the graphs (**Figures 8-10**), there is an increase in all vaccine antibody titers within 14 days post-vaccination, though the largest difference appears to be with the WNV IgG(a) between Days 1 and 14, and between days 29 and 42 for KLH IgG(a) and IgG(T) titers. The graphical results are consistent with the statistical difference in

antibody titers for the three vaccines (**Table 3**). WNV antibody levels differ significantly between days but neither cytokine expression nor inflammatory marker levels had an effect. In addition, the IgG(a) levels were numerically higher in the WNV untreated control group. KLH antibody levels differed not only between days, but they were also affected by SAA, haptoglobin and IFN-γ levels. On the other hand, treatment did not have an effect on the KLH titers. EHV antibody levels differed from day-to-day, but there was no effect of treatment, cytokines or inflammatory markers on the EHV antibody levels.

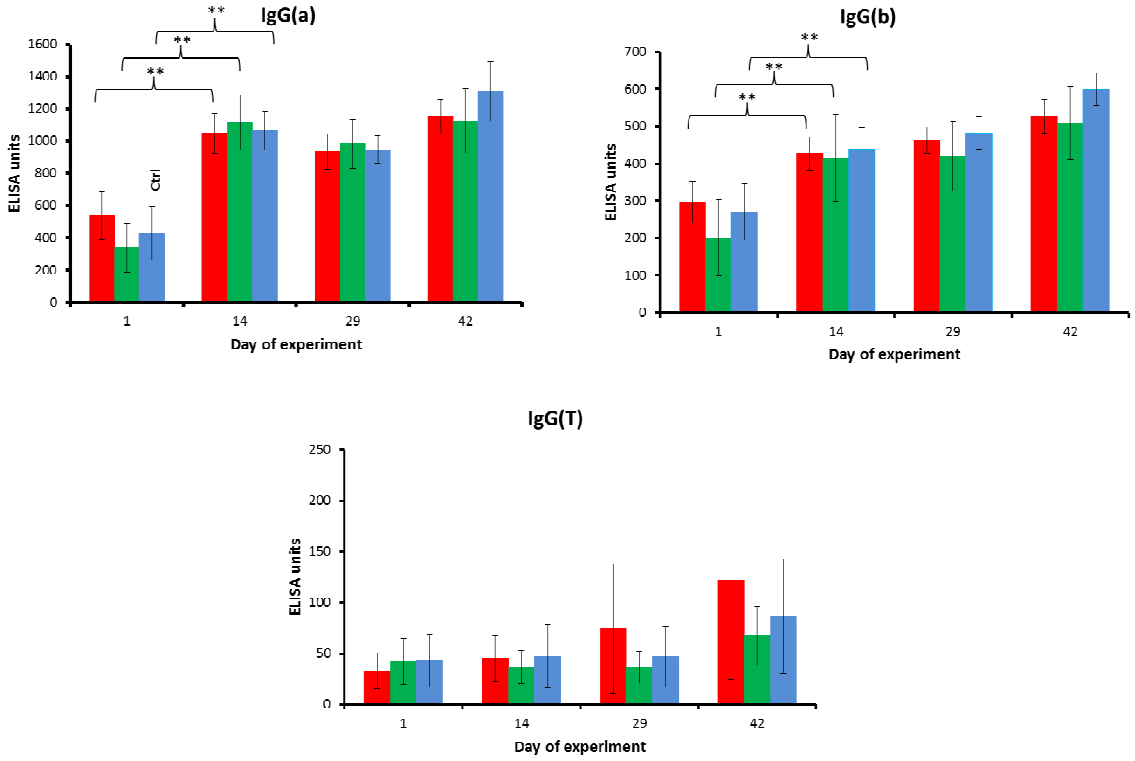

Figure 4.4*- EHV vaccine titer ELISA results. Red= ivermectin; green=pyrantel pamoate; blue=control. Error bars are the 95% confidence interval. ELISA units are defined as the OD ratio (sample OD/cut-off OD). Double asterisks signify where there was a significant difference between timepoints.*

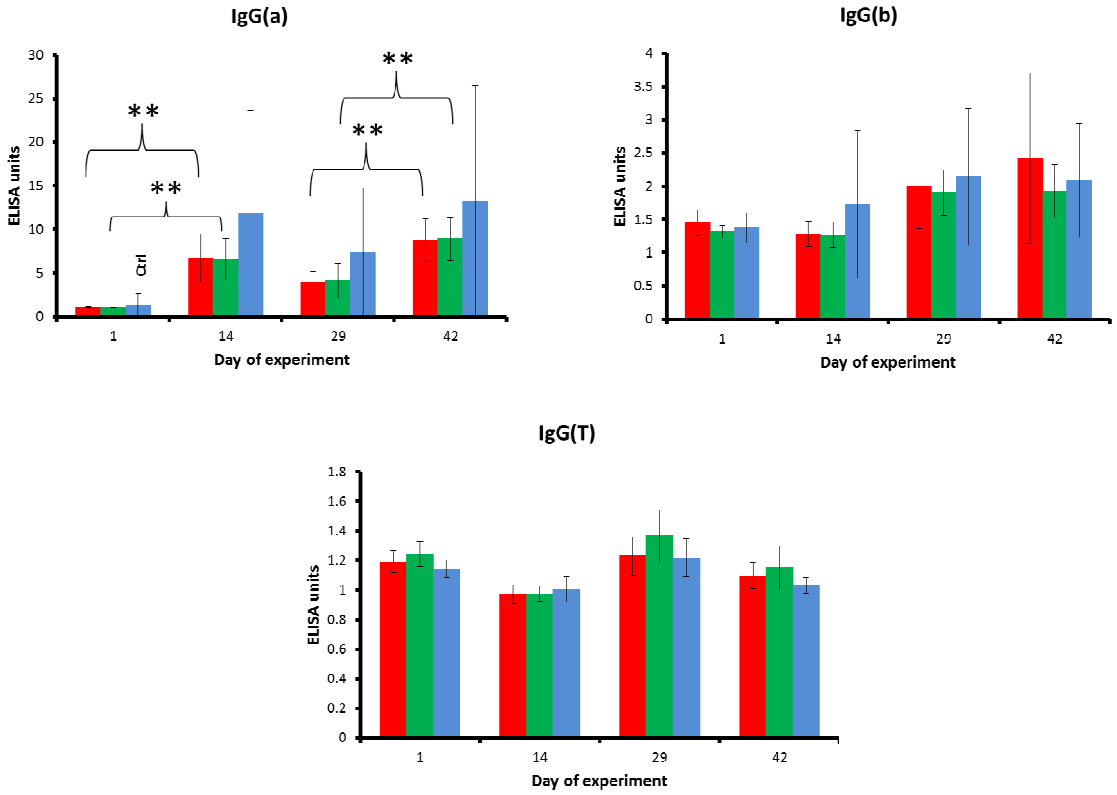

Figure 4.5*- WNV vaccine titer ELISA results. Red= ivermectin; green=pyrantel pamoate; blue=control. Error bars are the 95% confidence interval. ELISA units are defined as the OD ratio (sample OD/cut-off OD). Double asterisks signify where there was a significant difference between timepoints.*

