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The Surveillance of Gastrointestinal Parasitic Nematodes of Northwest Arkansas Dairy Cattle Using Traditional and Genetic Parasitological Identification Procedures

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The Surveillance of Gastrointestinal Parasitic Nematodes of Northwest Arkansas Dairy Cattle Using Traditional and Genetic Parasitological Identification Procedures

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by

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ABSTRACT

Traditional and genetic parasitological identification procedures were compared using natural and artificial nematode parasite infections in Holstein steer calves. The traditional parasitological procedures measured fecal egg counts, coprocultures with subsequent larval collection and adult nematodes collected at necropsy. The genetic identification procedures measured ITS-2 sequences extracted from different stages of nematode development: raw feces, concentrated nematode eggs, third stage larvae and adults. The primary nematodes observed were *Cooperia oncophora*, *Cooperia punctata* and *Ostertagia ostertagi*. The traditional techniques were not significantly different from one another, while the genetic sequencing showed variation amongst the different procedures. The raw feces sequences showed the most variation, displaying a wide array of sequences from nematode species that were not necessarily found in the other genetic procedures. There was good correlation between the traditional and genetic procedures as a whole, leading to the conclusion that traditional parasitological identification techniques are sufficient for the identification of parasitic nematodes of cattle.

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DEDICATION

It is with a genuine gratefulness and the greatest respect that I dedicate this work to my mother. She instilled in me the love of science and adventure, and gave me the support and strength to pursue both. Without her inspiration, my life would be empty.

I love you, Mama.

“A mother is the truest friend we have, when trials heavy and sudden, fall upon us; when adversity takes the place of prosperity; when friends who rejoice with us in our sunshine desert us; when trouble thickens around us, still will she cling to us, and endeavor by her kind precepts and counsels to dissipate the clouds of darkness, and cause peace to return to our hearts.” – Washington Irving

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1.1. Introduction

Evaluation of gastrointestinal parasitisms in cattle is an important factor in the production of healthy animals. Cattle with untreated internal parasitisms demonstrate reduce grazing time, forage intake and weight gain (Forbes *et al*., 2000), making surveillance of parasite burdens a vital aspect of the livestock industry. It has been established that co-infection of a singular host animal by multiple parasite genera and species is a common occurrence with natural infections (Viney & Graham, 2013). The parasitological techniques used to determine the presence and magnitude of parasite burdens have remained relatively unchanged for nearly a century, though genetic identification methods have only recently begun to emerge. Though this emergence of genetic identification has a promising future, the data acquired are limited to only being able to detect the presence of parasites, not the actual magnitude of the burden or burdens. These techniques also are somewhat unavailable to livestock producers, especially those who do not have a large operation. The techniques are costly and laborious, reinforcing the conjecture that traditional parasite surveillance techniques are still relevant. In this investigation, the two parasitological evaluation techniques (traditional vs. genetic) are compared and contrasted in order to determine the validity and practicality of traditional methods.

1.2. Traditional Parasitological Identification/Quantification Methods for Gastrointestinal Nematodes

Though human knowledge of parasites can be traced to ancient civilizations, the assessment of gastrointestinal nematode parasitisms in live animals has remained relatively unchanged since the standardization of the process in the early 20th century, though modifications to the procedure have been incorporated (Verocai & Chaudhry, 2020). Prior to this standardization, there were

many varying methods that parasitologists used to evaluate internal parasitisms in live animals. One popular method used by many scientists, that is still used today, was a simple fecal smear to determine the abundance of gastrointestinal parasite eggs in fecal samples (Stoll, 1923). Brazilian physician and epidemiologist, Adolfo Lutz ("Father of Tropical Medicine") was interested in making contributions to medical geography and published studies on *Ancylostoma duodenale* ("Old World Hookworm") and other important parasites that caused disease in humans, including *Strongyloides* (Benchimol, 2004). In 1885, Lutz developed a method of homogenizing 1-part feces with 3-parts water and counting 3 drops on a microscope slide (Looss, 1911). This method was deemed to be flawed due to the homogenate being too concentrated for accurate pipetting and counting (Stoll, 1923). German researcher Otto Leichtenstern, who contributed to the research on *Ancylostoma duadenale*, expanded on Lutz's method and developed a more accurate fecal evaluation method for assessing an internal parasite infection in the late 19th century. He came close to an answer in his attempt to standardize his method by weighing the feces (3-5 grams, weighed to the third decimal place) and measuring the amount of water (100-150 mL) that went into his homogenate. From this homogenate, 4-8 drops were placed on a microscope slide and the nematode eggs were counted (Looss, 1911). This method was deemed inaccurate due to the variation of feces measured from each fecal sample and the variation in the amount of water used (Stoll, 1923). A key piece of the puzzle came in the early 1900s, when American pathologist and bacteriologist, Samuel Taylor Darling replaced the water with brine in the homogenized sample. This introduction of a brine created a medium with a higher specific gravity than the helminth eggs, allowing for the separation of the eggs and the debris in the fecal sample, and thus, uniformity upon replication (Darling, 1922).

In 1923, the American parasitologist, Norman Stoll published the first paper using quantitative data on fecal egg counts from work he was conducting on human hookworms (Stoll, 1923). Stoll was a professor of medical research at Rockefeller University and conducted extensive research on the effects that parasites such as *Trichinella* spp. and *Necator americanus* had on people. Stoll's fecal flotation method, dubbed "Stoll Dilution Egg-Counting Technique", brought standardization to the estimation of gastrointestinal parasite infections using fecal samples. The method was adopted worldwide and is the basis for the current methods of fecal egg counting in parasitology. Stoll's 1930 complementary publication on the estimation of sheep nematodes was paramount in bringing this new quantitative diagnostic technique for internal parasites into the world of veterinary medicine (Stoll, 1930).

A significant modification was made in 1939 to Stoll's fecal egg counting technique by an Australian parasitology lab that processed a high volume of sheep fecal samples daily in order to assess parasite infections. Researchers H. V. Whitlock and Hugh Gordon were looking to streamline the fecal egg counting process by building on past methods, and, accordingly, developed a special microscope slide that did just this (Whitlock and Gordon, 1939). With some modifications that would arise by 1948, Whitlock eventually developed the Modified McMaster fecal egg counting technique and microscope slide that is the foundation for the varying techniques that are still used today (Whitlock, 1948). The Modified McMaster method is currently one of the most widely-used fecal egg counting techniques, regardless of the lack of sensitivity when detecting lower numbers of nematode eggs (Mes, 2003). It is advocated by the World Association for the Advancement of Veterinary Parasitology (WAAVP) for evaluating the efficacy of anthelmintic drugs in ruminants (Wood *et al*., 1995).

Since the standardization of fecal analysis for nematode eggs, researchers have made modifications to the Stoll's fecal egg counting technique, namely the Wisconsin flotation method (Cox & Todd, 1962). The Wisconsin flotation method can be achieved by using either a passive or a centrifugation technique. Passive flotation is the simplest of the procedures, and involves mixing a small amount of feces with flotation media, homogenization, straining and placing the filtrate into a container, typically a centrifuge tube. The container is brought to full volume using flotation media to form a slight positive meniscus and topped with a cover slip. The homogenate is allowed to sit for a certain amount of time to passively allow the buoyant nematode eggs to float to the top of the tube, after which the nematode eggs adhered to the slip are examined and enumerated. Passive flotation is essentially Stoll's method with modern flotation media. Direct flotations using centrifugation is the same technique as a passive flotation, only using an enhanced separation of nematode eggs from the fecal debris using high specific gravity coupled-with the centrifugation. The introduction of centrifugation added increased accuracy to the fecal egg counts (Dryden *et al*., 2005). The FLOTAC method is another modification to Stoll's method that has been recently utilized in veterinary parasitology. This flotation method uses centrifugation, as well as a specialized, chambered apparatus, called the FLOTAC, in order to determine the number of parasite eggs and oocysts present in a fecal sample. The FLOTAC protocol is somewhat complex and involves several centrifugation steps before analysis can occur (Cringoli, 2006).

Researchers also have made modifications to the McMaster method since its introduction to the field. These modifications include using different starting weights of feces, different volumes of flotation media, presence or absence of centrifugation, different centrifugation times and speeds, different flotation media, flotation length and total number of "sections" of the slide counted

(Pereckiene *et al.*, 2007). Regardless of method used to extract the nematode eggs, centrifugated samples result in a higher sensitivity than the non-centrifugated samples. However, in the absence of a centrifuge, which is a costly apparatus, McMaster fecal egg counting without centrifugation is a comparable substitution for the determination of internal helminthic parasitisms when compared to the Wisconsin Flotation Method (Pereckiene *et al*., 2007).

It should be noted that many inherent factors can influence the results of a fecal egg count, i.e. worm biology (species, prepatent period, fecundity, helminth populations), host physiology and immune status/competence, the time of the year, partial effect of anthelmintic use, etc. (Lyndal-Murphy, 1993). All of these are factors that must be considered when any fecal egg count is obtained. Additionally, fecal egg counts must be done with a competent, precise technique; a factor that may vary person to person.

Building on the brine introduced in the Stoll method, researchers also have exploited different media to utilize the buoyancy of nematode eggs. A few common flotation solutions include magnesium sulfate, zinc sulfate, sodium nitrate solution and Sheather's sucrose solution. All of the listed solutions are effective, easy to use, readily available and relatively inexpensive. However, failure to ensure the flotation media has the proper specific gravity for the type of parasite eggs under surveillance can result in flawed recovery of the target eggs (Dryden *et al*., 2005).

1.3. The Rise of Genetic Sequencing

The molecular structure of deoxyribose nucleic acid (DNA) was completed in 1953 by James Watson, Francis Crick and Rosalind Franklin, and has since been likened to the invention of the internet in its importance to the progression of the human race (Watson & Crick, 1953). This

discovery, though monumental, was limited to the molecular framework. Sequencing DNA was difficult, as known sequencing strategies used for proteins did not apply. Initial focus on sequencing nucleic acids was directed at relatively pure RNA (ribonucleic acid) preparation, such as the genomes from single-stranded RNA bacteriophages or microbial transfer RNA (Holley *et al*., 1961). These nucleic acids were advantageous to start with due to the ability to mass-produce them via culture, the lack of a complementary strand and their considerably shorter length than eukaryotic DNA. Another advantage to starting with RNA was RNase enzymes were already available and could be utilized; progress, however, was still slow (Holley *et al*., 1961).

In 1965, Fred Sanger and his colleagues developed a radiolabeled, two-dimensional fractionation method that allowed for the development of a pool of ribosomal and transfer RNA sequences that were available to researchers (Sanger *et al*., 1965). In 1972, using this method, the Walter Fiers' laboratory produced the first complete protein-coding gene sequence; the protein coat of a bacteriophage (Min-Jou *et al*., 1972). This same lab sequenced the complete genome of the bacteriophage in 1976 (Fiers *et al.*, 1976).

In 1977, Sanger and his colleagues at Cambridge University, and their competitor, Allan Maxam and Walter Gilbert at Harvard, developed the first generation of DNA sequencing technologies (Sanger *et al*., 1977; Maxam & Gilbert, 1977). The Sanger sequencing method ("chain termination method, dideoxynucleotide") uses one strand of double-helix DNA as a template to be sequenced and elongated using chemically modified nucleotides called dideoxy-nucleotides. Once the elongation process is completed, the DNA fragments are sorted using gel electrophoresis (Kchouck *et al*., 2017). Maxam-Gilbert sequencing ("chemical degradation method") involves chemically cleaving nucleotides via chemicals creating marked fragments that

can be separated; a technique that has the most success when working with small nucleotide polymers (Kchouck *et al.*, 2017). These sequencing techniques have low-throughputs, relatively short read-lengths of only 1000 base pairs (bp), and high costs of operation.

Despite the limitations, given the accuracy of sequencing (99.999%), the Sanger method sequencing technologies were utilized by researchers and biologists until 2005, when Second Generation Sequencing (SGS), or Next Generation Sequencing (NGS) technologies became available (Qiang-long *et al*., 2014). These technologies have a high-throughput capability of sequencing millions to billions of parallel reads from multiple samples in a single run, a reduced run-time (hours as opposed to days) and a reduced overall cost of operation (Kchouck *et al*., 2017). Next Generation techniques are divided into two approaches: sequencing by ligation (SBL) and sequencing by synthesis (SBS). Sequencing by ligation is a sequencing method that uses DNA ligase, an enzyme that joins together ends of DNA molecules by catalyzing the formation of a phosphodiester bond; that determines the nucleotide present at a given location in a DNA sequence. Sequencing by synthesis is a sequencing method that uses DNA polymerase, an enzyme that synthesizes DNA molecules from deoxyribonucleotides, and constructs DNA fragments after the amplification of a target fragment.

