Fecal Egg Counts, Nemabiomes, and Management Practices in Saskatchewan Horses

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By

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Abstract

The purpose of this thesis was to describe intensity (fecal egg counts, FEC) and diversity (using nemabiome ITS2 metabarcoding approaches) of gastrointestinal nematodes in Saskatchewan horses, their relationships with individual horse variables and management practices, and effectiveness of owner-administered treatments. We conducted fecal surveys in horses in fall 2021 (n=107) and spring 2022 (n=123) that had not been dewormed in the previous 6 months, most of which were adults (greater than 3 years old). *Parascaris* spp. eggs were rare, more commonly present in spring and in horses less than 3 years old. Strongyle prevalence and mean FEC were significantly higher in fall (76% and 964 eggs per gram of feces, EPG) than spring (66% and 593 EPG), and in pastured vs stabled horses. Age was significantly associated with strongyle FEC, with higher counts in young and old horses, especially older mares. Third stage larvae were cultured from 95 fecal samples containing >200 strongyle EPG, and 34 unique species were identified, including 28 small strongyles (dominated by *Cylicocyclus nassatus, Cyathostomum catinatum,* and *Cylicostephanus longibursatus)*, 2 large migratory strongyles (*Strongylus vulgaris* and *S. edentatus*), 3 non-migratory large strongyles (*Triodontophorus spp.* and *Craterostomum acuticaudatum)*, and the cattle nematode *Ostertagia ostertagi*. Nemabiome composition was significantly associated with age, sex, collection season, and FEC. In fall, fecal samples from 5 of 29 horses resampled 14 days after anthelmintic treatment administered by owners harboured strongyle eggs (from 11 to 775 EPG) with a mean treatment effectiveness of 96%; on a single property with 15 horses, mean effectiveness was 92%, excluding a horse that likely missed its dose. Three samples contained 18 small strongyle species before treatment, and seven species after, including the 3 dominant small strongyles. Only 23% of horse owners used FEC to determine need for treatment, 41% automatically dewormed twice per year, 26% relied

on appearance of the horse to calculate body weight, 55% used a product containing a macrocyclic lactone, and 39% of owners did not know what product they had most recently used. We report high strongyle FEC and diversity of strongyles for managed domestic horses, and evidence of lack of effectiveness of owner-administered treatments which may in part be related to suboptimal deworming practices as well as emerging resistance of small strongyles.

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Dedication

To my partner, for the support necessary final pushes, and my boys, for keeping me sane and inspiring this work.

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List of Abbreviations

FEC	Fecal egg count
EPG	Eggs per gram
ITS2	Second internal transcribed spacer
GI	Gastrointestinal
L3	Third-stage larvae
L4	Fourth-stage larvae
L5	Fifth-stage larvae
AAEP	American Association of Equine Practitioners
FECRT	Fecal egg count reduction testing
ID	Identification
DNA	Deoxyribonucleic acid
UCVM	University of Calgary Faculty of Veterinary Medicine
WCVM	Western College of Veterinary Medicine
ASV	Amplicon sequence variant
SG	Specific gravity

Chapter 1: Introduction and Literature Review

1.1 Relevance

In 2010, the Canadian horse industry contributed more than $19 billion to our economy as both an agricultural and sport sector, supporting more than 154,000 full time jobs (Evans, 2011). Furthermore, the physiological and mental benefits of horse-human interaction are increasingly recognized (Althobaiti et al., 2019; Baldwin et al., 2021). Equally well recognized are the detrimental effects of gastrointestinal parasitism on equine health and production, ranging from subtle subclinical effects on growth and performance to colic, emaciation, and even death (Matthews, 2011). Unfortunately, multi drug resistance is increasing in many equine nematode parasites, including the small strongyles (cyathostomes) and ascarids (Matthews, 2014).

1.2 Equine Gastrointestinal Nematodes

1.2.1 Strongyle species

Strongyle infections have been reported in horses worldwide. There exists over 50 species of these gastrointestinal parasites (Lichtenfels et al., 1998), all with identical eggs, and are typically classified in two different groups: small strongyles (cyathostomins) and large strongyles (*Strongylus* spp.), which can be migratory or non-migratory (based on larval migration routes).

Small strongyles are the most common parasites in horses (Collobert-Laugier et al., 2002). They infect horses of all ages in every part of the world, existing in every climate (Lyons et al., 1999). These nematodes are referred to as “small” as the adults are usually less than 2.5 cm in length (Corning, 2009). These parasites undergo direct life cycles involving significant pasture

transmission (Matthews, 2011); this, along with a short pre-patent period (2-3 months) and the lack of long-lived immunity (Reinemeyer & Nielsen, 2017), ensures the continuous reinfection of its host, despite treatment efforts. Infective L3 are ingested from where the horses graze and enter the mucosa and submucosa of the host's large intestine. Here, they develop through their early L3, late L3, and fourth larval (L4) stages. Emerging from the lumen, fifth stage larvae (L5) develop into adult nematodes, mate, and the females begin to lay eggs. These are excreted in the feces, often where the animals will be grazing. In warmer climates, the eggs can develop into infective L3 in three days (Corning, 2009) and can remain infective for up to nine months in cold weather (Nielsen et al., 2007). The host will ingest these larvae and the infection cycle will continue. Clinical signs of parasite infection range from subtle effects on growth, coat quality, weight, and performance, to colic and death. In the mucosa and submucosa, the L4 can at times undergo arrested development for up to two years (Corning, 2009; Nielsen & Lyons, 2017)), leading to their accumulation. The season during which inhibition occurs is dependent upon the climate; in temperate regions, larvae will encyst during cooler months. The simultaneous reactivation of these larvae in early spring (in temperate regions) leads to cyathostomiasis (Hodgkinson et al., 2003). This describes the inflammatory reaction that occurs in the caecum and colon, causing diarrhoea, protein losing enteropathy, weight loss, and colic, with a 50% fatality rate despite treatment (Love & Mckeand, 1997).

Large strongyles (*Strongylus* spp.) are considered to be the more pathogenic of the strongyle species, especially the three migratory species, *S. edentatus, S. equinus,* and *S. vulgaris*. The life cycle of these species differs from their smaller relatives due to their ability to migrate through extra-intestinal organs and for their much longer pre-patent periods (6 months to over a year). *Strongylus edentatus* third stage larvae leave the intestine by entering the portal

veins and traveling to the liver, then into the mesentery where they develop for months before re-entering the large intestine as L5 (Owen & Slocombe, 1985). In stallions, larval tracks and larvae have also been found in the spermatic cord and testes (Smith, 1973). Larvae of *S. equinus* enter the portal veins and travel to the liver, then use hepatic ligaments to travel to the pancreas resulting in disruption of the lobes, reduced parenchyma, and atrophied secretory cells (Farrar & Klei, 1985). *Strongylus vulgaris* (also called bloodworm) is widely considered to be the most pathogenic of the three large migratory species, as larvae migrate up the endothelium of the cranial mesenteric artery to the root adjacent to the aorta, develop for several months, and then return to the large intestine via the lumen of the artery. These larvae cause verminous arteritis (Owen & Slocombe, 1985) - inflammation, thrombosis, and vascular damage - and if infarcts are thrown into the intestinal vasculature, ischemic necrosis, colic, and even death.

Non-migratory strongyles (*Triodontophorus*, *Craterostomum* and *Oesophagodontus spp.)* are generally considered much less pathogenic, as larvae of these species undergo a simple mucosal migration, living only in the large intestine. *Triodontophorus* spp. will feed in groups to cause larger erosions in the mucosa than other strongyle species (Cullinane et al., 2006), possibly resulting in ulcers (Owen & Slocombe, 1985). *Trichostrongylus axei* is a trichostrongyle parasite of the stomach generally associated with cattle, but can also be found in other ruminants, horses, and even humans (Themes, 2017), potentially causing inflammatory lesions and ulcers in the gastric mucosa.

1.2.2 *Parascaris* spp.

Two species of *Parascaris* (ascarid nematodes), which produce identical eggs, have been described in horses: *P. univalens* and *P. equorum* (Nielsen et al., 2014), only differing in their

number of chromosomes. *Parascaris univalens* has one chromosomal pair, while *P. equorum* has two. Recent karyotyping work has revealed only the presence of *P. univalens* in Kentucky herds (Nielsen et al., 2014). The *Parascaris* spp. life cycle closely follows that of the small strongyles with some exceptions. Once the eggs are shed onto pasture, development takes longer (at least 10 days), and the infective stage is an egg containing the third stage large (L3) (Wright, 2020). These larvated eggs can remain infective on pasture and in stables for over 10 years, making the transmission cycle nearly impossible to stop. Following ingestion, larvae (L3) migrate to the liver, and within a week will travel through the lungs to the alveoli. They are then coughed up and swallowed, establishing in the small intestines where they mature, mate, and produce eggs within 10-12 weeks following ingestion.

Clinical symptoms of ascarid infections are generally only seen in young horses and include coughing, nasal discharge, poor hair coat quality, slowed growth, impaction colic, and intestinal rupture (Nielsen, 2016). Unlike with strongyles, immunity is developed to these parasites (Clayton & Duncan, 1979) and ascarids predominantly infect young horses (Armstrong et al., 2014), old horses with waning immunocompetence, or those that are immunocompromised (Belay & Teshome, 2016).

1.3 Current Diagnostics

1.3.1 Fecal Egg Counts

The fecal egg count (FEC) is the current standard for the diagnosis and non-invasive quantification of GI nematodes in horses. While there are variations in the methods of performing FECs, such as the Wisconsin or McMaster methods (each with their own advantages and disadvantages), the premise remains the same. A known amount of feces is mixed with a

solution having a higher specific gravity than that of the eggs. This will cause the eggs to float to the top of the solution, allowing them to be easily visualized and counted under a microscope. The McMaster method relies on passive, gravity-based separation and requires less specialized equipment than other tests, such as the Wisconsin, that make use of centrifugation (Williamson, 2014). However, centrifugal methods have increased sensitivity, as this step will further remove heavy debris that would obscure the view of the eggs. Generally, FEC testing is performed in horses in early fall (peak strongyle shedding) and spring (prior to pasture turn-out and when ascarid counts are high). Results are expressed as the number of eggs per gram (EPG) of feces. FEC are only a proxy for adult nematode intensities and truly only reflect the level of parasite egg shedding of the horse, and can vary widely depending on time of year and among individual horses. Suggested guidelines classify horses into different levels of strongyle egg shedding (Kaplan, 2010): low (< 200 EPG), medium (200-500 EPG) or high (> 500 EPG) shedders. Many sources use 200 EPG as the threshold above which a horse should receive anthelmintic treatment. To conserve efficacy of anthelmintics and to maximize untreated parasite refugia (proportions of the population not exposed to anthelmintics, and therefore not exposed to selection pressure for genetic resistance), it is currently recommended by the American Association for Equine Practitioners (AAEP) to treat only the medium and high shedders in an equine population. Fecal egg count reduction testing (FECRT)(Nielsen et al., 2018) is also recommended to be performed two weeks after treatment with anthelmintics to determine drug efficacy by calculating the percentage decrease in eggs shed after treatment. These rule of thumb guidelines are useful, but absolute FEC vary widely. For example, the median strongyle FEC for foals on two Saskatchewan breeding farms was 108 EPG, ranging from 0 to 1987, and was 212 EPG in mares, ranging from 0 to 2490 (Misuno et al., 2018). The mean FEC from German farms

was 202 EPG (Schneider et al., 2014), while the mean intensity from Irish horse farms was reported as 477 EPG (Elghryani et al., 2023). It is not known how applicable these guidelines are to horses in different regions and management systems. Furthermore, the effectiveness of owner administered anthelmintics in horses in Canada, and Saskatchewan specifically, has never been assessed. Unfortunately, since each strongyle species has identical eggs, the FEC gives no indication of what species are infecting the animals, nor can FECRT indicate which strongyle species are most resistant.

1.3.2 Morphological identification

Currently, genus or species level identification of equine strongyles from fecal samples requires coproculture of third stage larvae (L3), followed by morphological identification, which requires at least 7 days for culture and many hours at the microscope by a trained technician. Briefly, 30 g feces from each sample are mixed with vermiculite and kept moist at 26°C for 6 days. Larvae are extracted overnight and at least 200 L3 per horse are morphologically identified based on larval length and width, and number and distinctiveness of gut cells. As an example, for a recent investigation of parasite fauna of feral horses from Sable Island, 89 fecal samples were cultured, which required microscopic measurements of 17,381 larvae (Jenkins et al., 2020). This took the efforts of 3 people in the field to do the cultures over several weeks, and 2 months for a summer student and a trained technician to do the identifications. As a result, very few horse owners and veterinarians have any idea of what strongyle species are present in their horses, and the blanket treatment of all animals in a herd, regardless of FEC, with an arbitrary drug multiple times a year is still the norm among horse owners. This in turn has led to widespread anthelmintic resistance, especially in cyathostomes (small strongyles) and ascarids (*Parascaris*

spp.), including early signs of ivermectin resistance in ascarids in horses in SK (unpubl. data, associated with study by Misuno et al., 2018)

1.4 Emerging Drug Resistance

At the time of release of a new drug, effectiveness of anthelmintics was near 100% in equine ascarid and strongyle parasites. In 2005, when efficacy of ivermectin was tested in a group of foals inoculated with resistant Canadian isolates of *Parascaris* spp., worm numbers only decreased by 22% at necropsies (Reinemeyer et al., 2010). Of the 71 total studies published on equine strongyle anthelmintic resistance, benzimidazole resistance was reported in all studies, pyrimidine resistance in 92%, and macrocylic lactone resistance in 23% of studies (Nielsen, 2022). Benzimidazole resistance was still documented after 22 years of no treatment (Lyons et al., 2007), emphasizing that once resistance is present in a population, there is no reversing it. There is ongoing research on the mechanisms of anthelmintic resistance (Matthews, 2011); however, with no new drugs in production, new diagnostic methods and treatment strategies are urgently needed.

1.5 Molecular Diagnostics- The "Nemabiome"

DNA sequencing technology is rapidly advancing. Sanger sequencing, previously the gold-standard technique (Crossley et al., 2020), is effective in identifying single nucleotide polymorphisms and for single species identification (Seroussi, 2021). This method is fast and cost effective in a limited number of samples. However, this method usually only provides one consensus sequence of the most dominant species, and can produce overlapping chromatograms in mixed infections (de Koning et al., 2006). The evolution of next-generation sequencing (NGS)

technology, such as Illumina sequencing, has allowed for expanded genomic coverage with greater resolution of genomic variations in increasingly larger sample sizes (Rivas et al., 2011).

To address challenges in species level identification of morphologically similar eggs shed in host feces, collaborators at UCVM initially developed and validated DNA metabarcoding approaches for bovids (Avramenko et al., 2015). The “nemabiome” approach uses Illumina sequencing of the ITS2 region to gain a detailed picture of the entire gastrointestinal parasite community, and has now been validated in feral/untreated horse populations (Poissant et. al, 2020; Sargison et al., 2022), sheep (Redman et al., 2019), and bison (Avramenko et al., 2015). Here, the ITS2 region is chosen for sequencing due to its species specificity, with significantly lower variation within species (<1.5%) than between species (Yao et al., 2010). It is a short sequence, less than 300bp, so run time can be short and efficient.

In Illumina sequencing, libraries are created by adding specialized adapters to both ends of amplicons- these allow the amplicons to bind to the flow cell (a channel for adsorbing DNA fragments and facilitating sequencing reactions) (Modi et al., 2021). Multiple samples can be pooled and up to 96 samples can be sequenced in the same run (multiplexing) to save resources. Unique index sequences or ”barcodes” are added to each sample and are read by the Illumina software to distinguish between samples in data analyses. Libraries are loaded onto the flow cell and placed on the sequencer. The DNA is amplified, making millions of copies. Modified nucleotides, each containing a fluorescent tag, bind to the DNA template and signal which nucleotide has been added. The forward DNA strand is read and reads are washed away. This is repeated on the reverse strand, resulting in paired end sequencing. The instrument software identifies nucleotides, a process known as “base calling”, and reports the predicted accuracy of those base calls (Amarasinghe et al., 2020). Reads are demultiplexed, resulting in multiple

FASTQ files containing reads for each individual sample. These sequences are "cleaned" using a computer software system, where adapters and indexes are trimmed, low-quality reads are removed, and paired-ends may be merged.