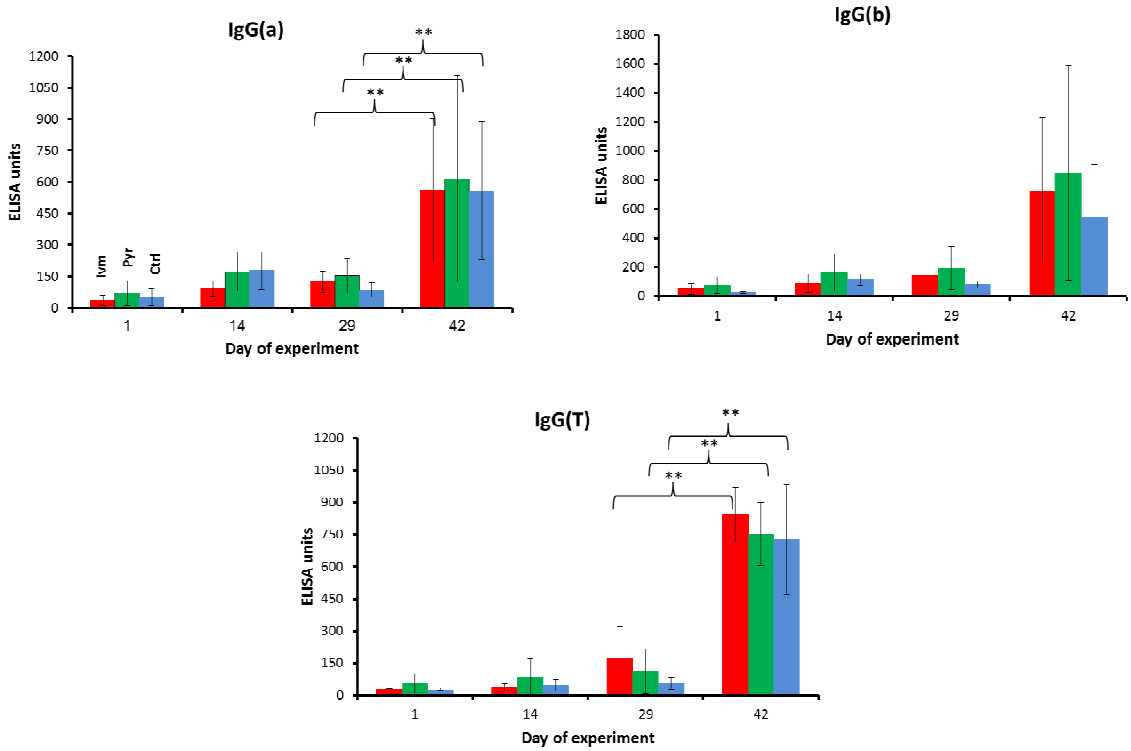

Figure 4.6*- KLH vaccine titer ELISA results. Red= ivermectin; green=pyrantel pamoate; blue=control. Error bars are the 95% confidence interval. ELISA units are defined as the OD ratio (sample OD/cut-off OD). Double asterisks signify where there was a significant difference between timepoints.*

Table 4.2*- Statistically significant (P≤ 0.05) associations between day, group, inflammatory markers and cytokine levels as calculated using a mixed linear model analysis.*

Response variable	Day/group	Inflammatory/immunologic
SAA	Day	Fibrinogen, TNF-α
Haptoglobin	Day, Group	Iron, Fibrinogen, IL-10, IFN-γ
Fibrinogen	Day, Group	SAA, Haptoglobin
Iron	Day	Haptoglobin, IL-10, IFN-γ
IL-1β	Group	Fibrinogen, Haptoglobin, IL-4, IL-10, TNF-α
IL-4	----	Haptoglobin, IL-10
IL-10	Group	IL-4, IL-1
IFN-γ	Day	Haptoglobin, Iron, IL-10
TNF-α	----	SAA, Fibrinogen, IL-1
WNV	Day	----
EHV	Day, Group*Day	----
KLH	Day	SAA, Haptoglobin, IFN-γ
EPG	----	Iron, IL-4, IL-10

5. Discussion

There were noteworthy effects of the administration of two different classes of anthelmintics on several acute phase markers, cytokines, and vaccine titers. We will discuss below those components which responded, both biologically and statistically, to the administration of anthelmintics, and those that had little to no response to anthelmintic treatment.

The acute-phase inflammatory marker data showed an increase in SAA, haptoglobin, and fibrinogen after each vaccination. At these same time points, the serum iron levels decreased, which is consistent with iron sequestration caused by induction of inflammation (Wessling-Resnick 2010, Andersen *et al.* 2012). These results are similar to data from Andersen *et al.* (2012). Their results showed that SAA and fibrinogen concentrations increased while those of iron decreased after inflammatory stimulation with both ISCOM and live recombinant vaccines (Andersen *et al.* 2012). In our study we administered all three vaccines (KLH, WNV, and EHV) at the same time; even with this difference, the results of the Andersen study and ours are similar: the overall trend, as expected, is that there is a decrease in iron and an increase in acute phase protein levels. Therefore, we can conclude that there was an inflammatory response to vaccination.