Next Generation sequencing technologies were further subdivided into five major sequencing platforms: Roche 454 pyrosequencing, Illumina (Solexa) HiSeq and MiSeq sequencing, SOLiD sequencing, DNA nanoball sequencing and Ion Torrent sequencing. Roche 454 is a platform that utilized pyrosequencing to generate sequence reads, a SBS approach developed by Pal Nyren and Mostafa Ronaghi at the Royal Institute of Technology in Stockholm (Ronaghi *et al*., 1996). This technology became available in 2005 and was the first commercially successful NGS. As of 2016, however, Roche has discontinued supply and service for this methodology.

Illumina (Solexa) HiSeq and MiSeq sequencing is an SBS sequencing platform, introduced in 2006, that is based on reversible dye-terminators technology and engineered polymerases (Bentley *et al.*, 2008). Today, it is one of the most successful sequencing systems, particularly with the HiSeq and MiSeq platforms (Kulski, 2016). Roger Tsien, Pepi Ross Margaret Fahnestock and Allan Johnston developed the base-by-base, or stepwise, DNA sequencing with a removable 3' blockers protocol (Tsien *et al*., 1991); Eric Kawashima, Pascal Mayer and Laurent Farinelli developed DNA colony sample preparation and random surface-polymerase chain reaction (PCR) arraying methods (Kawashima *et al*., 2005).

Applied Biosystems Instruments' Sequencing by Oligonucleotide Ligation and Detection (ABI SOLiD) is a sequencing procedure (SBL), available in 2006, that involves sequential annealing (joining two complementary strands of nucleic acid via hydrogen bond) of DNA probes to the nucleotide template and their subsequent ligation. This protocol has many disadvantages including 50-75 bp read lengths, run times of weeks and the need for costly computational infrastructure and personnel expertise for analysis of data (Kulski, 2016). SOLiD is currently unavailable, as this technology has been discontinued.

DNA nanoball sequencing (Beijing Genomics Institute Retrovolocity) is an SBL procedure that creates DNA nanoballs of small fragments of genomic DNA from circular templates by rolling-circle replication (Kulski, 2016). Beijing Genomic Institute affirms that in a five-year span, to have sequenced over 20,000 whole human genomes using the DNA nanoball sequencing platform, and provide public access to human genomes and cancer data on their website (Kulski, 2016).

Ion Torrent (Ion semiconductor sequencing) technology, available since 2010, is an SBS sequencing method where a complementary DNA strand is constructed based on the sequence of a template strand. It is based on a revised version of the 454 pyrosequencing, with methodical changes to the nucleotide detection and the implementation of a microchip in which the sequencing reactions occur (Kulski, 2016). This sequencing method is different than other SBS technologies, in that completion does not require modified nucleotides or optics. Though this platform has low costs and fast runs, it has a high rate of sequencing errors with homopolymer stretches and repeats (Kulski, 2016).

Third generation single molecule sequencing (TGS, "long-read sequencing") is a class of DNA sequencing that is still undergoing development (Bleidorn, 2015). This method of sequencing can be done without the need to create a DNA library (Thompson & Milos, 2011), thereby leading to easy sample preparation and lower operational costs. Third Generation sequencing can be divided into two main categories: 1. single molecule real time sequencing approach (SMRT) and 2. the synthetic approach. The SMRT sequencing is a technology that utilizes a zero-mode waveguide (Levene *et al*., 2003) and has many applications to medicine (Arduri *et al*., 2018). Pacific Biosciences developed a SMRT sequencing method by using the same fluorescent labeling from established technologies, but detects the signals as they are emitted in real time. The second category of TGS sequencing, the synthetic approach, determines the order of nucleotides in a nucleic acid sequence. Oxford Nanopore technology (ONT) is a synthetic sequencing technology and is one of the most widely used TGS method. Nanopore technology is a mobile sequencing technology that can sequence a single molecule of DNA/RNA in absence of PCR amplification or chemical labeling of the sample. This system has yet to become commercial, but great strides are being made in its development (Kulski, 2016).

Fourth-Generation Sequencing is an *in-situ* sequencing method that uses NGS technologies to read nucleic acid composition directly from tissues and fixed cells. Though in its infancy, this *in-situ* sequencing technique was demonstrated on mRNA for the first time for breast cancer tissues (Lee *et al*., 2014).

1.4. Genomic Diagnosis of Gastrointestinal Parasites in Veterinary Medicine

Helminth infections affect almost 1/2 of humanity, with some infections categorized as 'neglected tropical diseases' (eight of 13 pathogens are on this list are helminth parasites). With this known disregard for these types of infections in humans, one can rationally assume that helminths of veterinary importance have not received a great deal of attention either. The first nematode to have its genome sequences was *Caenorhabditis elegans* in 1998, by the *C. elegans* Sequencing Consortium. *C. elegans* is a free-living nematode that has been used as a 'model' for many biological investigations due to the ease of mass production and its utility for genetic analysis (Clare *et al*., 2000; Schafer, 2005; Kosinksi & Zaremba, 2007; Alcantar-Fernandez *et al*., 2018). Interest in this nematode and its biological processes opened a door for veterinary helminth research (Burglin *et al*., 1997; Geary & Thompson, 2001; Gilleard, 2004). The investigations into *C. elegans* led to the investigations of parasitic nematodes of veterinary importance. One of these nematodes was *Haemonchus contortus*, a hematophagic nematode has become a significant problem for the small ruminant industry (Vlassoff & Mckenna, 1994; Waller & Chandrawathani, 2005). *H. contortus* developed resistance to multiple anthelmintic classes ubiquitously on a global scale (Echevarria *et al*., 1996; van Wyk *et al*., 1997; Terrill *et al*., 2001). Genetic research conducted on *H. contortus* has been implemental in vaccine research (Roberts *et al*., 2013; Laing *et al*., 2013), new drug development (Laing *et al*., 2013) and identifying genetic changes that confer anthelmintic resistance (Gilleard, 2013). Due to the

intensive investigations into *Haemonchus*, other parasitic nematodes of veterinary importance that have similar lifecycles, biological processes and hosts have received attention. Two important parasitic nematode genera of cattle have received this interest, and which are central to this paper, are *Cooperia* and *Ostertagia*. These parasites affect cattle on a global scale and have been the subjects of genomic research. Studies to differentiate species (Newton *et al*., 1998), investigations into anthelmintic resistance (Njue & Prichard, 2004; de Graef *et al*., 2013) and genome mapping (van der Veer & de Vries, 2004; Amarante *et al*., 2014) have been conducted on *Cooperia*. *Ostertagia*, the more pathogenic of the two genera, got less attention from the molecular researchers than its counterpart. This could be due to *Cooperia* spp. having had the most attention due to its singularly high degree of anthelmintic resistance (Stromberg *et al*., 2012).The genome of *Ostertagia ostertagi*, the species that commonly affects cattle, has not been fully sequenced, and research with this nematode, for the most part, has been focused more in anthelmintic (Edmonds *et al*., 2010; Waghorn *et al*., 2016) and immunological (Claerbout *et al*., 2005; Bakshi *et al*., 2019) areas than in the genomic (Harmon *et al*., 2006).

The rise of genetic sequencing has allowed parasitologists to explore different techniques for diagnosis of gastrointestinal helminthiasis. Although new strides have been made to use genetic sequencing to diagnose gastrointestinal helminth infections in both humans (Pilotte *et al*., 2016) and food animals (Hoglund *et al*., 2013), the typical approach to diagnosis and survey livestock helminthiasis involves conducting a fecal egg count (FEC) to get an estimation of the intensity of intestinal helminthiasis. Egg counts are occasionally accompanied by a coproculture for larval speciation based on morphological features or conventional/real-time PCR (Durette-Desset *et al*., 1999; van Wyk, 2013; Roeber & Kahn, 2014). These methods of identification are time-consuming, prone to error and require specialized training (van Wyk, 2013; Valentini *et al*.,

2009). Russell Avramenko at the University of Calgary, supported by his colleagues, developed a next-generation deep amplicon sequencing (metabarcoding) method to explore the parasitic communities of nine common gastrointestinal nematodes of cattle (Avramenko *et al*., 2015) that typically exist, to varying degrees, as co-infections (Lello *et al*., 2004; Gasbarre, 2014; Serrano & Miller, 2014). This system introduced the concept of the 'nemabiome', which can be defined as the parasitic nematode "equivalent" to the bacterial microbiome. The development of this method was initiated by the researchers who explored the diagnostic use of the microbiome using next generation sequencing (Gloor *et al*., 2010; Rogers & Bruce, 2010), and revamped the procedure to eukaryotic organisms. This system has opened the door for mapping common nematode community compositions and intensities. Also, investigations into how parasite species are developing resistance to drug classes, and how the drugs affect the parasites and vice versa, are expanding.

Though this technology is very accurate and is becoming more available to researchers (Avramenko *et al*., 2017), it is not very accessible to the producers for diagnostics of livestock parasitisms. Producers shifting from simply using drugs to control the parasitisms of their livestock to conducting actual diagnostics to monitor the parasitisms long-term and implement strategic targeted treatments, has been a relatively recent occurrence, especially in cattle production (van Wyk *et al*., 2006; Kaplan & Vidyashankar, 2012). The uptick in the implementation of management protocols is directly related to the rise of anthelmintic resistance displayed by livestock helminth parasites on a global scale (Waller 1997; Sutherland & Leathwick, 2011; Kaplan & Vidyashankar, 2012). The push to educate producers on the parasites that affect their animals and different management strategies to control said parasites is a slow-moving giant. Traditionally, livestock producers have been somewhat resistant to changes in

husbandry practices, though the visualization of the economic impact of internal parasitisms has made it clear that change is the ideal path (Qamar *et al.*, 2013; Grisi *et al*., 2014; Rodriquez-Vivasa *et al*., 2017).

1.5. Study Objective

The purpose of this investigation is to compare and evaluate the accuracy both within and between traditional parasitological surveillance techniques (microscopic) and molecular identification techniques (ITS-2 metabarcoding genetic sequencing). The investigation was designed to compare and contrast the labor input, accuracy and applicability of the two identification/quantification methods.

Chapter 2. Materials and Methodology

2.1. Study Overview

Five calves were selected from a dairy farm based on qualifying criteria, detailed below and placed on concrete (day -1). Upon reception, a mass collection of feces from individual animals was conducted in order to cultivate artificial infections for inoculation on day 36 of the study. Fecal samples (200 grams) collected from the naturally-parasitized calves were obtained on days 0 and 7. The feces collected was divided into two 60 gram subsamples for traditional parasitological identification methods (fecal egg counts, coprocultures/L_3) and molecular parasitological identification methods (raw feces, floated/isolated nematode eggs, coproculture/L_3). All animals were given three anthelmintics of different classes on day 14. Fecal samples were collected to ensure negative fecal egg counts. The calves were given artificial infections using nematodes collected from their mass-coprocultures on days 36, 39 and 42. Fecal egg counts were conducted on days 52 and 57 in order to confirm successful artificial infections.

Fecal samples (200 grams) from the artificially-infected calves were obtained on day 64 and again at necropsy of the animals (days 76-79), samples that were divided into two 60 grams subsamples for traditional and molecular parasitological identification methods (listed above). Additionally, at necropsy, the contents of the abomasums and small intestines were collected and aliquots were designated to traditional (10%, 300 mL) and molecular (17%, 500 mL) identification methods each in order to collect and identify the adult parasites (Table 1).

2.2. Animals and Reception

Five, 6 to 8 months old Holstein calves, ranging in weight from 150-200 kg, were obtained from a local dairy farm in Washington County, Arkansas on 24 May 2018 (IACUC protocol # 18087). The calves were selected from a group of yearling Holstein calves based on preliminary fecal egg counts. Criteria for selection were fecal egg counts of at least 15 strongyle eggs per gram of feces (to ensure calves held parasitisms), as well as overall health and appearance. Beginning at arrival, the calves were housed individually on concrete and given ad libitum mixed-grass hay, minerals and water. Each calf also was given 0.5 kg of 16% protein grain daily.

2.3. Treatments and Inoculations

Animals were each administered oral anthelmintics from three different chemical classes, simultaneously, on day 14 of the investigation: moxidectin (Cydectin, 0.2 mg/kg BW), levamisole (Prohibit, 8 mg/kg BW) and oxfendazole (Synanthic, 4.5 mg/kg BW). Anthelmintics were administered at the 680 kg dosage. Fecal egg counts were conducted periodically throughout the next 20 days to ensure negative egg counts.

Feces was collected from individual animals for coprocultures (procedure detailed below) and harvest of L_3 for reinfections on day 36 of the investigation. Each animal was inoculated with its

"own" nematodes. Samples were carefully collected en masse rectally and from the cleaned concrete pad multiple times a day, continuing until a sufficient number of nematode larvae (~100,000 larvae for each animal) were collected. Animals were administered ~10,000 infective larvae on days 36, 39 and again on day 42 of the investigation (~30,000 total L_3 per animal). Fecal egg counts were conducted on days 52 and 57 to ensure that the artificial infections were successful.