The end product is a large data base of amplicon sequence variants, or ASVs. While traditional approaches use clustering, blurring similar sequences together, with ASVs the sequencer can differentiate millions of individual sequences, their rates and their confidence values (*Microbiome Informatics*, 2020). Because these are exact sequences, generated without clustering, ASV results can be readily compared among studies using the same target region. Additionally, a given target gene sequence should always generate the same ASV, and a given ASV, being an exact sequence, can be compared to a reference database at a much higher resolution allowing for more precise identification down to the species level and even beyond.

1.6 Trends in Parasite Management

Current recommendations for equine parasite management include 1-2 regular dewormings per year in adult horses, with additional treatments for those with high potential for transmission (Government of Canada, 2017). In addition to regular use of FEC, FECRT, and strategic and targeted deworming practices, the AAEP has recommended environmental management strategies to further improve parasite management such as cleaning of manure from pens at least twice weekly, regularly harrowing pasture to expose parasite eggs to the sun, and reducing the number of horses per acre. However, there are no records of current management practices in Saskatchewan horses, and reports from other regions are concerning. In Sweden, only 7.1% of horse owners report removing feces from horse pens at least twice weekly (Nielsen et al., n.d.), 36% of owners report harrowing their pastures, and only 1% regularly perform FECs (Lind et al.,

2007). In France, 42% of horse owners never perform FEC before treating (Sallé & Cabaret, 2015). A survey of 90 horse owners in Ethiopia showed that most had no knowledge about drug management techniques (Seyoum et al., 2017). Closer to home, most horse owners in the United states treat with anthelmintics 2-3 times a year regardless of horse age, less than 10% routinely use FEC, and less than 5% test anthelmintic efficacy (Becher et al., 2018). Currently, there exist no reports of current practices in Saskatchewan.

1.7 Study Area

Field work took place in fall 2021 and spring 2022, primarily around Saskatoon in central Saskatchewan with a maximum radius of 515 km. The most recent description of equine parasite intensity in this area included only two Saskatchewan farms, both of which focus specifically on breeding, with samples collected more than a decade prior (Misuno et al., 2018).

1.8 Thesis Objectives

1. Describe and compare strongyle and ascarid fecal egg counts (FEC) and strongyle diversity in Saskatchewan domestic horses in spring and fall
2. Determine overall and strongyle species-specific effectiveness of owner administered treatments
3. Describe current parasite management practices of horse owners
4. Determine relationships between FEC, strongyle diversity, horse variables and management practices

Chapter 2: How high is high? Fecal egg counts, anthelmintic effectiveness , and current parasite management practices in riding horses in Saskatchewan, Canada

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Abstract

Globally, equines are ubiquitously infected with gastrointestinal parasites, particularly those of the nematode family *Strongylidae* and the ascarid genus *Parascaris*; however, there are few reports on the prevalence and management of these infections in domestic horses in North America. We report McMaster fecal egg counts (FEC) of strongyles and ascarids in domestic horses in Saskatchewan, Canada, effectiveness of owner-administered anthelmintics, and current practices of parasite management based on owner survey. Fecal samples were collected from individual horses in fall 2021 (n=107) and spring 2022 (n=123), most of which were adults (greater than 3 years of age) and none of which had been dewormed in the previous 6 months. Strongyle prevalence and mean FEC were significantly higher in fall (76% and 964 EPG) than spring (66% and 593 EPG). Age was significantly associated with strongyle FEC, with higher counts in young and old horses; however, there was an association with sex, where FEC

decreased with age in geldings, but increased with age in mares. Strongyle FEC were significantly higher in horses housed solely on pasture versus stables. *Parascaris* spp. eggs were present in two horses in fall (both less than 3 years old) and five horses in spring (2 less than 3 years old and, unusually, 3 adult horses- 2 with no known co-morbidities). In fall, fecal samples from 6 of 29 horses resampled 14 days after anthelmintic treatment performed by owners harboured strongyle eggs (from 11 to 775 EPG) with a mean treatment effectiveness of 96%; on a single property with 15 horses, mean effectiveness was 92%, excluding a horse that likely missed its dose. This is in contrast with 100% efficacy in a study on Saskatchewan horses 10 years previously with veterinary administered treatment. Our survey (n=39) revealed that only 23% of horse owners used FEC to determine need for treatment, 41% automatically dewormed twice per year, 26% relied on appearance of the horse to calculate body weight, 55% used a product containing a macrocyclic lactone, and 39% of owners did not know what product they had used to deworm their horses. We report relatively high strongyle FEC for managed domestic horses and evidence of lack of effectiveness of owner-administered treatments which may in part be related to suboptimal deworming practices as well as emerging resistance of small strongyles.

2.1. Introduction

Globally, equines are ubiquitously infected with gastrointestinal parasites, particularly nematodes of the family *Strongylidae* (small and large strongyles) and the ascarid genus *Parascaris* (Saeed et al., 2019). Pasture transmission and lack of long-lived immunity ensures constant re-infection of horses of all ages with strongyles *(Reinemeyer & Nielsen, 2017)*. Ascarids are primarily shed by young, old, and immunocompromised horses, and the environmental resistance of eggs ensures transmission both at pasture and in indoor environments (Belay & Teshome, 2016). The detrimental effects of gastrointestinal parasites in horses range from subtle subclinical effects on growth and performance to colic, emaciation, and even death (Matthews, 2011).

Anthelmintic resistance is increasing in many equine parasites, including the small strongyles (cyathostomins) and ascarids (Matthews, 2014). The most commonly used drugs, macrocyclic lactones (moxidectin and ivermectin), benzimidazoles, and pyrimidines, have all shown reduced efficacy (<95% FECRT) across all regions of the United States (Nielsen et al., 2018). Fenbendazole and suspected ivermectin resistance are also reported in Northwest Ethiopian cart horses (Seyoum et al., 2017), and resistance to benzimidazoles is prevalent in the small strongyles of Danish horses (Bjørn et al., 1991).

There are approximately 50 species of small strongyles and 3 species of large strongyles infecting domestic horses, all of which produce similar eggs but vary in location of larvae and adults, and pathogenicity (Lichtenfels et al., 1998). Although FEC are only a proxy for adult nematode infection (Carstensen et al., 2013), and intensities vary widely depending on time of year and among individual horses (Jenkins et al., 2020), horses tend to consistently be low (< 200 eggs per gram, or EPG), medium (200-500 EPG) or high (> 500 EPG) shedders, as

described by guidelines set out by the American Association of Equine Practitioners (Nielsen et al., n.d.). To categorize horses by shedding category, FEC testing occurs in early fall during peak strongyle shedding, and spring, prior to pasture turn-out and when ascarid counts are high (Jenkins et al., 2020). To maximize untreated parasites in refugia (Singh & Swarnkar, 2008), and so conserve the efficacy of anthelmintics, it is currently recommended to treat only high, or medium and high, shedders in an equine population.

In 2010, Saskatchewan was estimated to be home to over 108,000 horses and over 18,000 horse owners (Equestrian Canada, 2010a). Over 72% of these were used for sport, pleasure riding, companionship or to generate owner income. The annual impact of the horse industry in Canada amounts to over $19 billion (Equestrian Canada, 2010b). Despite the importance of the horse industry and the potential impact of gastrointestinal parasites on horse health and performance, there is only one description of fecal parasite intensity in horses in Saskatchewan, from 2 breeding farms in 2010-11; at this time, veterinary-administered treatment with an ivermectin based product was 100% effective at reducing strongyle egg counts (Misuno et al., 2018). However, most horse owners do not rely on veterinary administration of anthelmintics, which can be purchased and administered without a veterinary prescription in Canada.

Therefore, for the first time, we sought to determine efficacy of owner administered anthelmintics in horses in Canada (Christie et al., 2004). We conducted fecal surveys in horses in fall and spring, pre- and post-owner administered treatments in fall, and knowledge-based surveys of participating equine owners to report on current practices for equine parasite management.

2.2. Materials and Methods

All procedures and protocols were reviewed and approved by the University of Saskatchewan's Human Behavioural Research Ethics Board ID #Beh 3028 and were exempt from animal research ethics review. Horse owners consented to the collection, analyses, and reporting of anonymized data.

2.2.1 Study population

Fecal samples were collected from 106 horses in fall 2021 and 122 horses in spring 2022 in central Saskatchewan, within a radius of 515 km of Saskatoon. Fall and spring sampling included unique and repeated horses- 27 individual horses were sampled both seasons. Ages of horses sampled in fall ranged from 0-27 years old and from 1-32 years old in spring. Ten horses were under three years of age. Two stallions, four stud colts, 124 geldings, and 98 mares were sampled. Horse breeds were reported by the owner for 164 horses: Quarter horse (n=49), Thoroughbred (n=36), Paint (n=12), Arabian (n=5), Fjord (n=3), Morgan (n=2), 1 Rocky Mountain horse, and 1 Saddlebred. Remaining breeds were grouped by breed type e.g., warmbloods (n=37), ponies (n=15), and draft horses (n=3).

All horses were either in current work for sport or pleasure, retired from work, or too young to be in work. All horses had been exposed to anthelmintic treatments at least once in their life, but not within six months prior to sample collection. Horse environments included stalls, pens, and pastures.

2.2.2 Fecal collection

Fresh fecal samples were collected from individually identified horses by owners or researcher and stored in a sealed bag in a cooler with ice for transportation to refrigeration (4°C) and analysis within a week of sample collection. Animals were chosen based on location, accessibility, herd size, owner volunteers, and no use of anthelmintics at least six months prior to sampling. Select horses were chosen for resampling in the fall two weeks after owner administered anthelmintic treatment on the basis of their initial FEC (>200 EPG), location, and availability.

2.2.3 Fecal egg counts

FECs were performed on all samples using the modified McMaster technique following the protocol outlined in the Paracount-EPG^{TM} Kit for equine samples (Chalex, USA). Briefly, 26 ml of Sheather's sucrose flotation solution (1.27 specific gravity) was measured into the provided tube and filled to the 30 ml mark with feces (equivalent to 4 g). The feces and sucrose solution were mixed and strained through cheese cloth to remove large particles and improve count accuracy prior to pipetting into the slide chamber. 1 ml of the solution was added to both chambers of the McMaster slide, ensuring there were no large air bubbles, and sat for at least two minutes. Strongyle and ascarid eggs were counted separately at 100x total magnification, and each count was multiplied by 25 to calculate eggs per gram (EPG) (limit of detection of 25 EPG). Post treatment, a second McMaster test along with a Wisconsin flotation (J. Martin, n.d.), due to the increased sensitivity (lower limit of detection LLOD), were performed as a FEC reduction test (FECRT), which for our purposes followed owner, versus researcher, administered

treatment. For the Wisconsin flotation, three grams of feces were weighed and mixed with 10 ml of Sheathers solution, strained into 15 ml glass tubes, and topped with flotation solution to form a meniscus. Cover slips were placed on tubes and centrifuged in a swing bucket rotor for 5 minutes at 500 g. Cover slips were placed on slides and examined under the microscope. All eggs were counted, and the total divided by 3 to calculate EPG.

2.2.4 Survey

Online surveys (Table 2.1) using Survey Monkey were sent to participating horse owners with questions on horse location, travel history, age, sex, breed, life-style/work, housing (stabled all or at least part of the day with some time spent in outdoor pens, or pastured, which included outdoor pens of unknown size), diet (alfalfa versus grass forage, feeding methods, and supplements such as grain or concentrates), previous FEC and anthelmintic products and history. Finally, owners were given the opportunity to comment on specific concerns about equine parasites and knowledge gaps in an open-ended format. Respondents were able to leave questions blank. For respondents with more than one horse that responded more than once the same way, duplicates were removed.

2.2.5 Data analyses

All data were entered into an Excel spreadsheet. Post-treatment samples were removed from FEC intensity and abundance calculations and when comparing results among groups. Reported FEC was calculated from the McMaster test for all samples. For FECRT, the McMaster

test was used, and followed up with a Wisconsin test when FEC was less than 25 EPG, due to the test's LLOD. A chi-square test was used to test for significant differences between group prevalences, and Kruskal Wallis was used to test for significant differences between group intensities. Numbers version 11.1 (7031.0.102) was used to plot shedding distributions and shedding per age category. In R, the "lm" function was used to fit linear regression models between multiple variables and calculate significance (Supplemental material 1). The interact plot from the ggplot2 (Wickham, 2016) package was used to plot these models (Supplemental material 2), along with the shedding distributions for individual horses in both seasons. Results were considered significant if $p < 0.05$.

Age was used as a categorical variable in single variable analysis and a continuous linear variable in multivariable regression. Due to the low number of intact male horses (n=6), these were excluded from analysis involving sex, which focused on mares and geldings. FECRT percent efficacy was calculated using pre (FEC1) and post treatment (FEC2) strongyle egg counts on McMaster as per (FEC1-FEC2) *100/FEC1. Lack of efficacy was defined as less than a 95% reduction in FEC. The eggCounts package (C. Wang et al., 2018) in R was used to compute FECRT, model the reduction in FEC with Bayesian hierarchical models, and plot the counts.

2.3. Results

2.3.1 High Strongyle FEC and Relationships with Horse Variables

Overall, including both spring and fall pre-treatment samples, strongyle egg prevalence was 71%, mean strongyle FEC was 551 EPG, and median strongyle FEC was 200 EPG. In fall, strongyle eggs were detected in 81 of 106 horses (76%), with a mean strongyle FEC intensity in positive horses of 964 EPG, median 575 EPG, and range from 25 to 4775 EPG. In spring, strongyle eggs were detected in 80 of 122 horses (66%), with a mean strongyle FEC intensity in positive horses of 593 EPG, median 375 EPG, and range from 25 to 2275 EPG (Figure 2.1). Strongyle FEC prevalence and intensity was significantly higher in fall than spring ($p= 0.001$, $p=0.00$). In fall, 42% of the horses fell into the low category (0-199 EPG), 15% into medium (200-500), and 42% into high (> 500 EPG). In spring, 57% fell into low, 15% into medium, and 29% into high categories (Figure 2.2).

Differences in strongyle FEC prevalence among eight farms in the fall and three in the spring with at least five horses sampled were statistically significant in fall ($p=0.001$), but not in spring ($p = 0.47$). Herd level shedding distributions were highly variable, ranging from 100% low shedders to 100% high shedders (Figure 2.2).

Not all data (sex, age, management, etc.) were available for all samples, so some analysis was done on a subset of data. We observed a strong relationship between age as a categorial variable and FEC ($p = 0.01$) (Figure 2.3, Table 2.2), with higher FEC in younger and older horses in fall and in spring. Prevalence of strongyle eggs was 100% in horses under 3 years of age. Age (plotted as a continuous linear variable) interacted with sex on FEC. FEC decreased

with age in geldings and increased in mares. ($F = 3.19$, $p = 0.0077$) (Figure 2.4a). A locally weighted running line smoother (LOESS) shows that the non-linear relationship between age and FEC was maintained in both sexes (Figure 4b).

Housing was also significant ($f = 6.06$, $p = 0.017$), with mean FEC for horses living only outdoors of 929 EPG (N=52) and stabled horses with a mean of 4 EPG (N=7). Diet, breed, and if a previous FEC had been done had no effect on FEC.