The cytokine expression data shows that anthelmintic treatment did have an effect on the immune response to vaccination. In the Edmonds *et al.* (2001) study, they tested whether immunity induced by KLH vaccination would be improved when the parasites were removed. Their results were that moxidectin-treated ponies had significantly higher levels of IL-4 than those that were treated with pyrantel. With pyrantel administration, the ponies had increased IFN-γ levels as compared to the other groups. Neither IL-5 nor IL-2 changed with any treatment as compared to the control group. They concluded that the decreased immune responses to KLH immunization may model the responses that may be seen with other vaccines, meaning that vaccine effectiveness of the vaccines will be diminished too (Edmonds *et al.* 2001). In comparison, our study showed a statistical difference between the treatment groups and the control group for IL-1β, another pro-inflammatory and lymphoproliferative cytokine like IFN-γ, which suggests that there was modulation of the inflammatory response as a result of treatment. Interestingly, there was no statistically significant effect of treatment on IFN-γ or TNF-α. Reasons for the differences in cytokine expression between this study and Edmonds' may be that 1) treatment was separated from vaccination in the Edmonds' study, 2) we measured gene expression by extracting RNA directly from blood, while Edmonds' study measured cytokine levels after culturing PBMCs, and 3) the Edmonds' study removed interaction with the acute phase reaction by concentrating on the worm burden effects. As a reminder, the Edmonds' study set out to assess the effect of increasing numbers of gastrointestinal parasites on immune response to vaccination. In addition, we did not measure cytokine levels on the "golden days" of their release, as described in section 2.6.2.

The hematology results showed that the percentage of neutrophils increased after each treatment. Previous studies involving intestinal inflammation found that cyathostomin infection tends to cause short-term neutrophilia due to decreased neutrophil turnover (Murphy and Love 1997). The increase in neutrophils is most likely a response to vaccination, which we expect, and it is possible that any intestinal reaction to the

worms or the treatment was overshadowed by the larger vaccine-associated inflammatory response.

The ELISAs showed that there was no significant difference between treatment and control groups, even when taking into consideration the high variability within the herd in response to the vaccines. As a side note, it is likely that the ponies were already exposed to EHV, because we see a larger increase in antibody levels by Day 1 but not at Day 29. The lower KLH IgG titers throughout the study in comparison to the two commercial vaccines were likely due to the lack of adjuvant in the protein vaccine and a lack of previous exposure.

The egg count cannot be used as a measure of the number of adult worms in the gastrointestinal tract; the accurate enumeration of adult worms is determined at necropsy. Though fecal egg counts are inaccurate for enumerating adult worm burdens, the World Association for the Advancement of Veterinary Parasitology (WAAVP) recommends this method for evaluating anthelmintic efficacy (Coles *et al.* 1992). It is apparent from the FECRT results that the ivermectin treatment was successful against strongyles after both Day 0 and Day 29. During this study, the efficacy of pyrantel is diminished after both treatment dates, suggesting that there is pyrantel resistance in this study cohort. As a consequence of the lack of efficacy, therefore a decreased ability to kill the worms, these ponies would most likely have a decreased local inflammatory response. As expected, the pyrantel-treated ponies had low egg count reductions and the ivermectin-treated ponies had >90% reductions, which is consistent with Lyons *et al.* (2008), in which they reported that on several farms in Central Kentucky, pyrantel was only able to reduce the strongyle egg count by an average of 27%, while ivermectin reduced strongyle egg counts by 100%.

6. Conclusions

The overarching conclusion of this study is that deworming does not affect vaccine IgG titers, but there is an effect of treatment on vaccine-induced inflammatory processes. Therefore, ceasing the practice of vaccinating and deworming on the same day does not appear to be necessary at this time. In addition to the effect of deworming on vaccine titers, we are able to determine whether ivermectin can act as an anti-inflammatory; we cannot conclude that ivermectin can act as an anti-inflammatory within the parameters of this study, since pyrantel and ivermectin treatments had similar biological effects on fibrinogen.

7. Future studies

As a follow-up to this study, I would follow Edmonds *et al.*'s model to determine the effect of the relative size of the parasite burden on the efficacy of a commercial vaccine. In this project, we did not concentrate on the effect the cyathostomins on vaccine efficacy, only the effect of the anthelmintics and the inflammatory reaction induced by the dying parasites. In the case of the proposed study, I would use EHV because it has been proven, not only in this study, but also in other studies, that of the three vaccines used in this thesis study, it induces a more significant antibody response in the horse than KLH or WNV (Patel *et al.* 2003, Van de Walle *et al.* 2010). In addition to the antibody response to the vaccine, the inflammatory reaction induced by this vaccine would be an increase in IFN-γ, signifying a pro-inflammatory response that would decrease with the presence of the worms (Gildea *et al.* 2013). I would allocate the horses or ponies into two groups: an ivermectin-treated group and an untreated group. Group size is determined using G*Power 3.1 analysis software (Faul 2009), with an α of 0.05 and a power of 0.40. Using the EHV IgG(a) naïve vaccine titers data for the ivermectin group and the control group from this thesis study, I am able to determine that we would need a total of 32 horses for the study, 16 per group. Pre-treatment and then every week after for three weeks, fecals would be taken to determine the anthelmintic efficacy. We should see a greater decrease in egg counts in the ivermectin group than the pyrantel group, and the control should have the highest EPG. On the same day the horses are dewormed, but before all participants receive a dose of EHV vaccine, blood will be taken to determine pre-vaccination IgG levels. At two weeks and at one month later, blood would be taken again to measure for vaccine-derived antibodies, along with IL-1, IL-10, IFN-γ, TNF-α, IL-4, and the acute phase markers (haptoglobin, fibrinogen, SAA, and iron). This study is useful because we can determine inflammatory effects for a single vaccine instead of all three used in this study, and also, we are concentrating on the effect of the worms rather than the treatment. If the parasites do have an effect on vaccine efficacy, then we should see an increase in antibody titers with the treatment groups, with the ivermectin-treated group having the highest levels of vaccine-derived antibodies.

We tested the effects of anthelmintics on inflammation in one year old ponies; testing the influence of anthelmintics in equids >1 year of age would be logical because as horses age, the production of pro-inflammatory cytokines such as IFN-γ and TNF-α increase in the peripheral blood (Adams *et al.* 2008, Hansen *et al.* 2013). Repeating this study in geriatric horses may be beneficial; as horses grow older, the immunological reaction to strongyle infection changes, resulting in differences in egg shedding levels and the number of cyathostomins found upon necropsy (Chapman *et al.* 2003, Lyons *et al.* 2012, Wood *et al.* 2013). In addition, as mentioned earlier in this thesis, the total serum IgG decreases and pro-inflammatory cytokine expression increases with age (Katepalli *et al.* 2008).