2.4. Fecal Collection

Fecal samples were collected rectally from the animals throughout the investigation in order to conduct the required coprology (fecal egg counts, coprocultures and egg harvest). Fecal samples were collected at four different time points for usage in traditional and molecular parasitological identification methods (days 0, 7, 64 and at necropsy [days 76, 77, 78, 79]). Fecal samples also were conducted on days 52 and 57 for assessment of treatment efficacy. Methodologies for both parasitological identification methods are described later in the text.

2.5. Traditional Parasitological Procedures

2.5.1. Fecal Egg Counts

Strongyle egg counts were obtained via direct centrifugation fecal flotations. For each fecal sample, one gram of feces was weighed and thoroughly homogenized with 10 mL of saturated $MgSO_4$ (flotation media). The homogenate was passed through a 1 mm aperture sieve and the filtrate was placed in a 15 mL centrifuge tube and each tube was brought up to volume with additional $MgSO_4$, creating a slight positive meniscus. The tubes were topped with a cover slip and then centrifuged at 2000 rpm for 3 minutes. The cover slip was placed on a microscope slide and examined at 40X magnification for egg quantification (Yazwinski *et al*., 2009).

2.5.2. Coprocultures

Coprocultures were conducted using 30 grams of feces homogenized with 2 grams of vermiculite, and supplemented to suitable consistency with water (procedure used was similar to Roberts and O'Sullivan [1959]). The covered coproculture cups were allowed to sit in a warm, dark room (23 C) for 14 days. Each coproculture cup was then flooded with water and inverted onto an inclined petri plate for 4 hours to allow the larvae to migrate from the culture into the lowest edge of the partially-floated plate. Collected larvae from one coproculture cup were siphoned into a 15 mL Pyrex centrifuge tube, killed with 10% formalin and stretched by transient boil. The samples were allowed to sit overnight in order to pellet the larvae at the bottom of the tube. The supernatant fluid was decanted and the precipitated L_3 were pipetted onto a microscope slide for identification and enumeration (Van Wyk *et al*., 2013). Larvae were identified at 40-100X magnification based on morphological features without the use of staining.

2.5.3. Aliquot and Digest Preparation

The calves were killed via captive bolt and exsanguination at the University of Arkansas parasitology farm in Fayetteville, AR. The intestinal contents were removed immediately following death and were processed for parasite collection. The abomasum and small intestine of each animal were ligated and separated for content collection. The individual organs were opened lengthwise, their contents emptied into a container and brought up to 3 L using water. Two aliquots were removed at this point: one 10% aliquot preserved with 10% formalin used for subsequent stereoscopic (10-70X magnification) identification and a 17% aliquot preserved by refrigeration used for molecular identification. The emptied abomasums were soaked overnight in water and the emptied small intestines sat in water for four hours. After soaking, the organs

were removed, washed, and the total wash/soak residue washed over a #200 sieve, with the total residue preserved with 10% formalin for subsequent traditional identification (Woods, *et al*., 1995).

Note: The large intestines and ceca of the calves were also processed using the methods detailed above, however no parasites were recovered.

2.5.4. Parasite Isolation and Quantification Procedures

The abomasal contents aliquot:

For each sample, the content aliquot was washed over a #120 (125 µm) sieve and all residue and filtrate were collected separately. The residue was collected via backwash and made up to 1 L (Residue 1). The filtrate was washed over a #200 (74 µm) sieve and the residue was backwashed, collected and made up to 1 L with water (Residue 2).

With homogenization, Residue 1 was stereoscopically viewed in 10-20 mL subsamples until the total volume was viewed. The same exact percentage analyzed was applied to Residue 2. All nematodes were collected, identified, quantified and recorded.

(Note: If the contents were too concentrated, then a lower aliquot was processed.)

The abomasal digest:

The collected digest fluid was made up to 4 L, and a 10% (400 mL) was removed during constant homogenization and washed over a #400 sieve (37 µm) sieve. The residue was collected via backwash and made up to 1000 mL. With homogenization, 10-20 mL subsamples were removed and viewed stereoscopically. This was continued until the total residue was viewed. All nematodes were collected, identified, quantified and recorded.

(Note: If the contents were too concentrated, then a lower aliquot was processed.)

The small intestine contents aliquot:

The same procedure used for abomasal contents aliquot was used for the small intestine contents aliquot.

(Note: If the contents were too concentrated, then a lower aliquot was processed.)

The small intestine digest:

The same procedure used for the abomasal digest detailed above was used to collect, identify, quantify and record the nematodes found in the small intestine digest.

(Note: If the contents were too concentrated, then a lower aliquot was processed.)

2.6. Molecular Parasitological Procedures

All samples below were collected for DNA identification.

2.6.1. Raw Feces

One subsample (~2 g) of each fresh feces (<30 minutes old) sample was placed in a 1.5 mL Eppendorf tube and immediately stored at -17°C until DNA extraction was carried out.

2.6.2. Fecal Strongyle Eggs

Six direct fecal flotations were carried out (detailed above) for each fecal sample collected from each animal, with 1-2.5 grams of feces used for the floatations, dependent on the fecal egg count conducted prior to flotations. The samples were spun at 2000 rpm for ten minutes. The cover slip was removed and washed into a 15 mL beaker. Next, 5 mL of centrifuged homogenate was

carefully siphoned from the surface of the centrifuge tube and placed into said beaker. The entirety of the beaker content was then placed into two clean centrifuge tubes and brought to full volume using water. Tubes were placed back into the centrifuge and spun at 2000 rpm for ten minutes in order to pellet the strongyle eggs at the base of the tube. After centrifugation, about 12 mL of water was siphoned off and the sedimented eggs were combined into one centrifuge tube, resuspended in water, and spun for another ten minutes at 2000 rpm. This washing process was repeated two more times. After the final spin, about 14 mL of water was siphoned off and the strongyle egg residue was placed into a 1.5 mL Eppendorf tube and frozen at -17°C until DNA extraction was carried out.

2.6.3. Third Stage Larvae Collection

Infective third stage larvae were collected from the coprocultures (detailed above). The live larval suspension was placed into a centrifuge tube. The larvae were pelleted using centrifugation at 2000 rpm. The excess water was siphoned and the larval pellet was placed into a 1.5 mL Eppendorf tube and frozen at -17°C until DNA extraction was carried out.

2.6.4. Adult Nematode Collections

At necropsy, ~500 mL of abomasum and small intestine contents were collected and washed through a 35 mm sieve (500µm) and put into a 1 L Nalgene container. All nematodes were collected, identified and enumerated, and then placed into a 1.5 mL Eppendorf tube, suspended in water, and frozen at -17°C until DNA extraction was carried out.

2.6.5. DNA Extractions

The DNA from samples that were collected for molecular work were extracted using the DNeasy PowerLyzer PowerSoil Kit (Qiagen) with the addition of Proteinase K. The raw feces samples and floated egg samples were extracted following the protocol provided in the kit, with 40µl of Proteinase K added before the bead beater step. The floated eggs samples were washed and pelleted via centrifugation twice prior to extraction. The infective larvae samples were also extracted using the kit-provided protocol, with the addition of 50 µL of Proteinase K; however, instead of using the bead beater machine, the samples underwent an "Alternate Bead Beater" step. In short, this method provided a gentler approach to disrupting the sheath of the nematode larvae, with alternation of heating and vortexing the samples. The adult nematodes were processed using the kit-provided protocol, with the addition of 50 µL of Proteinase K. Like the infective larvae, adult nematodes were processed using the "Alternate Bead Beater" step, in addition to being gently crushed using a pestle prior to the initiation of protocol. The nematodes were washed twice before being pelleted and crushed. In order to pellet the nematodes, the Eppendorf tubes were centrifuged and excess water siphoned off.

All extracted DNA samples were sent for sequencing to the Gilleard Lab at the Faculty of Veterinary Medicine, University of Calgary, TRW 2D10, 3280 Hospital Drive NW, Calgary, Alberta, T2N4Z6, Canada.

2.7. DNA Sequencing (University of Calgary)

2.7.1. PCR Amplification of ITS-2 Regions

The first step of sequencing the DNA target region for each sample was the amplification of the ITS-2 regions via PCR using the NC1 and NC2 primers (311-331 bp fragment) described by

Gasser et al. (1993) and Illumina adapters. Amplifications were carried out using the NEB Q5® High-Fidelity DNA Polymerase cat# M0491L using a 5 µl DNA template. The PCR conditions were 5 µL of Kapa HiFi reaction buffer, 0.75 µL of dNTPs (10 µM), 0.5 µL of Kapa HiFi polymerase, 12.24 µL dH_20, 0.75 µL of NC1 primer (10 µM), 0.75 µL of NC2 primer (10 µM), 0.1 µL of BSA (20 mg/mL) and 5 µL of diluted (1:10) lysate. The thermocycling parameters were 95ºC for 3 minutes, followed by 35 cycles of 98ºC for 20 seconds, 62ºC for 15 seconds, 72ºC for 15 seconds, followed by a final extension of 72ºC for 2 minutes.

2.7.2. PCR Product Purification

The PCR products were purified after the initial amplification (see above) and after the addition of barcoded regions (see below) using Agencourt AMPure XP magnetic Bead Based Purification Protocol. The protocol is as follows:

Bring AMPure XP beads to room temperature; prepare fresh 80% ethanol from absolute ethanol; centrifuge second amplification plate to collect condensation (290 x g for 1 minute at 20ºC); transfer 25 µL of the PCR product to a new 96 well MIDI plate; vortex AMPure XP beads for 30 seconds to evenly disperse the beads; add 25 µL of beads to each well of MIDI plate; mix up and down 10 times; incubate at room temperature for 5 minutes; place plate on magnetic stand for 2 minutes or until supernatant has cleared; remove and discard supernatant; while plate is still on magnetic stand, wash beads with 200 µL of fresh 80% ethanol (do not resuspend beads); incubate for 30 seconds or until clear; remove and discard supernatant; repeat 80% ethanol wash and 30 second incubation; remove and discard supernatant; allow beads to air-dry for 15 minutes; remove plate from magnetic stand; add 32.5 µL of pure water to each well; mix by pipetting up and down 10 times; incubate at room temperature for 2 minutes; place plate on

magnetic stand for 2 minutes or until supernatant has cleared; transfer 30 µL of the supernatant to new 96 well Nextera Library Plate.

2.7.3. Addition of Illumina Barcoded Regions

Step 3 of the sequencing protocol is the addition of Illumina barcoded regions and P5/P7 regions with pre-dispensed primer plates. This was carried out via low-cycle PCR using Kapa HiFi polymerase. The PCR conditions were 8.75 µL of dH_2O, 5 µL of Kapa HiFi buffer, 0.75 µL of dNTPs (10mM) and 5 µL of first round PCR product. The thermocycling parameters were 98ºC for 45 seconds, 7 cycles of 98ºC for 20 seconds, 63ºC for 20 seconds, 72ºC for 2 minutes, followed by an infinite hold at 10ºC.

2.7.4. Quantification of Individual PCR Products and Pooling

After another round of purification (see 3.5.2.), PCR products were quantified using a Nanodrop spectrophotometer. The individual samples were then pooled into a single tube in equal concentrations (50 ng/sample) in order to create a normalized library. The pooled library was quantified using a Nanodrop spectrophotometer, then diluted with molecular grade water until a concentration/volume of 8-10 ng/µL was achieved.

2.7.5. Library Quantification (qPCR)

Real-time PCR (qPCR) was performed to determine the final concentration of the library in order to amplify the ITS-2 regions using the KAPA Library Quantification Kit. The qPCR protocol was:

Dilute library (1:1000) with 10 mM Tris-HCl, pH 8 with 0.05% Tween-20 triplicate; using 1:1000 dilutions, set up 1:2 serial dilutions to achieve 1:2000, 1:4000 and 1:8000 dilutions; prior

to using Illumina library quantification kit for the first time, add 1 mL of primer premix (x10) to the 5 mL bottle of Sybr fast qPCR master mix (2x) and mix well; place all plasticware and water under UV light for 15 minutes; prepare qPCR plate (12 µL of Sybr fast qPCR master mix with added primers, 4 µL of molecular grade water and either 4 µL of diluted library, one of supplied standard for positive control or molecular grade water for negative control); place microseal over reactions; run qPCR. The qPCR thermocycling parameters were 95ºC for 5 minutes, then 35 cycles of 95ºC for 30 seconds, 60ºC for 45 seconds. Once the amplification was achieved, the data was confirmed to have a 90-100% reaction efficiency for samples and for standards. The library quantification was calculated using absolute quantifications against the 425 bp DNA standard. The qPCR products were purified with the MicroElute Cycle Pure Kit (OMEGA Bio-Tek, D6293-02). Sequences were aligned and trimmed using Geneious version 7.1.5 created by Biomatters. Available from http://www.geneious.com/.

