DETECTION AND CONTROL OF ANTHELMINTIC RESISTANCE IN GASTROINTESTINAL NEMATODES OF LIVESTOCK

by

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(Under the Direction of Ray M. Kaplan)

ABSTRACT

Parasitic gastrointestinal nematodes have a major impact on the health and productivity of livestock. Control of these pathogens is challenged by the widespread emergence of anthelmintic resistance. There is a pressing need for improved methods of detection and control of anthelmintic resistance.

Although the fecal egg count reduction test is labor and cost intensive and rarely used by cattle producers, this test is the only means available for detection of resistance in cattle at the farm level. To reduce the cost and improve acceptance among producers, we developed a method to use composite sampling as an alternative to individual sampling. Composite sampling reduced the number of fecal egg counts required for a test by 79%. The correlation coefficient was greater than 0.95 for drug efficacy between individual and composite sampling methods, demonstrating composite sampling is appropriate to diagnose anthelmintic resistance.

In vitro assays are more sensitive for detection of resistance and less labor and cost intensive than the fecal egg count reduction test. Motility is a commonly used *in vitro* phenotype for assessing anthelmintic activity of candidate compounds, and for

detecting anthelmintic resistance in nematodes. To evaluate the measurement of motility of third-stage larvae, we developed the Worminator, which quantitatively measures motility of parasites. Using the Worminator, differences in EC_{50} between third-stage larvae of avermectin/milbemycin susceptible and resistant isolates of *Cooperia* spp. and *Haemonchus contortus* were small, challenging the suitability of motility as a phenotype for detecting and measuring resistance to avermectin/milbemycin drugs in gastrointestinal nematodes of livestock.

Improved methods for control of multiple-anthelmintic resistant populations of gastrointestinal nematodes of livestock are urgently required. We replaced a multiple-anthelmintic resistant population of *Haemonchus contortus* in a sheep flock with a susceptible laboratory isolate. Surprisingly within 1.5 years following replacement, the population reverted to a resistant phenotype even under minimal drug selection pressure. We used molecular, *in vivo*, and *in vitro* methods to describe the population genetics and drug susceptibility following replacement, enabling improved insights into the epidemiology of anthelmintic resistance. This work contributes valuable information and methodologies for detection and control of anthelmintic resistance in gastrointestinal nematodes of livestock.

INDEX WORDS: anthelmintic; resistance; nematode; livestock; fecal egg count; composite samples; motility; L3; worminator; replacement

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CHAPTER 1

INTRODUCTION

Parasitic gastrointestinal nematodes have a major impact on the health and productivity of livestock throughout the world (Parkins and Holmes, 1989; Hawkins, 1993; Stromberg and Gasbarre, 2006). Control of gastrointestinal parasites is a fundamental component of livestock husbandry and management, and administration of anthelmintic drugs is the primary method of control employed by producers of livestock (Hawkins, 1993; Leathwick and Besier, 2014). Unfortunately, the development and widespread emergence of high levels of anthelmintic resistance to multiple classes of drugs in common parasites of small ruminants (Howell et al., 2008; Torres-Acosta et al., 2012 ; Playford et al., 2014) and cattle (Fiel et al., 2001; Anziani et al., 2004; Condi et al., 2009; Demeler et al., 2010b; Edmonds et al., 2010; Lyndal-Murphy et al., 2010; Rendell, 2010; Fazzio et al., 2014; Cotter et al., 2015; Geurden et al., 2015; Waghorn et al., 2016) has created a substantial challenge to the control of these important pathogens (Kaplan, 2004; Sutherland and Leathwick, 2011; Kaplan and Vidyashankar, 2012).

To effectively control gastrointestinal nematodes of livestock and minimize the development of anthelmintic resistance, sensitive methods to detect and monitor resistance are required (Gill et al., 1991; Taylor et al., 2002; Kaplan et al., 2007; Demeler et al., 2010a). There is a pressing need for improved and simplified methods to detect and monitor anthelmintic resistance as current methods are labor and/or cost intensive (Gill et al., 1991; George et al., 2017; George et al., 2018).

The fecal egg count reduction test (FECRT) is currently the preferred and most commonly used assay for detection of anthelmintic resistance in gastrointestinal nematodes of ruminants at the farm level (Coles et al., 1992; Coles et al., 2006). However, the FECRT has not been widely implemented by livestock producers for many reasons (USDA, 2010). FECRTs are expensive and producers view this test as uneconomical (Morgan et al., 2005), particularly given that many producers, especially cattle producers, have not perceived anthelmintic resistance to be a problem on their farm (Gasbarre et al., 2015). The FECRT is rather insensitive as this assay can only detect resistance once it is present at relatively high levels (Martin et al., 1989). Typically, only one a single drug dose is tested per assay and animals must be handled at the time of treatment and again approximately two weeks later to evaluate drug efficacy.

We proposed that a reduction in the cost of performing a FECRT may positively influence the acceptance of this test by producers and may facilitate an increase in its usage (Rinaldi et al., 2014). Composite sampling is a method where an equal quantity of feces are pooled from individuals and fecal egg counts are completed on the pooled sample rather than individuals to decrease the total number of fecal egg counts required (Baldock et al., 1990; Nicholls and Obendorf, 1994; Ward et al., 1997; Morgan et al., 2005). Composite sampling therefore reduces the laboratory expenses associated with a FECRT and may improve acceptance of the FECRT among livestock producers (Rinaldi et al., 2014).

The FECRT suffers from several technical and practical limitations. Alternatively, *in vitro* assays are a more efficient and cost-effective strategy to detect anthelmintic resistance (Taylor and Hunt, 1989; Gill et al., 1991). *In vitro* assays can test multiple

compounds and concentrations of anthelmintics which provides more granular data to describe the dose response and level of drug resistance present in a population of parasites (George et al., 2018). *In vitro* assays are designed to describe the effects of various concentrations and compounds of anthelmintics on nematode development, growth, behavior, or motility (Taylor et al., 2002). Although several *in vitro* assays have been developed and validated for detection of anthelmintic resistance in gastrointestinal nematodes of sheep including the egg hatch assay (Le Jambre, 1976), larval development assay (Taylor, 1990; Gill et al., 1995), larval feeding assay (Álvarez-Sánchez et al., 2005), and larval migration inhibition assay (Wagland et al., 1992; Kotze et al., 2006), there are no commercially-available or validated *in vitro as*says for detection of resistance to the avermectin/milbemycins in gastrointestinal nematodes of cattle. This dissertation aimed to further evaluate the measurement of motility of free-living stages of important gastrointestinal parasites of livestock as a measurement of anthelmintic resistance with hopes to develop an *in vitro* assay for detection of resistance in cattle.

Although there are scientifically validated and accepted methods to slow the development of anthelmintic resistance (van Wyk and Bath, 2002; Besier, 2012; Leathwick and Besier, 2014), the prevalence of small ruminant farms with multiple anthelmintic resistance and total anthelmintic failure is already very high and is increasing (Martínez-Valladares et al., 2013; Herrera-Manzanilla et al., 2017; Howell et al., 2017). Once anthelmintic resistance or multiple-anthelmintic resistance is present in a population, strategies to slow the development of resistance are less useful (Leathwick et al., 2012). Many farmers are left with few to no options for chemical control of gastrointestinal nematodes once multiple-anthelmintic resistance is present on their farm.

To provide an intervention strategy for this situation, we implemented a strategy to replace a multiple-anthelmintic resistant population of *Haemonchus contortus* (*H. contortus*) on a sheep farm with a susceptible laboratory isolate. Following replacement, we evaluated this strategy over several years using *in vivo*, *in vitro*, and molecular methods.

The specific aims of this dissertation are listed below.

Specific aim 1: Determine if composite sample and individual sample fecal egg count methods agree sufficiently to allow for the use of composite fecal egg counts to evaluate drug efficacy and to diagnose resistance

Specific aim 2: Evaluate the appropriateness of motility of third-stage larvae of gastrointestinal nematodes of livestock as an *in vitro* phenotype for detection of resistance to the avermectin/milbemycin class of anthelmintics

Specific aim 3: Evaluate changes in drug susceptibility and population genetic structure in *H. contortus* following worm replacement as a means to reverse the impact of multiple-anthelmintic resistance on a sheep farm

The outcomes of this dissertation provide several new insights into the diagnosis and management of anthelmintic resistance. First, we demonstrated a practical solution for livestock producers to reduce the cost and effort of diagnosing anthelmintic resistance on their farms. This dissertation also contributes valuable information regarding our understanding of *in vitro* phenotypes of gastrointestinal nematodes exposed to avermectin/milbemycin drugs relating to both the detection of resistance and the mechanism of action of these drugs. Finally, this work demonstrates the experimental power of using a multifaceted approach to studying the epidemiology of gastrointestinal

nematodes. By combining molecular, *in vitro*, and *in vivo* assays to describe phenotypic and genotypic changes in field populations of nematodes, we were able to determine the population-level changes in both drug susceptibility and genetics that occurred over time in our field study.

CHAPTER 2

LITERATURE REVIEW

Significance of gastrointestinal nematodes of livestock

Parasitic nematodes that infect the gastrointestinal tract of livestock present a major threat to the health and productivity of livestock (Parkins and Holmes, 1989; Hawkins, 1993; Stromberg and Gasbarre, 2006). Helminth infections of livestock are among the most economically important factors affecting productivity in grazing livestock (Waller, 2006), costing the global livestock industry billions of dollars annually in lost production and drug expenditures (Stromberg and Gasbarre, 2006). The economic losses associated with parasitism of the gastrointestinal tract of livestock are well documented and internationally accepted, including both clinical and subclinical losses in terms of appetite suppression, reduced average daily gain, reduced milk production, increased feed conversion ratio, reduced immune response to vaccination, reduced immunity to pathogens, and increased morbidity and mortality (Parkins and Holmes, 1989; Hawkins, 1993; Sutherland and Scott, 2010). Internal parasitism has been ranked as one of the most important infectious diseases impacting production livestock in multiple reviews and surveys across the globe (McLeod, 1995; Waller, 2006).

Impact of gastrointestinal nematode infections on cattle productivity

There were several randomized control studies completed in the 1980's and 1990's to evaluate the impact of parasite control as compared to untreated controls on productivity of livestock (Ciordia et al., 1987; Stuedemann et al., 1989; Hawkins, 1993).

The most commonly measured phenotype to describe the benefit of parasite control is weight gain (Hawkins, 1993). For grazing cattle, Ciordia et al. (1987) reported a 12.5 kg advantage in weight of Hereford/Brangus grazing calves treated with injectable ivermectin as compared to untreated controls over a 2-month period, however, the authors did not find a significant advantage in weight gain for cows treated with ivermectin compared to untreated cows. Stuedemann et al. (1989) reported 0.04 kg and 0.09 kg advantages in average daily gain of grazing Angus calves and cows, respectively, following treatment with fenbendazole over a 168-day study.

The impact of *Cooperia punctata* (*C. punctata*) on productivity of cattle in the feedlot sector was more recently evaluated in a randomized controlled trial (Stromberg et al., 2012). Cattle in the parasitized group were infected orally with 10,000 *C. punctata* L3 on day 0 and 8,250 *C. punctata* on day 14 while control animals where sham treated with water. Average daily gain was 1.47 kg vs 1.37 kg per day in the control versus infected animals, demonstrating that control cattle gained weight 7.4% more rapidly (P=0.02) than infected animals. In terms of daily dry matter intake, infected animals consumed 0.68 kg less feed than control cattle (P=0.02). This reduction in voluntary dry matter intake is one of the most commonly recognized features of infections with gastrointestinal nematodes in livestock (Parkins and Holmes, 1989).

The impact of a vehicle containing an extended release formulation of 5% eprinomectin was evaluated in 7 independent studies with grazing cattle (Kunkle et al., 2013). Animals were randomized and blocked based on body weight into 17 replicates of 4 animals. The eprinomectin treatment resulted in an increase of 43.9 lb per head compared to controls over a 120-day grazing period which is equivalent to an increase of

0.16 kg per day in average daily gain which is consistent with the results reported by (Ciordia et al., 1987).

In a performance study of naturally infected grazing Nellore calves, treatment with a combination of abamectin and ivermectin resulted in 84% fecal egg count reduction and 11.84 kg increase ($P<0.05$) in gain over untreated controls during a 112-day grazing period (Borges et al., 2013). This study may not reflect the full benefit of anthelmintic treatment as cattle from different treatment groups were pastured together and thus the results do not fully reflect the benefit of reduced pasture contamination as a result of effective anthelmintic treatment.

A recent feedlot study in Argentina consisting of 80 cattle carrying natural parasite infections that were randomly allocated to 4 groups showed that effective parasite control with a combination of ricobendazole and levamisole resulted in higher ($P<0.001$) live weights of 280 kg as compared to 266 kg in the untreated control group at the end of the 126-day study (Fazzio et al., 2014).

In a small, randomized study, grazing dairy heifers were allocated to three treatment groups, either untreated control, treated with moxidectin at 3 and 10 weeks post-turnout, or treated at turnout and 10 weeks later (Elsener et al., 2001). The heifers were exposed to naturally contaminated pastures over a 143-day grazing period. Both groups of treated cattle gained at least 15 kg more than the untreated controls, demonstrating a benefit on productivity of treatment with moxidectin. A limitation of this study is that there were no treatment (pen) replicates, outside of the individual animals.

Treatment with doramectin in stocker cattle resulted in an increase between 0.152-0.272 kg average daily gain as compared to untreated controls in a large 4-site

study where cattle were randomly allocated to treatment group (Ballweber et al., 1997). Further, a study in Minnesota found that calves strategically treated twice with fenbendazole gained 0.13 kg more per day as compared to untreated controls (Stromberg et al., 1997).

Recently, studies including untreated controls are less common as producers are not willing to leave animals untreated for research and thus the published literature has moved to comparing effective anthelmintics to less effective anthelmintics and measuring the difference in production parameters. Due to the emergence of resistance to the avermectin/milbemycins in several important species of gastrointestinal nematodes of cattle, these studies commonly use a combination of two classes of anthelmintics to achieve effective parasite control yielding a body of literature comparing single versus combination anthelmintics.

In a large study of yearling heifers in a feedlot setting, a combination of fenbendazole plus ivermectin pour-on was compared with ivermectin pour-on or doramectin injectable (Reinhardt et al., 2006). In comparison to both single anthelmintic treatments, the combination treated heifers had higher ($P<0.02$) live weights and higher dry matter intakes but no difference in gain to feed ratio could be measured. Overall, heifers treated with a combination of a fenbendazole and ivermectin pour-on consumed more feed, gained more weight, and produced heavier carcasses than heifers treated with a single action anthelmintic. In this study, it is very likely that the population of gastrointestinal nematodes was resistant to the avermectin/milbemycins and thus the addition of fenbendazole from the benzimidazole class of anthelmintics increased drug efficacy leading to the advantages in productivity and feed intake.

