Investigating the Efficacy of Fecal SIgA in High and Low Fecal Egg Shedding Horses

by

Gabrielle Bannister

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Degree in Pre-Veterinary Medicine with Distinction

May, 2024

Investigating the Efficacy of Fecal SIgA in High and Low Fecal Egg Shedding Horses

by

Gabrielle Bannister

Approved: ____

Dr. Amy Biddle, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: ____

Dr. Tara Gaab, V.M.D.
Committee member from the Department of Animal and Food Sciences

Approved: ____

Dr. Rolf Joerger, Ph.D.
Committee member from the Board of Senior Thesis Readers

Approved: ____

Dana Veron, Ph.D.
Chair of the University Committee on Student and Faculty Honors

ACKNOWLEDGMENTS

I want to start off by thanking Dr. Biddle for her never-ending support, advice, and insight. I have grown so much during this process and truly enjoyed the research process thanks to her mentorship. I also want to thank Kasey Hobert for helping me through this entire process and providing such helpful feedback.

Thank you to Dr. Gaab for agreeing to be a part of this process, I have enjoyed working with you and hearing your thoughts and input for this study. Thank you to Dr. Joerger as well, I appreciate the time spent meeting with me during our third reader meetings and hearing your advice and questions.

Jill, Gia, and Fran I want to thank you all for always being there and going through this process with me. I could not have imagined doing this without your support. I also want to thank the entire Biddle Lab for your help and support. Thank you to UD Envision for funding this project.

Thank you to all of the equine participants and their owners for letting me collect samples, there truly would not have been a study without you.

Finally, thank you to all of my friends and family for your continuous support during this process. I also want to thank my horse Tuesday. While she was not physically here during the end of my thesis process, I would not have gotten through undergrad without her and the piece of home and comfort she provided.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Parasite infection is an extremely prevalent topic with regards to equine health, especially when discussing cyathostomins. Cyathostomins are ubiquitous to horses and can pose a serious health threat. Cyathostomins are resistant to, or becoming resistant to, the few anthelmintic classes that are available for treating these persistent worms due to overuse of these medications. All horses are born with an innate natural immunity to cyathostomins that is existent throughout their life. The mechanism of how and why some horses are more immune to cyathostomins is not yet understood. Determining the mechanism behind horses' natural immunity could help in furthering an alternative treatment to cyathostomins other than anthelmintics. The objective of this study was to determine if there is a difference between the affinity of high and low fecal egg shedder fecal Secretory Immunoglobulin A for the surface of the cyathostomin larvae by imaging worms that were tagged using immunofluorescence. This study ultimately found no significant difference in the binding efficacy between high and low fecal egg shedder SIgA; however, the preliminary study did find fecal SIgA to be significant in regards to cyathostomin infection.

Chapter 1

INTRODUCTION

1.1 Cyathostomins

1.1.1 Overview

Cyathostomins, or small strongyles, are parasitic worms that are extremely infectious and omnipresent in horses [1]. They are most commonly less than 2.5cm in length, and are reddish in color [1]. Cyathostomins are one of the most prevalent parasites of concern found in horses.

1.1.2 Life Cycle

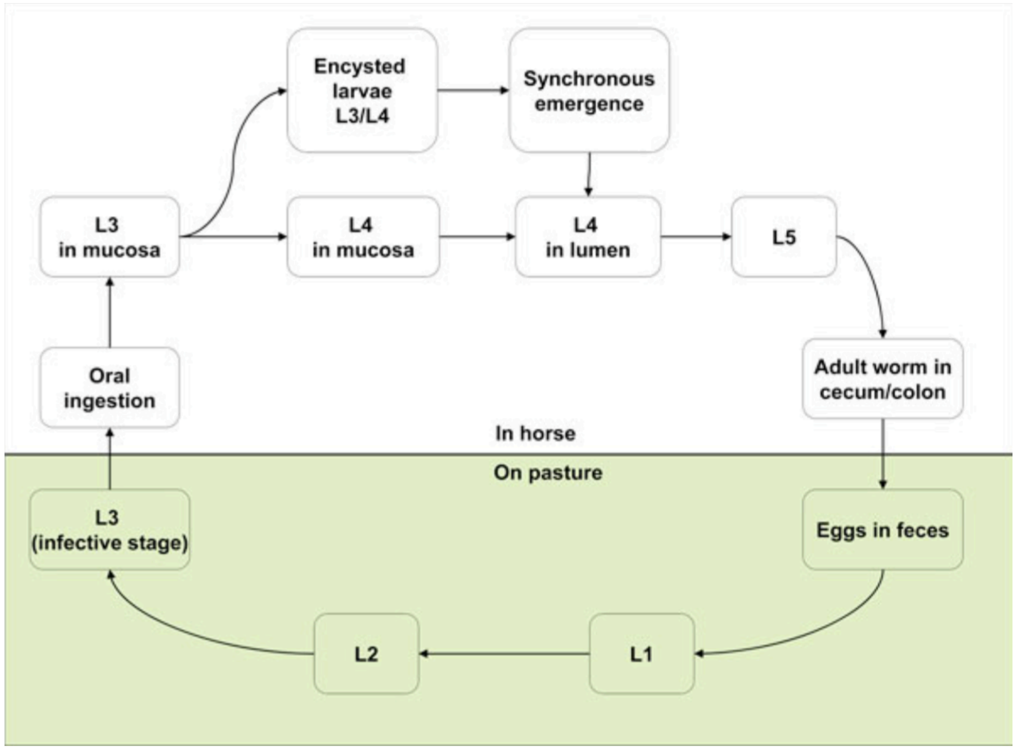

Figure 1. Diagram of Cyathostomin Life Cycle from S. Corning (2009).

Cyathostomins have a direct life cycle, meaning the parasite is transmitted directly to the host without an intermediate host or vector of another species [1]. After entrance into the horse, new eggs can be shed in just five to six weeks [1]. Cyathostomins are transmitted to horses from the pasture through grazing. Cyathostomins enter the horse's body system in the larval (L) 3 stage, develop into adult worms, and produce new eggs that are passed through the feces and onto pasture. The rate of development from the first larval stage, L1, to the L3 stage is directly related to temperature [2]. In warm, or approximately 86° weather, cyathostomin eggs can hatch and yield L3 in just three days [1]. Cyathostomins are infectious once they enter the L3 stage where they gain a protective membrane [1]. This allows them to survive the horse's gastrointestinal tract. This membrane also allows them to withstand freezing conditions, meaning they can survive on pasture for up to a year [3]. If eggs at the L1 or L2 stage are ingested, they are destroyed by the horse due to the lack of a protective membrane.

Once ingested, the L3 larvae develop into their L4 stage. These worms will either live as L4 in the mucosa, or become encysted. Encysted worms embed themselves into the mucosa of the intestinal wall and live there from four months up to two years [1]. At once, encysted worms will emerge and exit the horse. Temperature does play a role in when cyathostomins embed and emerge. In temperate climates, it is seen most often during the cooler months with emergence happening when the weather warms. In tropical climates it is the opposite. Cyathostomins tend to embed during the hot summer months, with emergence in the cooler fall [1]. However, it is not known why emergence occurs at specific times, as the worms can be embedded in the horse for years at a time before simultaneous emergence transpires.