2.7.6. Library Preparation and MiSeq Sequencing

The library was prepared for MiSeq sequencing using MiSeq Reagent Kit v2 (cat# MS-102-2002). Briefly, the preparation protocol was:

The library was diluted to 4 nM using molecular grade water (1 mL fresh 0.2M NaOH); the library was denatured by combining 5 µL of 4 nM library with 5 µL of 0.2 NaOH, briefly vortexing, centrifuging at 300 x g for 1 minute, incubating at room temperature for 5 minutes, then adding 990 µL of chilled hybridization buffer to produce 20 pM denatured library; the 20 pm library was diluted to 12 pM by combining 720 µL of 20 pM library with 480 µL of chilled hybridization buffer and inverted several times.

After the library was prepared, the sequencing control was prepared (PhiX library). The protocol was:

A 4 nM PhiX library was created by combining 2 uL of 10 nM PhiX library with 3 µL of 10 mM Tris-Cl, pH 8.5 with 0.1% Tween 20; the PhiX library was then denatured by combining 5 µL of 4 nM PhiX library with 5 µL of 0.2 M NaOH, briefly vortexing, centrifuging at 300 x g for 1 minute, incubating at room temperature for 5 minutes, then adding 990 µL of chilled hybridization buffer to produce a 20 pM denatured PhiX library; the library was then diluted from 20 pM to 12 pM by combining 180 µL of the PhiX library with 120 µL of chilled hybridization buffer and inverted several times.

The qPCR library (12 pM) and PhiX library (12 pM) were combined and loaded (600 uL) into the MiSeq cartridge. Products were directly sequenced on both strands using Sanger sequencing with the NC1 and NC2 primers.

2.8. Statistical Analysis

Weighted percent was calculated for each variable and were analyzed using the GLIMMIX procedures of SAS (SAS Inst. Inc., Cary, NC 2016). Animal served as the experimental unit for all dependent variables. The model included the laboratory method; means of infection, protocol, method by infection interaction, method by protocol interaction, infection by protocol interaction, or method by infection by protocol interaction. Means were separated using the F-protected *t*-test. When significant, the LINES option in the LSMEANS statement was used to display pairwise LS-means differences. All data are reported as weighted percentages, and for all analyses, significance was declared at $P \leq 0.05$.

Chapter 3. Results and Discussion

3.1. Traditional Parasitological Procedures

3.1.1. Fecal Egg Counts

Fecal egg counts (FEC) were conducted at four different intervals throughout the investigation for data collection. The results are expressed in nematode eggs per gram (epg) of feces. Strongyle counts are displayed in Table 2a, and *Nematodirus* spp. counts are displayed in Table 2b. From day 0 to day 7, with the exception of animal 4, the strongyle FEC for natural infections increased or remained relatively the same. Throughout the beginning of the investigation, animal 4 was affected by severe diarrhea, potentially due to a high burden of *Nematodirus* spp. A similar trend was seen with the artificial infections for animal 4, though *Nematodirus* spp was not found. From day 64 to necropsy, the strongyle FEC for all animals increased, indicating that the female nematodes were reaching fecundity at an increasing rate. This is to be expected at the parasite burdens matured. The exception to this was animal 5, whose FEC decreased to 1 epg on day 79. Upon necropsy, fecund female strongyle nematodes were recovered from all animals, so the low strongyle egg count of animal 5 reinforces the estimative nature of traditional fecal egg counts.

The FEC for *Nematodirus* spp can be found in Table 3. On day 0, only animals 4 and 5 had a positive count. Animal 4 had 89 epg, a count that represents a significant *Nematodirus* spp burden, and likely led to the loose fecal consistency throughout the investigation. On day 7, four of five animals held a positive *Nematodirus* spp FEC, with the epg ranging from 1-20. Animal 2 held the highest count on day 7.

Fecal egg counts were conducted after the animals were administered anthelmintic treatments on day 14. Counts were conducted on days 21, 28 and 30 to ensure that the animals had negative fecal egg counts (0 epg) and that the treatments were successful.

Post-infection fecal egg counts were conducted on days 52 and 57 to ensure that the artificial infections (administered on days 36, 39 and 42) were successful (Table 2a). There were no *Nematodirus* spp eggs found in the post-treatment fecal samples.

3.1.2. Coprocultures

Coprocultures were conducted from fecal samples collected in order to collect the L_3 and determine the larval compositions (Table 4a-e). The data are expresses in percentages of the total coproculture harvests. For animal 1 (Table 4a), the proportions of nematode species distribution were relatively similar for both natural and artificial infections, though there was a higher percentage *Ostertagia* spp. found on day 64 when compared to day 76. This could be due to a difference in overall *Ostertagia* eggs collected for the coproculture, as egg distribution in feces is not uniform (Michel, 1969). For animal 1, only three species of nematode were identified during natural and artificial infections.

The larvae recovered and identified for animal 2 is displayed in Table 4b. During the natural infections, the proportions of nematode species remained relatively the same. The artificial infection data showed that there was a flip-flop in the primary nematode identified. On day 64 and 77, the primary nematodes found were *Ostertagia* spp and *Cooperia punctata*, respectively. *Trichostrongylus* spp. was identified in the day 0 coproculture but was not found on any of the other collection dates.

The results for the larval proportions for animal 3 are displayed in Table 4c. Only three nematode species were identified across both natural and artificial infections. The natural infection proportions were similar from day 0 to 7. The artificial infection results were different when comparing day 64 to 78. On day 64, *C. punctata* was identified the most at 68%, and on day 78, only consisted of 28% of the sample. The proportions of species identified in the day 78 sample were fairly evenly distributed.

Animal 4 larval results are shown in Table 4d. Three species of nematodes were found in all 4 coprocultures, though on day 0, two additional species were recovered (*Haemonchus placei* and *Trichostrongylus* spp.) that were not found in the latter 3 collection dates. The natural infection proportions for *C. punctata* and *Ostertagia* spp. were reversed from day 0 to 7. The artificial infection results were similar for days 64 and 79, with *Ostertagia spp* and *C. punctata* holding the high and low proportions, respectively.

The results for animal 5 are displayed in Table 4e. The proportions for *Cooperia oncophora* were dissimilar from day 0 to 7, reversing from the top proportion to the bottom. The results for the artificial infection cannot be compared from day 64 to 79, due to the FEC at necropsy being 1 epg. The results for day 79 are expressed in absolute numbers of nematode larvae, rather than the proportions due to only 7 total larvae recovered from the entire coproculture. No *C. punctata* L_3 were observed in day 79 coproculture, though these results are dubious due to the miniscule number of recovered larvae. Images of the different species of nematode larvae are shown in Figures 1-4.

3.1.3. Abomasums

Adults collected from the abomasum of each animal at necropsy were quantified and are listed in Table 5. The data are expressed in both total calculated populations, as well as percentages. *O. ostertagi* was the prevalent species found in all of the animals, with a small population of *Ostertagi lyrata* present, as well. The two dimorphs were differentiated by the male's copulatory bursa. The female nematodes could not be individually differentiated, as the distinguishing features are less documented than that of the males. *Ostertagia* spp was the only gastrointestinal parasite genus recovered from the abomasum at necropsy from all of the animals. Images of the nematodes recovered from the abomasum are shown in Figure 5.

3.1.4. Small Intestines

Adults collected from the small intestines of each animal at necropsy were quantified and are listed in Table 6. The data are expressed in both total calculated proportions, as well as percentages. *C. oncophora* was the nematode species that was recovered in the highest proportion at necropsy for all animals. *C. punctata* was recovered the least (2 of the animals), and *Cooperia surnabada* was found in small numbers in 3 animals. The only gastrointestinal parasites recovered from the small intestines were in the genus *Cooperia*. The three species were determined based on morphological features of the male's copulatory bursa. The female nematodes had fewer distinguishing features and could not be individually differentiated. Images of the male nematodes recovered from the small intestines are shown in Figure 6.

3.2. Molecular Parasitological Procedures

3.2.1. Raw Feces

Extracted DNA from the raw feces samples was sequenced and are displayed in Tables 7a-e. The data are expressed in MiSeq reads, as well as percentages. For animal 1 (Table 7a), *C. oncophora* was the primary nematode species found across all study dates, followed by *O. ostertagi*. *H. placei* was sequenced from the feces collected on day 7, however the proportions were minute. The nematode species found in the lowest proportions on days 0 and 7 was Unclassified *Cooperia* (indicates that the ITS-2 sequences could not be specifically determined to the species level, rather grouped as "*Cooperia punctata/spatulata*"), followed by *C. punctata*. On days 64 and 76, *C. punctata* was found the least, followed by Unclassified *Cooperia*.

The sequencing data for animal 2, displayed in Table 7b. *C. oncophora* was the primary nematode sequenced on day 0, but *O. ostertagi* moved into the top spot for days 7 and 64. On day 77, the nematode species found in highest proportion was *Haemonchus contortus*, which was not recovered from the abomasum at necropsy. *H. contortus* and *Trichostrongylus colubriformis* were sequenced in minute numbers on day 7, holding the spots for the least amount of sequences recovered. The species sequenced the least on days 0 and 64 was Unclassified *Cooperia*; *C. punctata* was slightly higher. On day 77, eight total nematode species were sequenced, three of which being unique to this fecal collection when compared to previous dates. The species recovered in the lowest proportions were *Oesophagostomum asperum,* followed by *H. placei*. Neither of these nematodes were recovered at necropsy.

The results for animal 3 showed that sequences from a number of nematode species were found (Table 7c). Day 64 had the fewest nematode species sequences found (*C. oncophora, C.*

punctata, Unclassified *Cooperia* and *O. ostertagi*). The primary nematode species sequenced on days 0 and 64 was *O. ostertagi* and *C. oncophora*, respectively. On days 7 and 78, the primary nematode sequence found was *T. colubriformis*, followed by *H. contortus*, neither of which were recovered at necropsy. The nematodes that were recovered at necropsy (*Cooperia* spp. and *O. ostertagi*) were sequenced on day 78, but in low numbers. On day 0, *Cooperia spatulata* was the species found the least, followed by *H. contortus*. On day 7, the species found in the lowest quantity was *H. placei*, followed by Unclassified *Haemonchus* (indicates the genus *Haemonchus*). On day 64 and 78, the nematodes species found least were *O. ostertagi* and Unclassified *Haemonchus*, respectively. *T. colubriformis* and *Haemonchus* spp. were both found in 3 of the 4 fecal samples collected from animal 3, but none of these worms were recovered from the small intestine or abomasum at necropsy.

The sequencing results for animal 4 are displayed in Table 7d. On days 0 and 7, the primary nematode species found was *C. oncophora* and *O. ostertagi*, respectively. *Nematodirus helventianus* was found on both days 0 and 7 (the lowest proportion of species recovered for these days), which is to be expected as a good amount of eggs were found in the FEC corresponding to these days. As with animal 3, a variety of nematode sequences were recovered from animal 4 samples on days 64 and 79; only about half of these parasite species were recovered at necropsy. On day 64, *T. colubriformis* had the most sequences found, followed by *H. contortus*. On day 79, the nematode sequences recovered in the highest proportions were *O. ostertagi*, followed by *H. contortus*. Unclassified *Cooperia* was the species recovered the least on day 64, and *C. punctata* held this position for day 79.

The results for animal 5 are displayed in Table 7e. *C. oncophora* was the primary species sequenced on days 0, 7 and 64. On day 79, the nematode species found in the highest proportion

was *H. contortus*, followed by *O. ostertagi*; the third highest proportion belonged to that of *T. colubriformis*. Neither *H. contortus* nor *T. colubriformis* was recovered at necropsy. On days 0 and 7, the nematode species found in the lowest proportion was *H. placei* and *O. ostertagi*, respectively. On days 64 and 79, *C. punctata* was found the least.

The DNA sequenced from the raw feces samples, overall, had more variation when compared to the DNA sequenced from the floated eggs and larvae collected from the same fecal sample.

3.2.2. Floated Strongyle Eggs

Extracted DNA from the floated, isolated nematode eggs was sequenced and the resulting proportions are displayed in Tables 8a-e. The data are expressed in MiSeq reads and percentages. The sequencing data for animal 1 is displayed in Table 8a. Day 64 had no sequencing data. *C. oncophora* had the highest proportions of DNA sequenced for days 0, 7 and 76. *H. contortus* was found only in the day 0 sample, however, it was a minute amount. The nematode species sequenced are similar across all sample dates. The nematode species that had the fewest sequences recovered on day 0 was *H. contortus*, with higher reads for Unclassified *Cooperia* and *O. ostertagi*. On days 7 and 76, the species recovered least was Unclassified *Cooperia*, followed by *C. punctata*.