2.3.2 Strongyle FECRT Shows Reduced Effectiveness

In the fall, 5 of 29 horses from 2 properties with FEC > 200 EPG treated by the owner had strongyle eggs detectable in feces 14 days post-treatment at counts ranging from 11 to 775 EPG. Individual horse treatment effectiveness was 7%, 87%, 89%, 98.5%, 99.5%, and one horse showed an increase in egg counts following treatment giving an effectiveness of -41% (Figure 2.5). For all 29 horses, mean % reduction was 96% with a confidence level of 89%. Half (15) of these horses were from a single property, where strongyle FECRT was 83.5% following treatment with combined ivermectin and praziquantel. When the sample with an increase in FEC after deworming was discarded, the calculated efficacy was 92.4% (95% CI [66.1%, 99.8%]. Mean reduction was 99.9%, mode was 100%, and 50th percentile reduction was 100%.

2.3.3 *Parascaris* spp.

Eggs of *Parascaris* spp. were found in 2/106 horses in fall (300 and 575 EPG), both of which were less than 3 years old, and 5/122 samples in spring (50, 75, 150, 375 and 1675 EPG)

in 2 horses less than 3 years old, and 3 horses aged 7, 11, and 14 yrs. Following deworming of the 2 horses in fall, *Parascaris* eggs were not detected in feces on the McMaster; however, Wisconsin showed 3 EPG for one sample, down from 575 on the original McMaster test, and 1 in the other, down from 300 EPG, on two different farms, indicating *Parascaris* treatment efficacy of 99.5% and 99%.

2.3.4 Owner survey results

Online survey success rate in returns was 58%. Our results indicate that 23% of 39 horse owners deworm when indicated by FEC, 15% deworm automatically once a year, 41% twice a year, 5% three times a year, and 15% of horse owners responded with "other" (Figure 2.6). Prior to this study, 45% of 40 horse owners had never requested a fecal egg count, 20% had once in the past, 28% do them yearly, 5% twice a year, and 3% more than twice a year (Figure 2.7).

In response to the question of how owners determined the weight of a horse for deworming dose calculation, 26% of 42 respondents used the appearance of the horse, 2% relied on trainer advice, 10% relied on veterinary advice, 14% used a weigh scale, 33% used a weigh tape, and 14% responded with "other" in an open ended format which included: averaging the weight for deworming, comparing to horses with known weights, using previously known weights, giving the "whole tube" regardless of weight, and using the "Healthy horse calculator app" from the University of Minnesota.

Products were reported 71 times between open ended responses on the survey and through verbal conversations with owners, with a mix of brand names and the generic drug name; here we report only generic drug names. 30% of respondents used an ivermectin based

product, 14% a combined ivermectin and praziquantel product, 3% used fenbendazole, 3% used moxidectin alone, 3% reported using praziquantel alone, 6% used a combined moxidectin and fenbendazole product, 1% used combined moxidectin and praziquantel, 1% used pyrantel, 1% reported use of a vaccine against equine herpes and influenza viruses as a dewormer, and 39% did not know what dewormer they used (Figure 2.8). Some owners indicated administering injectable ivermectin orally.

16% of respondents indicated noticing a lack of effectiveness in their deworming products based on observations of tail rubbing, rough fur, and weight loss. 6% of owners reported having previously seen parasites in their horse's feces and 9% were unsure if they had. Average reported level of concern for parasite infections compared to other horse health issues was 4.3/10.

2.4. Discussion

2.4.1 Higher FEC and decreased efficacy of owner administered treatments

Here, we report higher median intensity (575 EPG) of strongyle eggs in fecal samples collected from adult horses in fall than 212 EPG reported in a previous study in Saskatchewan in 2010-11, as well as lower efficacy of owner administered treatments (96% efficacy, predominantly macrocyclic lactones) than 100% efficacy observed in a previous study with veterinary administered ivermectin treatment (Misuno et al., 2018). In one herd in our study, owner administered treatments had less than 95% efficacy, the working definition of resistance. As horses were dewormed by owners, using their current deworming programs, our results may not be considered evidence of resistance, but may in part reflect missed doses and/or suboptimal

deworming practices including underdosing and use of unknown or ineffective products, according to our survey results. Lack of efficacy is often an early warning sign of resistance, along with reduced egg reappearance periods, and both are documented in the global equine population (Lyons et al., 2010; von Samson-Himmelstjerna et al., 2007). Unfortunately, once anthelmintic resistance is demonstrated by FECRT, it is too late to reverse the resistance in the population (P. Martin et al., 1989). Therefore, it is essential to develop more sensitive, faster assays than the FECRT for anthelmintic resistance (Kotze et al., 2020). Current options include the larval migration assay (LMA) in which cultured L3 migrate though filters in the presence of increasing concentrations of paralytic anthelmintics (such as macrocyclic lactones e.g. moxidectin, ivermectin) to generate a dose-response curve (Matthews, 2011).

Our survey reveals a need for continuing education in the veterinary profession and the horse industry to address information gaps, including inaccurate methods of estimating weight, one-size-fits-all-horses practices ("giving the whole tube"), and lack of awareness of active ingredients or even brand of dewormer in over one third of owners. Knowledge translation could include publishing in lay journals, presenting to horse federations, and updating the educational material used to teach young horse owners, such as the Equestrian Canada and Canadian Pony Club handbooks.

2.4.2 High strongyle FEC intensity and seasonal patterns

Mean FEC in Saskatchewan horses in our study (550 EPG) was higher than horses in Germany (202 EPG) (Schneider et al., 2014), Italy (245 EPG) (Scala et al., 2020), and Ireland (477 EPG) (Elghryani et al., 2023), which may reflect differences in parasite diversity, care,

climate, study populations, and methods. We also had an unusually high proportion of high shedding horses (35%) compared to two managed populations of horses in Sweden (18% and 15%) (Hedberg-Alm et al., 2020). Our mean strongyle FEC in fall (964 EPG) are more comparable to an untreated herd in Kentucky (1000 EPG) (Steuer et al., 2022), and a high density, unmanaged feral horse population on Sable Island (1005 EPG) (Jenkins et al., 2020), demonstrating that both geographic differences and management practices result in vastly different parasite loads. The significance of FEC in terms of body condition and pathogenesis of these parasites is not well described in domestic horses, nor why some animals can tolerate significantly higher burdens than others.

We observed significantly higher prevalence and strongyle FEC in fall (76% and 964 EPG) than spring (66% and 593 EPG). Seasonal variations in parasite egg shedding are well documented, and FECs are especially unreliable in cold winter months in temperate climates, or hot, dry summers in arid regions, when many GIN undergo hypobiosis (Poynter, 1954). Therefore, FECs to determine individual horse shedding category should be performed in late summer and fall in western Canada, where strongyle shedding has been shown to be consistent July to September (Misuno et al., 2018).

2.4.3 Low prevalence of ascarids

Parascaris spp. prevalence (3%) was lower in our study when compared to an Italian study with 6% prevalence of *Parascaris* spp. (Scala et al., 2021). Interestingly, out of the 10 horses aged less than 3 in our study, only four were shedding ascarid eggs (20%), and in horses

older than 10 years of age, *Parascaris* spp. prevalence was 2%, similar to 1.2% in the same age group in Italy.

Clinical symptoms of ascarid infections include coughing, nasal discharge, poor hair coat quality, slowed growth, impaction colic, and intestinal rupture (Nielsen, 2016). Unlike with strongyles, immunity is developed to these parasites (Clayton & Duncan, 1979) and ascarids predominantly infect young horses (Armstrong et al., 2014), or those that are immunocompromised (Belay & Teshome, 2016). It is somewhat unusual for healthy adult horses to shed ascarid eggs - in our 3 positive adult horses, the history was known for two of them. One, a healthy 7 year old quarter horse mare, lives in a pasture with four other horses where feces are never removed, and is used for riding lessons and trail riding. The other, a 12 year-old thoroughbred gelding used for sport, lives in a stall that is cleaned daily, and is never exposed to other horses' feces. However, this gelding was diagnosed with an autoimmune disease and medications include azathioprine, steroids, NSAIDs, and oral and systemic antibiotics. There are previously documented cases of transmission of ascarids in a closed system (Lyons et al., 1996), and the horse's medical history could explain increased susceptibility to infection and shedding.

2.4.4 Risk factors linked to strongyle FEC

The majority of published literature reports a decrease in strongyle FEC throughout the horse's lifetime (Becher et al., 2010; Fritzen et al., 2010). In our population, age was significantly associated with strongyle FEC, with higher counts in young and old horses. As a single variable, sex played a small role in overall fecal egg counts; however, there was an association with age and sex, with FEC declining in geldings and increasing in mares over time

(Figure 2.4). Male sex bias in parasitism of animals (Schalk & Forbes, 1997) has, along with exposure variables, been attributed to immunosuppression by androgens, which are largely absent in geldings. Almost all males in our study were geldings, and their lower androgen levels may play a role in this observation. Regardless, the parabolic relationship between FEC and age was still maintained in both sexes when a LOESS line was used. Diet was not significantly associated with FEC in our study, suggesting adequate nutrition of most horses, since there are known associations between increased parasite burdens and malnutrition (Coop & Kyriazakis, 1999).

Strongyle FEC were significantly higher in horses housed solely on pasture versus stables, consistent with pasture transmission for most strongyle nematodes affecting horses. In addition to reducing access to pastures (which may not be feasible if that is the primary source of nutrition and exercise), owners seeking to reduce parasite transmission could also limit the number of horses in a turnout, remove feces daily, and target treatments to those horses spending more time outside. Horse turnouts can also be harrowed in the summer months when it is hot and dry to expose strongyles to heat and sun (Bearden et al., 2023). However, reports from other regions suggest that only 36% of respondents in Sweden reported harrowing their pastures, 6% reported the weekly removal of feces from grazing areas, and only 1% performed regular FECs (Lind et al., 2007). In Germany, only 10% of the studied farms removed feces to control parasites (Fritzen et al., 2010). The high proportions of medium and high shedders (Figure 2) in our study deviates from the AAEP guidelines that suggest 50-75% of a herd population will be low shedders, 10-20% medium, and 15-30% high. This suggests that equine management practices effective in controlling parasite shedding are not in wide-spread use in Saskatchewan.

2.4.5 Significance of our study findings for management

The American Association of Equine Practitioners (AAEP) best practice guidelines for targeted selective treatment suggest treating only medium-high shedders to maintain refugia; in Saskatchewan riding horses, we would be treating 57% of our horses in fall. Alternative options could include treating only those horses in the top 20-30% of shedding, with effective drugs determined for each farm through regular FECRT. It is appropriate to treat horses in fall, rather than when all of the worms are in the host (mid-winter), resulting in a higher selection pressure for resistant worms (Singh & Swarnkar, 2008), and this would also be well timed to target bots (*Gasterophilus* spp.) acquired over the summer. Although strongyle prevalence and FEC were lower in spring, owners may also wish to treat high shedders in spring to head off pasture contamination and reduce transmission, and to reduce *Parascaris* spp. egg shedding, which is higher in spring than fall. Results from our study suggest prioritizing treatment of young and old horses, especially young geldings (<8 years) and older mares (>16 years). Selective treatment (even random) is likely better than uniformly treating all animals in a herd, which has a proven positive correlation with drug resistance and decreased proportion of parasites in refugia (Singh & Swarnkar, 2008).

2.4.6 Study limitations

Our study examined real and current practices in the horse industry rather than a controlled study environment with researcher administered anthelmintics. We also used the most common and inexpensive FEC method (McMaster), with a detection limit of 25 EPG, meaning that low shedding horses may have been missed. Relying on horse owners to collect samples

greatly improved geographic range and sample size; however, samples may not have been properly stored at the correct temperature or sealed immediately after collection. Finally, a major study limitation is the inability to distinguish among the 50+ different strongyle species known to infect horses world-wide, which could be addressed through application of molecular diagnostic methods, such as nemabiome or metabarcoding approaches to characterize the complex equine gastrointestinal nematode community (Poissant et al., 2021).

2.4.7 Conclusions

Our research demonstrates high prevalence and FEC of strongyles and low prevalence of ascarids, decreased efficacy of owner-administered anthelmintics, and suboptimal current practices of parasite management in domestic horses in Saskatchewan, Canada. Current goals of parasite control programs are to minimize risk of disease, control parasite egg shedding, and maintain efficacious drugs and avoid future anthelmintic resistance (Kaplan & Nielsen, 2010). In practice, many owners make arbitrary decisions for deworming and many veterinarians find it difficult to advise horse owners on treatment practices especially in mixed ownership environments such as boarding stables. When developing tailored parasite control programs, veterinarians must take into account individual owner risk tolerance and use and value of the animals i.e., pastured horses versus high-performance horses that provide income. FECs and molecular testing to detect more pathogenic strongyles, such as *S. vulgaris*, is increasingly available and facilitates selective treatment of high shedding horses and those with more concerning parasites. It is encouraging to see a high level of interest and voluntary participation in this study by equine owners. This study can serve as evidence on which to base rational and

strategic practices such as testing in fall to find high shedders, treating the very young and the very old (especially mares), and changing management practices to decrease transmission and preserve anthelmintic efficacy.

Author Contributions

Toni-Anne Saworski: Conceptualization, Methodology, Formal analysis, Investigation, Research, Resources, Data Curation, Writing- Original Draft, Project Administration

John Gilleard: Conceptualization, Writing- Review and Editing

Jocelyn Poissant: Conceptualization, Writing- Review and Editing

Emily Jenkins: Conceptualization, Methodology, Resources, Writing- Review and Editing, Project Administration, Supervision, Funding acquisition

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Acknowledgements

Brent Wagner and Dr. Sarah Parker for their technical expertise on the project.

Tables

Table 2**Error! No text of specified style in document.**1 Owner Survey administered via Survey Monkey

What is your name?	(open)
What is your horse's name?	(open)
What barn are you stabled at, in/near what city?	(open)
How long has your horse been at this facility?	<6months 6 months-1 year 1-2 years 2+years
Where was your horse purchased from?	(open)
Where did your horse originate from? (USA, Europe, Alberta, Saskatchewan, etc.)	(open)
What is your horse used for? (companion, hunter, jumper, open show horse, eventing, barrel racing, lesson horse, etc.)	(open)
DURING THE COLLECTION SEASON does your horse live in a (select all that apply)	Stall Paddock Pasture other (please specify)
DURING THE COLLECTION SEASON How often is feces removed from their turnout?	Daily Weekly Biweekly Monthly Never

	Never, my horse lives in a pasture Twice a year Yearly Other (please specify)
DURING THE COLLECTION SEASON, how many horses is your horse turned out with?	(open)
DURING THE COLLECTION SEASON, what forage does your horse get? (check all that apply)	grazes on grass grass hay alfalfa hay alfalfa cubes grass hay cubes other (please specify)
Does your horses diet change at shows vs at home? ex: pasture at home, hay at shows. (if yes, please specify)	(open)
DURING THE COLLECTION SEASON How does your horse get its forage? Select all that apply	(open)
DURING THE COLLECTION SEASON Does your horse receive extra supplementary feed other than regular forage? (Briefly specify)	off the ground in a field off the ground in a paddock off of the stall floor from a trough from a hay net other (please specify)
Is there anything extraordinary about your horse's health or care that you think might affect his parasite burden?	(open)
Have you ever or do you normally do a fecal egg count on this animal	never done one besides this study

	have at least once in the past, not regularly done every 2 years yearly twice a year more than twice a year other (please specify)
How often do you deworm?	yearly twice a year three times a year four times a year only when indicated by egg count only when horse appears symptomatic never other (please specify)
What product do you normally use to deworm, and at what times of the year? (if this differs between times of the year, please specify) Is there anything specific you'd like to learn about parasites, or some knowledge you feel like is lacking in the indust	(open)
How do you determine weight for a dewormer dosage?	Estimate based on appearance Weight tape Weigh scale Based off of trainer recommendation

	Based off of veterinary recommendation Other (please specify)
Have you ever seen parasites in your horse's feces?	yes no unsure
Have you noticed lack of efficiency in your deworming products?	yes no
If you answered yes to the previous question, what did you notice? (poor body condition, tail rubbing, continued parasites, etc.) if you answered no, please just type no	(open)
From a scale of 1-10, how concerned are you with parasite infections as opposed to other health issues?	(scaled)
Is there anything specific you'd like to learn about parasites, or some knowledge you feel like is lacking in the industry?	(open)