1.2 Anthelmintic Resistance

Due to anthelmintic resistance, cyathostomins have been a major area of concern. Fifty years ago, the most important parasite of horses was *Strongylus* *vulgaris,* a large strongyle bloodworm [4]. A treatment plan using the anthelmintic class of benzimidazoles was set in place to specifically eliminate *S. vulgaris* [4]. The treatment was designed to kill *S. vulgaris* worms before they could mature and lay eggs. Treatment every two months ensured that the worms were destroyed [5]. This method was very successful in controlling *S. vulgaris*; however, it has since led to the treatment of horses who do not need to be treated which has allowed for resistant cyathostomins to survive and increase in frequency, driving resistance in the world of equine anthelmintics [4].

Three anthelmintic drug classes exist for the treatment of nematode parasites in horses, these are the benzimidazoles, the pyrimidines, and the macrocyclic lactones [5]. Macrocyclic lactones are the newest class, and were launched in the early 1980s [5]. Since then, no new anthelmintic classes have been introduced. Currently, cyathostomins are resistant to pyrimidines and benzimidazoles, with reports of resistance to macrocyclic lactones in recent years [6].

1.3 Cyathostomin Danger to Horse Health

Although these drugs have been helpful in the control of one parasite, their usage has had negative impacts on other parasites. Since there are only three drug classes, with no new ones being introduced, anthelmintic resistance in cyathostomins is a real threat. Without treatment, cyathostomins jeopardize horses' health. As with other nematodes, large numbers of adult cyathostomin worms can cause clinical symptoms such as lethargy, sudden weight loss, debilitation, and diarrhea [1]. However, larval cyathostomins pose an even greater risk. When the L3 larval stage embeds in the horse's intestinal mucosa, it can cause serious damage and reduce nutritional metabolism [7]. Substantial damage occurs from the L4 stage larvae when they emerge from the cysts. All embedded larvae may emerge at once in a condition called larval cyathostominosis This has serious health implications for the horse [7]. Not only can it damage the gut wall, but there is also potential for severe colic with a 50% mortality rate [1].

1.4 Equine Fecal Egg Shedding

Classification Based on Egg Shedding	**Egg Count (per gram of feces)**	**% of Horse Population**
Low	0-200	50%-70%
Moderate	200-500	5%-15%
High	>500	10%-30%

Table 1. Fecal egg shedding classification adapted from Kaplan and Nielson 2010**.**

All horses have a natural immunity to cyathostomins, which can be quantified through Fecal Egg Counts (FEC). As shown in Table 1, FEC's determine the amount of cyathostomin eggs per gram of feces (EPG) and can then be used to divide horses into three categories: low shedder, moderate shedder, and high shedder. Low egg shedding horses emit less than 200 eggs per gram, moderate egg shedding horses have EPGs between 200 and 500, and high egg shedding horses emit more than 500 eggs per gram [8]. Low egg shedding horses account for 50-70% of the herd, moderate egg shedding horses for 10-20%, and high egg shedding horses for 15-30% [8]. The physiology that makes one horse more immune than another is not yet understood but is a persistent trait throughout the horse's lifetime regardless of exposure.

1.5 Secretory Immunoglobulin A

While we do not understand the physiology behind parasite immunity, Secretory Immunoglobulin A (SIgA), is implicated in the differences in shedding status. SIgA is a naturally occurring mucosal antibody secreted by epithelial cells in the digestive system of mammals. SIgA is a form of Immunoglobulin A (IgA) that is bound and transported by the polymeric Ig receptor (pIgR) [9]. The pIgR binds to IgA which forms polymeric IgA (pIgA). PIgA is then joined by a polypeptide J chain [9]. Once fully bound the extracellular portion of pIgA is cleaved to form the secretory component (SC) [9]. The SC remains bound to the pIgA and provides SIgA with increased resistance to bacterial proteases [9]. SIgA acts as the first line of defense against pathogens by blocking pathogens' access to epithelial receptors, trapping them in mucus, and facilitating their removal [10]. SIgA has been used as a biomarker for gastrointestinal nematodes in sheep, but this relationship has not yet been established in horses. A study performed by researchers at the Hopkirk Research

Institute in New Zealand found that in sheep, intestinal mucosal anti-CarLA IgA levels in saliva were correlated with the immune response due to the parasite *T. colubriformis* [11].

A preliminary study was conducted in the summer of 2023 by Gabrielle Bannister and Gianna Metzger in collaboration with the Biddle Lab. The goal of this study was to identify any correlation between fecal and salivary SIgA and cyathostomin infection. This study used a total of 82 horse fecal samples to perform FEC's using the McMaster Method. The McMaster method uses flotation to separate the eggs from other debris in the sample based on density. Since the eggs are lighter, they float to the top of the counting chamber and can be quantified. Once shedding status for a sample was determined, the subject was either accepted as a participant or not on the need for their respective shedding status and cohabitation status with another participant. Equal groups of low, moderate, and high fecal egg shedding horses were selected for SIgA profiling. If accepted a saliva sample was collected. Enzyme Linked ImmunoSorbent Assay (ELISA) tests were then performed for both the salivary and fecal samples. ELISA testing measures the concentration of a specific antigen or antibody, in this case concentration of SIgA was measured. The results of the study showed that higher fecal SIgA concentrations are associated with lower egg shedders, but were not shown to have lower levels of salivary SIgA. This means that the immune response due to cyathostomin infection occurs later in the gastrointestinal tract of the horse. The findings of this preliminary study led to the question of if there are different forms of SIgA that are more or less efficient in regards to cyathostomin resistance. While different isoforms of horses have not yet been demonstrated, some human SIgA isoforms are known. There are two predominant forms of SIgA in humans, SIgA1 and SIgA2. They are formed by binding to either IgA1 or IgA2. IgA1 is responsible for immune homeostasis while IgA2 is responsible for inflammatory effects [12].

1.6 Objective

The objective of this study was to determine if there is a difference between the affinity for high and low shedder fecal SIgA on the surface of the cyathostomin larvae by imaging worms that were tagged using immunofluorescence.

Chapter 2

MATERIALS AND METHODS

2.1 Animals

Fecal samples were collected during January 2023 to August 2023 from 82 horses of various breeds, ages, and farms near the University of Delaware. Horses were selected based on prior FEC's, location, and number of horses living in the same area. All procedures were approved by the University of Delaware Agricultural Animal Care and Use Committee #129.