The sequencing data for animal 2 is shown in Table 8b. *O. ostertagi* had the highest proportions recovered for days 0 and 64, while *C. oncophora* had the highest for days 7 and 77. The species distribution had a somewhat similar trend across all sample dates, with the exception of *H. contortus* detected on day 64. The lowest proportion of sequences found were *C. punctata* on days 0 and 7. On day 64, the lowest proportion of sequences found were *H. contortus*, followed

by Unclassified *Cooperia*. *O. ostertagi* held the spot for the lowest proportions recovered on day 77.

The isolated nematode egg sequencing data for animal 3 is shown in Table 8c. On all collection dates, *C. oncophora* was the primary species sequenced. On day 0, the species that was recovered least was *H. placei*, and on day 7 was *O. radiatum*; both species were followed by Unclassified *Cooperia*. On day 64, *C. punctata* and Unclassified *Cooperia* were sequenced the least. *O. ostertagi* was the species sequenced the least on day 78.

The data for animal 4 is shown in Table 8d. On all sample dates, *O. ostertagi* was the nematode species sequenced the most. On day 0, *Nematodirus helventianus* had the lowest proportion of sequences, followed by Unclassified *Cooperia*. On days 7, 64 and 79, the nematode species recovered in the lowest proportion was Unclassified *Cooperia*, followed closely by *C. punctata*.

The sequencing data for animal 5 is shown in Table 8e. *O. ostertagi* was the nematode species that was sequenced most on all collection dates. *N. helventianus* was recovered least on day 0, followed by Unclassified *Cooperia*. On days 7, 64 and 79, Unclassified *Cooperia* was found in the lowest amount, followed closely by *C. punctata*.

3.2.3. Third Stage Nematode Larvae

Extracted DNA from the infective L_3 collected from coprocultures was sequenced and are displayed in Tables 9a-e. The data are expressed in MiSeq reads and percentages. The sequencing data from extracted larval DNA for animal 1 is displayed in Table 9a. The same four nematode species were found in all data collections. On day 0, the primary nematode species sequenced was Unclassified *Cooperia*. On day 7, *C. punctata* and *C. oncophora* were almost equally the primary nematodes found. For the artificial infection collection dates, *C. oncophora*

was the primary species sequenced; Unclassified *Cooperia* was sequenced the least. *C. oncophora* and *O. ostertagi* was sequenced in the least amount on days 0 and 7, respectively.

The sequencing data for animal 2 is shown in Table 9b. On day 0, the sequenced recovered were overwhelmingly *O. ostertagi*, and *C. oncophora* was sequenced the least. Day 7 had the highest range of nematode species (n=7) and the species sequenced the most on was *C. oncophora*. On days 64 and 77, *O. ostertagi* was the primary nematode sequenced. *C. oncophora* was sequenced the least on days 0 and 64. On days 7 and 77, the species found in the lowest proportion was Unclassified *Cooperia*.

The sequencing data for animal 3 is displayed in Table 9c. On day 0, the nematode species found in the highest proportion was *C. punctata*. Day 7 had the highest range of nematodes (n=5), with *C. oncophora* sequenced the most. The primary nematode species sequenced from the artificial infection collections was *C. punctata*. On days 0 and 7, the species least sequenced was *C. oncophora* and Unclassified *Cooperia*, respectively. *O. ostertagi* was the nematode found in the lowest proportion on day 64; *C. oncophora* held this spot for day 78.

Table 9d displays the ITS-2 sequencing data from larvae harvested from the feces of animal 4. The sequences from five species of nematodes were found, though not all 5 were found in any one sample. On all collection days, the species found in the highest proportion was *O. ostertagi*. On days 0 and 7, the nematode sequenced the least was *C. punctata* and *Cooperia spatulata*, respectively; *C. spatulata* was unique to day 7. On days 64 and 79, the nematode sequenced the least was Unclassified *Cooperia*, followed closely by *C. punctata*. The artificial infection data is more uniform than the natural infections data, as the same species are found in the same ranking order across both days.

The sequencing data for animal 5 is displayed in Table 9e. On days 0 and 7, the nematode species found in the greatest proportion was *C. oncophora* and *O. ostertagi*, respectively; Unclassified *Cooperia* was sequenced the least for both days. On day 64, *C. oncophora* was sequenced the most, and *O. ostertagi* the least. On day 79, two of the top three species sequenced were nematodes that were not recovered at necropsy (*H. contortus*, *T. colubriformis*). The species sequenced the least on day 79 was *C. punctata*.

3.2.4. Adult Nematodes

Extracted DNA from the adult nematodes collected from the abomasums and small intestines at necropsy were sequenced and displayed in Tables 10a-e. The data are expressed in MiSeq reads and percentages. The data is divided by organ from which the parasites were recovered and the percentage denotes what percentage of parasites were found per organ; all parasites collected from individual organs were combined prior to DNA extractions. With exception of animal 3, the primary nematode recovered from the small intestine was *C. oncophora* for all animals. *C. punctata* was the small intestine nematode sequenced the most from animal 3. All sequences obtained from abomasal nematodes were that of *O. ostertagi*.

3.3. Comparison of Methodologies

For procedure comparisons, nematodes that were found in few overall samples (n=6) were excluded in order to simplify the results. Traditional identification methods were able to determine that there were two different species of *Ostertagia* (*O. ostertagi* and *O. lyrata*), a distinction that genetic identification methods were unable to make. There is some disagreement as to whether the two species are, in fact, separate species or simply polymorphs (Zarlenga *et al*., 1998; Soll *et al*., 2013). *O. ostertagi* and *O. lyrata* adult nematode data identified by traditional

procedures were combined and compared to genetic procedures. Traditional adult identification was also able to determine that a third species of *Cooperia* was present (*C. surnabada*); genetic identification was unable to make this distinction, but was able to determine that an 'Unclassified *Cooperia*" species was present in these samples. This label indicates that the ITS-2 regions that were sequenced and categorized as such were either *C. punctata* or *C. spatulata*, though specificity ended there.

Within-Procedure comparisons

Larval and adult nematode identifications conducted using traditional parasitology are compared in Table 11. The proportions of *C. oncophora* ($p=0.3878$) and *C. punctata* ($p=0.7656$) found at the larval stage and the adult stage were not significantly different. The proportion of *O. ostertagi* larvae were significantly different from the proportion found at the adult stage ($p<0.00001$).

The within-method comparisons using genetic parasitology are shown in Table 12. The 'Raw Feces' samples showed the most overall variation; 11% of the sample was identified as something other than *Ostertagia* or *Cooperia*. For natural infections, the proportions of *C. oncophora*, *C. punctata* and *O. ostertagi* were not different across all genetic procedures (raw feces, floated eggs and infective larvae). The proportions of Unclassified *Cooperia* in the infective larvae samples were significantly different than the proportions found in the raw feces ($p=0.0193$) and floated eggs ($p=0.0256$) samples. For the artificial infections (Table 13), the proportions of *C. oncophora* was not significantly different across all genetic procedures (raw feces, floated eggs, infective larvae and adult nematodes). The proportions of *C. punctata* the in the raw feces was significantly different from the infective larvae ($p=0.0134$) and adults ($p=0.0384$). The proportions for Unclassified *Cooperia* found in the floated egg samples were

significantly different from proportions found in the raw feces (p=0.0517), larvae (p=0.0190) and adult nematode (p=0.0355) samples. These variations are likely due to this category of nematode sequences not being a definitive category; i.e. lumping two different species of *Cooperia* into one group that cannot be distinguished from one another. The proportion of *O. ostertagi* found in the raw feces samples were significantly different from the adult nematodes (p<0.0001), and all other comparisons not different. The raw feces samples showed the most variation, with 34% of the sample identified as something other than *Cooperia* or *Ostertagia*.

Across-Procedure Comparisons

The straight comparison of traditional and genetic parasitological identification methods proved difficult due to the variation of nematode species identified with each. There were only three nematode species that were comparable across methods: *C. oncophora, C. punctata and O. ostertagi.*

Comparing traditionally-identified larvae with genetically-identified raw feces are displayed in Table 14. The proportions of *C. punctata* data were significantly different for both natural (p<0.0001) and artificial (p=0.0003) infections. All other proportions compared were significantly not different.

Table 15 displays the data comparing proportions of nematodes found in traditionally-identified larvae with genetically-identified floated eggs. *C. punctata* was the only nematode whose proportions were significantly different for both natural (p<0.0001) and artificial (p=0.0177) infections. All other data were significantly not different.

The across-procedure comparisons of proportions of nematodes from infective larvae identified with both traditional and genetic procedures is shown in Table 16. All proportions of nematodes

for both natural and artificial infections are significantly not different, with the exception of *C. punctata* in the natural infection (p=0.0002).

The across-procedure comparisons showing proportions of nematodes found in adult samples are shown in Table 17, and all of the data are significantly not different.

Chapter 4. Conclusion

Observations of Procedures

Each parasitological identification method, traditional and genetic, has both advantages and drawbacks. The traditional procedures are tried-and-true methods that have been used by parasitologists for decades, though somewhat limited as to the information that can be ascertained. Fecal egg counts are, at best, an estimate of the overall parasite burdens, as speciation of the eggs cannot be done (strongyle nematodes cannot be morphologically distinguished). In addition, different species of nematodes display distinct egg laying tendencies, such as egg output per day, making it impossible to measure the exact level of parasite burden by conducting fecal egg counts (Michel, 1969). Morphologically distinguishing the subtle nuances of the larval features can be arduous and requires a trained eye. The enumeration method of L_3 can prove difficult, as a collection can have thousands of larvae with only a percentage identified, therefore, is an estimate of overall nematode burdens. Also, certain species of nematodes, have to be coprocultured using differing conditions than those used to culture strongyle nematodes. The collection of adult nematodes for identification and quantification requires a deceased animal, which can be a limitation with healthy animals that are not destined for slaughter. Though traditional parasitology had limitations, the capabilities used are readily accessible to the producer at an affordable cost. Fecal egg counts can be conducted for as little at

$18 per sample, and larval identifications can be conducted for $20 per sample (Oklahoma State University School of Veterinary Medicine). Genetic sequencing is much more costly (~$50-$75/sample), though, again, is not currently available at the diagnostic level to livestock producers. Even with the drawbacks of traditional parasitology, the affordability and reliability keep it a viable option for producers.

Genetic parasitology has made great headway in identifying gastrointestinal parasites via PCR, although not yet commercially available to producers. The sample collections for genetic parasitology relies on traditional methods to gather samples, (eggs, L_3 or adult nematodes). For the genetic portion of this investigation, fecal flotations had to be carried out in order to collect the floated eggs, coprocultures had to be conducted in order to collect the L_3 and necropsies had to be conducted in order to collect the adults. And though the DNA extraction protocols used in this investigation have not been verified, there was variation in the ITS-2 sequences extracted from different parasitic stages taken/cultured from the same fecal sample. Genetic sequencing is also limited in what parasites can be sequenced; if a particular species of nematode has not had its genome sequenced, then specific identification can prove problematic. For example, in this investigation, there were two species of nematodes, *C. surnabada* and *O. lyrata*, that were identified using traditional parasitology, but these sequences were not detected during the genetic identification. This is likely due to the fact that these are parasitic nematode of relatively minor importance and their ITS-2 genetic regions have not been sequenced; it should also be noted that *O. lyrata* is not considered a "true" species.

This project was designed to compare the techniques of traditional and genetic parasitology to identify parasitic cattle nematodes. Genetic identification procedures were able to detect nematode ITS-2 sequences in raw feces; a capacity not available with traditional procedures.

However, these sequences were not necessarily found in downstream genetic procedures (i.e. floated eggs, larvae and adults) using the same fecal sample, leaving room for identification errors. Genetic procedures were also unable to differentiate between species that were detected at the adult stage when using traditional procedures based on morphological features. This could be due to a number of reasons, with the most important factor being that accurate genetic identifications can only be conducted once the genome of an organism has been sequenced. Conducting genetic identifications of cattle nematodes is laborious and expensive, far greater than that of traditional methods. Though the technology is not yet available for commercial use, my own costs for these methods totaled to about $80 per sample. In order to collect the samples needed for genetic sequencing, traditional methods had to be conducted, doubling the labor input. The price-point for these two methods is almost incomparable, making traditional parasitology far more affordable and feasible for the average producer. It should also be noted that genetic sequencing is mostly unavailable to the average producer, giving traditional parasitology further advantage for routine parasitological monitoring. Based on the collected and analyzed data, it can be concluded that traditional parasitology is currently sufficient and practical for the identification of parasitic nematodes in cattle.

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Tables

Table 1. Schedule of events and distribution of collected samples for the comparison of traditional and genetic parasitology nematode identification methods.