In a small study of 80 calves consuming a feedlot ration that were naturally infected with an ivermectin-resistant population of gastrointestinal nematodes, calves treated with ricobendazole or a combination of ricobendazole and ivermectin had higher body weight as compared to untreated or ivermectin treated calves at 98 and 126 days on feed (Fazzio et al., 2014). This study demonstrated a live weight advantage of 11.0 kg over 126 days from the administration of a fully effective combination of ivermectin and ricobendazole versus an ineffective treatment with ivermectin.

Impact of gastrointestinal nematode infections on small ruminant productivity

It has been estimated that internal parasites of sheep cost the Australian sheep industry $369 million per year in expenses associated with productivity and treatment, representing the highest disease cost to the sheep industry (Sackett et al., 2006). This cost of gastrointestinal parasitism is not unique to Australia and there is a plethora of literature describing the impact of parasitism on productivity in sheep. The productivity losses generally associated with parasitism are often attributed to anorexia but additional costs such as increased metabolic activity, altered priorities for nutrient utilization, immunopathology, and turnover of immune cells and proteins may also contribute to the reduction in productivity.

The production cost of anthelmintic resistance in 280 lambs was evaluated based on live weight and body condition score (Sutherland et al., 2010). Lambs were allocated to 14 groups based on fecal egg count, live weight, and gender to form 7 groups that were treated with monepantel, an effective treatment, and 7 groups that were treated with an ineffective benzimidazole. At days 0, 28, and 56 there was no difference in fecal egg count or body condition score between groups but at day 84 ($P<0.005$) and day 112

(P<0.01) the albendazole treated group had higher fecal egg counts and lower body condition scores. At all time points tested there was no difference in live weight, except for d112 where monepantel treated lambs were 2.4 kg heavier (P<0.05) than albendazole treated lambs. Carcass weight of monepantel treated lambs was higher than that of albendazole (18.8 kg vs 16.0 kg). Due to differences in carcass quality and yield, it was estimated that average carcass price would be 14% higher for the monepantel treated lambs as compared to albendazole.

Further, measurement of productivity in terms of live weight may not accurately reflect the influence of parasitism on carcass productivity which is the primary economic driver in livestock produced for meat (Jacobson et al., 2009). Larval challenge with *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* resulted in an increase in the weight of the gastrointestinal tract as a proportion to live weight, decreasing dressing percentage by 1.3%, possibly due to stimulation of local immune response in response to parasitic infection (Jacobson et al., 2009). Thus, measurements of live weight would be expected to underestimate the full impact of parasitism on productivity, carcass weights, or cutability.

Biology of gastrointestinal trichostrongyles of livestock

The most important gastrointestinal nematodes that infect livestock are taxonomically classified as Kingdom Animalia, Phylum Nematoda, Class Secernentea, Order Strongylida, Superfamily Trichostrongyloidea, and Family Trichostrongylidae (Sutherland and Scott, 2010). The phylum Nematoda includes a vast number of extremely diverse multicellular organisms with divergent geographical, taxonomical, and ecological backgrounds (Hodda, 2007). There are profound differences in biology, pathogenicity,

and control between species within this phylum. However, there are also many similarities across species that can assist in making decisions regarding control efforts and understanding various attributes of nematode biology, pathophysiology, and genetics. The parasites within the Family Trichostrongylidae are slender, small, hair-like worms with simple, small mouthparts appropriate for mucosal browsing of the helminths (Sutherland and Scott, 2010). The most pathogenic and important genera of gastrointestinal nematodes that infect livestock include *Haemonchus, Ostertagia, Teladorsagia, Trichostrongylus, Nematodirus,* and *Cooperia* (Sutherland and Scott, 2010).

Grazing livestock are often infected with multiple species of gastrointestinal nematodes rather than monospecific infections. Infection with multiple species of gastrointestinal nematodes can produce a complex summation of pathophysiological symptoms which can present as parasitic gastroenteritis or remain subclinical. Certain species of gastrointestinal nematodes are suited for particular environments and thrive under specific climatic conditions. Additionally, species of gastrointestinal nematodes vary in drug susceptibility, for example, *Cooperia* is termed the 'dose-limiting' parasite of cattle as *Cooperia* can survive high levels of ivermectin as compared to other genera such as *Ostertagia a*nd *Trichostrongylus* which are more susceptible (West et al., 1994; Njue and Prichard, 2004).

Epidemiology of gastrointestinal nematodes of livestock

Gastrointestinal nematode infections are practically ubiquitous with prevalences reaching close to 100% in grazing livestock (Zajac, 2012). However, this shouldn't cause alarm or fear among livestock owners as most animals can manage parasite infections

without experiencing disease. Grazing livestock are continuously exposed to the infective stages of internal parasites that commonly contaminate pastures and infections are commonly successful leading to high prevalence levels in grazing herds.

Internal parasite infections in herds of livestock follow a negative binomial pattern of over-dispersion rather than a normal distribution. This negative binomial distribution is characterized by 20-30% of the animals in a herd or flock harboring 80% of the parasites while the other 70-80% of the animals in a herd only harbor 20% of the parasites (Barger, 1985; Sréter et al., 1994; Galvani, 2003). This highly aggregated pattern of parasite burdens in hosts means that a small portion of the population harbors the majority of parasite load and that a relatively small proportion of a herd of animals is responsible for the majority of parasitic egg excretion (Sréter et al., 1994). Variations and genetic heterogenicity in the immune responsiveness of hosts is one of the most important factors leading to overdispersion of trichostrongylid parasites of livestock (Barger, 1989).

Life cycle of gastrointestinal nematodes of livestock

Parasitic nematode species have evolved to infect specific hosts through defined life cycles, co-evolving with their respective host to find the most effective way to perpetuate their life cycle. The life cycle of most common gastrointestinal nematodes of livestock is simple and direct, meaning no intermediate host is required for development of the parasite (Olsen, 1962). Adult male and female parasites are located within the gastrointestinal tract of the host where they mate and females produce eggs that are passed out of the digestive tract in the feces of the host (Olsen, 1962). The fecundity of the adult female parasite varies between species, with some parasites such as *H. contortus*

producing thousands of eggs per day per worm and others such as *Ostertagia ostertagi* (*O. ostertagi*) producing just a few hundred eggs per day (Hansen and Perry, 1994). Pastures can become heavily contaminated through rapid deposition of eggs onto the pasture. Eggs in the early morula stage of development are passed in the feces and on the pasture, eggs become larvated and the embryo can reach three to four times the length of the egg (Veglia, 1916). Under moist conditions, most eggs usually hatch within 24-48 hours producing first-stage larvae. Directly subsequent to hatching, first-stage larvae exhibit intermittent periods of motility followed by periods of rest. Approximately one hour following hatching, the first-stage larvae begin to feed on bacteria present in the feces. Following the feeding period, first-stage larvae enter a state of lethargy for approximately 10 hours prior to awakening and shedding the outer cuticle. This act of ecdysis marks the transition to the second-stage larvae (Veglia, 1916). Second-stage larvae exhibit sinusoidal motility and increase in size as they continue to feed until entering a second period of lethargy approximately 60-65 hours following hatching. When conditions are favorable, larvae awaken from the second period of lethargy approximately 3 days following hatching. Unfavorable conditions such as reduced temperatures and low humidity delay hatching (Veglia, 1916). Development finally stops at the infective third-stage larvae (Olsen, 1962). Third-stage larvae do not feed and must subsequently survive on stored energy reserves, thus limiting the parasites life span on pasture but also representing a period of arrest in the nematode life cycle. The third-stage larvae also retain a second cuticle, protecting this stage from adverse environmental conditions (Olsen, 1962). Temperature and humidity are vital to the development of third stage larvae of gastrointestinal nematodes of livestock (Lettini and Sukhdeo, 2006). More

specifically, the optimal temperature for development of eggs and larvae ranges between 20°C and 35°C (Veglia, 1916). Infective third-stage larvae can survive for months on the pasture and it has well been established that parasite larvae can overwinter on pasture, even in unfavorable conditions (Lettini and Sukhdeo, 2006). Lettini and Sukhdeo (2006) demonstrated that third-stage larvae of *H. contortus* can survive repeat desiccation/rehydration cycles with no deleterious effects. However, humidity is required for development of eggs until they develop to third-stage larvae (O'Connor et al., 2006). In grasses that were divided into three height categories including 0-10 cm, 10-20 cm, and higher than 20 cm, higher numbers of third-stage larvae were identified in 10-20 cm and higher than 20 cm high grass as compared to 0-10 cm high grass, suggesting that third-stage larvae migrate to the upper regions of grasses, where they are more likely to be consumed by grazing livestock feeding on the upper portions of the grass (Silva et al., 2008).

Animals grazing a paddock can then ingest the infective stage, the ensheathed third-stage larvae. If the animal which ingested the third-stage larvae is a suitable host for the species of parasite, the animal can then become infected. Depending on the parasite species, exsheathment or removal of the second-stage cuticle will occur in different anatomical locations within the host dependent on the chemical conditions of the proximal gastrointestinal tract of the host (Lapage, 1933; Sutherland and Scott, 2010). It is generally accepted that exsheathment occurs in a segment of the gastrointestinal tract anterior to the site occupied by the adult form of the parasite. For example, exsheathment of *H. contortus* which resides in the abomasum occurs in the rumen in response to the near neutral pH of this stomach compartment as a result of the equilibrium between

bicarbonate and carbonic acid. This is one reason why *H. contortus* can be exsheathed by gassing with carbon dioxide and formation of carbonic acid (Conder and Johnson, 1996). However, for parasites that are exsheathed within the abomasum, it appears that the low pH and presence of pepsin and hydrochloric acid are important for the process.

The exsheathed third-stage larvae will then penetrate the epithelial layer of their appropriate digestive compartment and undergo further development to the fourth stage of development. The parasites emerge from the mucosal layer as late fourth stage larvae and continue to develop to the fifth and adult stages. Once the adult parasite has developed, they will continue the life cycle through sexual reproduction. The pre-patent period of the parasite is the time between ingestion of the infective stage by the host until the appearance of eggs in the feces. The pre-patent period of the parasite varies between species, but typically ranges between 2-4 weeks for trichostrongyles of livestock (Zajac, 2012). Depending on the parasite species, the worm may exit the mucosal layer or remain in a state of hypobiosis (Bremner, 1956).

Pathophysiology of *Haemonchus* spp.

There are three known species of *Haemonchus* found in domestic ruminants including *H. contortus*, *H. placei,* and *H. similis* (Bremner, 1956; Blouin et al., 1997). *H. contortus* primarily infects small ruminants, whereas *H. placei* primarily infects cattle, although, there is evidence for hybridization and cross-infections between species in the laboratory and field (Chaudhry et al., 2014).

H. contortus is the most pathogenic gastrointestinal nematode of small ruminants and exotic hoof stock, and is a major challenge for sheep and goat producers in tropical and subtropical regions throughout the world (Terrill et al., 2012; Bowman, 2013). In

sheep, clinical presentation of this disease is termed haemonchosis and presents with anemia due to blood loss as fourth and fifth-stage larvae and adults sequester blood from the host (Kaufmann, 1996). At peak infection, naturally acquired *H. contortus* infections may remove one fifth of the circulating erythrocyte volume per day in lambs (Bowman, 2013). An individual adult *H. contortus* can consume up to 0.5 mL of blood per day (Holmes, 1987). This loss of blood results in disturbances of organ-function, inducing alterations in normal physiology (Yacob et al., 2009). If the rate of blood loss exceeds the host's hematopoietic capacity, progressive anemia may lead to death (Bowman, 2013). Disease is also characterized by hypoproteinemia (Kaufmann, 1996). Loss of plasma protein associated with blood loss may result in anasarca, physically expressed in submandibular edema or bottle jaw. *H. contortus* has little effect on appetite and feces are usually well-formed in monospecific infections. This is an important species difference as infections of *Trichostrongylus* spp. and *Teladorsagia* spp. can be associated with diarrhea, while those only associated with *Haemonchus* are not associated with diarrhea.

In cattle, clinical symptoms of haemonchosis may include anemia, submandibular edema, rough hair coat, weight loss, or retarded growth. However, due to much larger blood volumes of cattle as compared to small ruminants, cattle are much more resilient to *Haemonchus* spp. infections as compared to small ruminants. The primary species associated with infection in cattle is *H. placei* (Blouin et al., 1997). Based on nucleotide difference in the internal transcribed spacer 2 region of ribosomal DNA between *H. contortus* and *H. placei*, it has been widely accepted that these are two distinct species (Blouin et al., 1997). The relative differences in pathology between *H. placei* and *H.*

contortus may be associated with host factors as described or differences between the parasite species themselves.

Differences in infection parameters of *H. contortus* between sheep and goats have been reviewed (Hoste et al., 2010). Goats are more susceptible to *H. contortus* infections than sheep, which is attributed to their evolution as browsing animals rather than grazers (Hoste et al., 2010). As browsers, goats have not likely evolved with high levels of exposure to parasitic nematodes and thus depend on their feeding behavior to limit ingestion of infective larvae as opposed to an immune response (sheep) as their primary strategy to regulate gastrointestinal nematode infections (Hoste et al., 2010).

Pathophysiology of *Cooperia* spp.

Cooperia spp. are parasites of the small intestine known to infect both small ruminants and cattle (Stewart, 1954). *Cooperia* spp. are considered the most prevalent parasite genera of young cattle (USDA, 2010) and are often the most common genera identified from feces of young cattle in the Southeastern United States. Heavy infections with *Cooperia* are known to reduce appetite of the host and cause poor nutrient utilization, weight loss, and diarrhea (Stromberg et al., 2012). *Cooperia curticei* is most commonly recovered from sheep, while *C. punctata, Cooperia pectinata,* and *Cooperia oncophora* (*C. oncophora*) are principally parasitic in cattle (Stewart, 1954).

Cooperia punctata is considered the most pathogenic species in this genera, most likely a result of its more invasive behavior as compared to other species. *Cooperia punctata* has a significant effect on cattle productivity, both reducing weight gain and decreased feed intake compared to uninfected controls (Stromberg et al., 2012). Mesenteric lymph nodes were substantially larger and the small intestinal mucosa was

thickened with an increased amount of mucous in cattle infected with *C. punctata* as compared to uninfected controls (Stromberg et al., 2012).