Table 2.2 Prevalence of strongyle infections by age over two seasons

	Season			
	Fall		Spring	
Age	N	Prevalence (strongyle)	N	Prevalence (strongyle)
20 +	11	90.9%	26	76.9%
16-19	15	93.3%	20	50.0%
12-15	26	57.7%	19	68.4%
8-11	23	69.6%	13	69.2%
4-7	19	89.5%	18	72.2%
≤3	5	100.0%	5	100.00%

Figures

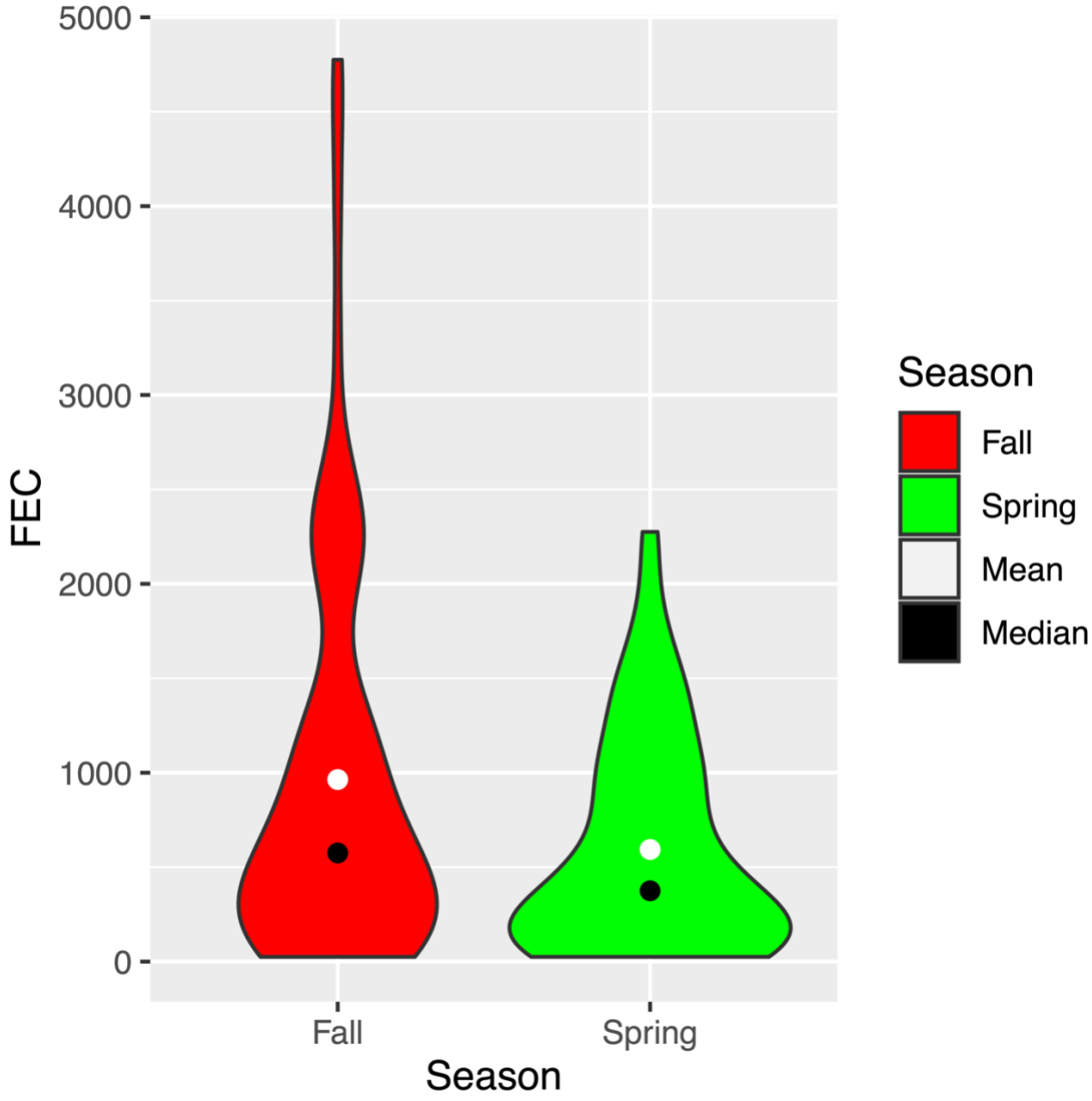

Figure 2.1: Strongyle fecal egg count (in eggs per gram of feces) distribution and measures of central tendency for 106 horses sampled in fall 2021 and 121 sampled in spring 2022 in Saskatchewan, Canada.

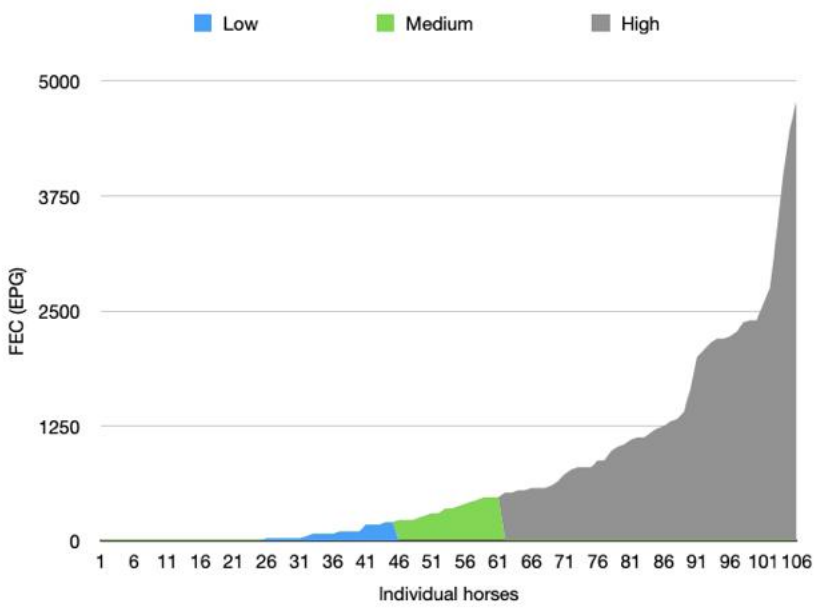

Figure 2.2 A

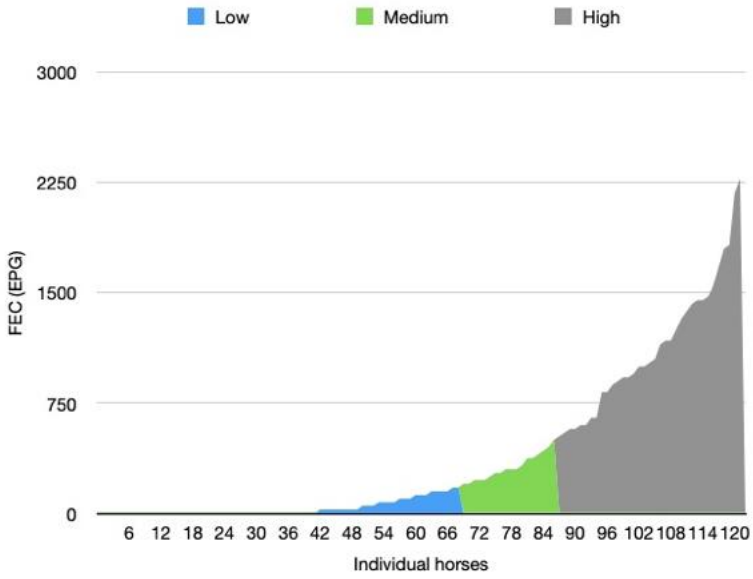

Figure 2.2 B

Figure 2.2: Individual horses classified by fecal egg count categories (low 0-199 eggs per gram of feces, or EPG; medium 200-500 EPG; or high >500 EPG) for 106 horses sampled in fall 2021 (A) and 121 sampled in spring 2022 (B) in Saskatchewan, Canada.

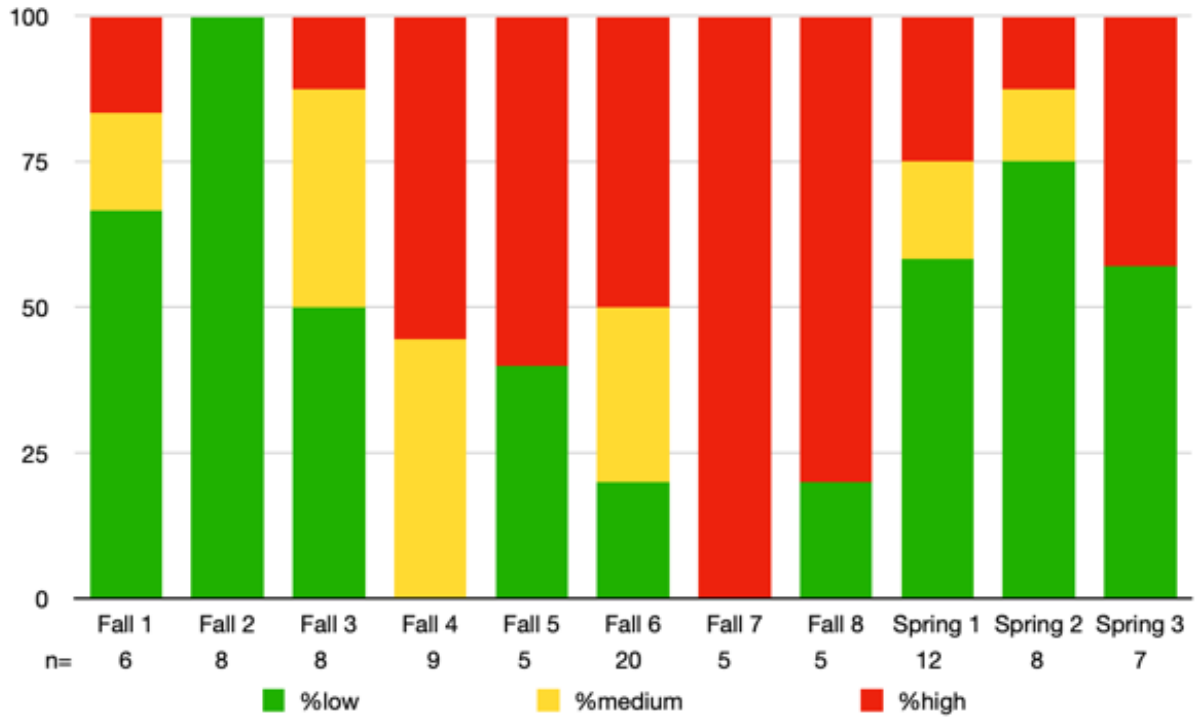

Figure 2.3: Proportional distributions of high (>500 EPG), medium (200-500 EPG) and low (<200 EPG) strongyle egg shedders on each premise (numbered) where a minimum of five horses were sampled in their labelled seasons.

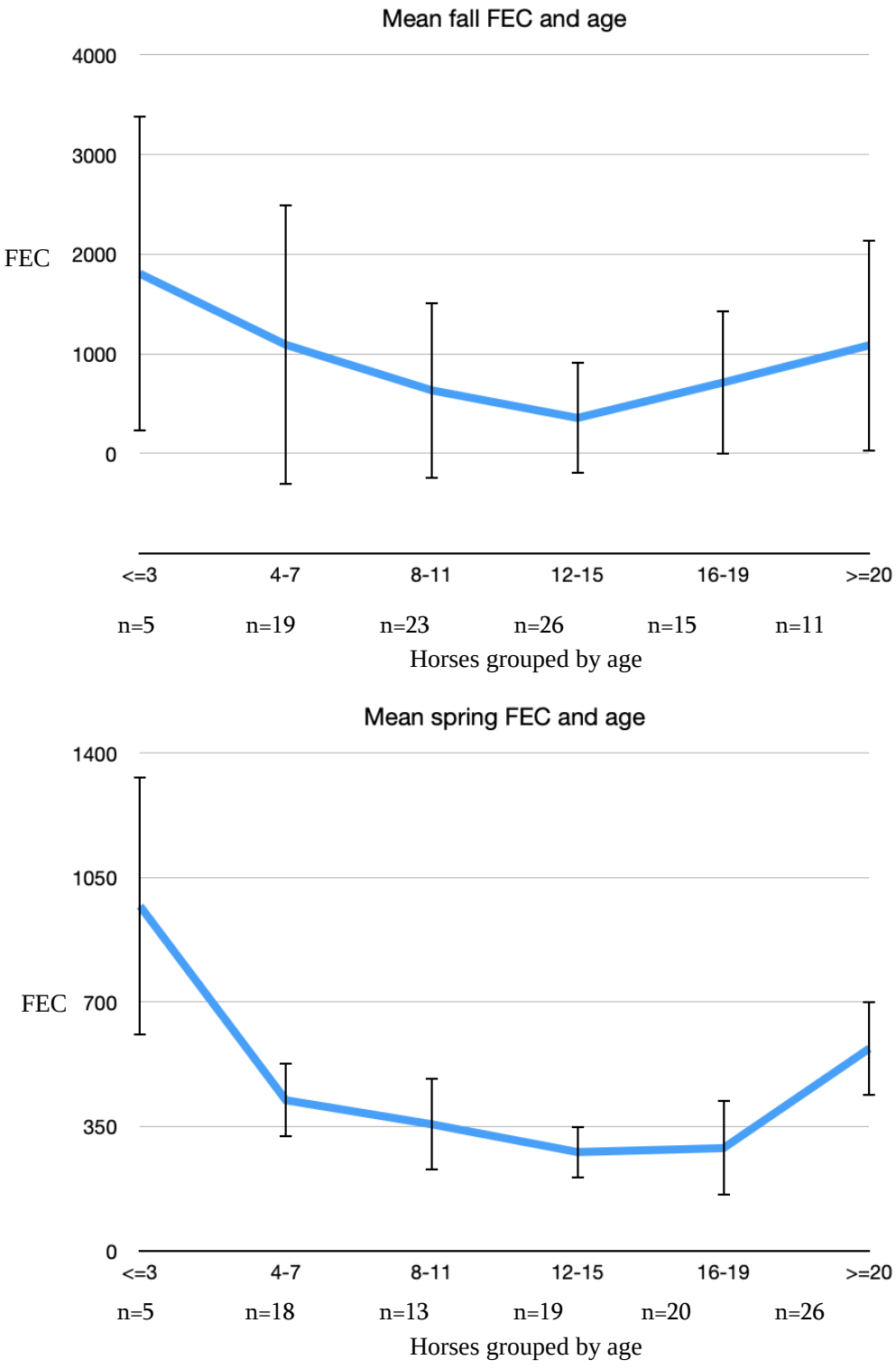

Figure 2.4: Mean strongyle fecal egg counts (including zero values) in different age groups of horses sampled in Saskatchewan, Canada in fall 2021 and spring 2022. Error bars represent standard deviation.

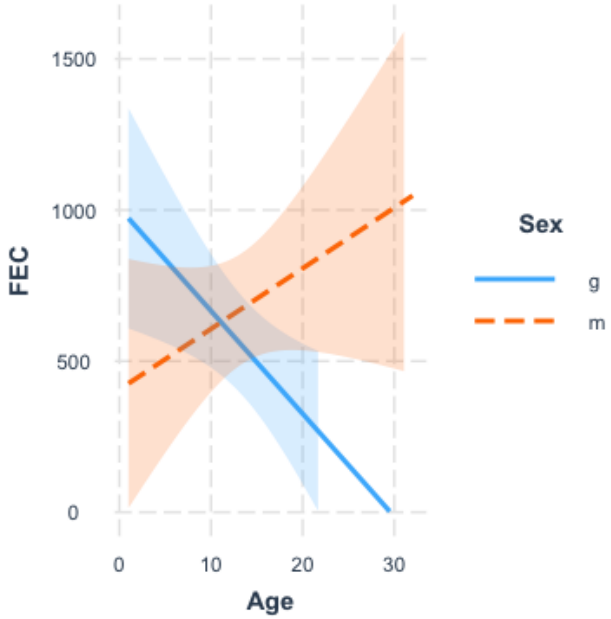

Figure 2.5a: Linear relationship between combined spring and fall FEC in mares (m) compared to geldings (g). Stallions and stud colts were excluded. Shaded areas indicate the confidence interval (95%).