Appendices

Appendix A: Quantitation of nematode eggs using the mini-FLOTAC method

We characterized and counted all eggs, but only carried out further quantitation and calculations using strongyle egg counts. The mini-FLOTAC method involves the homogenization of weighed 5± 0.1 grams of feces in 35±1 mL of flotation solution. The flotation solution was made by adding 600 mL of tap water to a 2 liter container. To this was added 250 g of solid NaCl (Amresco Corporation, Solon, Ohio) and 375 g of glucose monohydrate (Amresco Corporation, Solon, Ohio). Water was then added to 1 L and allowed to stir at 80 degrees C until dissolution, and then allowed to cool at room temperature. The resulting solution had a specific gravity equal 1.25. The mixture of feces and flotation solution in the Fill-FLOTAC was used to fill the two flotation chambers (Mini-FLOTAC seen in Figure 2). After 10 ± 2 minutes, the reading disc was rotated in order to make a single layer of eggs in each chamber. The two sides of the chamber were counted and the eggs per gram were calculated from the sum of the counted eggs by multiplying by 5.

Figure S1. *Setup of larval culture in makeshift humidity chamber. Picture courtesy of Holli Gravatte.*

Appendix B: Culturing third-stage strongyle larvae from fecal samples

Setup of cultures

Using a modified version of Henriksen and Korsholm (1983), we cultured feces from Day 0 and Day 60 in order to characterize the species of strongyles present in the samples. A humidity chamber was made to ensure the cultures stayed moist. In order to set up the humidity chamber for larval coprocultures, a plastic drinking cup approximately four inches in height was cut two-thirds of the way from the top. Another cup was filled with 100 mL of water, and the upper part of the cut cup was placed in the filled cup. The bottom of the cut cup was pierced with 4-5 holes in order to allow air into the culture. An approximately 8 in x 8 in square of cheesecloth was held in place to the water-filled cup by the open-ended part of the cut cup. Approximately 5 mL of water was added drop-wise to the feces-vermiculite mixture to make a cohesive mass, which was added to the cheesecloth. The cheesecloth was folded over the fecal mixture, and the bottom of the cut cup with the holes was placed upside down to keep the cheesecloth in place. The cultures were allowed to mature at 25°C in an incubator for 14 days, moistening the feces drop-wise directly every 2 to 3 days after setup.

Baermannizing

After 14 days of culturing, the larvae were collected using a modified Baermann technique (Olsen *et al.* 2003). The Baermann technique for harvesting the larvae involves attaching the cheesecloth-wrapped culture in its open-ended cup to a tea glass (Libbey Claret Footed Iced Tea Glasses, Model # 89594) filled with tap water using large paperclips (**Figure S2**). After the upper part of culture chamber was added to the glass, the cup filled with 100 mL of water is scraped to ensure any larvae that have escaped into the water during culturing are added to the apparatus. More tap water may have needed to be poured over the cheesecloth-wrapped culture until the bottom of the cheesecloth was covered in about a centimeter of water. The apparatus was left to sit at room temperature for 24 hours, at which point the cultures were removed, and 5 mL of fluid from the bottom of the wineglass was collected in a 15 mL tube, which was spun down 1140 x g* for 5 minutes in order to bring all larvae to the bottom. The water was removed with a dropper to a volume of 2 mL and stored at 4°C until the cultures were counted using nematode counting slides (Chalex Corporation, Grasonville, MD); the time of delay to counting could be immediately after harvest to up to 2 weeks. Prior work in this laboratory has determined that the ability of larvae to undergo heating is unchanged at 2 weeks post-harvest, as described in **Figure S3**.

Figure S2. *Setup of Baermann apparatus. Picture courtesy of Holli Gravatte.*

*conversion factor was found at www.sometechnology.com/pdfFiles/J_6B.pdf

Larval differentiation

The harvested, volume-reduced larval solution was mixed using a 5 mL disposable transfer pipette in order to make the mixture homogeneous. Then, using the same pipette, the entire 2 mL was taken up and added to the nematode slide. The slides were placed on a 55°C hotplate for 3 minutes and 30 seconds in order to ensure the larvae were no longer motile. If the larvae had continued motility, the slides were heated for an additional 30 seconds. The slides were viewed at 10× under a halogen backlit binocular microscope (Nikon Eclipse 80i, Nikon Corp., Tokyo, Japan). The third stage larval differentiation was made based on number of intestinal cells, presence of a visible cuticle, and shape of the esophagus. Even though there may be first and second stage larvae in the culture, we can't differentiate between first or second stage strongyle larvae.