While *C. oncophora* has generally be considered a mild pathogen of cattle (Coop et al., 1979), its importance as a species is increasing due to its propensity to develop anthelmintic resistance. Pathological changes associated with *C. oncophora* infection are restricted to areas surrounding the worm and consist of limited compression and distortion of the lateral margins of the intestinal villi in contact with the parasite (Coop et al., 1979). Coop et al. (1979) found no evidence of blunting or morphological alterations to the villi in calves experimentally infected with *C. oncophora*.

Cooperia spp. are the dose-limiting genera of parasite in cattle as the formulated dosage for the avermectin/milbemycins was based on the concentration required to kill this parasite genera (West et al., 1994; Njue and Prichard, 2004). This is an interesting concept as it indicates that *Cooperia* spp. are naturally able to survive increasing concentrations of avermectin/milbemycins as compared to other parasite genera. Reasons for this have not been explained in the literature, however, this is one reason that *Cooperia* spp. may develop resistance more rapidly than other parasite genera.

Pathophysiology of *Ostertagia* spp.

*Ostertagia s*pp. is the most economically important parasite of adult cattle in temperate regions and is also a significant parasite of young cattle. Production losses associated with *Ostertagia* spp. infections include reduced feed intake, gastrointestinal function, metabolism, and overall body condition (Fox, 1993). There are two morphological variants of *Ostertagia* spp. including the major morphotype, *O. ostertagi,* and the minor morphotype, *O. lyrata,* which do not differ in terms of ribosomal and

mitochondrial DNA and thus are considered the same species (Zarlenga et al., 1998). The adult stage of this parasite is 1.0 cm in length, slender, and brownish-red in color. Female *Ostertagia* spp. have low fecundity, only producing approximately 280 eggs per day which is substantially less than the 10,000 eggs per day produced by *H. contortus* (Smith et al., 1987; Verschave et al., 2014).

Larvae of *Ostertagia* spp. cause distension and hyperplasia of abomasal glands. Both acid-producing parietal cells and pepsinogen-producing chief cells are damaged and subsequently there is reduced acid production resulting in elevation of pH in the abomasum. Activation of pepsinogen to pepsin is reduced due to the elevated pH of the abomasum and thus digestion is inhibited leading to bacterial overgrowth and diarrhea (Stringfellow, 1977; Myers and Taylor, 1989).

Immunity to *O. ostertagi* develops slowly, with animals often remaining susceptible until over two years of age and carrying infections with this parasite for most of their lifetime (Gasbarre et al., 2001). Although animals remain susceptible to infection for an extended period of time, an immune response to this parasite is exhibited through delayed development, stunting of newly acquired parasites, and a reduction in fecundity of adult female worms (Gasbarre et al., 2001). Primary infection with *O. ostertagi* in naïve calves induces changes in regional abomasal lymph nodes including a rapid 30-40% reduction in the percentage of T-cells (Gasbarre, 1994) and an elevation in percentages of B-cells γδ-T cells and cells expressing the IL-2 receptor (Almería et al., 1997). Remarkably, there is a significant increase in size of regional lymph nodes that drain the abomasum within 3-4 days following infection and within 4-5 weeks following

infections lymph nodes can be 20-30 times the weight of lymph nodes from control animals matched for age and size (Gasbarre, 1986; Gasbarre, 1994)

Ostertagia spp. can enter a state of hypobiosis during periods of unfavorable environmental conditions for larval development on pasture (Raynaud et al., 1976). Verschave et al. (2014) reported that between 0.0% to 30.7% of larvae may enter a state of hypobiosis with an average hypobiosis rate of 4.1%. Fourth-stage larvae of *O. ostertagi* can enter a stage of arrested development where they can remain in the abomasal glands for up to 6 months.

There are three types of ostertagiasis, each with unique timelines of development and associated pathological timelines (Myers and Taylor, 1989). Type 1 ostertagiasis represents the usual biological process which occurs when large numbers of infective larvae are ingested in a short period of time and quickly develop to adult parasites and continue transmission. Type 1 is characterized by anorexia, weight loss, diarrhea, and hypoproteinemia. Pre-type 2 ostertagiasis occurs when fourth stage larvae burrow into the abomasum for a hypobiotic period of arrested development. Pre-type 2 ostertagiasis produces little pathology as long as the larvae remain inhibited and is not associated with disease. Arrested late fourth-stage larvae typically emerge progressively from the hypobiotic state. This most commonly occurs with no signs of disease. However, when large numbers of fourth-stage larvae emerge in a short period of time, this disease is termed Type 2 ostertagiasis. Type 2 ostertagiasis is associated with severe abomasal pathology with acute, profuse diarrhea, rapid weight loss, dehydration, and even death if left untreated. The primary lesions associated with both Type 1 and Type 2 ostertagiasis

are white, raised, hyperplastic nodules on the mucosal surface of the abomasum surrounded by severe mucosal inflammation and edema (Myers and Taylor, 1989).

Anthelmintic compounds for control of gastrointestinal nematodes of livestock

Parasite control efforts in livestock are primarily focused around the use of chemotherapeutic agents (anthelmintics). While management of livestock to reduce parasitic infection of livestock is a very important aspect of sustainable parasite control, most livestock owners rely on anthelmintic control as the primary means to control gastrointestinal nematode infections.

There are three primary classes of anthelmintics used in the United States for control of parasitic helminths including the benzimidazoles, the avermectin/milbemycins, and the imidazothiazoles/tetrahydropyrimidines. Anthelmintics within each class share a similar mechanism of action. The benzimidazole class of anthelmintics includes compounds such as fenbendazole, oxibendazole, albendazole, and oxfendazole. The avermectin/milbemycins include abamectin, doramectin, eprinomectin, ivermectin, and moxidectin. The imidazothiazoles/tetrahydropyrimidines include levamisole, pyrantel, and morantel. Although it may seem that there are a plethora of anthelmintic compounds, there are actually only three main classes available for use in livestock in the United States and thus there is a desperate need for novel anthelmintic classes to be discovered and developed (Geary et al., 2015).

Benzimidazoles

Thiabendazole was discovered in 1961 and represents the first of the benzimidazole class of anthelmintics (Lacey, 1990a; Dubey and Sanyal, 2010). This compound revolutionized parasite control being the first extremely safe, low-dose, broad-

spectrum anthelmintic (Lacey, 1988). Initially, the benzimidazole ring became a molecule of interest when it was found to be an essential component of vitamin B12 and extensive studies on the activity of this compound led to its discovery as an anthelmintic compound and later the development of many commercial products within this class (Dubey and Sanyal, 2010). Additional anthelmintics in the benzimidazole class include fenbendazole, oxfendazole, oxibendazole, and albendazole. Benzimidazoles were originally used as fungicides (Lubega et al., 1994). Benzimidazoles possess a bicyclic ring system with benzene fused to the 4 and 5 position of the hetero cycle. These compounds are crystalline with high melting points and poor solubility in water, which is why they are commonly formulated as oral therapeutics rather than pour-on solutions or injectables (Dubey and Sanyal, 2010).

The mechanism of action of the benzimidazoles involve their interaction with the eukaryotic cytoskeleton protein, tubulin which is a dimeric protein comprised of alpha and beta subunits (Lacey, 1990a). Tubulin is a subunit of the microtubules and the benzimidazoles bind to β-tubulin and prevent the polymerization of α-tubulin to β-tubulin, preventing further elongation of microtubules. Microtubules are in a constant state of association and dissociation and since the polymerization of tubulin into microtubules is inhibited and the microtubules continue to dissociate, this results in a net loss of microtubule length and subsequently a slow death of the parasite (Dubey and Sanyal, 2010). Thiabendazole is also known to irreversibly block glucose uptake in parasites leading to depletion of energy stores and eventually death in parasites (Papich, 2016). In summary, benzimidazoles work by inhibiting microtubule assembly (Prichard, 1994).

Avermectin/milbemycins

The avermectin/milbemycin drugs are the most important class of anthelmintics used in control of gastrointestinal nematodes of livestock. The avermectin/milbemycins are antiparasitic drugs that are endectocides that have transformed parasite control as they are extremely safe while harboring broad-spectrum antiparasitic activity against both endoparasites and ectoparasites (Prichard et al., 2012). Several avermectin/milbemycin drugs are registered for sheep and/or cattle including abamectin, eprinomectin, doramectin, ivermectin, and moxidectin (Kotze et al., 2014b). Ivermectin was introduced as an antiparasitic drug in 1981 and abamectin was introduced as both a pesticide and antiparasitic drug in 1985 (Campbell, 1989). Originally, a unique avermectin producing bacteria, *Streptomyces avermitilis* was discovered by Dr. Satoshi Omura and his research team at the Kitasato Institute in a microbial culture from a soil sample near a golf course in Japan (Burg et al., 1979). Over several years Dr. Omura's team sent thousands of fermentation broth cultures to Merck Research Laboratories in Rahway, New Jersey, USA to screen for use in the animal health industry (Molyneux and Taylor, 2015). Upon screening the fermentation broth containing *Streptomyces avermitilis,* the group identified antiparasitic activity and subsequently synthesized the active compound avermectin B1 and a derivative named ivermectin (Molyneux and Taylor, 2015). The avermectins were identified as a series of hydrophobic macrocyclic lactone derivatives, which have potent anthelmintic activity but lack antibacterial or antifungal activity. The avermectin complex was first screened in mice and shown to clear *Nematospiroides dubius* when fed to mice for 6 days (Burg et al., 1979).

Although all the avermectins and milbemycins contain a 16-member macrocyclic lactone ring, they are produced by different organisms during fermentation and have minor structural differences (Prichard et al., 2012). The milbemycins and their potent acaricidal and miticidal properties were actually discovered before the avermectins (Campbell, 1989). However, the anthelmintic properties of the milbemycins were not realized until after the discovery of the avermectins (Campbell, 1989). The milbemycins are fermentation products of *Streptomyces hydroscopicus* (milbemycin-oxime, 1967) and *Streptomyces cyaneogriseus* (nemadectin, moxidectin, 1983), while the avermectins are products of *Streptomyces avermitilis* (Burg et al., 1979). *Streptomyces avermitilis* actually produces four homologous pairs of avermectin analogs including avermectin A_1, A_2, B_1, and B_2. Avermectin B_{1A} is the most important analog as it has high potency against a broad spectrum of endo and ectoparasites of farm animals and serves as the starting material for the semisynthetic 22,23 dihydro analog which is used in generic ivermectin (Campbell, 1989). The macrocyclic lactone ring of these compounds is fused with benzofurane and spiroketal functions in a three-dimensional arrangement that have a high affinity for chloride ion channel receptors which contribute to the mechanism of action of these compounds (Prichard et al., 2012). The avermectins have sugar groups at C13 but the milbemycins are protonated at C13 (Takiguchi et al., 1980; Prichard et al., 2012). Moxidectin also has a methoxime at C23. Moxidectin also has a longer half-life as it is more lipophilic. In terms of analogs of avermectins, abamectin and ivermectin were the first analogs to be developed in the 1980s and subsequently doramectin, eprinomectin, and selamectin were developed.

Abamectin, the first avermectin/milbemycin developed, is a mixture of more than 90% avermectin B_{1a} and less than 10% avermectin B_{1B}. Ivermectin is a chemically reduced 22,23-dihydro derivative of abamectin with more than 90% 22,23-dihydroavermectin B_{1a} and less than 10% 22,23-dihydroavermectin B_{1B}. Ivermectin only differs from abamectin by the reduction of one double bond, resulting in a single methylene group at position 26 (Campbell, 1989).

The amino-avermectin eprinomectin was derived from avermectin B_1 with modified terminal oleandrose moiety called 4”-epiacetylamino-4”-deocy-avermectin B_1 (Prichard et al., 2012). This compound has favorable properties for milk production as the topical formulation requires 0 days milk withdrawal and thus is approved and used extensively in dairy cattle. Specifically, topically administered eprinomectin (0.5 mg/kg) is detectable in the milk of lactating dairy cattle at 0.109% the total administered dose of eprinomectin, a mean maximum concentration of 5.14 ng/mL, which does not exceed the maximum acceptable limit of 30 ng/mL and further supports the 0 days milk withdrawal period (Alvinerie et al., 1999). The pharmacokinetic profile of injectable eprinomectin in milk is similar to the topical formulation with a mean maximum concentration of 6.4 ng/mL and only 0.39% of the dose detected in milk (Baoliang et al., 2006).

Doramectin is another avermectin analog that is formulated as an injectable and pour-on for cattle. Doramectin is prepared by mutational biosynthesis and has a cyclohexyl group in the C25 position of the avermectin ring (Prichard et al., 2012). Doramectin is more similar to abamectin than ivermectin (Prichard et al., 2012).

The avermectins are highly lipophilic compounds and dissolve in most organic solvents such as alcohol, dimethylsulfoxide, chloroform, acetone, and cyclohexane

(Campbell, 1989). However, the solubility of the avermectins in water is low, only 0.006 to 0.009 mg/l (Campbell, 1989).

The milbemycins are unglycosylated when compared to the avermectins and lack a bisoleandrosyl moiety at C-13. The milbemycins are protonated at C-13, while the avermectins are hydroxylated at this position. The C-Nemadectin was isolated as an active fermentation product from *Streptomyces cyaneogriseus* in 1983. A methoxime moiety at C-23 was added to nemadectin to create moxidectin (Prichard et al., 2012). Moxidectin has a olefinic side chain at the C-25 and methoxime moity at the C-23 which are both specific to this compound and not found in any other commercial formulations.

The glutamate-gated chloride ion channel receptors are expressed in neurons and muscle cells of nematodes and are the putative biological targets of the drugs (Cully et al., 1994). The avermectin/milbemycins bind to these channels, causing irreversible opening of the channel and subsequently inhibit muscle contraction, causing rapid paralysis and death of the parasite (Kotze et al., 2014a). There is evidence that the avermectin/milbemycins also bind to gamma-aminobutyric acid and glycine receptors and may also contribute to the activity of these compounds, however, activity at these receptors requires much higher drug concentrations than the glutamate-gated chloride ion channel receptors (Kotze et al., 2014a).

Imidazothiazoles/tetrahydropyrimidines

The membrane depolarizers target the nicotinic acetylcholine receptors of nematodes. These receptors, typically found on somatic muscle cells, pharyngeal muscle, and nerve cells, are comprised of 5 subunits that form a transmembrane ion-channel that is typically opened by acetylcholine and subsequently allows for passage of sodium and

calcium ions into the cell (Kotze et al., 2014a). The selective nicotinic agonists, levamisole and pyrantel, selectively open these ligand-gated ion-channels found in the membranes of nerves and muscle of nematodes (Martin and Robertson, 2000).