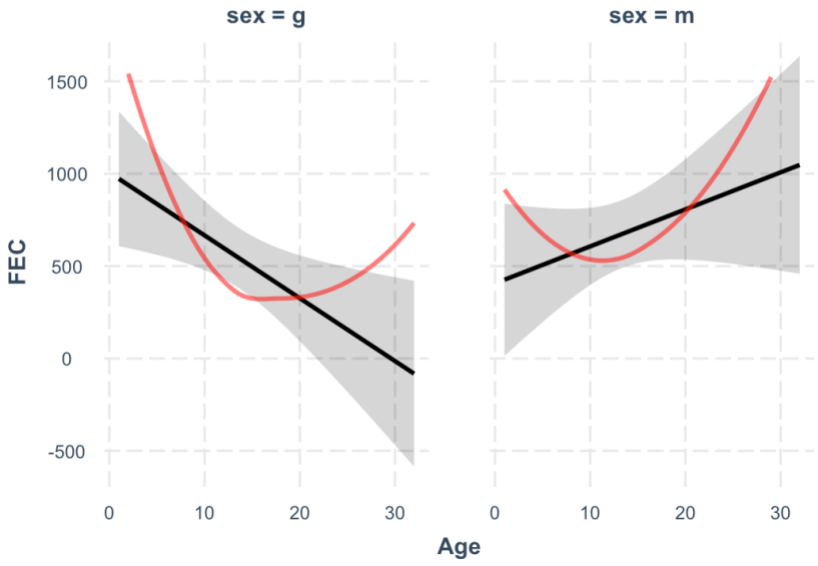

Figure 2.5b The superimposition of a LOESS line (red) to recover the trending decrease in FEC with age, followed by an increase at 12-15 years of age. While geldings are significantly more likely to have a higher FEC at a younger age compared to mares, geldings exhibited a statistically smaller increase in FEC after age 12-15 than mares.

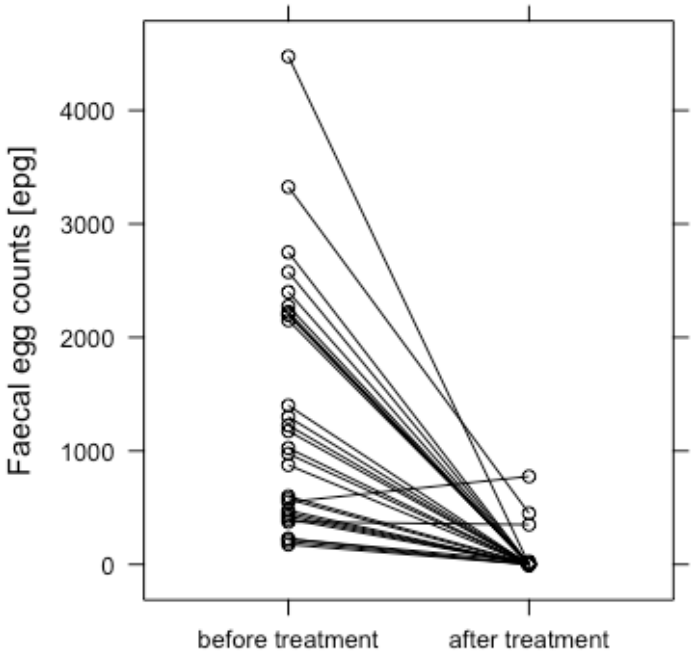

Figure 2.6: Fecal egg count reduction in 15 horses on the same premise before and 14 days after owner-administered treatment with combined ivermectin and praziquantel, with an overall treatment of efficacy of 92%, below the threshold generally accepted to indicate anthelmintic resistance (95%). Model was created using eggCounts (Wang et al., 2018) in R.

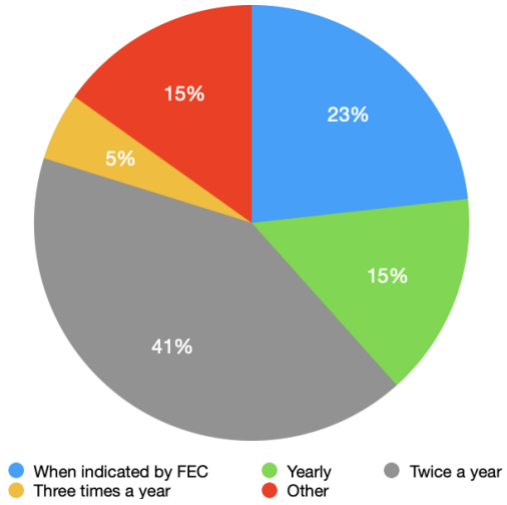

Figure 2.7: Deworming frequency based on owner survey.

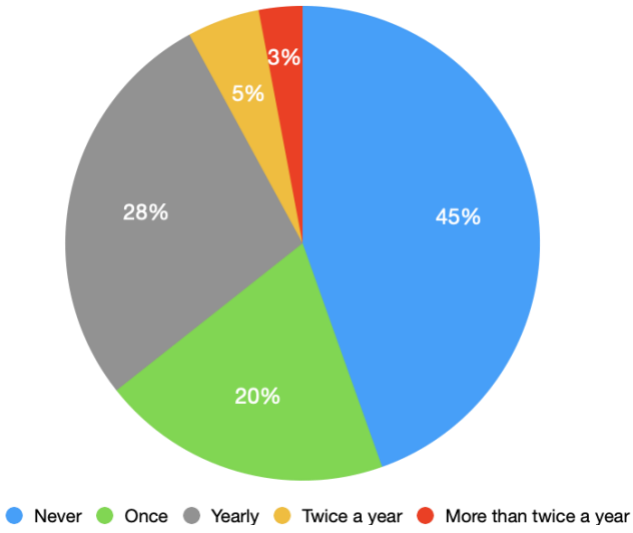

Figure 2.8: Frequency of FECs based on owner survey

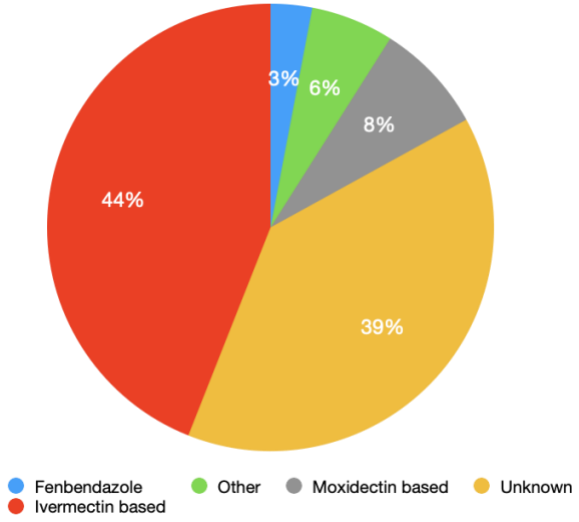

Figure 2.9: Description of owner administered anthelmintics based on owner survey. The "other" category includes non-traditional methods of treatment.

Transition Statement

In Chapter 2, I described high gastrointestinal nematode parasite prevalence and intensity, and reduced anthelmintic effectiveness , in Saskatchewan domestic horses. However, with at least 52 strongyle species infecting horses, all with identical eggs, FECs give no indication of diversity of species in these mixed infections, and therefore no insight into the pathogenicity and level of concern required for each parasite species. Therefore, in Chapter 3, I applied new nemabiome methods to enhance specificity and discriminatory ability of our diagnostic methods, and to determine which species of strongyles were surviving owner-administered treatments.

Chapter 3: Using and refining the ITS2 metabarcoding approach to describe high strongyle diversity in Saskatchewan working horses

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Abstract

Gastrointestinal parasites, especially those of the family *Strongylidae*, are of concern for equine health due to their ubiquitous nature, pathogenicity, and emerging resistance to anthelmintics. The current method of diagnosis is based on detection of eggs in feces; unfortunately, over 50 species of small strongyles and three species of large migratory strongyles produce morphologically identical eggs. Species level identification is time consuming and requires culture and morphological identification of third stage larvae (L3). Here, for the first time in managed domestic horses, we use a molecular metabarcoding approach to describe the gastrointestinal nemabiome of working horses in Saskatchewan, Canada. For species assignment, we used only forward reads and chose a confidence threshold of 50% (vs 80%) in order to capture maximal diversity. Fecal samples were collected from horses, none of whom had been exposed to anthelmintic treatment in the last 6 months, across the province over two seasons (fall

2021, spring 2022) to identify medium-high shedders (>200 eggs per gram (EPG). Pre-treatment, 34 unique species were identified, including 28 small strongyles (dominated by *Cylicocyclus nassatus, Cyathostomum catinatum,* and *Cylicostephanus longibursatus)*, two large migratory strongyles (*Strongylus vulgaris* and *S. edentatus*), 3 non-migratory large strongyles (*Triodontophorus spp.* and *Craterostomum acuticaudatum)*, and the cattle nematode *Ostertagia ostertagi*. In fall, fecal samples from 3/29 horses resampled 14 days after anthelmintic treatment administered by owners harboured 18 small strongyle species before treatment, and seven after, including the three dominant small strongyles. Nemabiome composition/species diversity was significantly associated with age (higher in older and younger horses), sex (higher in mares), collection season (higher in fall), and FEC, but was not associated with region or diet. This study demonstrates high strongyle diversity (number of species) within owner-managed working horses and concerning lack of efficacy of owner administered treatments in several small strongyle species, and lays the framework for future improvements in parasite diagnostics and management.

3.1 Introduction

There are over 50 strongyle species infecting horses, all having identical eggs (Clark, n.d.). Therefore, methods based on detection of eggs on fecal flotation give no indication of the diversity or pathogenicity of strongyle species, which is important, because some species, such as *S. vulgaris*, can cause colic, verminous arteritis, and death (Owen & Slocombe, 1985). Historically, species level identification of equine strongyles is done morphologically after culturing L3 for at least 7 days, which requires hours at a microscope and extensive knowledge

(Dunn, 1978). As a result, few veterinarians and horse owners know which strongyle species are present in their horses, leading to blanket treatment practices of all horses, or following more modern guidelines that leave parasites in refugia by treating only those horses classified as medium to high shedders (200-500+ EPG)(Equine Recommended Deworming Schedule, n.d.). In light of documented emerging anthelmintic resistance in equine gastrointestinal nematode parasites (R. M. Kaplan, 2004; Matthews, 2014), new research is urgently needed to improve the specificity and discriminatory ability of diagnostic methods.

Recently, new molecular techniques have been used to characterize the nemabiome of cattle (Avramenko et al., 2015), sheep (Redman et al., 2019), bison (Avramenko et al., 2018) and feral or untreated horse populations (Poissant et al. 2020; Sargison et al., 2022)) through PCR amplification and deep sequencing of the internal transcribed spacer 2 (ITS2 rDNA) region. We employ this method in Saskatchewan working horses with the objectives of describing the regional nemabiome in the province before and after owner administered treatment, and making connections between strongyle diversity, individual horse variables, and management factors.

3.2 Materials and Methods

Owner survey was reviewed and approved by the University of Saskatchewan's Research Ethics Board ID #Beh 3028, and protocols were exempt from animal research ethics review. Horse owners consented to the collection and analyses of data.

3.2.1 Horses, fecal and data collection

Fecal samples and horse information (age, sex, location within the province, and diet) are described in Chapter 2. Briefly, most were adult horses (greater than 3 years of age) currently or previously used for sport or pleasure riding, 44% mares and 56% geldings, from a range of housing/pasture environments, with diets that ranged from grass, supplemental alfalfa hay or extra dietary supplements. No horse had received anthelmintic treatment within the previous 6 months. Fresh fecal samples were collected from the ground from horses in fall (August to November) and spring (March-April). Fecal egg counts (FECs) were performed using the modified McMaster technique. In fall only, twenty-seven horses on selected farms, chosen by their pre-treatment FECs, availability, and herd size, had FEC done 14 days after deworming by the owner with their choice of product.

3.2.2 Larval culture

L3s were cultured from horses with FECs greater than 200 eggs per gram of feces (EPG): 58 horses in fall, including three horses with a positive FEC following treatment, and 40 horses in spring. Up to 40 g (as available) of feces from individual horses were weighed, mixed with equal volume of vermiculite in a glass tumbler, and moistened with tap water. A depression was made in the middle of the mixture and tumblers were covered with a plastic petri dish, with a strip of twisted paper towel separating the tumbler and dish to allow air flow. These were incubated at room temperature for 21 days, re-wetting every 3 days. L3 were harvested using the glass-over-petri-dish method (Dunn, 1978) into 2 ml Eppendorf tubes. L3 from each sample were stored in 2 ml of 70% ethanol at room temperature until DNA extraction.

3.2.3 Preparation of lysates from L3

Methods followed those of Poissant et al. (2020). All molecular grade water and plastics were UV treated for 15 minutes. Two 10 µl drops from each 2 ml sample were placed on a glass microscope slide and total L3 were counted, summed, and divided by 0.01 to obtain total number of L3. DNA was extracted from all ethanol-fixed L3 from an individual horse fecal sample, rather than normalizing each sample to 1000 L3 per tube as previously described (Avramenko et al., 2015). Ethanol fixed L3 were transferred to new labeled 2 ml Eppendorf tubes. These were centrifuged at 13000 × *g* for 4 minutes and supernatant was removed by pipetting. Lysis buffer (1 ml), prepared as per Avramenko et al. (2015), was added to each tube and vortexed to resuspend the L3. The centrifugation and buffer addition steps were repeated twice. Tubes were again centrifuged as above, and 900 µL of supernatant was removed, leaving 100 µL of lysis buffer with the L3. This was resuspended and topped up with 50 µL of lysis buffer. Tubes containing the L3 suspensions were incubated in a water bath (as we did not have a thermomixer as per the published method), at 95 °C for 15 minutes, with a brief vortex at 7 minutes.

Tubes were transferred to a -80°C freezer for a minimum of one hour. After freezing, they were defrosted on ice before adding 6 µL of proteinase K (20 mg/ml). These were placed in a 55 °C water bath for 120 minutes, briefly vortexing at 15 minute intervals. Tubes were then transferred to a 95 °C water bath for 20 minutes, vortexing after the first 10 minutes. Tubes were then placed on ice, and diluted 1:10 with molecular grade water into a 96-well PCR plate (5 µL lysate:45 µL H_2O), before storage at -80 °C.

3.2.4 ITS2 region amplification and sequencing

Methods are as described by Poissant et al. (2020) unless otherwise indicated. Briefly, we used NC1 and NC2 primer mix and KAPA Hifi Hotstart PCR Kit (Kapa Biosystems, Wilmington, Massachusetts, USA, KK2502). A reagent control, containing only the buffer and PCR reagents with no DNA template was used on each plate/run. Amplified DNA was covered with foil and stored at -20 °C until purification using nucleomagbeads (Takara Bio USA, San Jose, CA, USA) in each well of the plate which was covered and placed on a microplate shaker at 1800 rpm for 2 min. Success of the PCR and purification was confirmed by agarose gel electrophoresis and purified amplicon DNA was stored at -20 °C prior to indexing and sequencing. Illumina sequencing barcodes were added to purified amplicons and purified as above. The Qubit fluorometer (Thermofisher, Waltham, Massachusetts, United States) was used to quantify amplified DNA following the manufacturers protocol.

To prepare the combined library, we pooled 50 ng of indexed PCR product from each sample. 2 µL were added from any samples that contained insufficient DNA to be read by the Qubit, along with the reagent control. If the Qubit reading gave a high enough DNA concentration where less than 1µL of the sample would have been added, a 1:10 dilution of the sample was made and added to the library in a larger volume. Libraries were denatured and loaded on to a 500 cycle V2 nano flow cell for sequencing on a MiSeq instrument (Illumina I, Inc., San diego, USA).