Date	Trial Day	Event	Samples	Sample Distribution
25/May/18	-1	Reception Mass-coprocultures	Feces	+Collections for Artificial Infections
29/May/18	0	Fecal Collection 1	Feces	+Traditional Parasitology +Genetic Parasitology
05/June/18	7	Fecal Collection 2	Feces	+Traditional Parasitology +Genetic Parasitology
12/June/18	14	Anthelmintic Treatments		
04/July/18	36	Inoculation #1		10,000 L_3
07/July/18	39	Inoculation #2		10,000 L_3
10/July/18	42	Inoculation #3		10,000 L_3
20/July/18	52	FEC		
25/July/18	57	FEC		
01/August/18	64	Fecal Collection 3	Feces Abomasum Small Intestines	+Traditional Parasitology +Genetic Parasitology
13/August/18	76	Fecal Collection 4 Necropsy Animal 1	Feces Abomasum Small Intestines	+Traditional Parasitology +Genetic Parasitology
14/August/18	77	Fecal Collection 4 Necropsy Animal 2	Feces Abomasum Small Intestines	+Traditional Parasitology +Genetic Parasitology
15/August/18	78	Fecal Collection 4 Necropsy Animal 3	Feces Abomasum Small Intestines	+Traditional Parasitology +Genetic Parasitology
16/August/18	79	Fecal Collection 4 Necropsy Animals 4, 5	Feces Abomasum Small Intestines	+Traditional Parasitology +Genetic Parasitology

Table 2. Strongyle eggs per gram fecal counts conducted on calves during the investigation.

	Natural Infections		Artificial Infections			
Animal	**Day 0**	**Day 7**	**Day 52**	**Day 57**	**Day 64**	**Days 76-79**
1	258	320	6	34	231	260
2	204	449	13	26	109	130
3	51	160	14	47	88	302
4	141	21	0	8	176	342
5	59	54	0	8	94	1

Table 3. *Nematodirus* spp. eggs per gram fecal counts conducted on the calves during the investigation.

	Fecal Egg Counts	
Animal	**Day 0**	**Day 7**
1	0	1
2	0	20
3	0	2
4	89	11
5	3	0

Table 4a. Percent of parasitic cattle nematode larvae recovered from coprocultures from fecal samples taken from animal 1 throughout the investigation.

	Natural Infections		Artificial Infections	
Nematode	**Day 0**	**Day 7**	**Day 64**	**Day 76**
Cooperia oncophora	44	40	52	60
Cooperia punctata	36	36	16	24
***Ostertagia* spp**	20	24	32	16

Note: Larval results expressed in percentages of total larvae recovered.

Table 4b. Percent of parasitic cattle nematode larvae recovered from coprocultures from fecal samples taken from animal 2 throughout the investigation.

	Natural Infections		Artificial Infections	
Nematode	**Day 0**	**Day 7**	**Day 64**	**Day 77**
Cooperia oncophora	8	20	20	20
Cooperia punctata	30	30	20	52
***Ostertagia* spp**	60	50	60	28
***Trichostrongylus* spp.**	2	0	0	0

Note: Larval results expressed in percentages of total larvae recovered.

Table 4c. Percent of parasitic cattle nematode larvae recovered from coprocultures from fecal samples taken from animal 3 throughout the investigation.

	Natural Infections		Artificial Infections	
Nematode	**Day 0**	**Day 7**	**Day 64**	**Day 78**
Cooperia oncophora	50	42	12	32
Cooperia punctata	30	34	68	28
***Ostertagia* spp**	20	24	20	40

Note: Larval results expressed in percentages of total larvae recovered.

Table 4d. Percent of parasitic cattle nematode larvae recovered from coprocultures from fecal samples taken from animal 4 throughout the investigation.

	Natural Infections		Artificial Infections	
Nematode	Day 0	Day 7	Day 64	Day 79
Cooperia oncophora	2	8	32	28
Cooperia punctata	60	24	24	16
***Ostertagia* spp**	32	68	44	56
Haemonchus placei	2	0	0	0
Trichostrongylus spp.	4	0	0	0

Note: Larval results expressed in percentages of total larvae recovered.

Table 4e. Percent of parasitic cattle nematode larvae recovered from coprocultures from fecal samples taken from animal 5 throughout the investigation.

	Natural Infections		Artificial Infections	
Nematode	Day 0	Day 7	Day 64	Day 79
Cooperia oncophora	40	16	68	1*
Cooperia punctata	36	52	24	0
***Ostertagia* spp**	24	32	8	6*

Note: Larval results expressed in percentages of total larvae recovered.
Note: An (*) indicates that the coproculture counts corresponding to the fecal egg count are absolute numbers, not percentages.

Table 5. Calculated total number of adult nematode species collected from the combined abomasum contents and organ digests at necropsy.

Nematode	Animal 1	Animal 2	Animal 3	Animal 4	Animal 5
Ostertagia ostertagi	2866 (92%)	4403 (97%)	4480 (97%)	16803 (93%)	532 (92%)
Ostertagia lyrata	264 (8%)	147 (3%)	130 (3%)	1367 (7%)	48 (8%)

Note: Totals include mature female, mature male and early adult stages of the parasites.

Table 6. Calculated total number of adult nematodes collected from the combined small intestine contents and organ digests at necropsy.

Nematode	Animal 1	Animal 2	Animal 3	Animal 4	Animal 5
Cooperia oncophora	4971 (63%)	1331 (48%)	1415 (41%)	1758 (40%)	2915 (50%)
Cooperia punctata	1006 (13%)	915 (32%)	1388 (40%)	1648 (38%)	1237 (21%)
Cooperia surnabada	1952 (24%)	554 (20%)	667 (19%)	934 (22%)	1678 (29%)

Note: Totals include mature female, mature male and early adult stages of the parasites.

Table 7a. Nematode ITS-2 sequences identified from raw feces samples collected from animal 1 throughout the investigation.

	Natural Infections		Artificial Infections	
Nematode	**Day 0**	**Day 7**	**Day 64**	**Day 76**
Cooperia oncophora	18152 (46%)	11543 (56%)	31133 (94%)	25092 (64%)
Cooperia punctata	2773 (7%)	1717 (8%)	348 (1%)	1803 (4%)
Unclassified *Cooperia*	173 (4%)	748 (4%)	609 (2%)	1426 (4%)
Ostertagia ostertagi	16942 (43%)	6551 (32%)	1029 (3%)	10955 (28%)
Haemonchus placei	0	65 (<1%)	0	0

Note: Results are expressed in total MiSeq reads.
Note: "Unspecified *Cooperia*" indicates *Cooperia punctata/spatulata*.

Table 7b. Nematode ITS-2 sequences identified from raw feces samples collected from animal 2 throughout the investigation.

	Natural Infections		Artificial Infections	
Nematode	**Day 0**	**Day 7**	**Day 64**	**Day 77**
Cooperia oncophora	5030 (13%)	33360 (76%)	7442 (37%)	3356 (13%)
Cooperia punctata	1189 (3%)	1418 (3%)	1673 (8%)	2134 (8%)
Unclassified *Cooperia*	498 (1%)	324 (1%)	357 (2%)	1835 (7%)
Ostertagia ostertagi	33845 (83%)	7763 (18%)	10389 (52%)	3814 (14%)
Haemonchus contortus	0	57 (<1%)	0	6994 (26%)
Haemonchus placei	0	0	0	78 (<1%)
Unclassified *Haemonchus*	0	0	0	1304 (5%)
Oesophagostomum asperum	0	0	0	219 (1%)
Trichostrongylus colubriformis	0	81 (<1%)	0	6913 (26%)

Note: Results are expressed in total MiSeq reads.
Note: "Unclassified *Cooperia*" indicates *Cooperia punctata/spatulata*.
Note: "Unclassified *Haemonchus*" indicates the genus *Haemonchus*.

Table 7c. Nematode ITS-2 sequences identified from raw feces samples collected from animal 3 throughout the investigation.

	Natural Infections		Artificial Infections	
Nematode	**Day 0**	**Day 7**	**Day 64**	**Day 78**
Cooperia oncophora	16252 (30%)	3379 (19%)	33980 (86%)	681 (3%)
Cooperia punctata	3646 (7%)	929 (5%)	2964 (8%)	1536 (7%)
Cooperia spatulata	123 (<1%)	0	0	0
Unclassified *Cooperia*	1864 (3%)	561 (3%)	2229 (6%)	655 (3%)
Ostertagia ostertagi	22346 (41%)	1446 (8%)	164 (<1%)	846 (4%)
Haemonchus contortus	223 (<1%)	4159 (24%)	0	8702 (39%)
Haemonchus placei	4066 (7%)	306 (2%)	0	0
Unclassified *Haemonchus*	3631 (7%)	486 (3%)	0	203 (1%)
Oesophagostomum radiatum	894 (2%)	0	0	0
Oesophagostomum asperum	0	0	0	291 (1%)
Trichostrongylus colubriformis	1364 (3%)	6303 (36%)	0	9196 (42%)

Note: Results are expressed in total MiSeq reads.

Note: "Unclassified *Cooperia*" indicates *Cooperia punctata/spatulata*.

Note: "Unclassified *Haemonchus*" indicates the genus *Haemonchus*.

Table 7d. Nematode ITS-2 sequences identified from raw feces samples collected from animal 4 throughout the investigation.

	Natural Infections		Artificial Infections	
Nematode	**Day 0**	**Day 7**	**Day 64**	**Day 79**
Cooperia oncophora	26558 (82%)	0	477 (1%)	7574 (12%)
Cooperia punctata	0	470 (2%)	1115 (3%)	840 (1%)
Unclassified *Cooperia*	0	164 (1%)	300 (1%)	981 (2%)
Ostertagia ostertagi	3677 (11%)	18875 (95%)	981 (2%)	29406 (46%)
Haemonchus contortus	0	0	14477 (35%)	12632 (20%)
Haemonchus placei	1558 (5%)	0	0	1365 (2%)
Unclassified *Haemonchus*	0	0	653 (2%)	1394 (2%)
Oesophagostomum asperum	0	0	709 (2%)	0
Trichostrongylus colubriformis	0	0	22907 (55%)	9580 (15%)
Nematodirus helventianus	602 (2%)	404 (2%)	0	0

Note: Results are expressed in total MiSeq reads.

Note: "Unclassified *Cooperia*" indicates *Cooperia punctata/spatulata*.

Note: "Unclassified *Haemonchus*" indicates the genus *Haemonchus*.

Table 7e. Nematode ITS-2 sequences identified from raw feces samples collected from animal 5 throughout the investigation.

	Natural Infections		Artificial Infections	
Nematode	**Day 0**	**Day 7**	**Day 64**	**Day 79**
Cooperia oncophora	17326 (52%)	43827 (95%)	68227 (95%)	5049 (16%)
Cooperia punctata	0	0	1456 (2%)	812 (3%)
Unclassified *Cooperia*	0	0	2039 (3%)	2194 (7%)
Ostertagia ostertagi	15497 (46%)	650 (1%)	0	6883 (22%)
Haemonchus contortus	0	0	0	11924 (39%)
Haemonchus placei	504 (2%)	0	0	0
Oesophagostomum radiatum	0	1828 (4%)	0	0
Trichostrongylus colubriformis	0	0	0	3998 (13%)

Note: Results are expressed in total MiSeq reads.
Note: "Unclassified *Cooperia*" indicates *Cooperia punctata/spatulata*.
Note: "Unclassified *Haemonchus*" indicates the genus *Haemonchus*.

Table 8a. Nematode ITS-2 sequences identified from isolated nematode eggs collected from the feces of animal 1 throughout the investigation.

	Natural Infections		Artificial Infections	
Nematode	**Day 0**	**Day 7**	**Day 64**	**Day 76**
Cooperia oncophora	13895 (39%)	44906 (59%)	.	18519 (78%)
Cooperia punctata	1763 (5%)	5428 (7%)	.	774 (3%)
Unclassified *Cooperia*	1717 (5%)	4276 (6%)	.	762 (3%)
Ostertagia ostertagi	18512 (52%)	21505 (28%)	.	3816 (16%)
Haemonchus contortus	1 (<1%)	0	.	0

Note: Results are expressed in total MiSeq reads.
Note: "Unspecified *Cooperia*" indicates *Cooperia punctata/spatulata*.
Note: Day 64 has no data to display.

Table 8b. Nematode ITS-2 sequences identified from isolated nematode eggs collected from the feces of animal 2 throughout the investigation.