Levamisole is one of the primary drugs in this class and is an important drug in small ruminant parasite control. Levamisole is absorbed from the gastrointestinal tract and rapidly metabolized in the liver, however, levamisole has a relatively narrow margin of safety compared to anthelmintics in other classes.

Anthelmintic resistance

Anthelmintic resistance presents a major threat to livestock health and productivity (Kaplan, 2004). Resistance can be defined as the ability of a population or a proportion of a population to tolerate a therapeutic dose of a compound. Resistance is a heritable trait (Prichard et al., 1980). The presence of drug resistance in internal parasites of livestock is widespread and is the status quo rather than the exception in many species of parasitic nematodes (Kaplan, 2004; Kaplan and Vidyashankar, 2012). Strict reliance on anthelmintics for parasite control without regard to biological factors such as pasture management and parasite biology can lead to rapid selection for drug resistance (Williams, 1997).

This situation is well described in the small ruminant industry where anthelmintic resistance is a serious and widespread global problem (Kaplan, 2004; Howell et al., 2008; Kaplan and Vidyashankar, 2012). Anthelmintic resistance to multiple classes of drugs is a serious problem on small ruminant farms across the globe (Howell et al., 2008; Torres-Acosta et al., 2012; Playford et al., 2014), providing a significant challenge to effective parasite control (Kaplan and Vidyashankar, 2012). The prevalence of farms with

multiple-anthelmintic resistance and total anthelmintic failure is already high and is increasing (Martínez-Valladares et al., 2013; Herrera-Manzanilla et al., 2017; Howell et al., 2017).

In cattle, over the past fifteen years there have been increasing numbers of reports of anthelmintic resistance worldwide. Resistance to the avermectin/milbemycin class of drugs is most commonly reported in *Cooperia* spp. in cattle and has been documented in North America, South America, Europe, and Australasia (Fiel et al., 2001; Anziani et al., 2004; Condi et al., 2009; Demeler et al., 2010b; Edmonds et al., 2010; Lyndal-Murphy et al., 2010; Rendell, 2010; Fazzio et al., 2014; Cotter et al., 2015; Geurden et al., 2015). Although the prevalence of resistance is lower, there have also been published reports of resistance to avermectin/milbemycins in *Ostertagia* spp. in Australasia (Rendell, 2010; Waghorn et al., 2016), North America (Edmonds et al., 2010), and South America (Suarez and Cristel, 2007). Additionally, resistance in *Ostertagia* spp. has been reported to levamisole and benzimidazole in Australia (Rendell, 2010; Cotter et al., 2015).

Many of the parasitic nematodes species have biological characteristics that contribute to the development of resistance within the species, including but not limited to large population sizes and rapid genetic mutation rates which contribute to exceptionally high levels of genetic diversity within the species (Kaplan and Vidyashankar, 2012; Gilleard and Devaney, 2013). Even with this alarming rate of anthelmintic resistance development, development of novel classes of anthelmintics has been slow (Geary et al., 2015).

Detection of anthelmintic resistance

It has been accepted that effective nematode control programs designed to minimize the development of anthelmintic resistance must include effective and sensitive methods to detect and monitor anthelmintic resistance (Gill et al., 1991; Taylor et al., 2002; Kaplan et al., 2007; Demeler et al., 2010b). Numerous *in vivo* and *in vitro* assays have been developed to detect anthelmintic resistance to the avermectin/milbemycins, but each suffer from issues including cost, reliability, reproducibility, sensitivity, and ease of execution and interpretation (Taylor et al., 2002). Current *in vivo* tests for the detection of anthelmintic resistance include the controlled efficacy study and FECRT.

Fecal Egg Count Reduction Test

The Fecal Egg Count Reduction Test (FECRT) is the preferred assay for detection of resistance at the farm level in gastrointestinal nematodes of ruminants (Coles et al., 1992; Coles et al., 2006). However, true efficacy tests require animals to be treated and subsequently necropsied to enumerate worms that survive anthelmintic treatment as compared to untreated controls. These 'treat and slaughter' studies are not practical or economically viable in commercial or hobby livestock production and thus the FECRT is the preferred methodology to detect resistance.

A FECRT is conducted, by performing fecal egg counts (FEC) on samples from approximately 15–20 animals both prior to treatment and again following treatment (Coles et al., 1992). Subsequently, the reduction in fecal egg count after anthelmintic treatment can be calculated to evaluate the efficacy of the tested product. Although previous guidelines for fecal egg count reduction tests in sheep were published in 1992 (Coles et al., 1992), no guidelines currently exist for horses or cattle.

The FECRT is commonly used in livestock for research purposes, however it has not been widely adopted by producers. Specifically, the 2007–2008 USDA National Animal Health Monitoring Service report found that only 5.7% of beef cattle operations surveyed conducted any fecal egg counts (USDA, 2010). The expense of conducting a FECRT has been a limiting factor in the implementation of this test and producers often view testing as uneconomical (Morgan et al., 2005). However, the cost of anthelmintic resistance in sheep is about 10-15% of the lamb value at sale, suggesting that losses due to undetected resistance outweigh the cost of testing for anthelmintic efficacy (Leathwick and Besier, 2014).

The percentage fecal egg count reduction is calculated by using the following formula: [(Mean Pre-Tx FEC – Mean Post-Tx FEC)/Mean Pre-Tx FEC] x 100%. However, the mean percent fecal egg count reduction is not sufficient to diagnose resistance and it is necessary to calculate the 95% confidence interval around mean percent fecal egg count reduction to provide statistical relevance of the data (Wang et al., 2018).

In ruminants, a population is considered to be susceptible to an anthelmintic when there is greater than or equal to 95% mean reduction in fecal egg count and a lower 95% confidence interval greater than or equal to 90%. A resistant population is identified when both the mean reduction is less than 95% and the lower 95% confidence interval is less than 90%. Low resistance will be diagnosed if only one of these two criteria are met.

Martin et al. (1989) identified limitations in the sensitivity of the FECRT to detect resistance to the benzimidazoles if less than 25% of the parasite population is resistant. These results suggest the FECRT is not suitable for detecting low levels of resistance

such as when resistance is emerging in a population. More sensitive tests are required to detect low levels of drug resistance.

In vitro assays for detection of anthelmintic resistance

While the FECRT is currently the preferred method for detection of resistance on farm, it suffers from cost and practicality issues. Thus, focus has shifted to development of *in vitro* assays that measure the effects of anthelmintics on development, growth, behavior, or motility of nematodes (Taylor et al., 2002). Genotypic (molecular) methods for detection of resistance are highly attractive, however, the only class of drugs with validated genetic markers for resistance are the benzimidazoles (Kotze et al., 2014a).

In vitro assays are the most efficient and cost-effective strategy to detect anthelmintic resistance in nematode populations (Storey et al., 2014). The egg hatch assay (Le Jambre, 1976), larval development assay (Taylor, 1990; Gill et al., 1995), the larval migration inhibition assay (Wagland et al., 1992; Demeler et al., 2013), the larval feeding assay (Alvarez-Sanchez et al., 2005; Bartley et al., 2012), and most recently the Worminator (Storey et al., 2014) are all *in vitro* assays developed to detect anthelmintic resistance in gastrointestinal nematodes of livestock.

While several *in vitro* assays have been developed to measure anthelmintic resistance, few have been properly validated to determine the usefulness of the assay for detection of resistance in field isolates. Thorough and proper validation requires repeated biological replicates of assays to be completed on multiple, well-characterized susceptible and resistant isolates. These validation studies are rarely conducted as establishment and maintenance of research isolates of gastrointestinal nematodes of livestock is expensive, labor intensive, and requires animal ethics approval and

maintenance of livestock hosts. Although proper validation studies are essential to determine if an assay can distinguish susceptible and resistant isolates, these studies are rarely completed and often researchers complete single assays on field isolates or historic isolates to evaluate if an assay can distinguish isolates. Single assays, even with multiple technical (assay well) replicates, have little to no statistical power to provide any meaningful data regarding the ability of an assay to detect anthelmintic resistance.

The effective concentration 50 (EC_{50}) is the concentration of a drug or compound which yields 50% of the maximal effect after a specified period. The EC_{50} is commonly used to describe a drug's potency in a given population. Resistance ratios are commonly used to describe the suitability of an assay to detect resistance. Resistance ratios are calculated as the EC_{50} of the resistant isolate divided by the EC_{50} of the susceptible isolate. A resistance ratio of 5.0 is commonly regarded as the minimum threshold required for a test to detect resistance. However, this arbitrary threshold is completely inadequate as a single biological replicate comparing a susceptible and resistant isolate which yield a resistance ratio of 5.0, provides insufficient statistical power to validate an assay. Repeated replicates on multiple isolates are required to yield confidence interval around EC_{50}s and resistance ratios to yield the opportunity for statistical interpretation and testing of the assay. A resistance ratio of 2.0 could actually be completely appropriate to detect resistance as long as the level is highly repeatable. However, proper validation of *in vitro a*ssays are rarely completed in the parasitology community and thus much of the discussion will compare reported resistance ratios.

Egg Hatch Assay

The egg hatch assay was developed in 1976 to detect resistance to benzimidazole anthelmintics *in vitro* (Le Jambre, 1976). In this test, eggs are incubated in wells containing increasing concentrations of thiabendazole and the ability of thiabendazole to inhibit the embryonation and hatching of freshly collected nematode eggs is used to calculate an EC_{50}, or the concentration where 50% of egg hatching is inhibited (Coles et al., 1992).

This test relies on the ovicidal activity of the benzimidazoles which prevent hatching of eggs. Since the avermectin/milbemycins lack ovicidal activity, this assay is not appropriate for detection of resistance to this class. This assay is reliable and is consistently used in isolates of *H. contortus.*

Larval Development Assay

The larval development assay measures the development of nematode eggs to third-stage larvae in the presence of increasing concentrations of various analogues of anthelmintic compounds (Coles et al., 1988; Gill et al., 1995; Kotze et al., 2014b). Assay plates typically consist of a series of increasing drug concentrations dissolved in DMSO impregnated into 2% agar. Variations in the preparation of the assay plates exist with many laboratories making their own larval development assay agar plates. However, the only commercially available plates are those produced by Dr. Jenny Gill in Australia in a 96 - well format with increasing concentrations of thiabendazole, ivermectin aglycone, and levamisole. These plates consist of triplicate assay wells at a particular concentration and a minimum of 8 control wells.

Eggs are isolated from the feces of an individual animal or a pooled sample from a group of animals and dispensed into each well of the plate (50-100 eggs per well) and the plates are incubated overnight at 25 degrees Celsius. At 24 hours, the larvae are fed a nutritive media containing *Escherichia coli,* which provides a food source for larval development. After 7 days of incubation, Lugol's iodine is added to each well of the plate to kill the larvae. The number of eggs, L1, L2 and L3 are counted, however, in some laboratories only the L3 are counted to improve throughput of the assay particularly when completing this assay on known monospecific laboratory isolates (Howell et al., 2008; Kotze et al., 2014b). The third-stage larvae are identified and an EC_{50} for each class of anthelmintic is calculated. An EC_{95} for ivermectin aglycone is also calculated to provide further evaluation of moxidectin efficacy (Kaplan et al., 2007). This assay is a powerful tool for detection of anthelmintic resistance in sheep, goats, exotic hoofstock, llamas, and camelids (Kaplan et al., 2007; Kotze et al., 2014b).

The larval development assay is by far the most widely used *in vitro* assay for detection of resistance to the avermectin/milbemycin drugs, particularly in gastrointestinal nematodes of small ruminants. It has been used to distinguish isolates of *H. contortus* and *O. ostertagi* with varying levels of sensitivity to thiabendazole, ivermectin, and levamisole (Taylor, 1990). This test was further developed as a commercial assay, capable of simultaneous detection of anthelmintic resistance to all available classes of anthelmintics for parasites of small ruminants, particularly *H. contortus* (Lacey et al., 1990b). This assay was named the DrenchRite® Larval Development Assay and is currently completed as a diagnostic assay in the laboratory of Dr. Ray Kaplan at the University of Georgia. The DrenchRite® Larval Development

Assay has been shown to indicate the presence of resistance to the avermectin/milbemycins in *H. contortus*, *T. colubriformis*, and *T. circumcincta* (Gill et al., 1995; Gill and Lacey, 1998). However, reports of failure to detect avermectin/milbemycin resistance in field populations of *T. circumcincta* were reported at the 1998 Australian Veterinary Association meeting in Sydney and thus use of the DrenchRite® Larval Development Assay for detection of avermectin/milbemycin resistance on commercial farms has ceased in Australia (Kotze et al., 2006). Additionally, third-stage larvae of *T. colubriformis and T. circumcincta* have different response parameters to avermectin/milbemycins and cannot be differentiated without ex-sheathment and inspection of tubercles and thus cannot be differentiated on the agar plate of the larval development assay, further demonstrating the lack of suitability of this assay in Australia. However, low levels *T. circumcincta* are identified from coprocultures in the Southeastern USA and thus the larval development assay is very practical for detecting anthelmintic resistance in this region.

The avermectin/milbemycin analogues ivermectin, avermectin B1, avermectin B2, ivermectin aglycone and ivermectin monosaccharide were evaluated in the larval development assay against susceptible and resistant isolates of *H. contortus* (Gill et al., 1995). Dose response curves shifted to the right for resistant isolates as compared to the susceptible McMaster *H. contortus* isolate and resistance ratios from 1.4 to 11 were calculated for resistant isolates tested (Gill et al., 1995). It was shown that avermectin B2 was the most sensitive probe for ivermectin resistance in this assay and the larval motility assay (Gill et al., 1995). One explanation for this observation is that more polar analogues provide better discrimination between susceptible and resistant isolates. This observation

was supported in this study as hydrophobic avermectins such as avermectin B1 and ivermectin exhibited resistance ratios of less than 3.0, but more polar analogues such as avermectin B2 exhibited resistance ratios ranging from 5.0 to 17.0 depending on the resistant isolate assayed (Gill et al., 1995).