2.2 Preliminary Fecal Sample Analysis

2.2.1 Fecal Egg Counts

In the summer of 2023 FEC's were conducted on all fecal samples using the McMaster Method and the Paracount-EPG kit (Chalex, Wallowa, OR). First, 26ml of Fecasol (Vetoquinol, Fort Worth, TX) was dispensed into the calibrated mixing vial, then feces was added until filled to 30ml. The mixture was stirred until homogenous. Using a 1cc syringe, the liquid portion of the mixture was used to fill both chambers of the McMaster Counting Slide. The slide was allowed to rest for five minutes to allow the cyathostomin eggs to rise to the surface and any debris to sink. The slide was placed under a microscope and cyathostomin eggs were counted. The number of eggs counted was multiplied by 25 to get an accurate eggs per gram of feces. Based on the number of eggs per gram present in the sample, the respective horse was classified as a high, moderate, or low shedder.

After classification of shedding status, the fecal sample was either rejected or accepted as a study sample. The criteria for selection was on an as needed basis and based on horses in cohabitating groups. A total of 30 fecal samples were needed, 10 of each shedding classification. Once a shedding status was filled, no other samples were needed for that category. Out of the 82 samples collected, 32 were selected to proceed to ELISA testing. Ultimately only high and low fecal egg shedding horses became part of the study, this was due to there not being a large enough population of moderate fecal egg shedders to properly analyze.

2.2.2 SIgA Extraction

SIgA was extracted from each sample as follows: 1g of feces per sample was mixed with 5 ml of 1X Phosphate Buffered Solution (PBS), pH 7.5. All samples were shaken for 3 minutes and rested for 15 minutes. This shake and rest process was repeated twice. After both rounds of shaking and resting were completed, the samples were centrifuged at 16000 g for 20 minutes. 1mL of supernatant was then collected from each sample with a 0.2µl syringe filter. 10µl of a protease inhibitor cocktail was added to each sample and centrifuged at 3260 g for 15 minutes. 600µl of supernatant was collected and frozen until further analysis.

2.2.3 ELISA Testing

SIgA concentration was measured using the MyBioSource Horse Secretory Immunoglobulin A Enzyme Linked Immunosorbent Assay (ELISA) kit (MyBioSource, San Diego, CA). Thawed samples were first vortexed and centrifuged at 12000 g for 5 minutes. Using a 96 well plate, 50µl of each sample along with the six standards provided were placed into separate wells. Empty wells were left to be used as blank wells. 100µl of Horseradish Peroxidase (HRP)-conjugate was added to each sample and standard wells. The plate was covered with the provided closure plate membrane and incubated at 37°C. All liquid was dumped into a liquid waste container and the well plate was washed. The provided wash solution was added to all wells, including blanks, left for a minute, then dumped. This process was repeated four times. After the wash process was completed, 50µl of chromogen A, used as a substrate along with Chromagen B for the HRP-conjugate, was added to every well including blank wells. 50 µl of chromogen B was added to all wells including blank wells. The plate was immediately covered in tinfoil and incubated for 15 minutes at 37°C. After incubation, 50 µl of stop solution was added to each well. The plate was read using an ELISA reader set at 450 nm. The standard concentration values were used to extrapolate the SIgA concentration of each sample. The standard concentrations ranged from 0-100µg/mL. All sample concentrations were within this range.

2.3 Egg Concentration for Larval Culture

A fecal sample from a known high egg shedding horse was collected. A FEC was performed using the McMaster method to confirm. 25g of the sample was added to 40 mL of Fecasol and mixed until homogenous. The mixture was pressed through a mesh sieve, with the liquid being collected in a 250mL beaker. The solids in the mesh sieve were placed back into the original container and allowed to sit for 5 minutes. The process of mixing with Fecasol and straining was then repeated another two times. The liquid collected was centrifuged at 1000 rpm for 10 minutes. Once completed, the supernatant was removed and filtered through a 40µm filter. The filtrate was recovered by washing the filter with 25 mL of water. The recovered filtrate was centrifuged at 1200 rpm for 15 minutes. The supernatant was removed and discarded. The leftover solids were resuspended in PBS to a total volume of 5 mL and gently vortexed. This mixture was stored at 40°F until further use.

2.4 SIgA Extraction for Larval Culture

Prior to the beginning of the egg culture, SIgA was extracted in the same manner as stated in the previous section. SIgA was extracted from two samples, a high fecal egg shedding sample, and a low fecal egg shedding sample. These samples were run through the same MyBioSource Horse Secretory Immunoglobulin A ELISA kit, the protocol as stated in section 2.2.3.

2.5 Larval Culture

To start the larval culture, the previously concentrated eggs were placed into six tubes on day 0. Each tube was given 550µl of concentrated eggs. The eggs were then placed in the dark at room temperature for 2 days. On day 2, either 200µl of IgA or 200µl of PBS was added. The amount of SIgA added to each tube was determined through concentration given by the ELISA test mentioned in section 2.2.3. The high shedder SIgA concentration was 12.75µg/mL and the low shedder concentration 12.8µg/mL. Since the concentrations were nearly identical, no dilution was needed. Three tubes were given the SIgA extracted from the high fecal egg shedding sample, three tubes were given the SIgA extracted from the low fecal egg shedding sample, and three samples were given PBS. On day 3, all samples were centrifuged for 90s at 400 g. The supernatant was removed and 1mL of 100% isopropanol was added to fix the samples. All samples were vortexed and allowed to fix for 10 minutes. The samples were then left in the refrigerator until needed.

2.6 Immunofluorescence Microscopy

A Horse IgA antibody Fluorescein isothiocyanate (FITC) conjugate (Bethyl Laboratories, Montgomery, TX) was used to tag the cyathostomin worms in order for them to be viewed fluorescently as follows: 20µl of larvae from the previously cultured cyathostomin eggs were placed on a microscope slide. This was repeated a second time on the same slide. The two pools of liquid were allowed to dry before

40µl of FITC was added to each pool of liquid. A microscope slide cover was placed on top and the slide was incubated at room temperature in the dark for one hour. The same procedure was repeated for control slides of just the high and low fecal egg shedder SIgA that was added to the cultures. After the incubation period, the microscope slides were viewed under the microscope at 20x with and without a 49002 Et-EGFP (FITC/Cy2) filter (Bellows Falls, VT). Eighty-two photographs were taken of the larvae and areas of interest on the slides; 41 of these images were taken without the FITC filter, the other 41 images are the same images but with the FITC filter. Each photograph pictures between 1-3 larva. These pictures were uploaded to ImageJ.

2.7 ImageJ

ImageJ was used to calculate the fluorescence of photographed worms. The values ImageJ calculated were the area, mean fluorescence, and the integrated density. First selected photographs were uploaded into ImageJ. Once a picture was uploaded, all worms pictured were photographed. The freehand selections tool was used to outline the worms, then the measurements were taken. This process was repeated for all photographs and all worms in the photographs. This process was also used for the control SIgA slides except measurements were only taken for the background of the slide. Once all measurements were collected, the data were transferred to Google Sheets. Using the data collected from ImageJ, the average fluorescence for each worm and its background was measured. For the worm, the fluorescent mean gray value was subtracted from the non-fluorescent mean gray value. For the background, the average of the fluorescent mean gray value was subtracted from the average of the nonfluorescent mean gray value.