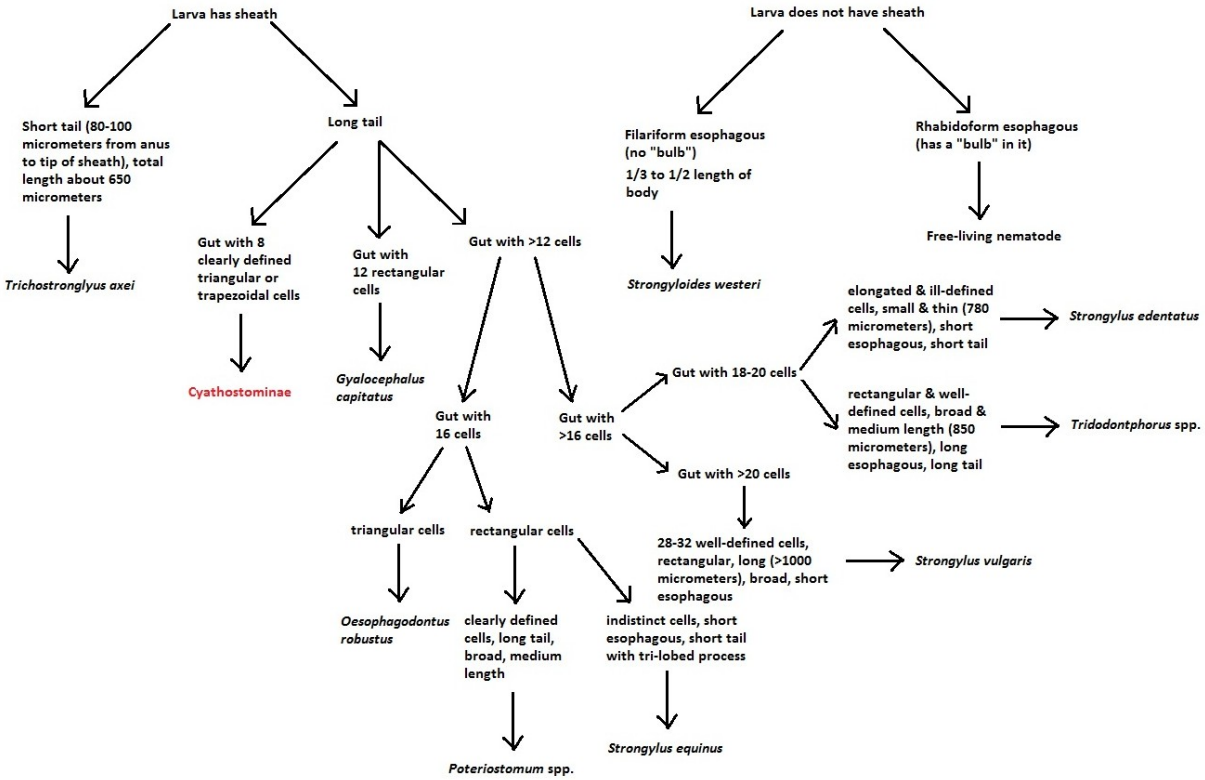

Figure S3*-Differentiation of larvae grown in coprocultures. Identification is performed according to Russell (1948).*

The larvae were characterized as seen in **Table S1**; the majority of larvae were found to be third stage cyathostomins, identified by their 8 triangular intestinal cells, filariform esophagus, sheath, and long tail (**Figure 1**). In addition, several *Poteriostomum* spp. and *Strongylus vulgaris* were found using identification parameters defined by Zajac and Conboy (2012), as seen in **Figure S3**.

Table S1*- Larvae characterized in each sample at pre-trial and post-trial dates.*

Pre-trial

Pony	**Cyathostomin**	Poteriostomum	*S. vulgaris*	**Total**	% cyathostomins
M66	**55**	0	0	**55**	100.00
M67	**315**	0	0	**315**	100.00
M69	**22**	0	0	**22**	100.00
M71	**86**	3	0	**89**	96.63
M73	**0**	0	0	**0**	100.00
M74	**121**	3	0	**124**	97.58
M77	**981**	0	0	**981**	100.00
M78	**1**	13	1	**15**	6.67
M79	**382**	0	0	**382**	100.00
M81	**388**	0	0	**388**	100.00
M83	**9**	0	0	**9**	100.00
M85	**10**	1	0	**11**	90.91
M86	**131**	4	0	**135**	97.04
M88	**486**	0	0	**486**	100.00
M90	**48**	0	0	**48**	100.00
M91	**106**	0	0	**106**	100.00
M92	**65**	0	0	**65**	100.00

Post-trial

Pony	**Cyathostomin**	Poteriostomum	*S. vulgaris*	**Total**	% cyathostomins
M66	**107**	0	0	**107**	100.00
M67	**66**	0	0	**66**	100.00
M69	**0**	0	0	**0**	100.00
M71	**7**	0	0	**7**	100.00
M73	**0**	0	0	**0**	100.00
M74	**267**	1	0	**268**	99.63
M77	**303**	0	0	**303**	100.00
M78	**160**	0	0	**160**	100.00
M79	**1**	0	0	**1**	100.00
M81	**229**	2	0	**231**	99.13
M83	**90**	0	0	**90**	100.00
M85	**32**	0	0	**32**	100.00
M86	**2**	0	0	**2**	100.00
M88	**14**	0	0	**14**	100.00
M90	**115**	0	0	**115**	100.00
M91	**29**	0	0	**29**	100.00
M92	**49**	0	0	**49**	100.00

Appendix C: Abbreviations

AMV= avian myeloblastosis virus
cDNA= complementary DNA
C_T= cycle threshold; proportional to –log of the concentration
CTRL= control
EHV= equine herpes rhinopneumonitis virus
epg= eggs per gram of feces
FEC= fecal nematode egg count
FECRT= fecal nematode egg count reduction test
GABA= gamma-aminobutyric acid
IFN-γ= interferon-gamma
IgG= immunoglobulin G
IL-1= interleukin 1
IL-4= interleukin 1
IL-10= interleukin 10
ISCOM= Immune Stimulating COMplex
IVM= ivermectin
KLH= keyhole limpet hemocyanin
L1= first larval stage
L2= second larval stage
L3= third larval stage; infective stage; on pasture
EL3= early third larval stage; in horse large intestine
LL3= late third larval stage; in horse large intestine
L4= fourth larval stage
L5= early fifth stage (adult)
nACh= nicotinic acetylcholine
NFR= NADH-fumerate reductase
PBMC= peripheral blood mononuclear cell
PYR= pyrantel pamoate
SAA= serum amyloid A
Th1= type 1 helper T-cell
Th2= type 2 helper T-cell
TNF-α= tumor necrosis factor alpha
WNV= West Nile virus

References

Adams, A.A., *et al*. (2008) Advanced age in horses affects divisional history of T cells and inflammatory cytokine production. *Mech. Ageing Dev*. **129**, 656-664.

Alverez, L. I., et al. (2009) Formulations, Dosages, and Routes of Administration. Veterinary Pharmacology and Therapeutics. J. E. Riviere and M. G. Papich. Ames, Iowa, John-Wiley and Sons, 1081.

Andersen, S. A., *et al.* (2012) Vaccination elicits a prominent acute phase response in horses. *Vet. J.* **191**, 199-202.

Andersen, U.V. *et al.* (2014) Physiologic and systemic acute phase inflammatory responses in young horses repeatedly infected with cyathostomins and *Strongylus vulgaris*. *Vet. Parasitol.* **201**, 67-74.

Barda, B.D. *et al.* (2013) Mini-FLOTAC, an Innovative Direct Diagnostic Technique for Intestinal Parasitic Infections: Experience from the Field. *PLoS Neg. Trop. Dis.* **7**, e2344.

Berger, A. (2000) Th1 and Th2 responses: what are they? *BMJ* **321**, 424.