	Natural Infections		Artificial Infections	
Nematode	**Day 0**	**Day 7**	**Day 64**	**Day 77**
Cooperia oncophora	2258 (4%)	22629 (56%)	11194 (19%)	15721 (42%)
Cooperia punctata	948 (2%)	2375 (6%)	7680 (13%)	8487 (23%)
Unclassified *Cooperia*	832 (2%)	2070 (5%)	4613 (8%)	6619 (18%)
Ostertagia ostertagi	47457 (92%)	13617 (33%)	31996 (55%)	6332 (17%)
Haemonchus contortus	0	0	2463 (4%)	0

Note: Results are expressed in total MiSeq reads.
Note: "Unclassified *Cooperia*" indicates *Cooperia punctata/spatulata*.

Table 8c. Nematode ITS-2 sequences identified from isolated nematode eggs collected from the feces of animal 3 throughout the investigation.

	Natural Infections		Artificial Infections	
Nematode	**Day 0**	**Day 7**	**Day 64**	**Day 78**
Cooperia oncophora	12123 (64%)	32419 (74%)	54394 (98%)	13269 (34%)
Cooperia punctata	1026 (5%)	3182 (7%)	592 (1%)	9081 (23%)
Unclassified *Cooperia*	751 (4%)	2478 (6%)	703 (1%)	13544 (35%)
Ostertagia ostertagi	5037 (27%)	5720 (13%)	0	3165 (8%)
Haemonchus placei	31 (<1%)	0	0	0
Oesophagostomum radiatum	0	24 (<1%)	0	0

Note: Results are expressed in total MiSeq reads.
Note: "Unclassified *Cooperia*" indicates *Cooperia punctata/spatulata*.

Table 8d. Nematode ITS-2 sequences identified from isolated nematode eggs collected from the feces of animal 4 throughout the investigation.

	Natural Infections		Artificial Infections	
Nematode	**Day 0**	**Day 7**	**Day 64**	**Day 79**
Cooperia oncophora	4311 (10%)	6203 (15%)	9158 (26%)	12895 (41%)
Cooperia punctata	4851 (11%)	419 (1%)	417 (1%)	719 (2%)
Unclassified *Cooperia*	2280 (5%)	94 (<1%)	257 (1%)	602 (2%)
Ostertagia ostertagi	32465 (74%)	34646 (84%)	25395 (72%)	17532 (55%)
Nematodirus helventianus	247 (1%)	0	0	0

Note: Results are expressed in total MiSeq reads.
Note: "Unclassified *Cooperia*" indicates *Cooperia punctata/spatulata*.

Table 8e. Nematode ITS-2 sequences identified from isolated nematode eggs collected from the feces of animal 5 throughout the investigation.

	Natural Infections		Artificial Infections	
Nematode	**Day 0**	**Day 7**	**Day 64**	**Day 79**
Cooperia oncophora	32590 (67%)	17641 (42%)	6827 (27%)	.
Cooperia punctata	1769 (4%)	2411 (6%)	7280 (28%)	.
Unclassified *Cooperia*	1031 (2%)	1587 (4%)	7790 (30%)	.
Ostertagia ostertagi	13288 (27%)	19930 (48%)	3800 (15%)	.

Note: Results are expressed in total MiSeq reads.
Note: "Unclassified *Cooperia*" indicates *Cooperia punctata/spatulata*.
Note: Day 79 has no data to display.

Table 9a. Nematode ITS-2 sequences identified from infective nematode larvae collected from coprocultures comprised of feces from animal 1 throughout the investigation.

	Natural Infections		Artificial Infections	
Nematode	**Day 0**	**Day 7**	**Day 64**	**Day 76**
Cooperia oncophora	4437 (12%)	11020 (33%)	23117 (61%)	19077 (66%)
Cooperia punctata	9144 (25%)	11043 (33%)	5065 (13%)	596 (2%)
Unclassified *Cooperia*	13422 (36%)	7080 (21%)	3950 (10%)	510 (2%)
Ostertagia ostertagi	10211 (27%)	4318 (13%)	5745 (15%)	8629 (30%)

Note: Results are expressed in total MiSeq reads.
Note: "Unspecified *Cooperia*" indicates *Cooperia punctata/spatulata*.

Table 9b. Nematode ITS-2 sequences identified from infective nematode larvae collected from coprocultures comprised of feces from animal 2 throughout the investigation.

	Natural Infections		Artificial Infections	
Nematode	**Day 0**	**Day 7**	**Day 64**	**Day 77**
Cooperia oncophora	20 (<1%)	44749 (57%)	5245 (14%)	5168 (23%)
Cooperia punctata	1937 (5%)	3815 (5%)	11418 (30%)	5229 (23%
Unclassified *Cooperia*	449 (1%)	1750 (2%)	7204 (19%)	2520 (11%)
Ostertagia ostertagi	34923 (94%)	23904 (30%)	14394 (38%)	9759 (43%)
Haemonchus contortus	0	2345 (3%)	0	0
Unclassified *Haemonchus*	0	367 (<1%)	0	0
Trichostrongylus colubriformis	0	1937 (2%)	0	0

Note: Results are expressed in total MiSeq reads.
Note: "Unclassified *Cooperia*" indicates *Cooperia punctata/spatulata*.
Note: "Unclassified *Haemonchus*" indicates the genus *Haemonchus*.

Table 9c. Nematode ITS-2 sequences identified from infective nematode larvae collected from coprocultures comprised of feces from animal 3 throughout the investigation.

	Natural Infections		Artificial Infections	
Nematode	**Day 0**	**Day 7**	**Day 64**	**Day 78**
Cooperia oncophora	1064 (3%)	7664 (75%)	5116 (13%)	1438 (4%)
Cooperia punctata	17637 (44%)	485 (5%)	21095 (54%)	17218 (47%)
Unclassified *Cooperia*	11798 (29%)	338 (3%)	10126 (26%)	9170 (25%)
Ostertagia ostertagi	9551 (24%)	387 (4%)	2512 (6%)	9062 (25%)
Oesophagostomum radiatum	0	1315 (13%)	0	0

Note: Results are expressed in total MiSeq reads.
Note: "Unclassified *Cooperia*" indicates *Cooperia punctata/spatulata*.

Table 9d. Nematode ITS-2 sequences identified from infective nematode larvae collected from coprocultures comprised of feces from animal 4 throughout the investigation.

	Natural Infections		Artificial Infections	
Nematode	**Day 0**	**Day 7**	**Day 64**	**Day 79**
Cooperia oncophora	2364 (13%)	0	5681 (16%)	8293 (18%)
Cooperia punctata	792 (4%)	5537 (14%)	3401 (10%)	930 (2%)
Cooperia spatulata	0	779 (2%)	0	0
Unclassified *Cooperia*	4241 (24%)	4934 (13%)	3255 (9%)	651 (1%)
Ostertagia ostertagi	10228 (58%)	27927 (71%)	22997 (65%)	35830 (78%)

Note: Results are expressed in total MiSeq reads.
Note: "Unclassified *Cooperia*" indicates *Cooperia punctata/spatulata*.

Table 9e. Nematode ITS-2 sequences identified from infective nematode larvae collected from coprocultures comprised of feces from animal 5 throughout the investigation.

	Natural Infections		Artificial Infections	
Nematode	**Day 0**	**Day 7**	**Day 64**	**Day 79**
Cooperia oncophora	22949 (67%)	8105 (17%)	31684 (84%)	2916 (13%)
Cooperia punctata	1401 (4%)	565 (1%)	2374 (6%)	2364 (10%)
Unclassified *Cooperia*	789 (2%)	397 (1%)	3025 (8%)	0
Ostertagia ostertagi	9361 (27%)	38336 (81%)	474 (1%)	5447 (24%)
Haemonchus contortus	0	0	0	7094 (31%)
Trichostrongylus colubriformis	0	0	0	5293 (23%)

Note: Results are expressed in total MiSeq reads.

Note: "Unclassified *Cooperia*" indicates *Cooperia punctata/spatulata*.

Table 10a. Nematode ITS-2 sequences identified from adult nematodes collected from the organ contents from animal 1 at necropsy.

Nematode	Organ	Day 76
Cooperia oncophora	Small Intestine	28140 (97%)
Cooperia punctata	Small Intestine	303 (1%)
Unclassified *Cooperia*	Small Intestine	610 (2%)
Ostertagia ostertagi	Abomasum	25054 (100%)

Note: Results are expressed in total MiSeq reads.
Note: "Unspecified *Cooperia*" indicates *Cooperia punctata/spatulata*.

Table 10b. Nematode ITS-2 sequences identified from adult nematodes collected from the organ contents from animal 2 at necropsy.

Nematode	Organ	Day 77
Cooperia oncophora	Small Intestine	13534 (51%)
Cooperia punctata	Small Intestine	6793 (26%)
Unclassified *Cooperia* spp	Small Intestine	6096 (23%)
Ostertagia ostertagi	Abomasum	28315 (100%)

Note: Results are expressed in total MiSeq reads.
Note: "Unspecified *Cooperia*" indicates *Cooperia punctata/spatulata*.

Table 10c. Nematode ITS-2 sequences identified from adult nematodes collected from the organ contents from animal 3 at necropsy.

Nematode	Organ	Day 78
Cooperia oncophora	Small Intestine	4648 (12%)
Cooperia punctata	Small Intestine	22081 (58%)
Unclassified *Cooperia* spp	Small Intestine	11210 (30%)
Ostertagia ostertagi	Abomasum	3708 (100%)

Note: Results are expressed in total MiSeq reads.
Note: "Unspecified *Cooperia*" indicates *Cooperia punctata/spatulata*.

Table 10d. Nematode ITS-2 sequences identified from adult nematodes collected from the organ contents from animal 4 at necropsy.

Nematode	Organ	Day 79
Cooperia oncophora	Small Intestine	27552 (83%)
Cooperia punctata	Small Intestine	2598 (8%)
Unclassified *Cooperia* spp	Small Intestine	3104 (9%)
Ostertagia ostertagi	Abomasum	30215 (100%)

Note: Results are expressed in total MiSeq reads.
Note: "Unspecified *Cooperia*" indicates *Cooperia punctata/spatulata*.

Table 10e. Nematode ITS-2 sequences identified from adult nematodes collected from the organ contents from animal 5 at necropsy.

Nematode	Organ	Day 79
Cooperia oncophora	Small Intestine	26944 (85%)
Cooperia punctata	Small Intestine	2204 (7%)
Unclassified *Cooperia* spp	Small Intestine	2435 (8%)
Ostertagia ostertagi	Abomasum	38683 (100%)

Note: Results are expressed in total MiSeq reads.
Note: "Unspecified *Cooperia*" indicates *Cooperia punctata/spatulata*.

Table 11. Within-procedure comparisons of nematode species found in using traditional parasitological procedures during artificial infections.

Nematode	Infective L_3	Adults
Cooperia oncophora	34%[a]	27% [a]
Cooperia punctata	27% [a]	13% [a]
Ostertagia ostertagi	39% [a]	45% [b]
Other genera/species	0	15%

Note: Different superscripts between columns indicates a significant difference ($p<0.05$).
Note: "Other genera/species" indicates percentage of nematode genera/species recovered but not comparable.

Table 12. Within-procedure comparisons of nematode species found during natural infections using genetic parasitological procedures.

Nematode	Raw Feces	Floated Eggs	Infective L_3
Cooperia oncophora	47%[a]	42% [a]	27% [a]
Cooperia punctata	4% [a]	5% [a]	14% [a]
Unclassified *Cooperia*	8% [a]	3% [a]	14% [b]
Ostertagia ostertagi	30% [a]	48% [a]	43% [a]
Other genera/species	11%	2%	2%

Note: Different superscripts between columns indicates a significant difference ($p<0.05$). Note: "Other genera/species" indicates percentage of nematode genera/species recovered but not comparable.

Table 13. Within-procedure comparisons of nematode species found during artificial infections using genetic parasitological procedures.

Nematode	Raw Feces	Floated Eggs	Infective L_3	Adults
Cooperia oncophora	41%[a]	47% [a]	31% [a]	34% [a]
Cooperia punctata	5% [a]	12% [ab]	20% [b]	14% [b]
Unclassified *Cooperia*	6% [a]	13% [b]	11% [b]	10% [b]
Ostertagia ostertagi	14% [a]	27% [ac]	33% [ac]	42% [bc]
Other genera/species	34%	1%	5%	0

Note: Different superscripts between columns indicates a significant difference ($p<0.05$).
Note: "Other genera/species" indicates percentage of nematode genera/species recovered but not comparable.

Table 14. Comparisons of nematode species of infective larvae identified using traditional parasitological procedures with raw feces using genetic parasitological procedures.

	Natural Infections		Artificial Infections	
Nematode	Traditional (L_3)	Genetic (Raw Feces)	Traditional (L_3)	Genetic (Raw Feces)
Cooperia oncophora	27%[a]	47% [a]	34% [a]	41% [a]
Cooperia punctata	37% [a]	4% [b]	27% [a]	5% [b]
Ostertagia ostertagi	35% [a]	30% [a]	39% [a]	14% [a]
Other genera/species	1%	19%	0	40%

Note: Different superscripts between columns indicates a significant difference ($p<0.05$).
Note: "Other genera/species" indicates percentage of nematode genera/species recovered but not comparable.