More recently, commercial formulations have been evaluated for their use in larval development assays. Commercial formulations of abamectin, doramectin, eprinomectin, ivermectin, and moxidectin were used to distinguish between susceptible and resistant isolates of *H. contortus* (Kotze et al., 2014b). A resistance ratio of 69.2 was exhibited with commercially formulated eprinomectin in this assay, demonstrating the greatest ability to discriminate among susceptible and resistant *H. contortus* among the commercially available products tested (Kotze et al., 2014b). However, commercially formulated moxidectin only exhibited a resistance ratio of 4.0, exhibiting the poorest discrimination among isolates of varying resistance status (Kotze et al., 2014b). Overall, the commercial formulations of anthelmintics ranked from highest to lowest in terms of resistance ratio in the following order eprinomectin, doramectin, abamectin, ivermectin, and moxidectin (Kotze et al., 2014b). However, there was no statistical difference between doramectin, abamectin, and ivermectin in terms of resistance ratio (Kotze et al., 2014b). This study also compared ivermectin aglycone, the analog used in the DrenchRite® Larval Development Assay to eprinomectin and found that ivermectin aglycone produced a resistance ratio of 216 while eprinomectin demonstrated 69, showing that ivermectin aglycone displayed a greater ability to distinguish between avermectin/milbemycin susceptible and resistant *H. contortus* (Kotze et al., 2014b). However, commercially available eprinomectin is much cheaper and more readily

available than ivermectin aglycone and these advantages demonstrate the acceptability of its use in future larval development assays (Kotze et al., 2014b).

A DrenchRite® Larval Development Assay performed on two laboratory isolates of *H. contortus* demonstrated the ability to detect resistance to ivermectin with a resistance ratio of 60 using ivermectin aglycone as the analog of choice (Williamson et al., 2011). The larval development assay has been evaluated against numerous susceptible and resistant isolates of *H. contortus* and provides a powerful tool of detection of resistance to the avermectin/milbemycins, particularly with the analogs ivermectin aglycone and eprinomectin.

The larval development assay has also been applied for detection of resistance to avermectin/milbemycins in other important species of gastrointestinal nematodes. Recently, highly ivermectin-susceptible and highly ivermectin-resistant (0% efficacy with ivermectin) isolates of *H. placei* were subjected to the larval development assay using commercially available formulations of abamectin, doramectin, eprinomectin, ivermectin, and moxidectin. Very low resistance ratios of less than 3.5 were calculated and there was no statistical difference between the analogues assayed in terms of resistance ratio (Kotze et al., 2014b). However, the gold standard of analogues for the larval development assay, ivermectin aglycone, produced a resistance ratio of 6.90 as compared to that of 3.33 for eprinomectin in *H. placei* (Kotze et al., 2014b). In this same publication, resistance ratios of 216 and 69 for ivermectin aglycone and eprinomectin, respectively were calculated for *H. contortus* (Kotze et al., 2014b).

Ivermectin failed to produce a normal sigmoidal dose response curve in a larval development assay with *T. colubriformis*, but did exhibit a shift in response characteristic

of the decreased sensitivity to ivermectin in laboratory selected ivermectin resistant *T. colubriformis* (Gill et al., 1991). Resistance ratios ranging from 7 to 32 in *T. colubriformis* and 1.13 to 4.21 in *T. circumcincta* were calculated in larval development assays with five avermectin/milbemycin analogs but there was no obvious pattern in which analog was best able to discriminate between susceptible and resistant isolates and 95% confidence intervals of EC_{50} across analogs overlapped (Demeler et al., 2013).

The larval development assay was also applied to isolates of *Cooperia oncophora* and yielded a resistance ratio of 5.30 using ivermectin (Demeler et al., 2010b). Overall, the larval development assay is an effective *in vitro* assay for detection of resistance in laboratory and field isolates of *H. contortus* and has shown to be effective in discrimination of susceptible and resistant isolates of other important gastrointestinal nematodes of livestock. It seems that eprinomectin and ivermectin aglycone are the most appropriate avermectin/milbemycin analogues for differentiation of susceptible and resistant isolates.

Larval Migration Inhibition Assay

The larval migration inhibition assay measures the ability of third-stage larvae to migrate through a fine-mesh as an indicator of larval health and drug effectiveness (Wagland et al., 1992). Larvae that are affected by the compound tested will not be able to migrate through the mesh while larvae that are not affected by the compound will migrate through the mesh. An EC_{50} can be calculated as a measure of where 50% of larval migration is inhibited as compared to maximum migration in control wells.

The larval migration inhibition assay was evaluated across laboratories and shown to exhibit low levels of variability when standardized protocols, techniques, and reagents

were used (Demeler et al., 2010b). However, many laboratories still believe this assay lacks consistency and reproducibility across laboratories. It appears that various research laboratories develop unique adaptations to *in vitro* assays to either 1) make the assays easier or more economical with the resources they have available or 2) yield improvements to the assay to better detect resistance. These minor changes in assays make it difficult to directly compare data from various publications and laboratories since the assays are not standardized on a global scale. As an example of these adaptations, modifications to the larval migration inhibition assay were made to conduct the assay in a 96-well format and used to detect resistance to macrocyclic lactones in *H. contortus* (Kotze et al., 2006). Briefly, infective third-stage larvae are exposed to drug for 24 hours in the dark and then allowed to migrate through an agar and filter mesh system for an additional 48 hours. Some laboratories use agar mesh and other laboratories only use a filter mesh, however, it has been demonstrated that the agar mesh greatly improved the sensitivity of the assay for resistance detection (Kotze et al., 2006). This migration through agar and mesh has been described to potentially allow for greater discrimination between twitching movement of avermectin/milbemycin affected worms and coordinated movement to move in one direction through the agar layer, improving the sensitivity of the assay (Kotze et al., 2006). Addition of 0.125% agar reduced the EC_{50} of the susceptible Kirby isolate and left the resistant isolate unchanged allowing for greater discrimination between susceptible and resistant *H. contortus* (Kotze et al., 2006). While adaptations of current assays are required for optimization, these changes make it difficult to compare data from various studies.

Unlike the larval development assay, which evaluates inhibition of development through multiple life stages from the egg to third-stage larvae, the larval migration inhibition assay only measures inhibition of motility of third-stage larvae and thus only evaluates resistance mechanisms present in this life stage. While the larval migration inhibition assay can detect differences in susceptible and resistant *H. contortus*, resistance ratios are lower than those of the larval development assay and may be associated with the differences in life stages targeted by each assay. The concentration of anthelmintic compounds required to inhibit motility of L3 has been shown to be more than 100 times what is required to prevent larval development (Geary et al., 1993; Gill et al., 1995). This characteristic may provide information on why it is difficult to discriminate between susceptible and resistant isolates of gastrointestinal nematodes using motility of L3.

In comparison to the larval development assay, resistance ratios obtained with the larval migration inhibition assay are low regardless of the analogue of avermectin/milbemycin tested. Eprinomectin was demonstrated to best distinguish between susceptible and resistant *H. contortus* as compared to abamectin and ivermectin (Kotze et al., 2006). The analogue eprinomectin was previously shown to be able to best detect anthelmintic resistance in the larval development assay. Demeler et al. (2013) found moxidectin demonstrated the highest resistance ratio of 6.74 in *H. contortus* while ivermectin, ivermectin B1 a, and ivermectin B1b showed resistance ratios of less than 1.5 and milbemycin A3 and milbemycin A4 showed higher EC_{50} values for susceptible isolates than resistant isolates.

In *Cooperia oncophora*, a resistance ratio of 5.05 for ivermectin was calculated in the larval migration inhibition assay (Demeler et al., 2010b). No resistance ratios were

reported for isolates of *C. oncophora* evaluated in the larval migration inhibition assay but higher EC_{50} for two farms with known avermectin/milbemycin resistance were calculated (Demeler et al., 2012). Unfortunately, no level of resistance was reported for the resistant isolate used by Dememler et al., (2010b) other than 'insufficient fecal egg count reduction'. This highlights one of the common problems in this field of research where isolates are poorly characterized (Redman et al., 2008b) and then used to describe the usefulness of *in vitro a*ssays. There is an urgent need for more thorough characterization of research isolates of gastrointestinal nematodes of livestock to provide the minimum resources to conduct validation studies for *in vitro* assays to detect anthelmintic resistance.

Although a dose response could be demonstrated in both *T. colubriformis* and *T. circumcincta*, the modified larval migration inhibition assay was unable to detect differences between avermectin/milbemycin susceptible and resistant *T. circumcincta* or *T. colubriformis* using abamectin, ivermectin, or eprinomectin, demonstrating its lack of practicality in the field (Kotze et al., 2006). Demeler et al. (2013) found that moxidectin exhibited the highest resistance ratios for *T. colubriformis* (7.28) and *T. circumcincta* (22.45) with the larval migration inhibition assay, however, the resistance ratio for *T. colubriformis* is much lower than that in the larval development assay.

Relative potencies of analogues of avermectin/milbemycins were also evaluated for larval migration inhibition assays. In a susceptible laboratory isolate of *H. contortus*, avermectin B1 and ivermectin were more potent than avermectin B2 which was much more potent than ivermectin monosaccharide and ivermectin aglycone (Gill et al., 1991). These results agreed with those of the resistant *H. contortus* isolate evaluated except that

avermectin B1 was slightly less potent than ivermectin (Gill et al., 1991). Abamectin was more potent than ivermectin that was more potent than eprinomectin in a larval migration inhibition assay with *H. contortus* (Kotze et al., 2006). Ivermectin and milbemycin A3 and A4 were more potent than moxidectin or either component B1a or B1b of ivermectin against a susceptible isolate of *H. contortus* and moxidectin and ivermectin B1a and B1b were more potent than ivermectin or either milbemycin with the resistant isolate of *H. contortus* in a larval migration inhibition assay (Demeler et al., 2013). This same variation in potency between isolates was demonstrated in *T. colubriformis*, and *T. circumcincta* (Demeler et al., 2013).

Larval Feeding Assay

Larval feeding assays measure the activity of anthelmintics on pharyngeal pumping which is a known target of the avermectin/milbemycins. Insulin uptake was quantified in adults of *T. colubriformis* and the analogues eprinomectin, ivermectin, ivermectin monosaccharide, and ivermectin aglycone were shown to decrease the frequency and amplitude of pharyngeal pumping events (Sheriff et al., 2002). In terms of pumping frequency, differences between analogues were attributed to differences in drug uptake (Sheriff et al., 2002). Ivermectin was evaluated in a larval feeding assay with *H. contortus* and *T. circumcincta*, and demonstrated resistance ratios of 7.46 and 7.75, respectively (Alvarez-Sanchez et al., 2005). However, this assay was again applied to isolates of *H. contortus* and a resistance ratio of only 1.4 was generated with ivermectin demonstrating the lack of reproducibility with this assay (Bartley et al., 2009).

The Worminator

The Worminator system objectively quantifies motility of microscopic larval stages of gastrointestinal parasites of livestock and has provided a technology to study the interaction of larval stages of parasites of livestock and anthelmintic compounds (Storey et al., 2014). This technology is comprised of an inverted microscope connected to a video camera which provides output to a software program which can quantify displacement of pixels between video frames as a measure of parasite motility within a recorded well (Storey et al., 2014). This technology is capable of producing repeatable dose-response curves in response to incubation with avermectin/milbemycins or L3 of *H. contortus* and *C. punctata*. However, this technology has not been able to detect differences between susceptible and resistant isolates of *C. punctata* and *H. contortus* (George et al., 2018). Resistance ratios of 1.28, 1.55, and 0.98 have been observed for eprinomectin, ivermectin, and moxidectin, respectively in *H. contortus* (George et al., 2018). The most potent analog was ivermectin in both isolates of *H. contortus* (George et al., 2018). For the *C. punctata* isolates, relative potency of analogs against the susceptible isolate are abamectin > ivermectin > doramectin > eprinomectin > moxidectin (George et al., 2018). Abamectin was also the most potent against the resistant isolate and moxidectin was the least potent against the susceptible isolate (George et al., 2018). Resistance ratios were less than 1.5 for all analogues tested against the *C. punctata* isolates (George et al., 2018).

Pyrosequence genotyping for benzimidazole resistance

Pyrosequence genotyping was developed as an alternative to whole-genome sequencing and is based on sequencing by synthesis (Ronaghi et al., 1998). This method is based on the detection of nucleotide incorporation (Ronaghi et al., 1998).

In several species of gastrointestinal nematodes of livestock, resistance to the benzimidazoles is caused by single nucleotide polymorphisms in the isotype-1 β-tubulin gene (Samson-Himmelstjerna et al., 2009b). Originally, a substitution of a tyrosine for a phenylalanine at codon 200, which was encoded by a change from TTC to TAC was demonstrated and is now the most common single nucleotide polymorphism associated with benzimidazole resistance (Kwa et al., 1994). While the F200Y single nucleotide polymorphism in the beta-tubulin gene is the most common polymorphism associated with benzimidazole resistance, further work has shown the contribution of single nucleotide polymorphisms at codons 167 (F167Y) and 198 (E198A) (Silvestre and Cabaret, 2001).

Pyrosequence genotyping of these polymorphisms allows for quantitative evaluation of allele frequencies associated with benzimidazole sensitivity and resistance and can be completed on pooled samples of DNA representing various ratios of alleles associated with resistance (Samson-Himmelstjerna et al., 2009b). Pyrosequence genotyping of polymorphisms associated with resistance to benzimidazoles is suitable for routine diagnosis of benzimidazole resistance of field populations and this assay is more sensitive than the egg hatch assay and less time-consuming than current *in vivo* or *in vitro* methods for detection of resistance (Samson-Himmelstjerna et al., 2009b).

Although more in depth studies are required to provide an accurate level of detection sensitivity for pyrosequence genotyping for benzimidazole resistance, 10% is a conservative estimate for the sensitivity of this assay's ability to detect resistance meaning that this assay should detect resistance alleles once the frequency of that allele is at least 10% in the population (Samson-Himmelstjerna et al., 2009b). This suggests that pyrosequence genotyping is more sensitive than the FECRT or egg hatch assay for detection of resistance to the benzimidazoles.

Mechanisms of anthelmintic resistance

The most significant progress in terms of determining the mechanism of anthelmintic resistance has been in the benzimidazole class of anthelmintics. We know that a single nucleotide polymorphism in the isotype 1 β-tubulin gene is associated with resistance to these drugs in most, if not all, trichostrongylid nematode species (Kwa et al., 1994). Originally, a substitution of a tyrosine for a phenylalanine at codon 200, which was encoded by a change from TTC to TAC was demonstrated and is now the most common single nucleotide polymorphism associated with benzimidazole resistance (Kwa et al., 1994). The F200Y single nucleotide polymorphism in the beta-tubulin gene is most commonly associated with benzimidazole resistance in trichostrongylid nematodes, however, other single nucleotide polymorphisms and drug efflux pathways have been identified as potential mechanisms of anthelmintic resistance (Kotze et al., 2012a). This F200Y polymorphism has been found in many countries across the globe and occurs at very high frequencies in some populations (Hoglund et al., 2009; Redman et al., 2015). Since those discoveries, further work has shown the contribution of single nucleotide polymorphisms at codons 167 (F167Y) and 198 (E198A) (Silvestre and Cabaret, 2001).