Berridge, M. J. (2012) Module 11: Cell Stress, Inflammatory Responses and Cell Death. *Cell Signaling Biology*, 1-29.

Betancourt, A., *et al.* (2014) Characterisation of the inflammatory cytokine response to anthelmintic treatment in ponies. *Equine Vet. J*. **Epub ahead of print**.

Breathnach, C.C. *et al.* (2006) Foals are interferon gamma-deficient at birth. *Vet. Immunol. Immunopathol.* **112**, 199-209.

Canever, R. J., *et al.* (2013) Lack of Cyathostomin sp. reduction after anthelmintic treatment in horses in Brazil. *Vet. Parasitol*. **194**: 35-39.

Castro, G. A., Ed. (1996) Chapter 86 Helminths: Structure, Classification, Growth, and Development. Medical Microbiology. Galveston, TX, University of Texas Medical Branch at Galveston.

Chapman, M.R. *et al*. (2003) Prevalence of strongyle nematodes in naturally infected ponies of different ages during different seasons of the year in Louisiana. *J. Parasitol*. **89**, 309-314.

Chu, K.M. *et al.* (2013) Childhood Helminth Exposure Is Protective Against Inflammatory Bowel Disease: A Case Control Study in South Africa. *Inflamm. Bowel Dis.* **19**, 614-620.

Claerebout, E. and J. Vercruysse (2012a) Macrocyclic Lactones. . In: Aiello, S.E., Moses, M. (Eds.) The Merck Veterinary Manual (online version). Merck Sharpe & Dohme Corp., Whitehouse Station, NJ.

Claerebout, E. and J. Vercruysse (2012b) Mechanisms of Action of Anthelmintics. In: Aiello, S.E., Moses, M. (Eds.) The Merck Veterinary Manual (online version). Merck Sharpe & Dohme Corp., Whitehouse Station, NJ.

Claerebout, E., Vercruysse, J. (2012c) Benzimidazoles, In: Aiello, S.E., Moses, M. (Eds.) The Merck Veterinary Manual (online version). Merck Sharpe & Dohme Corp, Whitehouse Station, NJ.

Coles, G.C. *et al.* (2006) The detection of anthelmintic resistance in nematodes of veterinary importance. *Vet. Parasitol*. **136**, 167-185.

Costa, A.J., Barbosa, O.F., Moraes, F.R., Acuna, A.H., Rocha, U.F., Soares, V.E., Paullilo, A.C., Sanches, A. (1998) Comparative efficacy evaluation of moxidectin gel and ivermectin paste against internal parasites of equines in Brazil. *Vet. Parasitol*. **80**, 29-36.

Cringoli, G. *et al.* (2010) FLOTAC: new multivalent techniques for qualitative and quantitative copromicroscopic diagnosis of parasites in animals and humans. *Nat. Protoc.* **5**, 503-515.

Cunha, C.W. *et al.* (2006) Development of Specific Immunoglobulin Ga (IgGa) and IgGb Antibodies Correlates with Control of Parasitemia in *Babesia equi* Infection. *Clin. Vaccine Immunol.* **13**, 297-300.

Davidson, A. J., *et al.* (2005) Cytokine responses to Cyathostominae larvae in the equine large intestinal wall. *Res. Vet. Sci*. **78**, 169-176.

Demeulenaere, D., Vercruysse, J., Dorny, P., Claerebout, E. (1997) Comparative studies of ivermectin and moxidectin in the control of naturally acquired cyathostome infections in horses. *Vet. Rec.* **141**, 383-386.

Eckersall, P.D. (1995) Acute phase proteins as markers of inflammatory lesions. *Comp. Haematol. Int.* **5**, 93-97.

Edmonds, J.D. *et al.* (2001) Altered immune responses to a heterologous protein in ponies with heavy gastrointestinal parasite burdens. *Equine Vet. J.* **33**, 658-663.

Faul, F. *et al.* (2009) Statistical power analyses using G*Power 3.1: Tests for correlation and regression analyses. *Behav. Res. Methods* **41**, 1149-1160.

Gause, W.C. *et al.* (2013) Type 2 immunity and wound healing: evolutionary refinement of adaptive immunity by helminths. *Nat. Rev. Immunol.* **13**, 607-614.

Gibson, T.E. (1953) The Effect of Repeated Anthelmintic Treatment with Phenothiazine on the Faecal Egg Counts of Housed Horses, with Some Observations on the Life Cycle of *Trichonema* spp. in the Horse. *J. Helminthol.* **27**, 29-40.

Gildea, S. *et al.* (2013) Humoral response and antiviral cytokine expression following vaccination of thoroughbred weanlings—A blinded comparison of commercially available vaccines. *Vaccine* **31**, 5216-5222.

Guguère, S. and Prescott, J.F. (1999) Quantitation of equine cytokine mRNA expression by reverse transcription-competitive polymerase chain reaction. *Vet. Immunol. Immunopathol.* **67**, 1-15.

Hansen, S. *et al.* (2013) Age-related changes in intracellular expression of IFN-γ and TNF-α in equine lymphocytes measured in bronchoalveolar lavage and peripheral blood. *Dev. Comp. Immunol.* **39**, 228-233.

Harn, D.A. *et al.* (2009) Modulation of host immune responses by helminth glycans. *Immun. Rev.* **230**, 247-257.

Harris, N. and Gause, W.C. (2011) B cell function and immune response to helminths. *Trends Parasitol.* **32**, 80-88.

Harty, J.T., *et al.* (2000). $CD8^+$ T Cell Effector Mechanisms in Resistance to Infection. *Annu. Rev. Immun.* **18**, 275-308.

Henriksen, S.A. and Korshalm, H. (1983) A method for culture and recovery of gastrointestinal strongyle larvae. *Nord. Vet. Med.* **35**, 429-430.

Hepburn, J.J. *et al.* (2009) Technical note: Copper chaperone for copper, zinc superoxide dismutase: A potential biomarker for copper status in cattle. *J. Anim. Sci*. **87**, 4161-4166.

Hiemstra, I.H. *et al.* (2014) Excreted/secreted *Trichuris suis* products reduce barrier function and suppress inflammatory cytokine production of intestinal epithelial cells. *Mol. Immunol.* **60**, 1-7.

Holden-Dye, L. and R. J. Walker (2007) Anthelmintic drugs. WormBook 1-13.