2.8 Statistical Analysis

Difference in fluorescence using the FITC conjugate was analyzed in Excel using a pairwise t-test. Calculations, with significance at p-value <0.05, were performed for control culture versus high egg shedding culture, control culture versus low egg shedding culture, and low egg shedding culture versus high egg shedding culture. Calculations were also performed with significance at p-value <0.05 for the control high fecal egg shedder SIgA versus the control low fecal egg shedder SIgA.

Chapter 3

RESULTS

3.1 Animals

The animal samples used for this study are shown in Table 2. In total 27 samples were used with 15 being low fecal egg shedding horses and 12 being high fecal egg shedding horses. The horses used are all located at four different private farms near the University of Delaware, UD's Webb Farm, and Winbak Farm.

Low Fecal Egg Shedder			**High Fecal Egg Shedder**		
Name	**Location**	**FEC Average (EPG)**	**Name**	**Location**	**FEC Average (EPG)**
Darwin	UD Webb Farm	0	244 Real Drama	Winbak Farm	575
Caleb	Private Farm #1 Elkton, MD	0	125 Sniper Hanover	Winbak Farm	825

Austin	Private Farm #3 Elkton, MD	0	142 Misty Kristy	Winbak Farm	1000
Profit	Private Farm #3 Elkton, MD	0	Rayne	Private Farm #2 Elkton, MD	1050
Regal	Private Farm #2 Elkton, MD	0	R68 Faith Healer	Winbak Farm	1100
Claire	Private Farm #4 Louisville, PA	0	R51 Finely Tune	Winbak Farm	1150
R136 Best Diva	Winbak Farm	0	PJ	Private Farm #4 Louisville, PA	1287.5
234 Not Enough	Winbak Farm	0	Maggie	UD Webb Farm	1575
Magic	UD Webb Farm	12.5	Tallulah	UD Webb Farm	1837.5

380 Sea Gypsy	Winbak Farm	25	R97 Buy One Get One	Winbak Farm	2125
952 Bankers Audit	Winbak Farm	50	507 Turn the Tab	Winbak Farm	2525
Hank	Private Farm #4 Louisville, PA	62.5	R85 Art Chic	Winbak Farm	3475
27 Lady Lynnly	Winbak Farm	100			
Nimbus	UD Webb Farm	112.5			
Pec	Private Farm #4 Louisville, PA	212.5			

Table 2. Animal samples used, their location, and their resulting FEC

3.2 Preliminary Fecal Sample Analysis

Preliminary fecal egg counts for all eighty-two samples collected can be found in Appendix A. Table 2 in the above section shows all the samples that were selected to continue on to ELISA testing. ELISA results for both fecal and salivary SIgA from the preliminary study were analyzed using a Pearson correlation test with rho significance at 0.3, the salivary SIgA was statistically insignificant with a rho value of -0.165. The fecal SIgA however was statistically significant with a rho value of -0.397. Since the fecal SIgA's r value was significantly different from 0 we concluded that it was significant whereas the salivary SIgA's r value was not significantly different from 0. ELISA results for Fecal SIgA for all selected samples. The average low fecal egg shedder SIgA concentration in µg/mL was 38.59065726, the average high fecal egg shedder SIgA concentration in µg/mL was 28.55129756.

A scatterplot of the samples selected to move onto ELISA testing and their resulting FEC and fecal SIgA can be seen below in Figure 2. The line of best fit shows the negative relationship between FEC and fecal SIgA.

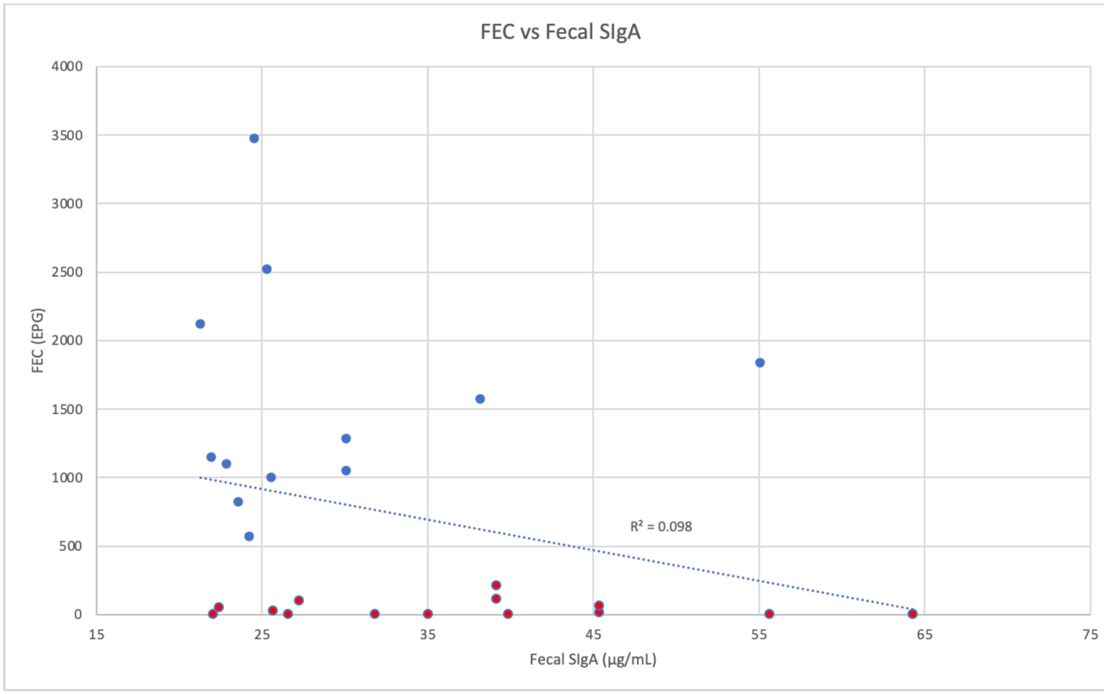

Figure 2. Scatter plot of animal samples used and their resulting FEC and fecal SIgA. Red points indicate low fecal egg shedders and blue points indicate high fecal egg shedders.

3.3 SIgA Extraction for Larval Culture

The ELISA results for the two low and high fecal egg shedding samples used in the larval culture is shown below in Table 3. The samples used for SIgA extraction were Caleb as the low shedder and Caitlyn as the high shedder. Both SIgA concentrations were nearly identical with the low shedder SIgA being 12.84µg/mL and the high fecal egg shedder SIgA being 12.75µg/mL.

Low Fecal Egg Shedding SIgA (µg/mL)	High Fecal Egg Shedding SIgA (µg/mL)
12.84	12.75

Table 3. ELISA SIgA concentration results for low and high fecal egg shedding samples.