Recently, Redman et al. (2015) reported similar frequencies of the F167Y and F200Y polymorphisms in six out of seven farms surveyed in the United Kingdom, suggesting the F167Y mutation is also highly important in some populations and regions. The E198A single nucleotide polymorphism of the beta-tubulin gene is associated with the highest levels of resistance to thiabendazole under *in vitro* selection for resistance (Kotze et al., 2012a). These mutations have been evaluated in field studies (Barrère et al., 2013). However, the relative contributions of these single nucleotide polymorphisms to phenotypic resistance and additional mutations associated with very high levels of resistance to benzimidazoles remain to be determined (Kotze et al., 2012a). Further evaluation of the relative contributions of additional single nucleotide polymorphisms in the beta tubulin gene must also be addressed and although less common contributions of single nucleotide polymorphisms in the isotype 2, 3, and 4 of this gene may also contribute to resistance and should be further evaluated. It has been demonstrated that a TAC frequency of at least 10% at the P200 of Beta-tubulin isotype 1 is sufficient for sensitive detection of benzimidazole resistance (Samson-Himmelstjerna et al., 2009b). While these results have substantial evidence, implementation of this diagnostic testing in the field is very rare or almost absent outside of research and thus efforts to reduce the cost and improve the practicality of this assay must be a focus of research efforts.

For the avermectin/milbemycins, the evidence describing the mechanism of resistance is not defined and proposed mechanisms are described below. Overall, the genetic basis of resistance to the avermectin/milbemycin class of anthelmintics in parasitic helminths is poorly understood (Urdaneta-Marquez et al., 2014). Early reports of the mechanism of resistance to ivermectin indicated mutations in the alpha subunit of the

glutamate gated chloride channel receptors with particular emphasis on the L256F mutation, however, no field isolates have been documented to express this mutation (Kotze et al., 2014a). In *Caenorhabditis elegans* (C. *elegans*) null mutations of ivermectin-sensitive glutamate gated chloride genes resulted in more than 4,000 fold increase in resistance to ivermectin (Cully et al., 1994). There are not yet any mutations identified that can explain avermectin/milbemycin resistance phenotypes in the field of most parasitic nematode species (Kotze et al., 2014a).

Currently, it is thought that resistance to avermectin/milbemycins involves multiple genes. It is possible that the dye-filling 7 genes and the ligand-binding domain of GLC-1, the alpha subunit of a glutamate gated chloride channel that has been identified in *C. elegans*, are involved in resistance to avermectin/milbemycins in parasitic nematodes (Kotze et al., 2014a). Recently, it was shown that selected isolates for avermectin/milbemycin resistance were enriched with a *Hco-dyf-7* haplotype that was rare in the drug naïve isolate and that this haplotype correlated with sensory neuron dye filling defects (Urdaneta-Marquez et al., 2014). These avermectin/milbemycin resistant isolates also showed decreased expression of *dyf-7* and single nucleotide polymorphisms in the *dyf-7* gene of *H. contortus* (Urdaneta-Marquez et al., 2014). While it is important to notice that these mutations are in the non-coding region and thus are unlikely to be the mechanism of resistance, it is also important to note that they may be linked to resistance (Urdaneta-Marquez et al., 2014). Recently, Elmahalawy et al. (2018) concluded that genetic variants in the *dyf-7* gene were not drivers for ivermectin resistance in *H. contortus*. In field isolates of *H. contortus* that were well-characterized for ivermectin resistance using the FECRT there was no correlation between *dyf-7* and level of

ivermectin resistance, providing conclusive evidence that *dyf-7* is not a suitable marker for ivermectin resistance in field isolates of *H. contortus*.

Additional studies of avermectin/milbemycin resistance have focused on drug efflux pumps and detoxification enzymes as potential mechanisms of resistance. The avermectin/milbemycins are transported by drug efflux pumps such as ABC transporters that are present both in mammalian and parasite cell membranes (Lespine et al., 2008). Multidrug resistance pumps and elevated expression of P-glycoproteins have been associated with elevated levels of resistance to avermectin/milbemycins in several parasite species (Lespine et al., 2006; Lespine et al., 2008; Lespine et al., 2012; Prichard et al., 2012). The *mdr-1* ABC transporters are known to prevent ivermectin from crossing the blood-brain barrier in mammals (Schinkel et al., 1994). Drug transporters such as the p-glycoproteins are of particular interest in anthelmintic resistance and have been considered as markers for resistance and as targets to improve anthelmintic activity through using inhibitors of these proteins (Kotze et al., 2014a). However, the interaction of moxidectin with p-glycoproteins is much weaker than that with ivermectin, which raises the question of differences in resistance mechanisms between the avermectins and milbemycins (Lespine et al., 2008). The current consensus is that neither the glutamate gated chloride genes nor the ABC transporter genes fully explain the genetic basis of resistance to the avermectin/milbemycin class of anthelmintics in parasitic helminths of livestock.

One additional potential mechanism of resistance to the avermectin/milbemycins are the cytochrome P450 enzymes which are a large superfamily of enzymes found in almost all living organisms that catalyze a variety of reactions involving both endogenous

and exogenous substrates. These enzymes are known to be involved in detoxification of exogenous substrates including drugs and insecticides (Laing et al., 2015). The relationship between cytochrome P450 expression and drug resistance is well established as a single gene, cyp6g1, responsible for resistance to multiple insecticides in *Drosophila melanogaster* (Daborn et al., 2007; Laing et al., 2015). Previously, it was suggested that oxidative metabolism was not a process in parasitic nematodes and activity of cytochrome P450 enzymes was absent (Precious and Barrett, 1989). However, with genomic advances in the world of helminth research we now know otherwise. The model helminth *C. elegans* genome encodes 80 cytochrome P450s and several are induced upon exposure to xenobiotics. A cytochrome P450 inhibitor piperonyl butoxide recently was found to potentiate the effect of ivermectin in larval stages of *C. oncophora* and *O. ostertagi* and even eliminate differences between ivermectin susceptible and resistant larval stages of *C. oncophora* (AlGusbi et al., 2014). Recently, constitutive cytochrome P450 enzyme expression was compared across *H. contortus* life stages with RNA-seq and quantitative real-time PCR including eggs, L1, sheathed L3, ex-sheathed L3, L4, and adults (Laing et al., 2015). Overall expression of cytochrome P450s was low across life stages, but expression levels in individual cytochrome P450s varied dramatically amongst life stages, and some genes showed a distinct and interesting expression profile (Laing et al., 2015). This mechanism of resistance should be evaluated among isolates. It is also important to note that both permeability glycoproteins and cytochrome P450 enzymes may be involved in anthelmintic resistance across drug classes.

Levamisole resistance has primarily been studied in *C. elegans* but the mechanism within parasitic nematodes is not understood very well and is quite complex, involving

multiple genes (Kotze et al., 2014a). Truncated acr-8 mRNA splice variants of the acr-8 gene were expressed in several levamisole resistant *H. contortus* isolates, identifying this gene as a potential marker for levamisole resistance (Williamson et al., 2011; Barrère et al., 2014). Overall, it is thought that levamisole resistance involves multiple genes and changes in expression of the subunits of the nicotinic acetylcholine receptor, truncated subunits, and mutations in subunits (Kotze et al., 2014a).

Population genetics

In the pursuit to understand the genetic basis of anthelmintic resistance, information regarding genomic sequence variability and population genetic structure can provide valuable insights. Variation in genomic sequence of parasitic helminths of different resistance profiles, species, or genetic background can help to identify potential markers for anthelmintic resistance (Gilleard, 2006; Grillo et al., 2007). However, some species of parasitic helminth have extremely high levels of genome variation which can create difficulties in interpretation of variation in sequences between susceptible and resistant isolates.

At this point, the primary methodology to identify mutations responsible for anthelmintic resistance has focused on study of candidate genes, particularly those associated with the mechanism of action of the drug or mutations identified in resistant model organisms (Gilleard, 2006). But, as sequencing technologies capable of whole helminth genome sequencing become more affordable, these approaches can be applied to identify genetic loci associated with anthelmintic resistance (Gilleard and Beech, 2007). However, interpretation of whole genome approaches and evaluation of candidate-gene studies require a thorough understanding of population genetics and parasite

biology. Information regarding how mutations associated with resistance arise and are distributed across a population is essential for interpretation of the implications of genomic variations associated with resistance. Limited knowledge of how resistance mutations arise and spread in parasitic helminths greatly inhibits our ability to develop effective control measures (Grillo et al., 2007; Redman et al., 2012).

Original studies of the population genetics of parasitic nematodes were dominated by the use of single locus markers (Blouin, 1998; Redman et al., 2008b; Belanger et al., 2011). However, much work has been completed to develop neutral polymorphic microsatellite loci for several species of parasitic nematodes (Grillo et al., 2006; Redman et al., 2008a; Belanger et al., 2011). The usefulness of microsatellite markers for studies of population genetics in gastrointestinal nematodes of livestock will be further discussed. As a whole, the number of studies on the population genetics of parasitic nematodes is low and this is an area of research that warrants further work (Gilleard, 2006; Grillo et al., 2007).

Microsatellite genotyping

Microsatellite markers are short, tandem repeats of 2-8mer nucleotide motifs which occur frequently and randomly throughout the genomes of eukaryotes (Tautz and Renz, 1984). Microsatellite markers are also known as simple sequence repeats. Polymorphic microsatellite markers are simple and inexpensive to sequence using polymerase chain reaction and fragment analysis via capillary electrophoresis (Redman et al., 2008b).

In *H. contortus*, the first microsatellites were published in 1997 by Hoekstra et al. and consisted of six polymorphic loci. To develop a more extensive panel of

microsatellite markers for use in monitoring experimental populations of *H. contortus*, Otsen et al. (2000) developed an additional 30 markers. Otsen et al. (2000) used the panel of microsatellites to discriminate between well-characterized isolates of *H. contortus* and evaluate purity of the isolates. Otsen et al. (2000) were able to use microsatellite genotyping to identify contamination of an inbred isolate of *H. contortus.* These results demonstrate the power of microsatellite genotyping to monitor and evaluate purity of laboratory isolates used for research purposes.

To build upon these findings and to provide further information regarding genetic structure of populations of *H. contortus,* Redman et al. (2008b) developed two additional polymorphic microsatellite loci and combined these with six markers developed by Otsen et al. (2000). Redman et al. (2008b) used principle coordinate analysis of the 8 microsatellite markers to understand the differences between the isolates tested and were able to both statistically and visually describe the marked differences between isolates. Adults and third-stage larvae yielded similar microsatellite fingerprints and pools of larvae were similar to adults. These results suggest that rapid fingerprinting using microsatellite genotyping of pools of third-stage larvae may serve as a simple tool to compare populations of *H. contortus* (Redman et al., 2008b).

Redman et al. (2015) used ten microsatellite loci to examine differences in population genetic structure and genetic diversity in seven field populations of *H. contortus* and *T. circumcincta*. Results of this study showed that *T. circumcincta* had higher genetic diversity but lower genetic differentiation between farms than *H. contortus* in the United Kingdom (Redman et al., 2015). This study demonstrated the power of using microsatellite genotyping to describe differences in field populations of

gastrointestinal nematodes of livestock and provides framework and methodologies for future studies of population genetics of field populations.

Frequency and relatedness of isotype-1 β-tubulin haplotypes

Sequencing and further phylogenetic and statistical analyses of susceptible and resistant haplotypes of the isotype-1 β-tubulin gene can provide valuable insights regarding how resistance emerges and spreads in a population (Chaudhry et al., 2015; Redman et al., 2015). It is possible to describe the genetic diversity of the gene using measures such as the number of polymorphic sites, total number of haplotypes, and pairwise differences for nucleotide and gene diversity. The level of genetic diversity in resistant versus susceptible haplotypes can provide information regarding selection of resistance. The frequency of various haplotypes in a population over time can be used to describe changes in that population and if resistance mutations were introduced to the population from an external source or if the mutations developed over time within the population. Phylogenetic relationships between these haplotypes can describe the level of relatedness. If resistance haplotypes and susceptible haplotypes are mapped together and resistant haplotypes are scattered across the network, it can be inferred that resistance mutations have arisen on different ancestral susceptible haplotypes which is the case in both the United Kingdom and India for the F200Y mutation (Chaudhry et al., 2015; Redman et al., 2015). It is clear from this discussion that the frequency and relatedness of haplotypes of the isotype-1 β-tubulin gene are valuable in field studies of gastrointestinal nematodes of livestock.

CHAPTER 3

UTILIZATION OF COMPOSITE FECAL SAMPLES FOR DETECTION OF ANTHELMINTIC RESISTANCE IN GASTROINTESTINAL NEMATODES OF CATTLE [1]

[1] George M.M., Paras, K.L., Howell, S.B., and Kaplan, R.M., 2017. Veterinary Parasitology 240, 24-29. Reprinted with the permission of the publisher.

Abstract

Recent reports indicate that anthelmintic resistance in gastrointestinal nematodes of cattle is becoming increasingly prevalent worldwide. Presently, the fecal egg count reduction test (FECRT) is the only means available for detection of resistance to anthelmintics in cattle herds at the farm level. However, the FECRT is labor and cost intensive, and consequently is only rarely performed on cattle farms unless for research purposes. If costs could be reduced, cattle producers might be more likely to pursue drug resistance testing on their farms. One approach to reducing the cost of the FECRT, is the use of composite fecal samples for performing fecal egg counts (FEC), rather than conducting FEC on fecal samples from 15 to 20 individual animals. In this study FECRT were performed on 14 groups of cattle using both individual and composite FEC methods To measure how well the results of composite sampling reproduce those of individual sampling, Lin's Concordance Correlation Coefficient was utilized to describe both the linear relationship between methods and the slope and y-intercept of the line relating the data sets. There was little difference between the approaches with a concordance correlation of 0.99 for mean FEC between methods. Mean FEC based on individual counts ranged between 0 and 670.6 eggs per gram of feces, indicating that the results of this study are applicable to a wide range of FEC levels. Standard error of the mean FEC and range of FEC are reported for each group prior to and following treatment to describe the variability of the data set. The concordance correlation was greater than 0.95 for drug efficacy between individual and composite sampling methods, demonstrating composite sampling is appropriate to evaluate drug efficacy. Notably, for all groups tested the efficacy calculated by composite sampling was within the 95% confidence interval for

efficacy calculated using individual sampling. The use of composite samples was shown to reduce the number of FEC required by 79%. These data demonstrate that pooling fecal samples from a group of cattle and then performing repeated FEC on that composite sample yields results very similar to performing individual FEC on those same animals, while substantially reducing the cost of performing a FECRT as compared to individual fecal samples. Furthermore, we have developed suggested methods for using composite samples in a FECRT, provided a cost comparison for this methodology, and described potential issues associated with the use of composite samples that must be considered.