Hopster-Iversen, C. *et al.* (2011) Influence of mechanical manipulations on the local inflammatory reaction in the equine colon. *Equine Vet. J. Suppl.* **43**, 1-7.

Ihler, C.F. (2010) Anthelmintic resistance. An overview of the situation in the Nordic countries. *Acta Vet. Scand*. 52, S24.

Hsu, W. H. and R. J. Martin (2013). Chapter 16- Antiparasitic Agents. Handbook of Veterinary Pharmacology, 379-412.

Jacobsen, S. *et al.* (2005) Use of serum amyloid A and other acute phase reactants to monitor the inflammatory response after castration in horses: a field study. *Equine Vet. J.* **37**, 552-556.

Johnson, E. E. and M. Wessling-Resnick (2012) Iron metabolism and the innate immune response to infection. *Microbes Infect.* **14**, 207-217.

Katepalli, M.P. *et al.* (2008) The effect of age and telomere length on immune function in the horse. *Dev. Comp. Immunol*. 32, 1409-1415.

Killi, R. (2008) Assessment of the Ability of Two Differently Constructed Equine Viral Vaccines to Induce Systemic Inflammation. University of Georgia, Athens, Georgia.

Koyasu, S. and K. Moro (2011). Type 2 innate immune responses and the natural helper cell. *Immunology* **132**, 475-481.

Krista, K.M. *et al.* (2013) Evaluation of neutrophil apoptosis in horses with acute abdominal disease. *Am. J. Vet. Res.* **74**, 999-1004.

Kydd, J.H., Townsend, H.G.G., Hannant, D. (2006) The equine immune response to equine herpesvirus-1: The virus and its vaccines. *Vet. Immunol. Immunop*. **111**, 15-30.

Lester, H. E. and J. B. Matthews (2013) "Faecal worm egg count analysis for targeting anthelmintic treatment in horses: Points to consider." *Equine Vet. J.* **46**, 139-145.

Lewis, M.J. *et al.* (2008) The different effector function capabilities of the seven equine IgG subclasses have implications for vaccine strategies. *Mol. Immunol.* **45**, 818-827.

Lichtenfels, J.R. (1975) Helminths of Domestic Equids: Illustrated Keys to Genera and Species with Emphasis on North American Forms. *P. Helm. Soc. Wash.* **42**, 1-78.

Lichtenfels, J. R., *et al.* (2008) Illustrated identification keys to strongylid parasites (strongylidae: Nematoda) of horses, zebras and asses (Equidae). *Vet. Parasitol.* **156**, 4-161.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods* **25**, 402-408.

Love, S., *et al.* (1999) Pathogenicity of cyathostome infection. *Vet. Parasitol.* **85**, 113-122.

Lyons, E. T., *et al.* (1999) Historical perspective of cyathostomes: prevalence, treatment and control programs. *Vet. Parasitol.* **85**, 97-112.
Lyons, E. T., *et al.* (2008) Evaluation of parasiticidal activity of fenbendazole, ivermectin, oxibendazole, and pyrantel pamoate in horse foals with emphasis on ascarids (*Parascaris equorum*) in field studies on five farms in Central Kentucky in 2007. *Parasitol. Res.* **103**, 287-291.
Lyons, E.T., *et al.* (2009) Probable reason why small strongyle EPG counts are returning "early" after ivermectin treatment of horses on a farm in Central Kentucky. *Parasitol. Res.* **104**, 569-574.
Lyons, E.T., *et al.* (2011) Field tests demonstrating reduced activity of ivermectin and moxidectin against small strongyles in horses on 14 farms in Central Kentucky in 2007-2009. *Parasitol. Res*. **108**, 355-360.
Lyons, E. T., *et al.* (2011) Observations on development of natural infection and species composition of small strongyles in young equids in Kentucky. *Parasitol. Res.* **109**, 1529-1535.
Lyons, E.T. *et al.* (2012) Investigation of strongyle EPG values in horse mares relative to known age, number positive, and level of egg shedding in field studies on 26 farms in Central Kentucky (2010-2011). *Parasitol. Res.* **110**, 2237-2245.
Mangan, N.E. *et al.* (2004) Helminth Infection Protects Mice from Anaphylaxis via IL-10-Producing B Cells. *J. Immunol.* **173**, 6346-6356.
McCulloch, J., Williamson, S.A., Powis, S.J., Edington, N., 1993. The effect of EHV-1 infection upon circulating leucocyte populations in the natural equine host. *Vet. Microbiol.* **37**, 147-161.
McKay, D.M. (2009) The therapeutic helminth? *Trends Parasitol.* **25**, 109-114.
Motomura, Y. *et al.* (2008) Helminth antigen-based strategy to ameliorate inflammation in an experimental model of colitis. *Clin. Exp. Immunol.* **155**, 88-95.
Nielsen, M.K., Chambers, T., Horohov, D.W., Jacobsen, S. (2013). The interaction between anthelmintic treatment and vaccination (University of Kentucky and University of Copenhagen), pp. 1-36.
Olsen, S.N. *et al.* (2003) Recovery of live immature cyathostome larvae from the faeces of horses by Baermann technique. *Vet. Parasitol.* **116**, 259-263.
Omura, S., *et al.* (2001) An anthelmintic compound, nafuredin, shows selective inhibition of complex I in helminth mitochondria. *PNAS* **98**, 60-62.
Osborne, L.C., *et al.* (2014) Virus-helminth coinfection reveals a microbiota-independent mechanism of immunomodulation. *Science* **345**, 578-582.
Paillot, R. *et al.* (2005) Equine interferon gamma synthesis in lymphoytes after *in vivo* infection and *in vitro* stimulation with EHV-1. *Vaccine* **23**, 4541-4551.
Patel, J.R. *et al.* (2003) Equid herpesvirus (EHV-1) live vaccine strain C147: efficacy against respiratory diseases following EHV types 1 and 4 challenges. *Vet. Microbiol.* **92**, 1-17.
Peón, A. N., *et al.* (2013) Immunoregulation by *Taenia crassiceps* and Its Antigens. *BioMed Res. Int.* **2013**, 1-13.
Peregrine, A. S., *et al.* (2005) Larval cyathostominosis in horses in Ontario: An emerging disease? *Can. Vet. J.* **46**, 80-82.