3.2.5 Bioinformatics

Processing of the demultiplexed ITS2 amplicon sequences and taxonomic assignment followed the previously published R script for the equine nemabiome (v2), which includes merging R1 and R2 reads and amplicon sequence variant (ASV) formation with DADA2 (Poissant, et al. 2020). For quality filtering, truncation quality was set to 2 with a minimum length of 200 base pairs. Taxonomic classification was assigned using IDTaxa and a curated equine parasitic nematode ITS2 database (v1.3) (Workentine et al., 2020). Additionally, to mitigate the possibility of failing to detect ITS2 sequences that were too long to provide adequate overlap of R1 and R2 for merging or in cases where poor quality R2 reads would prevent merging, we generated and classified ASV from the R1 and R2 reads separately by modifying the published pipeline after de-noising, skipping the merging step. Only the R1 feature table and classification results were used in subsequent analysis. For species assignment, with confidence thresholds of both 50% and 80% being reported in literature (Poissant, et al. 2020), we chose a confidence threshold of 50% in order to capture maximal diversity. To describe differences between the merged and unmerged reads, reads were merged by concatenating rather than overlap.

3.2.5 Data visualization and analyses

Feature tables containing read counts for each ASV in each sample were imported into Numbers version 11.1 (7031.0.102). For species level analysis, read counts of ASV with the same species identification were summed. Seqkit was used to determine amplicon sequence variant (ASV) lengths. The Maaslin2 package (version 1.12.0) in R (version 4.2.0) (Mallick et

al., 2021) was used for determining multivariable associations between horse variables (age, sex, region, and diet) and parasite species abundance. Maaslin2 was used to calculate significance using season and age as fixed effects and to plot the associations. Season was used as a categorical value (fall vs spring) and age as a linear continuous variable. When determining the relationship between FEC and species composition, age, sex, and season were fitted as random variables. Horse ID was set as a random variable, and treatment was a fixed effect, using the pre-treatment proportions as the reference.

Maaslin2 was used to investigate significant change in proportion of ITS2 sequences for species in pre and post treatment samples. Due to the low sample size (n=3) the q-value for significance was relaxed to 0.5 (default = 0.25) in order to obtain plots. Horse ID was set as a random variable, and treatment was a fixed effect, using the pre-treatment proportions as the reference.

3.3 Results

3.3.1 Sequencing outputs

3,414,361 raw read pairs from 95 samples (55 in fall and 40 in spring) were generated, ranging from 4 to 273,931 per sample, with a median of 27,457 per sample. Number of larvae per sample ranged from 12-6800 ($\bar{x}$ = 1489, $\tilde{x}$ = 800). Five samples with fewer than 1000 reads were removed from further analysis. Three samples collected after anthelmintic treatment were analyzed separately. The reagent negative control gave no reads.

3.3.2 DADA2 processing

After filtering and taxonomic assignment, the merged output gave a total of 2,290,847 reads, which formed 558 (ASVs) representing 34 unique species. The R1 pipeline assigned 3,183,624 reads to 1022 ASVs, corresponding to 39 species. Results from the two approaches were similar overall except that proportional abundance of ASVs for two species, *Cylicocyclus elongatus* and *C. radiatus,* were significantly higher (p = 0.00) across both seasons in the R1 vs merged reads. As well, neither *Cyathostomum tetracanthum* (with a confidence level of up to 54) or *Cylicocyclus elongatus* (with a confidence level of up to 76) were detected in the merged reads. Species belonging to the order *Rhabditida* were found in the R1 pipeline with high levels of confidence (>80%) at the taxonomic level but were not found in any merged output. When reads were concatenated instead of merged by overlap, *C. elongatus* was identified with a confidence level of up to 74.

3.3.3 Taxonomic assignment

At 50% confidence, 870 of the 1022 ASVs were assigned to 34 species from 14 genera across both sampling seasons (Figure 1). Proportions of ASV per sample that were assigned to species with 50% confidence ranged from 35.7% to 100%.

3.3.4 Nemabiome compositions

Each sample contained at least 3 and up to 20 assigned species (mean=12) (Figure 3.1). Each species was present in up to 89 (95%) samples. 31 species were identified in each season;

however, composition varied between fall and spring. *Strongylus edentatus, S. vulgaris*, and *Ostertagia ostertagi* were only detected in the fall, while *Gyalocephalus capitatus*, *Cylicodontophorus bicoronatus*, and *Cylicocyclus ultrajectinus* were only detected in spring.

Cylicocyclus nassatus (fall 30%, spring 22%), *Cyathostomum catinatum* (fall 30%, spring 22%), and *Cylicostephanus longibursatus* (12% in both fall and spring), had the highest average proportions of ITS2 reads per sample across both collection seasons. *Cylicocyclus nassatus, C. longibursatus, C. catinatum, Cyathostomum pateratum*, and *Cylicostephanus goldi* were the most prevalent species in both seasons, present in 86-100% of samples (Table 3.1).

Of the species identified, 28 were small strongyles. In addition, two species of large migratory strongyles were found on one farm in three different horses: *Strongylus vulgaris* and *S. edentatus* were each found once in separate animals, and once in a co-infection. *Strongylus equinus* was not detected in any samples. Three non-migratory large strongyles (*Triodontophorus nipponicus, T. serratus,* and *Craterostomum acuticaudatum*) were detected in a total of 45 samples and the cattle nematode *Ostertagia ostertagi* was detected in two samples. As well, three ASVs were assigned to three different species in the order *Rhabditida* with at least 80% confidence at the order level.

3.3.4.1 Post-treatment samples

Cultured L3s from three positive fecal samples (2 from the same herd) collected two weeks after owners reported orally administering 120 mg of ivermectin (and 600 mg of praziquantel) were sequenced. Number of ITS2 reads decreased in each species for all three samples. Both groups only included small strongyle species. 18 species were identified between the three samples before treatment, and seven after-*Cylicostephanus calicatus, C. goldi, C.*

longibursatus, Cylicocyclus ashworthi, C. nassatus, Cyathostomum pateratum, and *C. catinatum*. However, *C. catinatum* and *Cylicostephanus longibursatus* were the only species where proportions of reads increased after treatment (coefficients= 3.36, 2.26) (Figure 3.2) and other species reads were found in relatively small amounts.

3.3.5 Relationship of nemabiome composition to horse characteristics

Age and sex were known for 81 of the horses with nemabiome sequence data. The proportions of *Cylicocyclus insigne, Cylicostephanus minutus, Coronocyclus labiatus,* and *C. labratus* reads significantly decreased with age (p = 0.007, 0.021, 0.025, 0.048) (Figure 3.3). Seasonal differences include a significantly higher proportional abundance of *C. conoratus, Cylicostephanus calicatus,* and *Cylicocyclus radiatus* in spring compared to fall (p = 0.00, 0.00, 0.01). In addition to season, age and sex, but not region or diet, were significantly associated with abundance of individual ASVs. Age was negatively correlated with 69 ASVs and positively with 5 ASVs (Supplemental Table 3). ASVs having significant correlations with age included those from species *Coronocyclus labiatus, C. labratus, Cyathostomum catinatum, C. pateratum, Cylicocyclus ashworthi, C. insigne, C. leptostomum, C. nassatus, Cylicostephanus calicatus, C. goldi, C. longibursatus,* and *C. minutus.* In all 57 ASVs where a correlation was found with sex, mares were more likely to be infected than geldings. FEC was significantly associated with the proportions of reads of individual species, with a direct relationship between FEC and proportions of reads assigned to *Coronocyclus radiatus, C. labiatus, Cylicocyclus insigne, C. leptostomum, Cylicostephanus calicatus,* and *C. coronatus* (p = 0.00, 0.01, 0.00 ,0.00, 0.02, 0.04).

3.4 Discussion

3.4.1 Species composition and diversity

3.4.1.1 Small strongyle dominance and decline of large strongyles

This study represents the first time the nemabiome metabarcoding approach has been used on managed, working horses routinely exposed to owner administered anthelmintics. The dominance of small strongyles in our study is preferable to large migratory strongyles, which have increased pathogenicity. However, little is known about the pathogenic differences among species of small strongyles. DNA metabarcoding can now be used to determine which species of small strongyles are of the greatest concern for causing larval cyathostomiasis: mass emergence of hypobiotic L4 from the mucosa into the intestinal lumen, potentially resulting in malabsorptive diarrhoea, colic, and death (Corning, 2009). Therefore, molecular diagnostics are an essential tool to discriminate among small strongyle species, study their pathogenesis, and track emerging anthelmintic resistance.

We detected two large migratory strongyles at low prevalence and only on one property, including *S. vulgaris* which is of particular concern due to its ability to migrate through the cranial mesenteric artery, causing colic, verminous arteritis, and even death (Owen & Slocombe, 1985). However, no large strongyles were detected in any post treatment sample. This is inconsistent with other studies demonstrating higher prevalence of large strongyles in managed horse populations with mixed results on the effectiveness of treatment on large strongyles (Nielsen et al., 2012; Tydén et al., 2019).

3.4.1.2 High diversity relative to other populations

Our herds had a higher parasite diversity (34 species), most similar to nemabiome studies in feral horses in Alberta (31 species), which is closest geographically, as compared to those in Kentucky, USA (12 species), Sable Island, Canada (20 species), and Scotland (20 species) (Poissant et al., 2020; Sargison et al., 2022). However, the Alberta herd had a higher range of diversity within a single animal (max= 26) compared to ours (max=20). Comparing our results with those from unmanaged, untreated feral horse populations (Poissant et al. 2020) shows the effects of anthelmintics and other management practices on parasite diversity. In our study, cyathostomin reads dominated, similar to Kentucky, Alberta, and Scotland. In contrast, no cyathostomin species on Sable Island was responsible for more than 3% of the sequences.

Large migratory strongyles (*Strongylus* spp.) were only seen in 3.2% of our samples, and accounted for 0.15% of the fall, and none of the spring ASV counts. In contrast, *Strongylus* spp. were the primary parasites seen in Sable Island horses, representing 86% of all amplicons. The complete lack of *S. equinus* in our samples, compared to 50% of the sequences in the Sable Island herd, supports the theory that modern anthelmintics have virtually eradicated it from domestic horses. While present in lower numbers, *S. vulgaris* and *S. edentatus* sequences were still found in significant amounts in the unmanaged herds in both Alberta (9.5%, 7.8%) and Kentucky (11.1%, 9.4%). These herds, living in similar environments to ours and only differing in the care they receive, clearly demonstrate the effectiveness of anthelmintic treatment on Saskatchewan herds in the control of large strongyles.

3.4.1.3 Non migratory strongyles and other nematodes

Numerous parasites other than small strongyles and *Strongylus* spp. were present in our samples, including *O. ostertagi* (2 horses), *Triodontophorus nipponicus* (3 horses), *T. serratus* (3 horses) and *Craterostomum acuticaudatum* (6 horses). Commonly found in cattle, *O. ostertagi* was found in samples from a 7 year-old thoroughbred gelding, and a 5 year-old thoroughbred mare, both used for English sport riding. This species has also been reported in humans (Ghadirian & Arfaa, 1973) and is the cause of ostertagiasis in cattle, with epidemiological similarities to cyathostomiasis. The detection of ITS sequence of the cattle abomasal nematode *O. ostertagi* is enigmatic. The horses in question were not co-pastured with cattle and this parasite has not, to our knowledge, been described in horses. It is also possible that these ASVs represent another closely related parasite with identical ITS sequence to *O. ostertagi*, one of the challenges of using short, highly conserved regions for metabarcoding studies.

We did not detect any *Trichostrongylus axei*, a generalist nematode that can infect horses, ruminants, and humans (Themes, 2017). A similar study in horses in Scotland found seven *Trichostrongylus* species which can be explained by co-grazing with sheep (Sargison et al., 2022). *Triodontophorus nipponicus* and *T. serratus* are large non-migratory strongyles more often seen in thoroughbreds compared to other breeds (Saeed et al., 2019). However, out of the six horses positive for *Triodontophorus* spp. in our study, only one was a thoroughbred, four were quarter horses, and one was of unknown breed. *Triodontophorus nipponicus* is rare (Tolliver, 2000), previously reported a few times in Kentucky. *Craterostomum spp.* was identified in six horses, ages ranging from 1-21. Infected breeds included one warmblood, three quarter horses, one Morgan, and one Connemara. The clinical significance is unknown (Themes, 2017). Finally, we detected some ASVs in the order Rhabditida which likely represents

contamination of ground collected fecal samples with free-living nematodes in the environment, with the possible exception of *Strongyloides westeri*, which has a parasitic component to the life cycle and is pathogenic in foals. Unfortunately, this species' ITS2 sequence is not available.

3.4.2 Preliminary evidence for lack of effectiveness of owner administered treatment for small strongyles

This project is the first time that metabarcoding has been used to observe nemabiome composition before and after anthelmintic treatment in horses. Following owner administered treatment, lack of effectiveness was observed in three horses. This may signify early anthelmintic resistance and/or suboptimal deworming practices which may well lead to resistance (Chapter 2). Metabarcoding revealed that the species that survived treatment included *Cylicostephanus longibursatus* and *Cyathostomum catinatum*, among the most common small strongyles detected in the untreated population. Interestingly, the ASVs present in the post treatment samples differed from those in the pre-treatment population, which may indicate strains with reduced susceptibility to anthelmintics. Species specific anthelmintic resistance has been described, such as the reduced susceptibility to treatment of immature *Cylicocyclus* spp. in horses (Kooyman et al., 2016), and in ovine GI nematodes using the nemabiome metabarcoding methods (Queiroz et al., 2020).

3.4.3 Horse variables and impact on diversity

Horse sex, age, collection season, density, and FEC were all significantly associated with equine gastrointestinal community composition. The increased associations between unique

ASVs, indicating unique strains or species and increased overall increased diversity, in mares compared to geldings is surprising in light of the proposed male bias in parasitism of animals (Schalk & Forbes, 1997), thought to be caused by immunosuppressing androgens. Our male population was almost entirely composed of geldings, with low levels of androgens, which may explain this unusual result.

Infection intensity and diversity are expected to decrease with horse age (Becher et al., 2010; Fritzen et al., 2010). Interestingly, two dominant small strongyles showed different patterns. Proportions of reads assigned to *Cylicostephanus longibursatus* were highest in horses between the ages of 12 and 15, while *Cylicocyclus nassatus* decreased until the geriatric years (20+ years of age). This cannot be explained by spatial-niche competition (Stancampiano et al., 2010) since *Cylicostephanus longibursatus* inhabits the dorsal colon, while *Cylicocyclus nassatus* inhabits the ventral colon (Tolliver, 2000). Nemabiome methods open new opportunities to explore interspecies relationships among nematodes.

Variations between spring and fall ASVs could indicate seasonal epidemiological differences- those found primarily in spring may be more likely to over winter in hypobiosis, and those found in fall may be more resistant to heat and drought in summer. The presence of three species of small strongyles (*Gyalocephalus capitatus, Cylicodontophorus bicoronatus,* and *Cylicocyclus ultrajectinus*) uniquely in spring could be due to emergence after wintering as hypobiotic larvae. The two *Strongylus* spp. were likely acquired from pasture transmission, and if present at low abundance, fall would be the most likely season to detect their presence.

Horses living in a higher herd density were more likely to be infected with *Cylicostephanus goldi* ($p=0.006$). Due to the low FEC in horses living indoors, it was not

possible to compare species composition to those living on pasture. No relationship was found between diet and nemabiome composition.