3.4 Immunofluorescence of Cyathostomin Larvae

Eighty-two photographs of worms were taken using ImageJ, 41 without using the fluorescent filter, the other 41 being the same image but with the fluorescent filter. Seventeen images were taken of the control larval culture, 21 images were taken of the high shedder SIgA culture, 25 images were taken of the low shedder SIgA culture. The six images below (Figure 3,4,5) are examples of each of the different cultures both with and without the fluorescence filter. The letter in the image name corresponds to which culture it is from, C for control, H for high, and L for low.

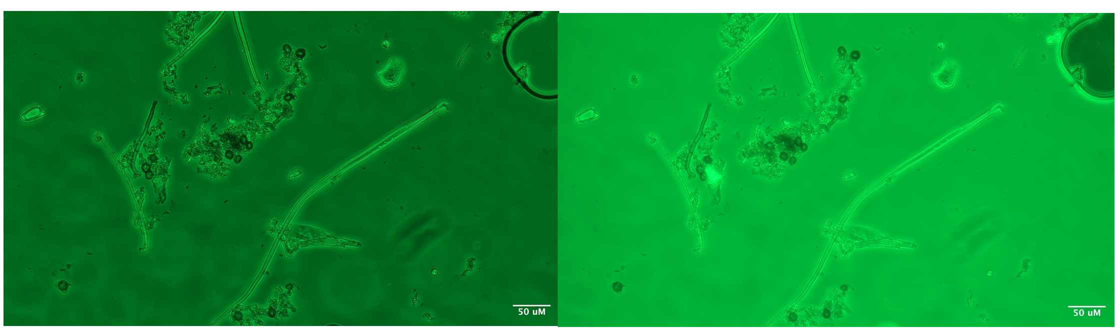

Figure 3. Image C2 without (left) and with (right) fluorescent filters.

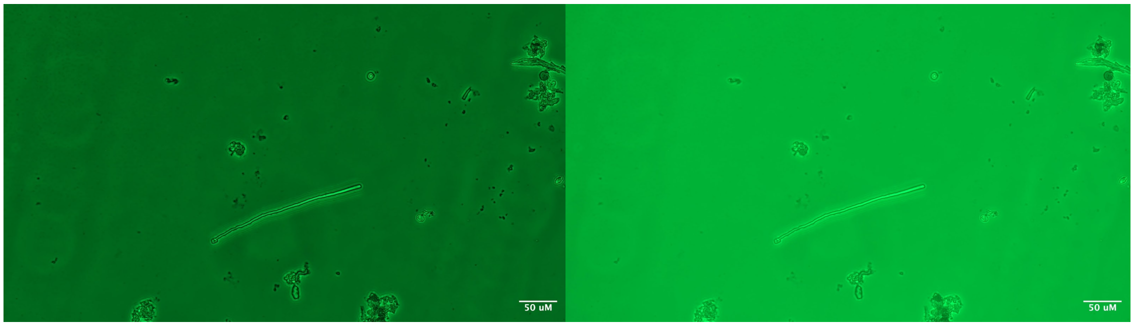

Figure 4. Image H3 without (left) and with (right) fluorescent filters.

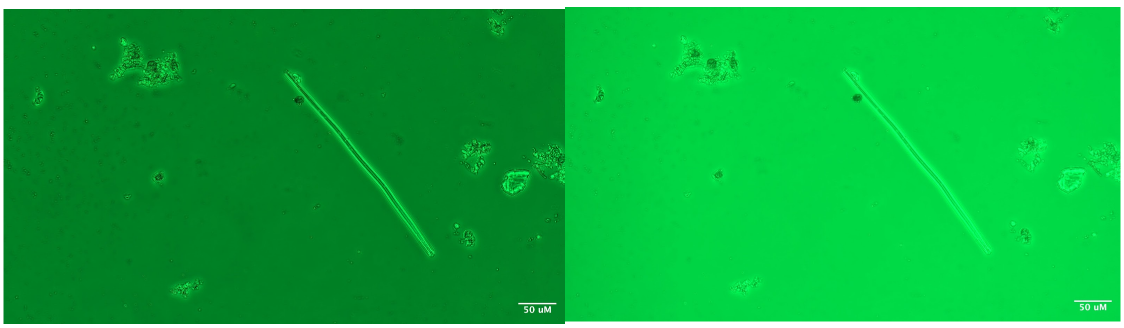

Figure 5. Image L10 without (left) and with (right) fluorescent filters.

The mean gray value results from ImageJ were used to find the average mean gray value of each worm. These values were averaged to find the average mean gray value of each culture. Those values are shown in Figure 6.

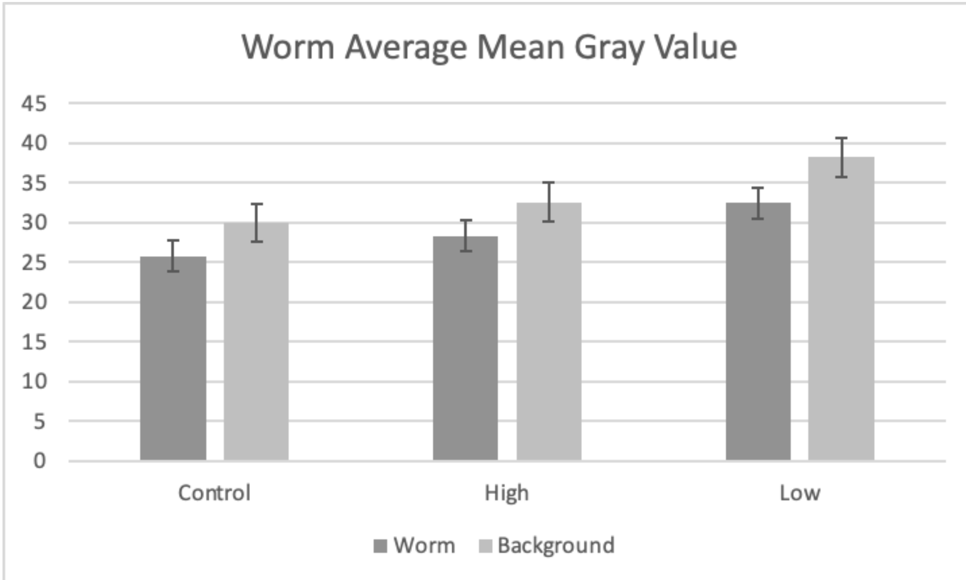

Figure 6. Worm average mean gray value for all three cultures.

3.5 Immunofluorescence of SIgA

An additional 16 images were taken of control high and low shedder SigA. The mean gray value results from ImageJ were used to find the average mean gray value of both the high and low shedder SigA. These values were averaged to find the average mean gray value of both types of SigA. These values are shown in Figure 7.