Introduction

Control of gastrointestinal parasites is a fundamental component of cattle health and productivity, and use of effective anthelmintics is the primary means of achieving this goal (Hawkins, 1993; Leathwick and Besier, 2014). Historically, cattle producers have reaped the economic benefits of internal parasite control through the availability and use of extremely safe, broad-spectrum, high-efficacy anthelmintics (Williams, 1997; Gasbarre et al., 2015). However, strict reliance on anthelmintics for parasite control without regard to biological factors such as pasture management and parasite biology can lead to rapid selection for drug resistance (Williams, 1997). This situation is well described in the small ruminant industry where anthelmintic resistance is a serious and widespread global problem (Kaplan, 2004; Howell et al., 2008; Kaplan and Vidyashankar, 2012). In cattle, over the past fifteen years there have been increasing numbers of reports of anthelmintic resistance worldwide. Resistance to the avermectin/milbemycin class of drugs is most commonly reported in *Cooperia* spp. in cattle, and has been documented in North America, South America, Europe, and

Australasia (Fiel et al., 2001; Anziani et al., 2004; Condi et al., 2009; Demeler et al., 2010b; Edmonds et al., 2010; Lyndal-Murphy et al., 2010; Rendell, 2010; Fazzio et al., 2014; Cotter et al., 2015; Geurden et al., 2015). Although the prevalence of resistance is lower, there have also been published reports of resistance to avermectin/milbemycins in *Ostertagia* in Australasia (Rendell, 2010; Waghorn et al., 2016), North America (Edmonds et al., 2010), and South America (Suarez and Cristel, 2007). Additionally, resistance in *Ostertagia* has been reported to levamisole and benzimidazole in Australia (Rendell, 2010; Cotter et al., 2015). These reports indicate that it should not be assumed that anthelmintics are highly effective and cattle producers and their advisors should determine which anthelmintic classes are effective on their operations.

The Fecal Egg Count Reduction Test (FECRT) is the preferred assay for detection of resistance at the farm level in gastrointestinal nematodes of ruminants (Coles et al., 1992; Coles et al., 2006). While several *in vitro* assays have been developed for detection of resistance in gastrointestinal nematodes of cattle, none of these have been validated for field samples comprised of multiple species and for multiple classes and combinations of anthelmintics (Coles et al., 2006; Demeler et al., 2012). Additionally, there are serious logistical and practicality issues, as there are very few labs with the expertise necessary to perform *in vitro* diagnostic assays, and even fewer that offer them as a diagnostic service. Thus, the FECRT remains the preferred method to assess drug efficacy in livestock. A FECRT is conducted, by performing fecal egg counts (FEC) on samples from approximately 15–20 calves both prior to treatment and again following treatment (Coles et al., 1992). Subsequently, the reduction in fecal egg count after anthelmintic treatment can be calculated to evaluate the efficacy of the tested product.

The FECRT is commonly used in cattle for research purposes, however it has not been widely adopted by cattle producers. The reason for this lack of implementation by cattle producers is multifactorial. First, anthelmintic resistance in gastrointestinal nematodes of cattle is a relatively new issue and most producers have not perceived resistance to be a problem on their farm (Gasbarre et al., 2015). It is extremely uncommon for cattle producers to conduct fecal egg counts or monitor anthelmintic efficacy as pointed out in the 2007–2008 USDA National Animal Health Monitoring Service report which found that only 5.7% of beef cattle operations surveyed conducted any fecal egg counts (USDA, 2010). Additionally, the expense of conducting a FECRT has been a limiting factor in the implementation of this test and producers often view testing as uneconomical (Morgan et al., 2005).

It is recommended that cattle farms perform FECRT to determine the efficacy of drugs used on their farm, however, the test is rarely used by cattlemen for several reasons outlined above (McArthur and Reinemeyer, 2014). Reducing the cost of performing this test may facilitate an increase in its usage (Rinaldi et al., 2014). The aim of this paper is to determine if composite (pooled) sample and individual sample FEC methods agree sufficiently to allow for the use of composite FECs to evaluate drug efficacy. In sheep, the use of composite fecal egg count methods to estimate the mean fecal egg count of a group of individuals has been evaluated and was highly correlated (Baldock et al., 1990; Nicholls and Obendorf, 1994; Morgan et al., 2005). The first evaluation of composite sampling in cattle found that the mean of individual counts and a composite count of 1.0 g of feces from 5 animals displayed a high level of agreement (Ward et al., 1997). However, these studies were completed 10–25 years ago and this methodology of

composite sampling is still rarely used. Recently, Rinaldi et al. (2014) reported a correlation of 0.94 between the composite FEC and mean FEC of individual samples in sheep regardless of pool size or analytical sensitivities, however, the authors were only able to assess FECR in flocks with efficacy greater than 95% FECR and thus did not evaluate the correlation between composite and individual samples in regards to drug efficacy. Subsequently, a correlation of greater than 0.90 between FECR using composite or 20 individual FECs was found in a study of 5 sheep farms in Scotland across a range of anthelmintic efficacies (Kenyon et al., 2016). The present study aimed to further evaluate the use of composite sampling in cattle to estimate both mean fecal egg count and anthelmintic efficacy using common FEC methods and across a broad range of efficacy levels. Methods were developed to describe a proper technique for sampling and preparation of fecal samples. This study provides a thorough evaluation of composite sampling in cattle and serves as a practical guide for conducting composite sampling for FEC and FECRT.

Materials and Methods

Study design & guidelines for composite sampling

This study was designed to compare individual and composite FEC methods to detect mean FEC and FECR in beef calves. Sampling was completed between September 2015 and January 2016. FECRTs were completed on 14 groups of cattle. Fecal samples were collected from the rectum of a minimum of 15 animals per group, whenever possible, and stored at approximately 4 °C to prevent egg hatching (Coles et al., 1992). Of the 14 FECRTs performed, 9 were completed using the Mini-FLOTAC (5 eggs per gram sensitivity), 4 with the modified-McMaster (12.5 eggs per gram sensitivity, i.e. 2–

25 egg per gram sensitivity slides), and 1 with the modified-Wisconsin (5 eggs per gram sensitivity) technique.

For Mini-FLOTAC analysis, five grams of feces were placed into a Fill-FLOTAC homogenizer (Dr. Giuseppe Cringoli, University of Naples, Italy) and suspended in 45 mL of sodium nitrate flotation solution (specific gravity =1.25 − 1.30, FECA-MED, Vedco, Inc., St. Joseph, Missouri, USA). Homogenization of sample and sodium nitrate solution, slide preparation, and counting were completed as previously described (Noel et al., 2017).

For modified-McMaster analysis, four grams of feces were placed into a cup with 26 mL of sodium nitrate flotation solution (specific gravity = 1.25 − 1.30, FECA-MED, Vedco, Inc., St. Joseph, Missouri, USA). Homogenization of sample and sodium nitrate solution, slide preparation, and counting were completed as previously described (Noel et al., 2017). Importantly, 25 egg per gram sensitivity modified-McMaster fecal egg counts were completed in duplicate resulting in a sensitivity of 12.5 eggs per gram.

For modified-Wisconsin analysis, five grams of feces were placed into a cup with 75 mL of water, homogenized by stirring with a tongue depressor, and then strained through a single layer of cheese cloth. The strained suspension was mixed using a figure-8 pattern and 3.0 mL of the suspension was transferred into a 15 mL conical centrifuge tube with 12 mL of tap water. The 15 mL suspension was centrifuged for 10 min at 1500 RPM and the supernatant was removed. The sediment was mixed with 10 mL of Sheather's solution (specific gravity = 1.28), filled to obtain a positive meniscus, covered with a cover slip, and centrifuged for 10 min at 1500 RPM. The cover slip was placed on a glass slide and a secondary cover slip was placed on the conical centrifuge for an

additional 10 min on the bench top. This secondary cover slip was removed and placed on the glass slide. All eggs were counted under both cover slips and this modified-Wisconsin technique resulted in a sensitivity of 5 eggs per gram.

FECs were completed prior to and 10–14 days following treatment on each of the 14 groups of cattle, thus providing 28 sample sets for evaluating the relationship between mean FEC of the individuals and the composite samples. The mean number of samples that were pooled was 15.7 with a range of 9 −19. The same animals that were sampled prior to treatment were sampled post-treatment. The methods for conducting composite FECs and FECRTs are provided in Fig. 3.1.

For composite sampling prior to treatment, replicate reading discs (Mini FLOTAC) or slides (McMaster or Wisconsin) were prepared and counted until a minimum of 140 eggs were counted on the composite sample. If 140 eggs were counted prior to finishing reading a slide, the entire slide was counted until completion. The total number of slides required to count 140 eggs prior to treatment was recorded and the same number of slides or devices were prepared and counted following treatment. The required number of 140 eggs counted under the microscope pre-treatment was selected based on simulation experiments suggesting this as the minimum number of eggs required to obtain a statistically valid measure of efficacy (Levecke et al., 2015; unpublished data). A minimum of 140 eggs were counted under the microscope prior to treatment for both individual and composite sampling methods.

Statistical analyses

Analysis of mean FEC & FECR in individual samples

The mean FEC of individual samples was calculated as the arithmetic mean of the individual FECs. Analysis of FECR and associated 95% confidence intervals using individual samples were completed in the statistical software R version 3.3.2 (R Core Team, 2016) according to the previously described delta method for calculation of variance for group-based FECR using the arithmetic mean (Levecke et al., 2014; Levecke et al., 2015).

The FECR(%) was calculated according to the following equation:

FECR(%) = [1 − (arithmetic mean FEC post treatment ÷ arithmetic mean FEC pre treatment)]x100%

The code used to calculate FECR(%) and associated 95% confidence interval in R is included as Appendix A. Resistance status was assigned using the current WAAVP recommendations; mean efficacy<95% and lower 95% CI<90% (Coles et al., 1992).

Analysis of mean FEC & FECR in composite samples

The mean FEC of the composite sample was calculated as the arithmetic mean of the repeated FECs completed on the composite sample as described in Fig. 3.1. The FECR(%) was calculated as the percent reduction in composite FEC following anthelmintic treatment. An accurate 95% confidence interval for FECR(%) of composite samples cannot be calculated as the number of slides counted for composite samples is too low to provide a statistically valid confidence interval.

FEC & FECR: agreement between individual and composite sampling methods

Lin's concordance correlation coefficient (ρc) and the corresponding 95% confidence interval were calculated to assess the agreement between the two methods. Additionally, the nonparametric Spearman correlation coefficient (rs), corresponding 95% confidence interval, and p-value were calculated in GraphPad Prism 6 to measure the correlation between the two methods.

Results

Correlation and agreement of individual and composite samples for measuring FEC

The mean FEC was highly correlated for the two different approaches (rs =0.9741, 95% CI rs = 0.9429 to 0.9884, $p < 0.001$; Fig. 3.2). However, a high level of correlation does not always reflect a high level of agreement. Thus, Lin's Concordance Correlation Coefficient was utilized to measure agreement and also resulted in a substantial level of agreement (ρc= 0.9887, 95% CI ρc= 0.9761, 0.9947).

Correlation and agreement of individual and composite samples for measuring FECR

The anthelmintic efficacy measured as FECR(%), displayed a substantial level of agreement (ρc= 0.9586, 95% CI ρc= 0.8700, 0.9872), and a high positive correlation (rs = 0.9282, 95% CI rs = 0.7498, 0.9808, $p < 0.001$) between individual and composite sampling methods (Fig. 3.3, Table 3.1). FECRT that resulted in less than 0% FECR were not included in this analysis, but are displayed in Fig. 3.4. These tests were removed from the primary analysis because variations in FEC are not a result of drug efficacy, but may represent host or environmental changes and are outside the focus of this experiment. Interestingly, when all FECRT were included in the analysis, even those with efficacy

less than 0%, the Lin's Concordance Correlation Coefficient increased to a substantial level of agreement (ρc =0.9782, 95% CI ρc= 0.9431, 0.9917; Fig. 3.4). Further, the classification of a population as resistant or susceptible was consistent for both methods for all 14 groups of cattle tested; for all groups with positive FECR, the mean percent reduction for the composite was within the 95% CI calculated for the individual samples.

Discussion and Conclusion

The results of this study provide strong evidence that composite sampling of fecal samples in beef calves is a scientifically valid approach for evaluating anthelmintic efficacy and detecting resistance. The correlation and agreement of individual and composite sampling methodologies were analyzed for estimation of mean fecal egg count and anthelmintic efficacy. While a high correlation between mean FEC and composite FEC has previously been found in beef cattle (Ward et al., 1997), to our knowledge, this is the first published comparison of individual and composite sampling for testing drug efficacy in beef calves.

A high level of correlation ($r_s = 0.9741$) in mean FEC was found between composite and individual sampling. Importantly, the mean FEC based on individual counts ranged between 0 and 670.6 EPG, highlighting that the results of this study can be applied across a wide range of fecal egg count levels commonly seen in beef calves. Similar levels of correlation (Pearson Correlation Coefficient ≥ 0.94) were recently reported in sheep (Rinaldi et al., 2014). The Spearman Correlation Coefficient as opposed to the Pearson Correlation Coefficient was used in the present study as it assesses correlation using rank as opposed to raw values (Andrijana et al., 2015), which is more

appropriate with variables that do not follow a normal distribution such as fecal egg counts. This strong correlation between individual and composite mean FEC is not surprising as two methods, i.e. composite and individual sampling, describing the same variable, i.e. mean FEC, are expected to be highly correlated. The Spearman's Correlation Coefficient describes the linear relationship between two data sets, however, in this situation it is more appropriate to measure the perpendicular departure from the 1:1 line of agreement between a gold standard and a candidate method. This value is termed the level of agreement and is described by Lin's Concordance Correlation Coefficient. In the present study, Lin's Concordance Correlation Coefficient measures how well the results of composite sampling reproduce those of individual sampling by describing both the linear relationship between the two methods and the slope and y-intercept of the line relating the two data sets. Lin's Concordance Correlation Coefficient is calculated in reference to a line with a y-intercept of zero and a slope of 1. The Lin's Concordance Correlation Coefficient resulted in a substantial level of agreement between methods ($\rho c = 0.9887$).