Peregrine, A. S., *et al.* (2014) Anthelmintic resistance in important parasites of horses: Does it really matter? *Vet. Parasitol.* **201**, 1-8.
Romagnani, S. (1999) Th1/Th2 cells. *Inflamm. Bowel Dis.* **5**, 285-94.
Reid, S.W.J. *et al.* (1995) Epidemiological risk factors associated with a diagnosis of clinical cyathostominosis in the horse. *Equine Vet. J.* **27**, 127-130.
Reinemeyer, C. R. (1999) Current concerns about control programs in temperate climates. *Vet. Parasitol.* **85**, 163-172.
Reinemeyer, C.R., *et al.* (2007) Nematodes. *Equine Infectious Diseases.* D. C. Sellon and M. T. Long. St. Louis, Saunders Elsevier**:** 80-90.
Reinemeyer, C.R. and Nielsen, M.K. (2012) *Handbook of Equine Parasite Control*, 1st edn., Wiley-Blackwell, Hoboken, NJ.
Relf, V.E. *et al.* (2014) Anthelmintic efficacy on UK Thoroughbred stud farms. *Int. J. Parasitol.* **44**, 507-514.
Romero-Cabello, R. *et al.* (2012) Hyperinfection with *Strongyloides stercoralis*. *BMJ Case Rep.* 2012.
Russell, A.F. (1948) The Development of Helminthiasis in Thoroughbred Foals. J. Comp. Pathol. Therap. **58**, 107-127.
Sandborn, W.J. *et al.* (2013) Randomised clinical trial: the safety and tolerability of *Trichuris suis* ova in patients with Crohn's disease. *Aliment. Pharmacol. Ther.* **38**, 255-263.
Schutz, J.S. *et al.* (2012) Effects of gastrointestinal parasites on parasite burden, rectal temperature and antibody titer responses to vaccination and infectious bovine rhinotracheitis virus challenge. *J. Anim. Sci.* **90**, 1948-1954.
Spellberg, B. and Edwards, J.E. (2001) Type 1/Type 2 Immunity in Infectious Diseases. *Clin. Infect. Dis.* **32**, 76-102.
Steinbach, T., *et al.* (2006) Small strongyle infection: Consequences of larvicidal treatment of horses with fenbendazole and moxidectin. *Vet. Parasitol.* **139**: 115-131.
Stratford, C. H., *et al.* (2014) An investigation of anthelmintic efficacy against strongyles on equine yards in Scotland. *Equine Vet. J.* **46**: 17-24.
Taylor, M.A., Coop, R.L., Wall, R.L. (2013) *Veterinary Parasitology*, 3rd Edition. Blackwell Publishing, Oxford, UK.
Traversa, D., Samson-Himmelstjerna, G.v., Demeler, J., Milillo, P., Schurmann, S., Barnes, H., Otranto, D., Perrucci, S., Regalbono, A.F.d., Beraldo, P., Boeckh, A., Cobb, R. (2009) Anthelmintic resistance in cyathostomin populations from horse yards in Italy, United Kingdom and Germany. *Parasite. Vector.* **2**, S2.
Urban, Jr., J.F., *et al.* (2007) Infection with parasitic nematodes confounds vaccination efficacy. *Vet. Parasitol.* **148**: 14-20.
U.S. Food and Drug Administration (2013a) Antiparasitic Resistance. Retrieved 19 March, 2014.
U.S. Food and Drug Administration (2013b) Antiparasitic Resistance in Cattle and Small Ruminants in the United States: How to Detect It and What to Do About It. Retrieved 19 March, 2014.

Van de Walle, G.R. *et al.* (2010) A vectored equine herpesvirus type 1 (EHV-1) vaccine elicits protective immune responses against EHV-1 and H3N8 equine influenza virus. *Vaccine* **28**, 1048-1055.

Walsh, K.P., *et al.* (2009) Infection with a Helminth Parasite Attenuates Autoimmunity through TGF-beta-mediated Suppression of Th17 and Th1 Responses. *J. Immunol.* **183**, 1577-1586.

Wesseling-Resnick, M. (2010) Iron Homeostasis and Inflammatory Response. *Annu. Rev. Nutr.* **30**, 105-122.

Wood, E.L. *et al.* (2013) Variation in fecal egg counts in horses managed for conservation purposes: individual egg shedding consistency, age effects and seasonal variation. *Parasitology* **140**, 115-128.

Yan, S. *et al.* (2011) Anti-inflammatory effects of ivermectin in mouse model of allergic asthma. *Inflamm. Res.* **60**, 589-596.

Zajac, A.M. and Conboy, G.A. (2012) Helminth ova and protozoan cysts as found in freshly voided feces of horses. In: *Veterinary Clinical Parasitology*, 8th edn., John-Wiley and Sons, Ames, Iowa. pp 115-127.

Zhu, J., *et al.* (2010). Differentiation of Effector CD4 Cell Populations. *Annu. Rev. Immunol.* **28**, 445-489.

EMILY F. RUBINSON

EDUCATION

Smith College, Northampton, MA, August 2005-May 2009, BA, Biochemistry.

Georgetown University, Washington, DC, June-August 2008, no degree awarded.

George Washington University, Washington, DC January 2011-May 2012, no degree awarded.

University of Kentucky, Lexington, KY, September 2012-present, M.S. to be awarded December 2014.

POSITIONS HELD

Graduate Research Assistant, Department of Microbiology, Immunobiology and Molecular Genetics/Department of Veterinary Science, University of Kentucky, Lexington, KY (July 2013-present)

Volunteer Laboratory Technician, Department of Chemistry, Georgetown University, Washington, DC (September 2010-September 2011)

CQL Program Participant, Department of Molecular Pathology, Walter Reed Army Institute of Research, Silver Spring, MD (Summers 2006 and 2007)

Volunteer Lab Technician, Department of Biochemistry, Georgetown University, Washington, DC (Summer 2004 and January 2006)

PUBLICATIONS

Nielsen, M.K., Vidyashankar, A.N., Bellaw, J., Gravatte, H.S., Cao, X., **Rubinson, E.F.**, Reinemeyer, C.R. (2014) "Serum Strongylus vulgaris-specific antibody responses to anthelmintic treatment in naturally infected horses." *Parasitol Res.* 2014 Oct 31. [Epub ahead of print].