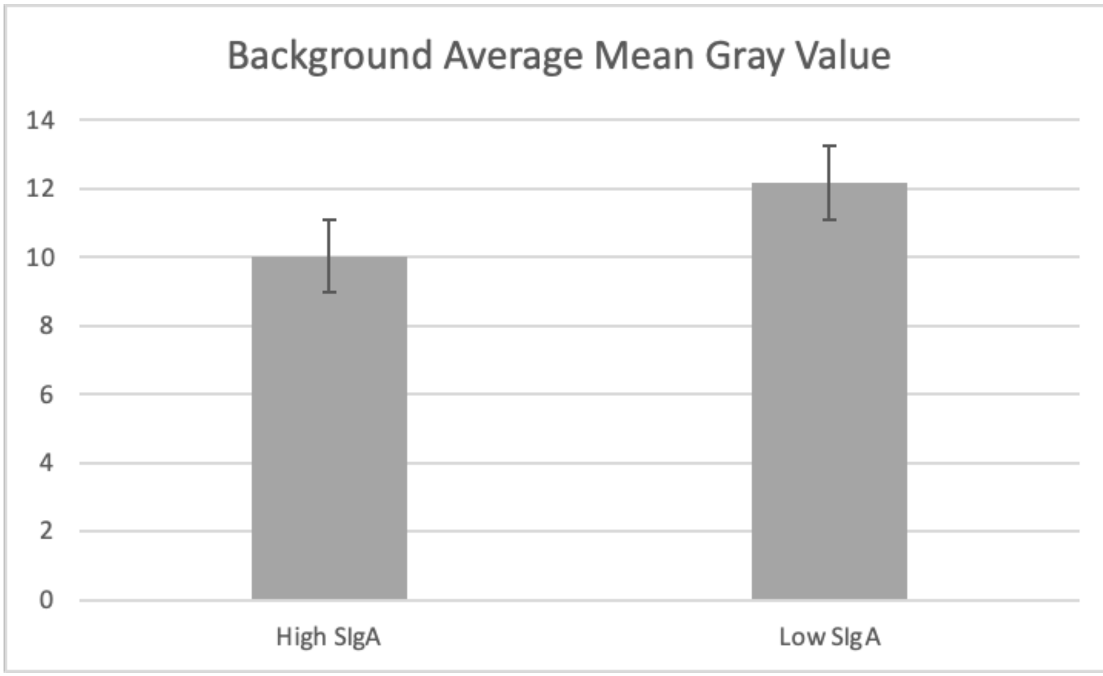

Figure 7. Control high and low shedder SigA average mean gray value.

3.6 Statistical Analysis

T-test p-value results from the control, high shedder, and low shedder larval cultures are shown below in Table 4.

	Control v High	**Control v Low**	**High v Low**
Worm	0.0513	0.0103	0.1025
Background	0.1615	0.2772	0.9559

Table 4. Pairwise t-test results of culture averages.

T-test p-value results from the control high and low fecal egg shedder SigA are shown below in Table 5.

	High vs Low SigA
Background	0.4212

Table 5. Pairwise t-test results of control high and low fecal egg shedder SigA.

Chapter 4

DISCUSSION

The mechanism of natural resistance is a topic of interest because cyathostomins can be incredibly harmful to horses and are resistant to, or on the verge of resistance, to the few anthelmintic classes. Previous studies in sheep have found that SigA has an effect on the level of gastrointestinal nematodes in sheep [11]. The preliminary 2023 summer study found fecal SigA amounts to be statistically significant with respect to cyathostomin infection. This led to the question of why some horses display a greater immunity towards cyathostomins and thus if SIgA binding efficacy plays a role.

This study ultimately found that there was not a statistically significant difference between the high fecal egg shedding samples and the low fecal egg shedding samples with respect to fecal SIgA binding efficacy. This means that the high shedder SIgA and the low shedder SIgA did not bind to the cyathostomin larvae differently, therefore they both have similar binding efficacy. Consequently, this is not a reason why fecal SIgA impacts high and low fecal egg shedders differently.

While the high versus low fecal egg shedding samples were not statically significant to each other, there was a significant relationship between the control sample worms and the low fecal egg shedding sample worms. We can thus conclude that the SIgA is binding to the cyathostomin larvae, just not binding differently between the different shedding classifications. The relationship between the control sample worms and the high fecal egg shedding sample worms was almost significant with a p-value of 0.051. Perhaps if we had more data collected this p-value would change and we would see it being statistically significant like the low fecal egg shedding sample.

A pairwise t-test was also run against the background values, the p-value for the high fecal egg shedding background versus the low fecal egg shedding background was 0.96. This value makes sense as the background between the two samples should be almost identical since they were both in a culture of the same materials and were fixed with identical protocols. The control sample background and the low fecal egg shedding sample background as well as the control sample background and the high fecal egg shedding sample background were also statistically insignificant.

The low R^2 value seen in Figure 2 is supported by the negatively correlated rho values determined by the Pearson correlation test in the preliminary study. Since the Pearson correlation test indicated low significance between fecal SIgA and level cyathostomin infection, we expect a lower R^2 value. While these numbers are still significant, their low values could be due to other immune stimulating factors that influence SIgA beyond cyathostomin infection.

It was also found that the control high versus low fecal egg shedder SIgA were statistically insignificant with respect to each other. This control shows that when not bound to cyathostomin worms, high and low fecal egg shedder SIgA is similar with respect to fluorescence.

While different isoforms of SIgA in horses have not yet been demonstrated, it has been in some human cases. There are two predominant forms of SIgA in humans, SIgA1 and SIgA2. They are formed by binding to either IgA1 or IgA2 which have functions related to immune homeostasis and inflammatory effects [13].

Limitations of this study include autofluorescence and species of cyathostomins that may have different affinities for SIgA . When viewed under the FITC fluorescent filter, there was substantial autofluorescence, this was most likely due plant material leftover in the samples and thus being added into the culture. For future studies, this could be minimized by autofluorescence quenching using a chemical such as Sudan Black [14]. Other questions that this study leads to include whether or not SIgA affects the various species of cyathostomins differently, if other parasites that horses may harbor lead to differences in SIgA, and if there is a difference in SIgA concentration due to age.

Chapter 5

CONCLUSION

Horses have an innate immunity to cyathostomins that can be quantified as being a high, moderate, or low shedder. While we know that this immunity is a persistent trait throughout a horse's life, the mechanism of how and why is still not yet understood. Understanding the mechanism of natural immunity to parasites may provide clues for more effective ways to manage parasites like cyathostomins. This is especially important nowadays since the worms are becoming resistant to anthelmintics. This study did not find statistically significant differences in the binding affinity of SIgA from high fecal egg shedders and low fecal egg shedders; however, we do know that fecal SIgA is significant in regards to cyathostomin infection level. Future studies should look at other ways in which SIgA could differ from high to low fecal egg shedding horses such as if there is a difference in where SIgA is being produced in the gastrointestinal tract and if horses having parasites other than cyathostomins plays a significant role in SIgA production.

REFERENCES

[1] S. Corning, “Equine cyathostomins: a review of biology, clinical significance and therapy,” *Parasit. Vectors*, vol. 2, no. 2, p. S1, Sep. 2009, doi: 10.1186/1756-3305-2-S2-S1.