Table 15. Comparisons of nematode species of infective larvae identified using traditional parasitological procedures with floated eggs using genetic parasitological procedures.

	Natural Infections		Artificial Infections	
Nematode	Traditional (L_3)	Genetic (Eggs)	Traditional (L_3)	Genetic (Eggs)
Cooperia oncophora	27%[a]	42% [a]	34% [a]	47% [a]
Cooperia punctata	37% [a]	5% [b]	27% [a]	12% [b]
Ostertagia ostertagi	35% [a]	48% [a]	39% [a]	27% [a]
Other genera/species	1%	5%	0	14%

Note: Different superscripts between columns indicates a significant difference ($p<0.05$).
Note: "Other genera/species" indicates percentage of nematode genera/species recovered but not comparable.

Table 16. Comparisons of nematode species of infective larvae identified using traditional parasitological procedures with infective larvae identified using genetic parasitological procedures.

	Natural Infections		Artificial Infections	
Nematode	Traditional (L_3)	Genetic (L_3)	Traditional (L_3)	Genetic (L_3)
Cooperia oncophora	27%[a]	27% [a]	34% [a]	31% [a]
Cooperia punctata	37% [a]	14% [b]	27% [a]	20% [a]
Ostertagia ostertagi	35% [a]	43% [a]	39% [a]	33% [a]
Other genera/species	1%	16%	0	16%

Note: Different superscripts between columns indicates a significant difference ($p<0.05$).
Note: "Other genera/species" indicates percentage of nematode genera/species recovered but not comparable.

Table 17. Species comparisons of adult nematodes identified using traditional parasitological procedures with adult nematodes identified using genetic parasitological procedures.

Nematode	Traditional	Genetic
Cooperia oncophora	27%[a]	34% [a]
Cooperia punctata	13% [a]	14% [a]
Ostertagia ostertagi	45% [a]	42% [a]
Other genera/species	15%	10%

Note: Different superscripts between columns indicates a significant difference ($p<0.05$).
Note: "Other genera/species" indicates percentage of nematode genera/species recovered but not comparable.

Figures

Figure 1. 400X magnification of *Cooperia* spp third stage infective larvae.
Top to bottom: *Cooperia* spp head, *Cooperia punctata* tail, *Cooperia oncophora* tail.

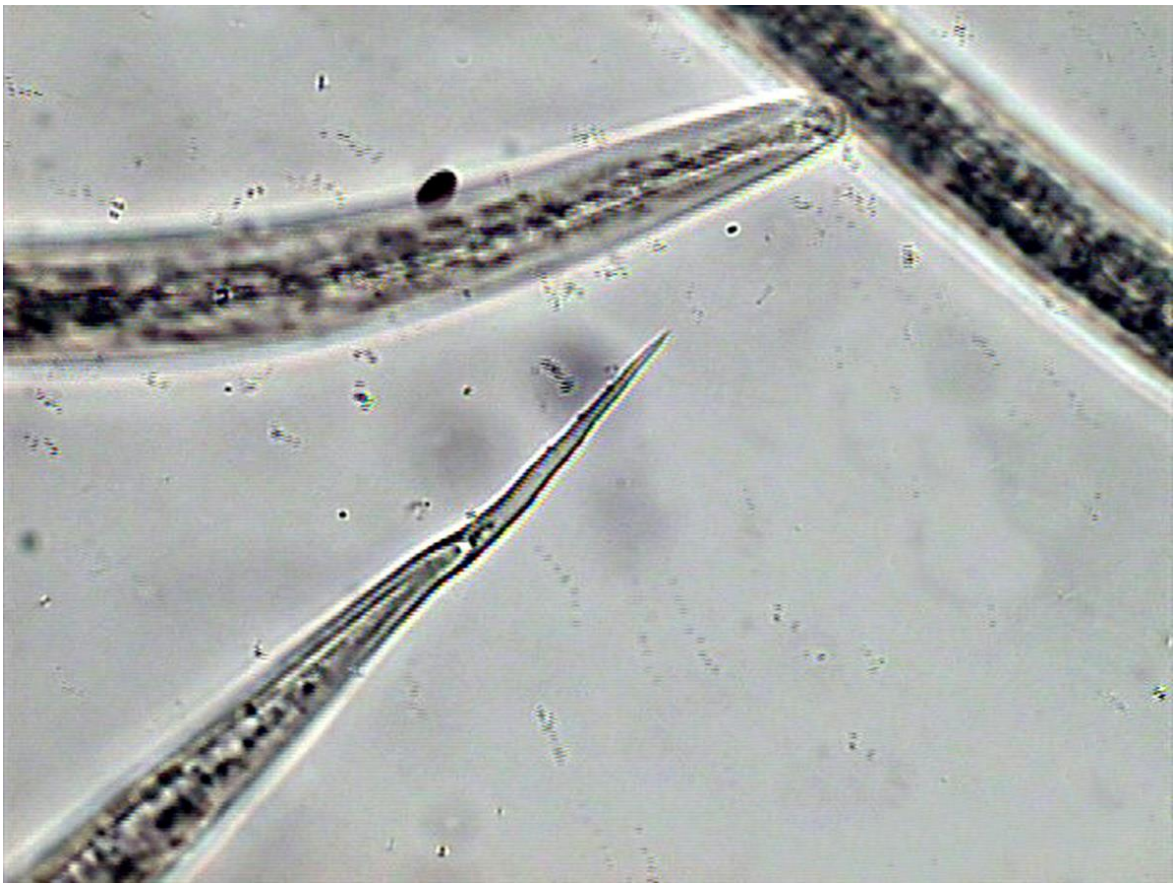

Figure 2. 400X magnification of *Ostertagia* spp third stage larvae.

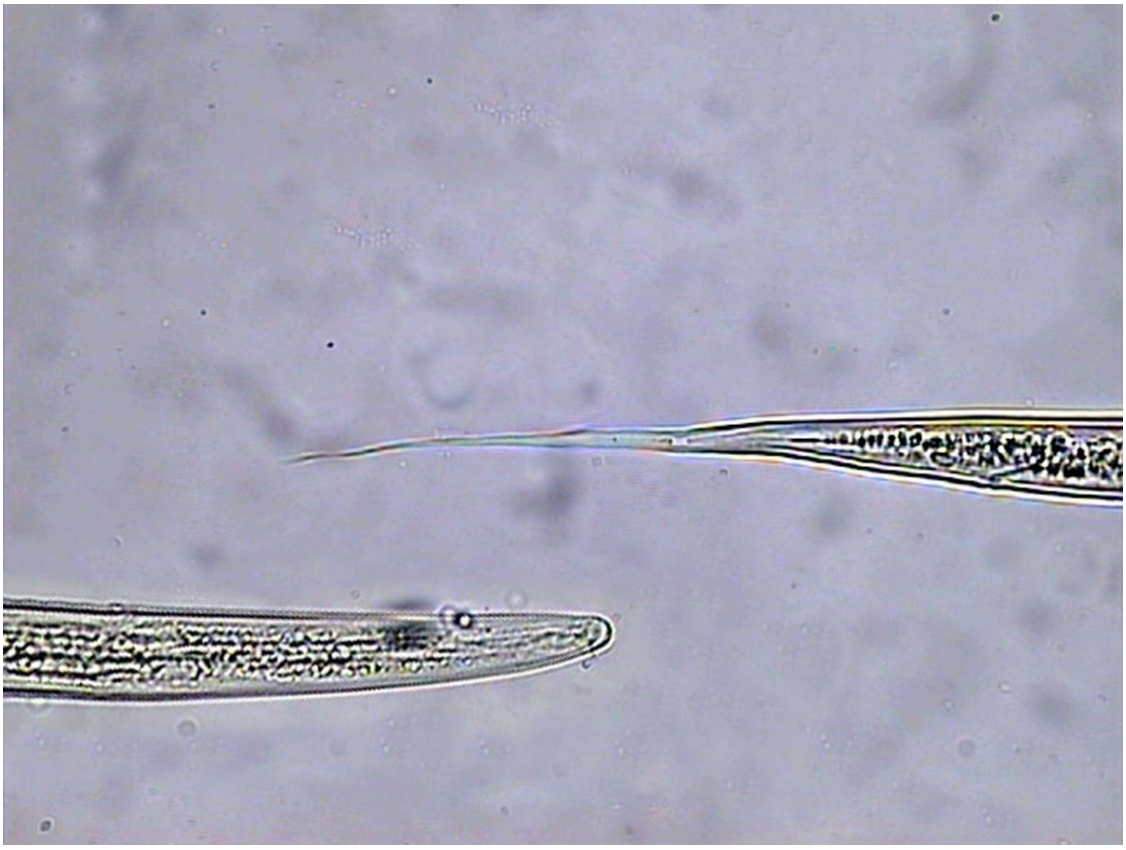

Figure 3. 400X magnification of *Haemonchus placei* third stage larvae.

Figure 4. 400X magnification of Trichostrongylus axei third stage larvae.

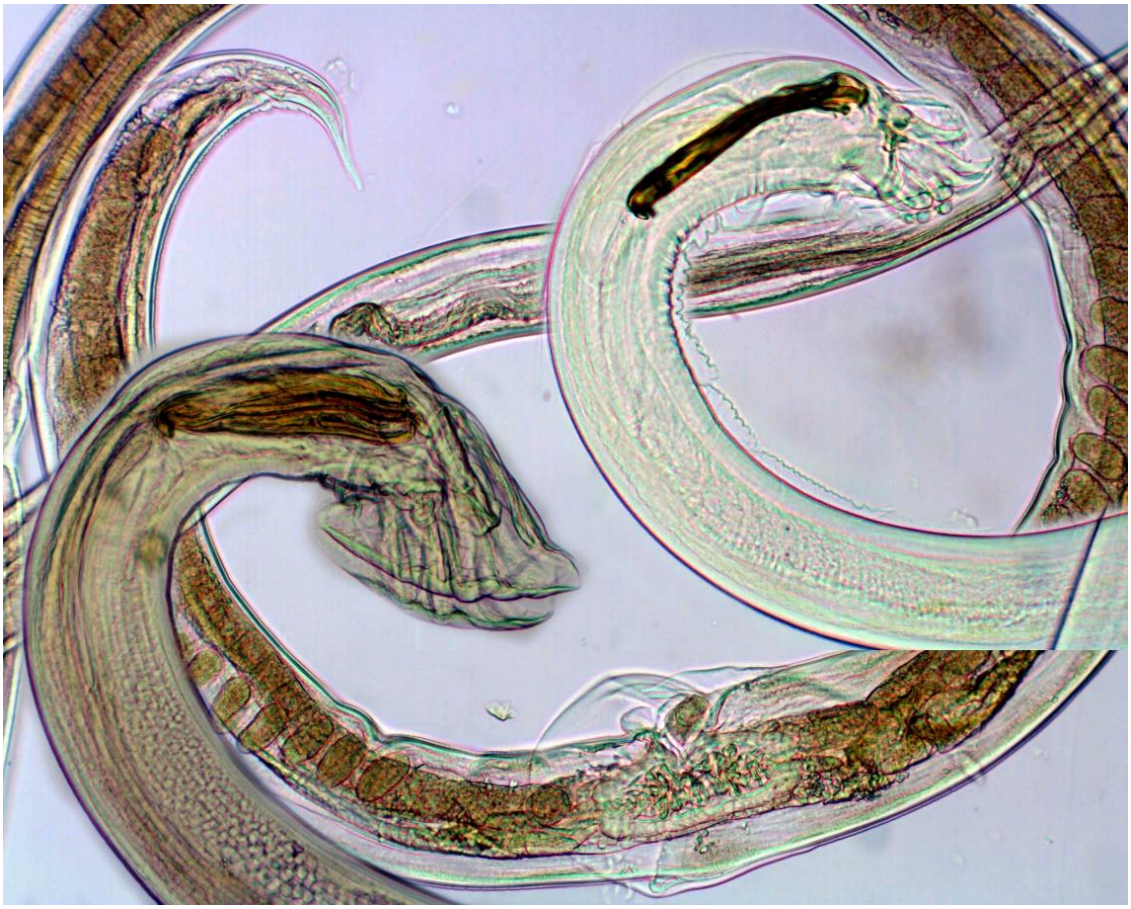

Figure 5. 100X magnification of adult *Ostertagia* spp.
Left to right: *Ostertagia* spp female, *Ostertagia ostertagi* male, *Ostertagia lyrata* male.

Figure 6. 100X magnification of adult male *Cooperia* spp.
Top to bottom: *Cooperia oncophora*, *C. surnabada*, *C. punctata*.