3.4.4 Refining and applying the nemabiome approach

Modifying the DAD2 pipeline to examine only the R1 reads instead of merged reads resulted in detection of significantly more counts of *C. elongatus* and *C. radiatus* ($p = 0.00, 0.00$) across both seasons. Furthermore, merging resulted in loss of nearly half of the ASVs (1,023 down to 558) and the loss of several species IDs, including *Cyathostomum tetracanthum* and *Cylicocyclus elongatus*, across both seasons. The significantly longer average read length in the merged pipeline offers an explanation for this- the merging step requires a minimum of 20 base pairs of overlap between reads (Gaspar, 2018). This suggests that longer ITS-2 sequences, with shorter reads, are being lost due to the inability to merge. This is supported by the presence of *C. elongatus* in the results when forward and reverse reads were concatenated instead of merged by overlap in DADA2. In addition, the higher Guaninine/Cystine (GC) content (>50%) in reads from the order *Rhabditida*, compared to other reads in our study, may contribute to the loss of the order when merging. Previous work has demonstrated poor PCR efficiency and loss of species IDs when sequencing targets with very high or low GC content (Benjamini & Speed, 2012; Chen et al., 2013). As is typical with sequencing, the reverse strand will be more affected by such biases, further reducing its quality. In such a case, taxonomic assignment will be limited to the forward strand. Further work is clearly required to account for the variable length and content of the ITS2 region.

Finally, it is important to note that our methods (using forward reads only and 50% vs 80% confidence for taxa assignment) probably in part are responsible for the higher diversity

detected in our study compared to others. The decision to use the unmerged pipeline reflects our objective of describing the diverse nemabiome in our study population, which also drove our decision to use a 50% bootstrap support threshold. While less stringent filtering can increase the number of incorrect species assignments, it can also increase the number of correct assignments. In a clinical setting, the risk of a false negative diagnosis greatly outweighs the drawbacks of a false positive. An animal is not generally harmed by administering anthelmintic treatment, but not treating an animal infected with a pathogenic nematode can lead to poor health outcomes.

3.4.5 Conclusions and future work

For the first time, we have described the strongyle parasite diversity in Saskatchewan working horses, laying the framework for future improvements in parasite research and management. Differences in geography, horse breeds and uses, and management practices within the province of Saskatchewan made it difficult to conduct statistical analyses but did generate hypotheses on the links between these variables and horse parasite diversity that can be explored in future. We have described potentially emerging species-specific anthelmintic resistance to owner administered treatments and explored new ways to assess the ITS2 sequencing outputs from the equine nemabiome. This project is the first time the ITS2 DNA metabarcoding approach has been used to investigate parasite risk factors, management practices, and treatments and provides valuable information on species composition for future research on parasite pathogenesis and anthelmintic treatment effectiveness . This includes the expansion to other targets and into metagenomics to look for known resistance genes, such as the amino acid substitutions in beta-tubulin responsible for resistance to benzimidazoles (Beech et al., 2011),

and increased P-glycoprotein expression, observed in parasite species demonstrating ivermectin resistance (Turnbull et al., 2018).

Continuous efforts should be made to improve and refine the molecular methods, including re-evaluating our process of data handling after sequencing as demonstrated by our alternative pipeline. Furthermore, phylogenetic work on small strongyles will help to improve the accuracy of our database. Future work should focus on increasing the availability of molecular diagnostics for horse owners and veterinarians to effectively diagnose and treat large strongyle infections and follow up on the potential for species-specific anthelmintic resistance.

Author Contributions

Toni-Anne Saworski: Conceptualization, Methodology, Formal analysis, Investigation, Research, Resources, Data Curation, Writing- Original Draft, Project Administration, Software

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Figures

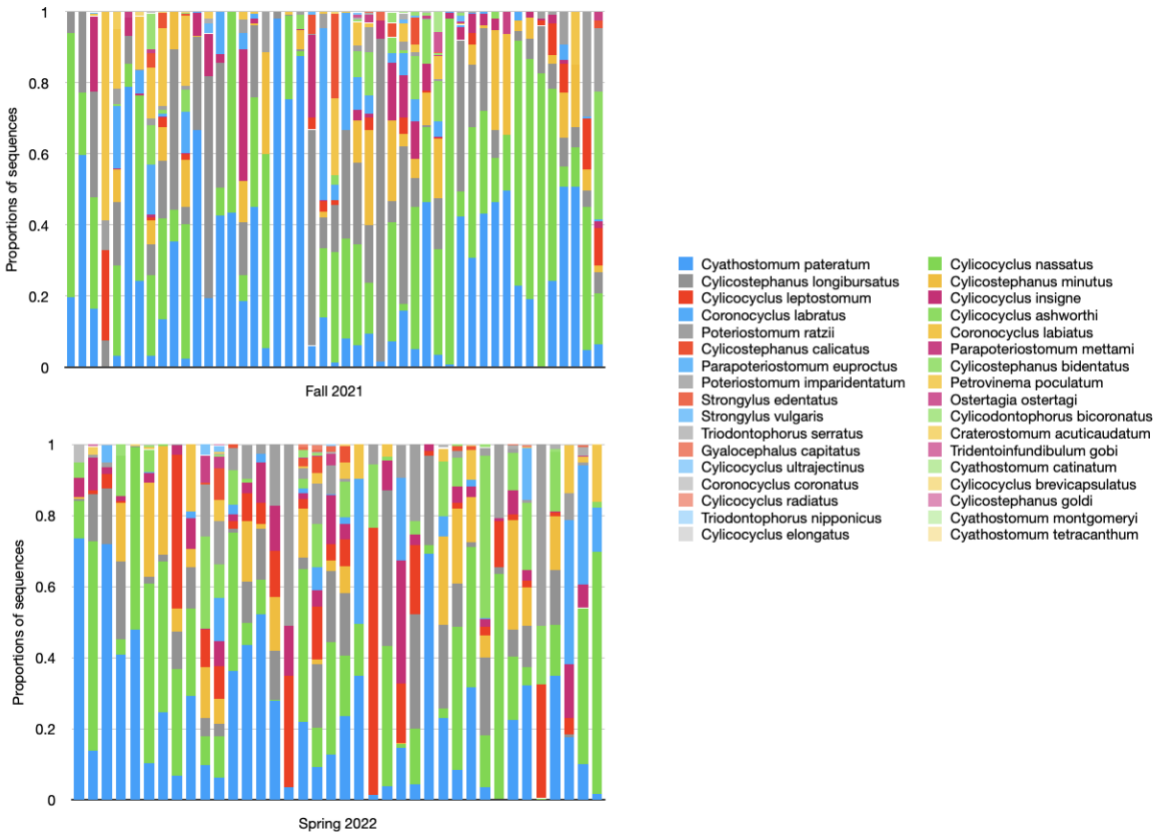

Figure 3.1: Compositions of nemabiomes of domestic Saskatchewan horse fecal samples collected in fall 2021 and spring 2022. Read 1 amplicon sequence variants were assigned to species using a 50% confidence threshold.

Table 3.1 Species prevalence. Percentage of samples containing at least one ITS2 read for each species at 50% confidence

	Fall	Spring
Cylicocyclus nassatus	85	90
Cylicostephanus longibursatus	85	93
Cyathostomum pateratum	85	95
Cylicostephanus minutus	82	90
Cylicocyclus insigne	82	93
Coronocyclus labratus	76	75
Coronocyclus labiatus	62	60
Poteriostomum ratzii	56	88
Cylicocyclus ashworthi	55	78
Cylicocyclus leptostomum	55	83
Cyathostomum catinatum	40	43
Parapoteriostomum mettami	33	35
Cylicostephanus calicatus	25	30
Craterostomum acuticaudatum	25	75
Cylicostephanus bidentatus	18	25
Parapoteriostomum euproctus	15	13
Strongylus edentatus	13	20
Poteriostomum imparidentatum	11	25
Strongylus vulgaris	11	5
Ostertagia ostertagi	10	0
Cylicostephanus goldi	4	10
Petrovinema poculatum	4	10
Cylicocyclus brevicapsulatus	4	3
Triodontophorus serratus	4	3
Cylicodontophorus bicoronatus	4	0
Gyalocephalus capitatus	4	15
Coronocyclus coronatus	2	8
Cylicocyclus radiatus	2	33
Cyathostomum montgomeryi	2	3
Cyathostomum tetracanthum	2	0
Cylicocyclus ultrajectinus	2	5
Cylicocyclus elongatus	0	3
Triodontophorus nipponicus	0	3
Tridentoinfundibulum gobi	0	5

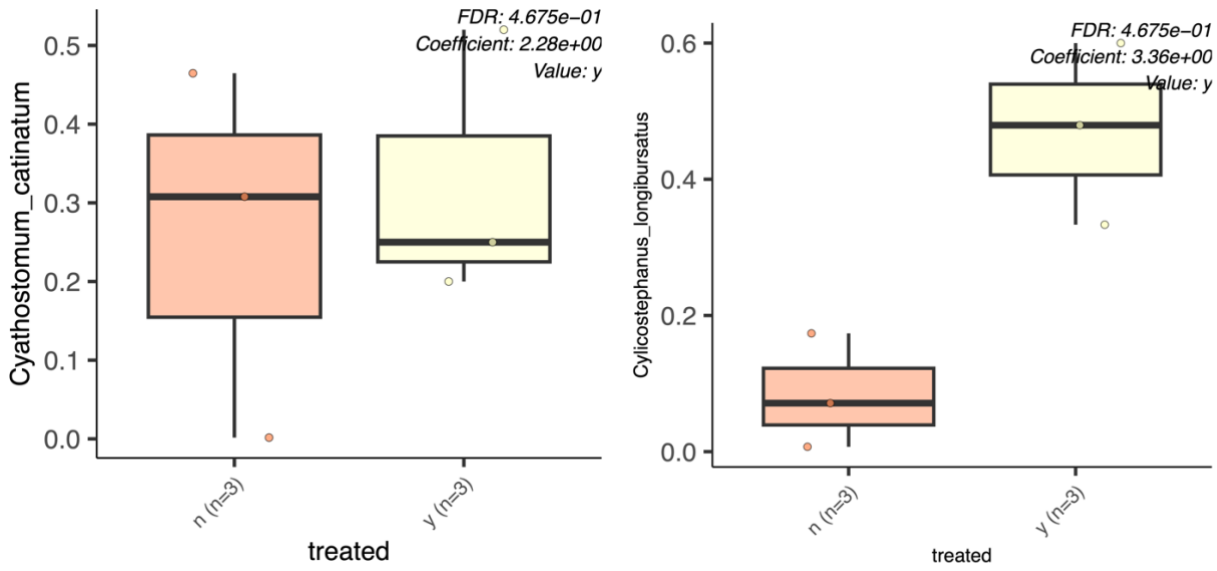

Figure 3.2 Average proportions of ITS2 reads assigned to two small strongyle species in three horses before (n) and 2 weeks after treatment with oral ivermectin (y).

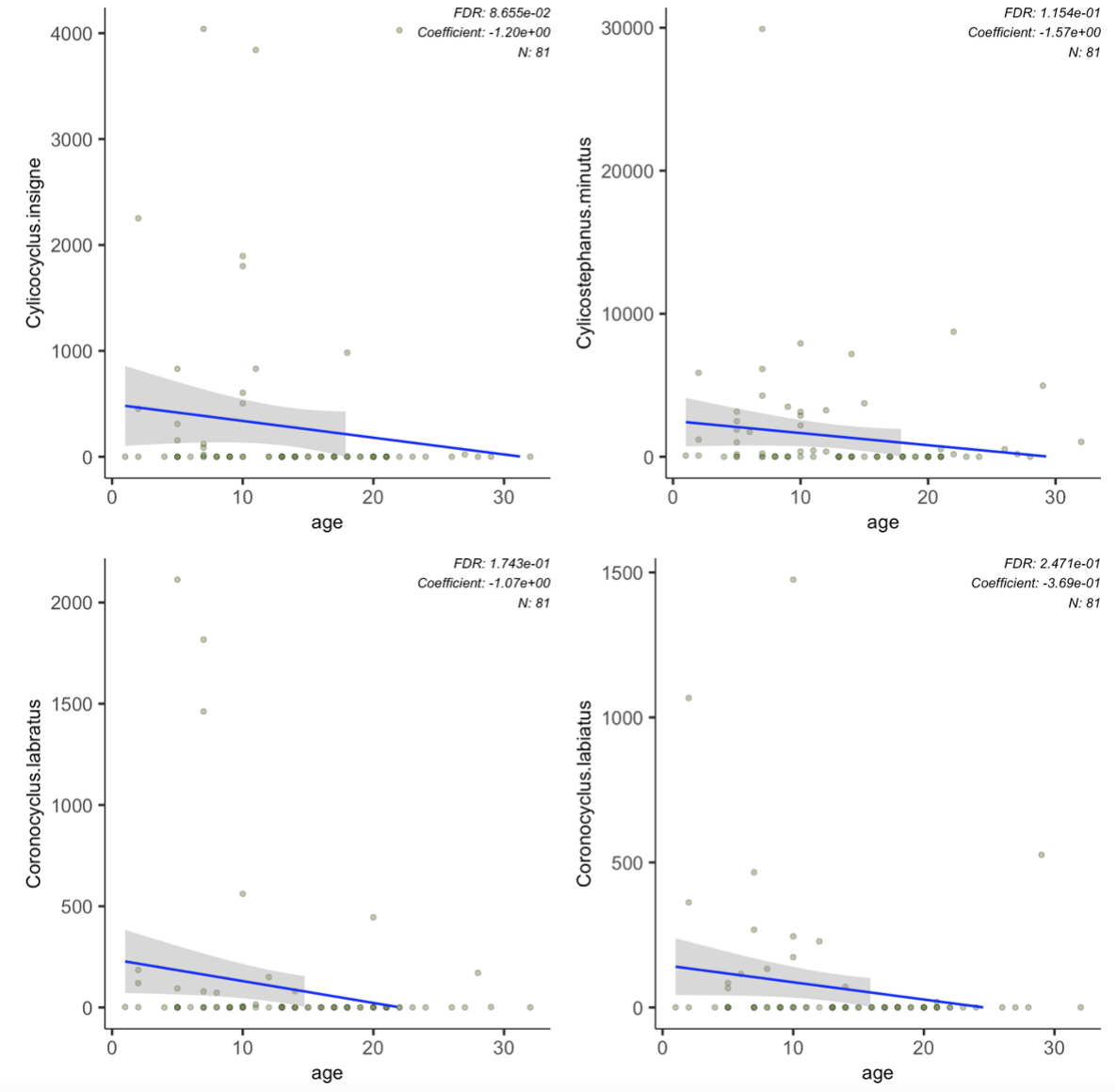

Figure 3.3: Relationship of abundance of small strongyles and horse age. Graphs and p-values were generated using Maaslin2 in R with age as a fixed effect in 81 samples. The blue line is the smoothed relationship of y (proportions of species reads relative to the animal's entire nemabiome) proportional to x (age). Dark grey area indicates the confidence threshold (95%)

Chapter 4: Discussion and Conclusions

This thesis investigated prevalence and fecal intensity of gastrointestinal nematodes infecting Saskatchewan working horses, and for the first time in North America, applied cutting edge DNA techniques to describe gastrointestinal nematode diversity in managed domestic horses. We also gained insight into demographic and management variables that affect the intensity and diversity of an individual animal's parasite community and created a record of current management practices within the industry. We also demonstrated lack of effectiveness of owner administered anthelmintics in a small proportion of horses, which may signify early anthelmintic resistance and/or suboptimal deworming practices which may well lead to resistance.

4.1 Parasite epidemiology

Understanding the relationship between individual horse variables, seasonal changes, FEC, and nematode diversity allows us to work towards a more targeted parasite management plan- both in deciding which horses to target, and how to manage their infections with pharmaceuticals as well as management practices that can reduce exposure to infective parasites.

Previous descriptions of the effects of seasonal changes on FEC have varied, with very few having been done in North America. In Kentucky, previous work demonstrated no statistical differences in parasite egg shedding across seasons (Steuer et al., 2022). However, this region does not experience the same cold winters as in northern regions. Locally in Saskatchewan, strongyle FEC has been shown to peak in July and August, and remain high into September in adult horses (Misuno et al., 2018). In our study, seasonal differences include a significant increase in *Coronocyclus conoratus, Cylicostephanus calicatus,* and *Cylicocyclus radiatus* reads

in spring (p = 0.00, 0.00,0.01) and the prevalence and intensity of strongyle shedding was significantly higher in fall than in spring. Large migratory strongyles, *S. vulgaris* and *S. edentatus,* were only found in fall, and not spring.