[2] “Effect of temperature on the development of the free-living stages of horse cyathostomins - ScienceDirect.” Accessed: Apr. 14, 2024. [Online]. Available: https://www.sciencedirect.com/science/article/abs/pii/S240593902200003X

[3] C. R. Reinemeyer, “Controlling Strongyle Parasites of Horses: A Mandate for Change” AAEP Proceedings 2009 vol. 55, p. 352-360.

[4] R. M. Kaplan and M. K. Nielsen, “An evidence-based approach to equine parasite control: It ain’t the 60s anymore,” *Equine Vet. Educ.*, vol. 22, no. 6, pp. 306–316, 2010, doi: 10.1111/j.2042-3292.2010.00084.x.

[5] M. Nielsen *et al.*, “AAEP Parasite Control Guidelines.” AAEP Infectious Disease Committee. [Online]. Available: https://aaep.org/sites/default/files/Guidelines/AAEPParasiteControlGuidelines_0.pdf

[6] M. K. Nielsen, “Anthelmintic resistance in equine nematodes: Current status and emerging trends,” *Int. J. Parasitol. Drugs Drug Resist.*, vol. 20, pp. 76–88, Oct. 2022, doi: 10.1016/j.ijpddr.2022.10.005.

[7] A. S. Peregrine, B. McEwen, D. Bienzle, T. G. Koch, and J. S. Weese, “Larval cyathostominosis in horses in Ontario: An emerging disease?,” *Can. Vet. J.*, vol. 47, no. 1, pp. 80–82, Jan. 2006.

[8] M. Lejeune *et al.*, “Evaluation of Fecal Egg Count Tests for Effective Control of Equine Intestinal Strongyles,” *Pathogens*, vol. 12, no. 11, p. 1283, Oct. 2023, doi: 10.3390/pathogens12111283.

[9] M. J. Lewis, B. Wagner, R. M. Irvine, and J. M. Woof, “IgA in the horse: cloning of equine polymeric Ig receptor and J chain and characterization of recombinant forms of equine IgA,” *Mucosal Immunol.*, vol. 3, no. 6, pp. 610–621, Nov. 2010, doi: 10.1038/mi.2010.38.

[10] N. J. Mantis, N. Rol, and B. Corthésy, “Secretory IgA’s Complex Roles in Immunity and Mucosal Homeostasis in the Gut,” *Mucosal Immunol.*, vol. 4, no. 6, pp. 603–611, Nov. 2011, doi: 10.1038/mi.2011.41.

[11] R. J. Shaw, C. A. Morris, M. Wheeler, M. Tate, and I. A. Sutherland, “Salivary IgA: A suitable measure of immunity to gastrointestinal nematodes in sheep,” *Vet. Parasitol.*, vol. 186, no. 1, pp. 109–117, May 2012, doi: 10.1016/j.vetpar.2011.11.051.

[12] U. Steffen *et al.*, “IgA subclasses have different effector functions associated with distinct glycosylation profiles,” *Nat. Commun.*, vol. 11, no. 1, p. 120, Jan. 2020, doi: 10.1038/s41467-019-13992-8.

[13] A. Bonner, A. Almogren, P. B. Furtado, M. A. Kerr, and S. J. Perkins, “The Nonplanar Secretory IgA2 and Near Planar Secretory IgA1 Solution Structures Rationalize Their Different Mucosal Immune Responses,” *J. Biol. Chem.*, vol. 284, no. 8, pp. 5077–5087, Feb. 2009, doi: 10.1074/jbc.M807529200.
[14] O. N. Shilova, E. S. Shilov, and S. M. Deyev, “The effect of trypan blue treatment on autofluorescence of fixed cells,” *Cytometry A*, vol. 91, no. 9, pp. 917–925, 2017, doi: 10.1002/cyto.a.23199.

Appendix A

COMPLETE LIST OF EQUINE PARTICIPANTS

Shedding Status	Name	Location	Breed	Age	Average FEC
Low	Caleb	Private Farm #1 Elkton, MD	Saddlebred	24	0
	Regal	Private Farm #2 Elkton, MD	Thoroughbred	27	0
	Bitsy	Private Farm #2 Elkton, MD	Connemara x Thoroughbred	22	0
	River	Private Farm #2 Elkton, MD	Morgan	15	0
	Claire	Private Farm #4 Louisville, PA	Irish Sport Horse	7	0
	Magic	Private Farm #4 Louisville, PA	Holsteiner	20	0
	Tuesday	Private Farm #5 Nottingham, PA	Paint	21	0
	Magic	UD Webb Farm	Quarter Horse	14	0
	Darwin	UD Webb Farm	Thoroughbred	14	0
	Diva	Private Farm #4 Louisville, PA	Thoroughbred	7	0
	Profit	Private Farm #3 Elkton, MD	Standardbred	-	0

Low	Poncho	C-Line Stables	Paint	14	0
	Topsy	C-Line Stables	Thoroughbred	16	0
	25 Bobbi Jo's Ex	Winbak Farm	Standardbred	15	0
	234 Not Enough	Winbak Farm	Standardbred	17	0
	355 Michelle's Ideal	Winbak Farm	Standardbred	11	0
	366 Kimberly's Baby	Winbak Farm	Standardbred	10	0
	395 Marantha Hanover	Winbak Farm	Standardbred	16	0
	438 Princess Christine	Winbak Farm	Standardbred	8	0
	453 Marietta Hall	Winbak Farm	Standardbred	19	0
	465 Purrfect Bags	Winbak Farm	Standardbred	11	0
	495 Commando Queen	Winbak Farm	Standardbred	18	0
	522 Drag n Sand	Winbak Farm	Standardbred	10	0
	525 Ideal Talker	Winbak Farm	Standardbred	8	0
	585 Kims the Best	Winbak Farm	Standardbred	8	0
	829 Winbak Bet	Winbak Farm	Standardbred	14	0
	892 End to End Hanover	Winbak Farm	Standardbred	10	0

Low	909 Michiko	Winbak Farm	Standardbred	27	0
	910 Lady of Lust	Winbak Farm	Standardbred	23	0
	911 Sas Newton	Winbak Farm	Standardbred	29	0
	914 Bit of Blarney	Winbak Farm	Standardbred	27	0
	930 Sierra Fortune	Winbak Farm	Standardbred	24	0
	974 Tug River Della	Winbak Farm	Standardbred	25	0
	R25 Free Swag	Winbak Farm	Standardbred	14	0
	R84 By the Zip	Winbak Farm	Standardbred	14	0
	R136 Best Diva	Winbak Farm	Standardbred	4	0
	209 O Narutac Kaiulani	Winbak Farm	Standardbred	11	25
	R96 Bag of Jewels	Winbak Farm	Standardbred	6	25
	380 Sea Gypsy	Winbak Farm	Standardbred	14	25
	388 Rock N Roll Wishes	Winbak Farm	Standardbred	14	25
	275 Travelin Supergirl	Winbak Farm	Standardbred	18	50
	952 Bankers Audit	Winbak Farm	Standardbred	25	50
	104 Guard My Heart	Winbak Farm	Standardbred	6	50

Low	Rumble	C-Line Stables	Thoroughbred	26	50
	Hank	Private Farm #4 Louisville, PA	Quarter Horse	23	87.5
	Nimbus	UD Webb Farm	Thoroughbred	6	100
	27 Lady Lynnly	Winbak Farm	Standardbred	9	100
	498 Starter Up	Winbak Farm	Standardbred	11	125
	961 Musical Memory	Winbak Farm	Standardbred	32	125
	983 Cool Flying Filly	Winbak Farm	Standardbred	31	125
	Vinny	Private Farm #4 Louisville, PA	Thoroughbred	9	125
	442 Break the Deal	Winbak Farm	Standardbred	-	150
	PJ	Private Farm #4 Louisville, PA	Connemara/Warmblood	5	175
	R34 Empowerment	Winbak Farm	Standardbred	4	175
Moderate	Caitlyn	Private Farm #1 Elkton, MD	Standardbred	21	233.33
	214 Lady with Credit	Winbak Farm	Standardbred	-	250
	Frankie	Private Farm #4 Louisville, PA	Irish Sport Horse	7	300
	Slick	UD Webb Farm	Standardbred	25	325

	372 Young Michelle	Winbak Farm	Standardbred	-	375
	R98 Safe n Free	Winbak Farm	Standardbred	-	425
High	244 Real Drama	Winbak Farm	Standardbred	12	575
	Pec	Private Farm #4 Louisville, PA	Thoroughbred	19	625
	Tallulah	UD Webb Farm	Quarter Horse	16	655
	Flash	Private Farm #4 Louisville, PA	Thoroughbred	4	700
	93 Free Flight Fran	Winbak Farm	Standardbred	9	700
	Maggie	UD Webb Farm	Arabian	16	716.67
	R06 Lisa	Winbak Farm	Standardbred	16	750
	Okie	C-Line Stables	Quarter Horse	27	800
	Rayne	Private Farm #2 Elkton, MD	Morgan	19	812.5
	125 Sniper Hanover	Winbak Farm	Standardbred	5	825
	953 Siobhan	Winbak Farm	Standardbred	26	865
	142 Misty Kristy	Winbak Farm	Standardbred	5	1000
	R68 Faith Healer	Winbak Farm	Standardbred	5	1100
	Ego	C-Line Stables	Quarter Horse	31	1500

	R97 Buy One Get One	Winbak Farm	Standardbred	4	2125
	507 Turn the Tab	Winbak Farm	Standardbred	-	2525
	R85 Art Chic	Winbak Farm	Standardbred	5	3475

Table A.1. Complete list of equine participants, their location, breed, age, and FEC.

Appendix B

IMAGEJ CYATHOSTOMIN WORM DATA

	Image Name: Worm Number	Worm Mean Gray Value	Worm with Fluorescent Filter Mean Gray Value	Background Mean Gray Value (average)	Background Fluorescent Filter Mean Gray Value (average)	Worm Fluorescence Calculated	Background Fluorescence Calculated
Control	C1:1	64.683	92.796	43.691	78.565	28.113	34.874
	C2:2	57.79	87.008	43.114	76.699	29.218	33.585
	C2:3	58.608	90.12	42.423	77.284	31.512	34.862
	C3:4	66.108	93.662	45.497	77.342	27.554	31.845
	C3:5	64.138	89.824	45.075	76.749	25.686	31.674
	C3:6	63.778	90.394	46.271	76.706	26.616	30.435
	C4:7	60.358	82.978	42.354	70.206	22.62	27.853
	C4:8	53.419	76.223	42.244	69.722	22.804	27.478
	C5:9	62.279	82.815	42.307	68.309	20.536	26.002
	C6:10	56.932	81.899	44.675	72.299	24.967	27.625
	C6:11	59.623	84.339	44.166	72.861	24.716	28.695
	C6:12	57.72	81.649	43.485	71.01	23.929	27.525
	C7:13	61.032	84.356	45.519	72.501	23.324	26.982
	C7:14	51.75	76.904	46.118	74.213	25.154	28.094

	C8:15	57.242	86.747	42.408	74.398	29.505	31.989
High	H1:1	51.928	74.763	42.317	66.983	22.835	24.666
	H1:2	38.764	64.177	42.955	67.691	25.413	24.736
	H2:3	53.926	83.394	42.875	76.375	29.468	33.499
	H3:4	55.897	86.857	43.947	78.179	30.96	34.232
	H4:5	56.126	86.857	43.111	78.094	30.731	34.983
	H5:6	66.588	96.227	44.156	77.970	29.639	33.814
	H5:7	63.571	91.875	45.091	79.992	28.304	34.901
	H6:8	61.752	94.313	44.710	80.579	32.561	35.868
	H6:9	52.121	82.876	43.965	78.455	30.755	34.49
	H6:10	61.338	87.917	43.578	78.089	26.579	34.512
	H6:11	63.872	89.774	43.904	76.304	25.902	32.4
	H7:12	64.437	96.842	45.684	83.667	32.405	37.984
	H8:13	59.805	84.124	43.853	71.413	24.319	27.559
	H10:14	64.104	88.409	43.384	73.711	24.305	30.328
	H11:15	59.73	90.486	43.193	77.665	30.756	34.473
Low	L1:1	59.417	101.107	40.909	90.256	41.69	49.347
	L2:2	60.351	92.282	42.284	80.326	31.931	38.042

Low	L2:3	61.076	91.488	42.109	79.108	30.412	36.999
	L3:4	63.631	99.556	42.719	86.247	35.925	43.528
	L3:5	52.871	90.539	42.066	84.212	37.668	42.145
	L4:6	63.911	85.658	42.176	69.84	21.747	27.663
	L5:7	42.112	57.05	31.293	50.486	14.938	19.192
	L6:8	38.151	38.151	22.4	67.467	0	45.067
	L6:9	29.293	67.67	22.054	64.489	38.377	42.436
	L8:10	74.603	103.029	54.933	90.163	28.426	35.23
	L9:11	75.047	105.277	54.624	87.728	30.23	33.104
	L10:12	75.542	108.846	54.936	94.685	33.304	39.749
	L12:13	76.448	112.113	54.895	99.306	35.665	44.411

Table B.1. ImageJ data of the control, high, and low larval cultures.

Appendix C

IMAGEJ CONTROL SIGA DATA

	Image Name: Background Number	Background Mean Gray Value (average)	Background Fluorescent Filter Mean Gray Value (average)	Background Fluorescence Calculated
High SIgA	HS2:1	44.111	60.160	16.049
	HS3:2	43.9343	50.432	6.497
	HS4:3	42.185	49.708	7.523
Low SIgA	LS1:1	43.263	58.209	14.945
	LS2:2	43.077	56.480	13.403
	LS3:3	43.068	51.927	8.859
	LS4:4	42.601	54.038	11.437

Table C.1. ImageJ data of the control high and low fecal egg shedder fecal SIgA.