Ambient temperature in the summer in Saskatchewan compared to warmer climates could explain differences in peak shedding times. When temperatures exceed 29ºC, in the absence of nutrient ingestion, L3 die on pasture (Nielsen et al., 2007). As such, Saskatchewan horses are more likely to continue to be exposed to infective stage larvae throughout the grazing season than those living in warmer climates. At temperatures below 7 º C, which are well established in Saskatchewan winters, strongyle eggs will not hatch and larvae will not develop until warmer temperatures (Kuzmina et al., 2006). This results in the lower FEC seen in early spring. Strongyle species found in higher proportions in spring compared to fall could likely represent those more likely to enter hypobiosis, emerging in spring after overwintering in the horse. Likewise, the fact that *Strongylus* spp. do not encyst supports their absence in spring, and their longer pre-patent period means that if these parasites are acquired in August, they will not begin to shed eggs for up to a year.

4.2 What was not found

Our results were compared to the few other published equine nemabiome studies, from feral horses on Sable Island, Alberta, and untreated domestic horse herds in Kentucky (Poissant et al. 2020) and Scotland (Sargison et al., 2022), to compare among regions and to explore the effects of anthelmintics and domestication on parasite diversity. Most notably, large migratory strongyles (*Strongylus* spp.) were the primary parasites seen on Sable Island, representing 86% of all amplicons. In contrast, *Strongylus* spp. were only seen in three fall samples in horses in

Saskatchewan in our study and was not found at all in spring. While *S. equinus* was the most common species found on Sable Island, we did not find *S. equinus* in our study, consistent with lack of detection in most North American/European horses (Jürgenschellert et al., 2022; Nielsen, Baptiste, et al., 2010), supporting the theory that modern anthelmintics have virtually eradicated this large strongyle from domestic herds.

Trichostrongylus axei can infect humans, ruminants, and horses (*Trichostrongylus axei* Infection in Horses - Digestive System, 2019), but we did not detect it in any horses in our study. This parasite is more commonly found in horses co-pastured with cows. Its absence could therefore be due to lack of exposure to cattle, but also because our method relied on eggs that could be easily cultured in vitro. Previous work has clearly shown that despite extensive efforts, *T. axei* does not culture well in laboratories (Leland, 1963). In cattle, this parasite has demonstrated its ability to overwinter in air temperatures as low as -32.5 °C in western Canada (Wang et al., 2020). This suggests that the absence of this parasite in our study may not be due to its lack of presence in our climate but was quite possibly due to its inability to culture. This is further supported by the considerably small number of amplicons from this species present in previous equine nemabiome studies (Poissant et al. 2020).

4.3 Horse management

Housing affected egg count – stabled horses (N=7; $\bar{x}$ FEC= 998 EPG) had a lower egg count than those living outside (N=54; $\bar{x}$ = 4) (p = 0.014), and higher counts were observed in owners reporting infrequent feces removal from pens and stables. Density-dependent transmission was also observed, with FEC increasing with additional horses in a horse's turnout pen. These results, along with the clear differences seen among different populations in our

study, clearly indicate that horse factors and their management can greatly affect parasite load and diversity.

4.4 Individual horse tolerance and susceptibility

FEC range (0-4475 EPG) was wide in our study population, which is not surprising given what we know about aggregated distribution of parasites within host populations and reports from other equine studies (Carstensen et al., 2013; Lester et al., 2018; Jenkins et al., 2020). As mentioned, the highest FEC in our study was from fall 2021 at 4475 EPG- more than eight times higher than the mean across both seasons- recorded from a 5 year-old competition gelding in ideal body condition and on a high nutritional plane (Figure 4.1). This greatly contrasts with the situation in feral horses on limited forage, where a negative correlation between FEC and body condition was recognized (Debeffe et al., 2016). Inter-individual variation of FEC is also linked to genetics (Kornaś et al., 2015). While age will typically play a role in FEC, a study in Welsh ponies found their cyathostomin susceptibility level to remain consistent over a period of 10-years and identified new biomarkers to indicate the need for treatment (Sallé et al., 2021). A better understanding of these phenomena could predict individual horse susceptibility to infection and allow more individualized treatment plans.

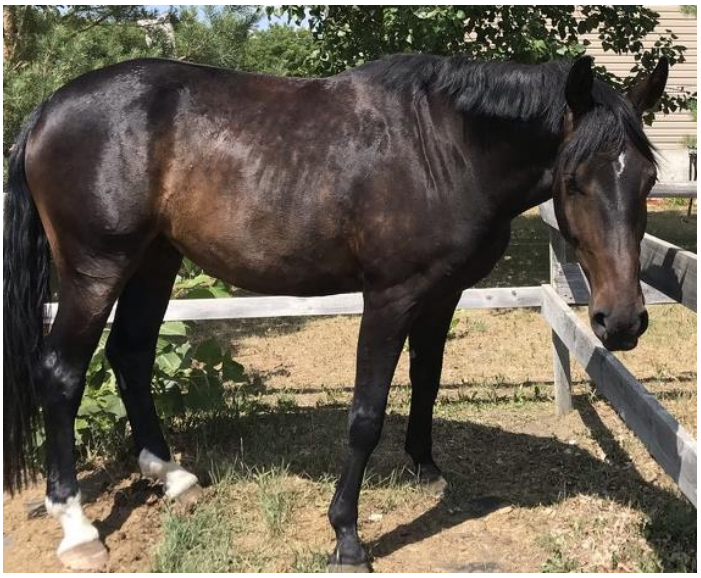

Figure 4.1: A healthy horse in peak training with a FEC of 4475 EPG, eight times the mean in our current study

4.5 Horse owner education

Responses to our survey varied and the volunteers likely represent horse owners with an interest in parasite research; owners ranked parasitism relatively highly compared with other health concerns (4.5/10). We report that only 23% of local horse owners use FECs to decide when to treat, and 45% of horse owners have never ordered a fecal egg count. Currently, many horse owners are not aware of what kind of dewormer they are giving their horses, which makes efforts to track drug specific anthelmintic resistance impossible.

The comments received on the open-ended responses varied and many expressed genuine interest in learning about parasites and their management. Many horse owners reported using a "good" dewormer, "whatever was available", "whatever's cheapest", or a "different coloured box" for anthelmintic treatment. Some also reported using whatever was available due a lack of access to dewormers following the start of the COVID-19 pandemic. Non-traditional methods of treatment were reported, such as administering injectable ivermectin orally, using Calvenza as a

dewormer, only deworming under a full moon, and breaking up a deworming dose over a week. Many owners indicated that they would like more access to FEC testing, and increased availability of information on evidence-based treatment and expressed genuine concern for horse welfare and the lack of access to FECs.

Throughout the project, we frequently received questions and comments from horse owners about their horses having been diagnosed with "verminous arteries" following an appointment with an equine osteopath. Diagnosis is not made on the basis of FECs or visualization of damage to any arteries, but by the observation of reduced spinal mobility on one side. The recommended treatment they received after diagnosis was to administer a double dose of Panacur for 5 days, then give Quest, followed by Quest Plus. We also had the opportunity to discuss this with veterinarians, one of whom has been following up on these cases to ultrasound for arterial damage and has found no evidence of these infections. In our study, *S. vulgaris* was only found on one farm in very few horses. Our results do not support its widespread transmission and we sampled at times when they should be shedding eggs- in spring, six months after the last pasture exposure (the pre-patent period), and in fall during peak strongyle egg shedding.

The responses received from horse owners only serve to emphasize the dire need to disseminate these results and improve the quality, accessibility, and comprehensibility of information on anthelmintic use and deworming protocols.

4.6 New deworming guidelines

Currently, AAEP guidelines are moving towards a more targeted approach to treat each animal as an individual, given the wide range of FECs in adult horses (AAEP). However, an "acceptable" limit of strongyle EPG based on FEC for horses remains a debate. Goals of a parasite control program are:

- to minimize risk of disease,
- control parasite egg shedding,
- and maintain efficacious drugs and avoid future anthelmintic resistance.

Targeted selective treatment is based on knowing the FEC of individual horses in order to make a decision for deworming based on their shedding level. Without knowing individual FECs or what is "normal" in a region or equine population, it is difficult for veterinarians to advise horse owners on the need for treatment of an individual or group of horses. In Saskatchewan, this is problematic as only 23% of our survey respondents use FECs, and the mean in our study population is much higher than what would be considered high in an individual horse (500 EPG) using the AAEP guidelines, even in spring when egg counts were lower. Furthermore, traditional egg based diagnostic methods cannot identify large migratory strongyles such as *S. vulgaris*, known to migrate through the cranial mesenteric artery causing colic and verminous arteritis (Owen & Slocombe, 1985). While there are PCR tests for *S. vulgaris* (Nielsen et al., 2021), these are not locally available. Regardless of how well supported and intentioned any guidelines are, they cannot be effective in controlling parasites and protecting anthelmintic efficacy if the information is not disseminated to horse owners and if diagnostic and management tools are not easily accessible.

Seasonal variations in parasite egg shedding are typical and FECs may be further unreliable when parasites reduce egg shedding in cold winter months or hot, dry summers (Poynter, 1954)- both typical in Saskatchewan, yet our high FECs indicate that parasites are thriving here. Therefore, guidelines should give clear recommendations on seasonal timing of sampling to ensure accurate diagnosis and reduce false negatives. Strongyle shedding in Saskatchewan has been shown to be consistent July to September (Misuno et al., 2018), indicating that these would be ideal times to test horses. Further emphasis should be placed on region-specific environmental management – for example, leaving a pasture in Saskatchewan to sit over the winter may not be effective in reducing strongyle transmission, as infective larvae can live up to 9 months in cold weather (Nielsen et al., 2007). Furthermore, previous experiments have demonstrated that infective larvae can survive Saskatchewan winters (Polley, 1986). Instead, horse turnouts should have manure removed daily if possible, or should be harrowed in the summer months when it is hot and dry to expose strongyles to heat and sun, an effective combination.

Current AAEP guidelines suggest treating only medium-high shedders to maintain refugia; in our population, that would mean treating 57% of our horses every fall. We recommend instead treating, with effective drugs determined by farm through FECRT, the top 30% of horses which would reduce overall egg shedding by over 80%. Our study provides evidence to regionally refine guidelines for our study area i.e., a high shedder in SK might be 1000 eggs per gram of feces instead of 500. Timing is important for the goal of maintaining refugia- anthelmintics should be given to head off pasture contamination ion spring and/or in late summer/fall when egg shedding peaks and to kill small strongyle larvae before they encyst for the winter. This is preferable to treating in winter when pasture transmission is not occurring

(during the pre-patent periods) which results in a higher selection pressure for resistant worms (Singh & Swarnkar, 2008). However, additional research is still needed to determine optimal levels of refugia to balance horse health and anthelmintic efficacy i.e., a skinny or unhealthy animal with low FEC should still be treated.

Still, the differences in pathogenicity between the common cyathostomins and the migratory species of strongyles are not addressed using the current diagnostic methods. As such, individual owner risk tolerance must be discussed with the veterinarian and considered when deciding on treatment strategies- the owner of a pastured companion horse may tolerate more risk than the owner of a high-performance horse that provides a source of income.

Future work should include ways to increase access to continuing education on best practices in parasite management in the horse industry and support advancements in diagnostics, such as PCR for "high-threat" species such as *S. vulgaris* and anthelmintic resistant small strongyles. Suggestions could include publishing in layman's journals, presenting to horse federations, and updating the educational material used to teach young horse owners, such as the Equestrian Canada and Canadian Pony Club handbooks.

4.6.2 When the FEC isn't easy

When FECs are not easily accessible due to geographical locations, time constraints, or owner finances, our study identifies other factors that should be considered when deciding which horses to treat (i.e., young horses, older mares) and management practices that reduce FECs, such as limiting the number of horses in a turnout, removing feces regularly, and living in stalls. On a large farm with mixed housing methods, only those horses living in herds outside may need intervention. This is especially true for strongyles (versus ascarids), as their transmission occurs

primarily through pastures rather than in stalls (Nielsen, Fritzen, et al., 2010). Treating or separating groups of young and old horses from the rest of the herd, especially young geldings (<8 years) and older mares (>16 years), can reduce egg transmission between animals in pastures. Selective treatment based on individual horse factors (ideally along with FECs) should still be advised over treating all animals in a herd, which has proven positive correlation with drug resistance and reduced anthelmintic efficacy (Singh & Swarnkar, 2008).

4.7 Anthelmintic resistance genes

Future work is also needed to identify genes responsible for anthelmintic resistance; for example, targeting the small strongyle species that we identified as surviving owner administered treatments including *Cylicostephanus calicatus, C. longibursatus, C. goldi, Cylicocyclus ashworthi, C. nassatus, Cyathostomum pateratum,* and *C. catinatum*. Specific amino acid substitutions in beta-tubulin is one of the sequences responsible for resistance to benzimidazoles, and sequence substitutions in ion channels can lead to macrocylic lactone resistance (Beech et al., 2011). Especially concerning are the changes in gene expression associated with resistance to multiple drug classes (Lespine et al., 2011). Increased P-glycoprotein expression, which functions as an anti-absorption mechanism, can limit drug access to the site of action within the parasite, and has already been observed in numerous parasite species demonstrating ivermectin resistance (Turnbull et al., 2018). Decreased ion-channels and loss of receptors can remove the drug target all together (Wolstenholme, 2011). We have identified multiple ASVs belonging to the same species of nematodes, possibly representing different strains. By increasing our sample size and using whole genome sequencing on any strongyles that survive anthelmintic treatment, we could identify which strains or species are of the most concern, examine their genetics, and choose more effective treatment methods.

4.8 Strengths, limitations, and future directions

This study follows the previous groundwork laid by Misuno et al. (2018), who initially investigated strongyle and ascarid egg shedding in Saskatchewan horses on two breeding farms more than a decade ago, serving as a baseline for comparison. As an active member of the equine community, I was able to expand on this work by using my connections to reach a large number of horse owners from across the province. A strength of our study was the ability to use my connections to collect detailed information on each horse, which we could then relate to their FEC and GI nemabiome composition.

Eggs were collected across two shedding seasons (spring and fall) to address seasonal shedding patterns (Steuer et al., 2022). At each farm, fecal samples were collected both at times convenient for the owners and when the horse was observed defecating, increasing participation, and ensuring accurate sample identifications. Still, we were unable to control the effects of circadian rhythm on FEC results or day to day differences in FEC. Within-day variation seems less of a concern (Carstensen et al., 2013;Lester et al., 2018), and while variation exists day to day in FEC over a single season, the majority of horses still remain in the same treatment category regardless of their sampling time (Warnick, 1992). Further work capitalizing on nemabiome methods could investigate species-specific variations in shedding within and between days.

By having the owners administer the treatment with their usual choice of drug, we were able to examine the true effectiveness of deworming programs in our communities. However, because of this, we cannot be sure if each animal received an appropriate dose of the drug, so our observed lack of efficacy cannot be defined as resistance but rather effectiveness of real-life practices.

Fortunately, previous work by Poissant et al. (2020) had already validated the DNA metabarcoding methods to characterize the equine nemabiome to ensure the accuracy and repeatability. We capitalized on this pioneering work and were able to compare multiple bioinformatics pipelines to analyze our data. Keeping in mind our goal of describing the local equine GI parasite community, along with considering the risks of a false negative diagnosis of more pathogenic species in a clinical setting, we chose to only evaluate the forward reads in our bioinformatics pipeline, compared to merging the forwards and reverse, resulting in greater species and ASV diversity. However, there is still much to be done in the field. Evaluating how our data is handled and updating reference databases to improve their reach and accuracy will allow for more precise identifications and further analyses of these reads.

4.5 Final conclusions

To my knowledge, this is the largest study done on the equine nemabiome, and the only one to be performed in domestic, managed horses. Combined with our FEC and survey results, we have provided a real world, applicable picture of what is actually happening in our horse community; a useful tool for future research and treatment plans.

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