A high level of similarity has previously been documented between a composite count and an arithmetic mean of 10 sheep samples using sign test analysis (Nicholls and Obendorf, 1994). Thus, the high level of correlation and agreement in FEC between methods that were found in the present study were expected. These results suggest that composite sampling is a valid approach to evaluate the arithmetic mean fecal egg count and make clinical decisions based on results. These findings were consistent among the various FEC methods used in our study.

The primary goal of this work was to determine if use of composite samples is an accurate method of performing FECRT in cattle. We found a high level of correlation (rs =0.9282) in the estimated drug efficacy between composite and individual sampling, suggesting that composite sampling is appropriate for estimation of drug efficacy as compared to the current gold standard. The Lin's Concordance Correlation Coefficient resulted in a substantial level of agreement between methods (ρc= 0.9586). This study tested anthelmintics with varying levels of effectiveness, ranging between −29.2% and 100.0% FECR, providing evidence that composite sampling is appropriate for evaluating efficacy across a wide spectrum of efficacy levels. (Rinaldi et al., 2014) found a similar level of agreement in FECR between composite and individual sampling but only evaluated anthelmintics with very high efficacy (> 98.0%) on sheep farms in Italy. Importantly, Rinaldi et al. (2014) highlighted that composite sampling should be further evaluated at an efficacy level below 95% FECR. Subsequently, Kenyon et al. (2016) found a correlation greater than 0.90 between FECR calculated from individual and composite samples in sheep using anthelmintics ranging in efficacy from less than −100% FECR to 100% FECR.

The present study adds important information to the body of scientific literature that is currently available regarding composite sampling in cattle, demonstrating that mean FEC and FECR determined using composite sampling agreed with the results of values generated from individual samples. Anthelmintic resistance is considered to be present when (i) mean FECR(%) is less than 95% and (ii) the lower 95% confidence interval for FECR(%) is less than 90% (Coles et al., 1992). If only one of the two criteria is met, resistance is suspected (Coles et al., 1992). Using these criteria with data from

individual sampling, 13 of the 14 groups were diagnosed as being resistant and 1 of the 14 groups was diagnosed as being susceptible.

An important limitation of composite sampling is that a 95% confidence interval for FECR cannot be calculated from the few composite FECs that are completed. Since parameters for precise classification of resistance status require calculation of a 95% confidence interval (Coles et al., 1992), resistance status determined by composite sampling must be interpreted with some caution. Based on the present results, a population of parasites yielding greater than 95% FECR using composite sampling could be reasonably classified as susceptible and a population with less than 80% FECR could be reasonably classified as resistant. When FECR is between 80 and 90% resistance would seem highly likely, however, with no ability to calculate 95% CI, it is possible that a given result was due to chance. In all cases, when FECR is between 90 and 95% results should be interpreted with caution as this is a gray zone where variability can easily impact the correct interpretation.

Still, composite sampling does provide a cost-effective and simplified method to determine if a drug is effective or not. In this study, composite sampling reduced the number of FECs required to evaluate mean fecal egg count or drug efficacy by 79.2%, thereby greatly reducing the expense of laboratory testing. For example, a FECRT including 15 animals requires 30 FECs, one before and one after treatment per animal. On average, a FECRT using composite sampling on this same group of animals requires 6 FECs, which presents genuine cost and time savings. Thus, composite sampling can significantly reduce the labor and cost associated with determining mean FEC and/or

performing FECRT for groups of beef calves (Nicholls and Obendorf, 1994; Ward et al., 1997).

A further limitation of composite sampling is the inability to identify high-shedding individuals that may require specialized treatment or care. It has been well established that FECs follow an overdispersed pattern within a herd as the majority of animals shed low numbers of eggs and a few animals shed high numbers of eggs (Kaplan and Vidyashankar, 2012). This information can be used to identify individuals that warrant treatment. However, cattle producers tend to treat an entire herd or group of animals regardless of FEC, and rarely if ever employ targeted selective treatment. Thus, the inability to identify high-shedding individuals would not be a concern for the vast majority of cattle producers. Overall, the present results demonstrate that composite sampling is a resource efficient methodology for assessing mean FEC and drug efficacy with minimal practical limitations.

In conclusion, composite sampling of fecal samples from beef calves provides a cost-efficient and practical methodology for cattle producers and veterinarians to employ parasitological testing. There was both a high correlation and substantial level of agreement in FEC and FECR between individual and composite sampling methods, confirming the validity of composite sampling. We also provide methods for conducting composite sampling to assist in implementation and consistency of this procedure. Thus, this work provides both a valuable and practical tool for cattle producers to assess anthelmintic resistance in a cost-efficient manner.

Declaration of conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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Methods for Composite Fecal Egg Counts & Efficacy Testing

1. Collection: Collect fecal samples via the rectum from a minimum of 15 animals, whenever possible.
2. Paired Sampling: The same animals must be sampled pre- and post-treatment. Animals that were not sampled or did not produce a fecal sample prior to treatment should not be sampled post-treatment. An animal that produced a sample prior to treatment may not produce a fecal sample post-treatment and thus will not be included in the post-treatment composite sample.
3. Storage: Individually collect and store feces in a cool environment (~4℃) to prevent egg hatching.
4. Homogenization: Mix individual samples for approximately 15 s to homogenize the sample.
5. Composite Sample Preparation: Weigh 1.0 g of feces from each individual sample and combine into a container holding the composite sample. For 15 samples, the final composite sample should weigh 15.0 g. Homogenize for approximately 1 min to ensure uniformity of the sample.
6. Fecal Egg Count: Prepare FEC slides/chambers using standard methods such as the McMaster, Wisconsin, FLOTAC, or mini-FLOTAC procedures. A minimum of 140 eggs should be counted prior to treatment to provide statistical validity of the mean FEC. Thus, repeated FEC slides/chambers should be read until 140 eggs have been counted, with all eggs counted on the final slide or chamber. The number of FEC slides/chambers required to count 140 eggs should be recorded and the exact same number of FEC slides/chambers should be counted post-treatment. The greater the detection sensitivity of the FEC method, the fewer the number of FEC needed to reach the 140 eggs counted threshold.
7. Infection Intensity Analysis: N is the number of FECs (slides) completed. Eggs is the number of eggs per FEC.

   Composite FEC = [($\sum_{i=1}^{N} Eggs\ i$ = $Eggs_1$ + $Eggs_2$ + … + $Eggs_N$) x detection sensitivity of FEC method] ÷ N
8. Efficacy Analysis:

   % FECRT = [1 – (Post-Treatment Composite FEC ÷ Pre-Treatment Composite FEC)] x 100%

Example: An 8 EPG high-sensitivity McMaster slide was prepared. 72 eggs were counted on slide 1 ($Eggs_1$=72).
$Eggs_1$<140 so a second slide was counted. ($Eggs_2$=74). $Eggs_1$ + $Eggs_2$ > 140, so there was no need to count additional slides.
Total number of eggs counted was $\sum_{i=1}^{N} \text{Eggs}\ i$ = $Eggs_1$ + $Eggs_2$ = 72 eggs +74 eggs = 146 eggs.
Pre- Treatment Composite FEC = [($\sum_{i=1}^{N} Eggs\ i$) x Sensitivity of FEC method] ÷ N = (146 eggs x 8 EPG) ÷ 2 = 584 EPG
Assume Post-Treatment Composite FEC = 80 EPG
% FECRT = [(Pre-Treatment Composite FEC – Post-Treatment Composite FEC) ÷ Pre-Treatment Composite FEC] x 100%
% FECRT = [(584-80) ÷ 584] x 100% = 86.3%

Figure 3.1. Methods for composite fecal egg counts and efficacy testing

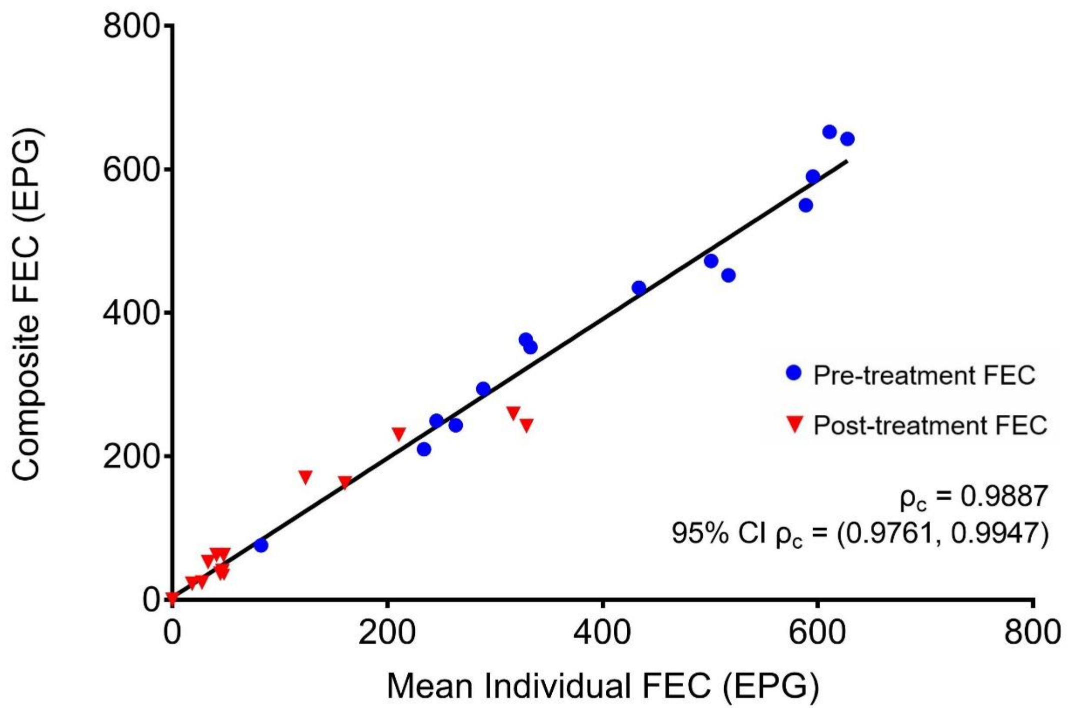

Figure 3.2. Correlation between mean of individual fecal egg counts and composite fecal egg count in 28 groups of cattle. ρc: Lin's Concordance Correlation Coefficient; 95% CI =95% Confidence Interval.

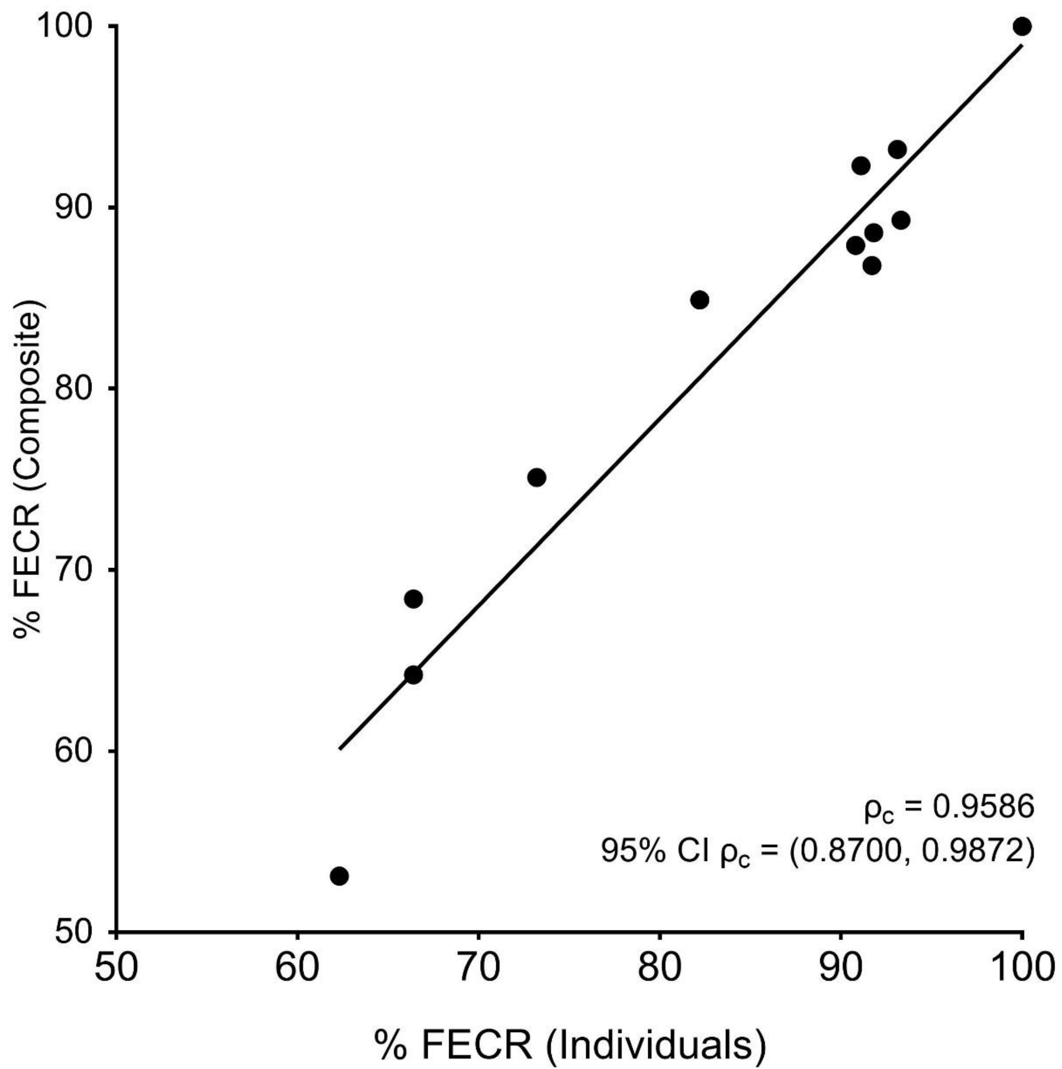

Figure 3.3. Correlation between fecal egg count reduction using individual counts and composite counts. Fecal egg count reduction tests were performed on 14 groups of cattle. Tests which yielded negative fecal egg count reduction were not included in this graph. ρc: Lin's Concordance Correlation Coefficient; 95% CI = 95% Confidence Interval.

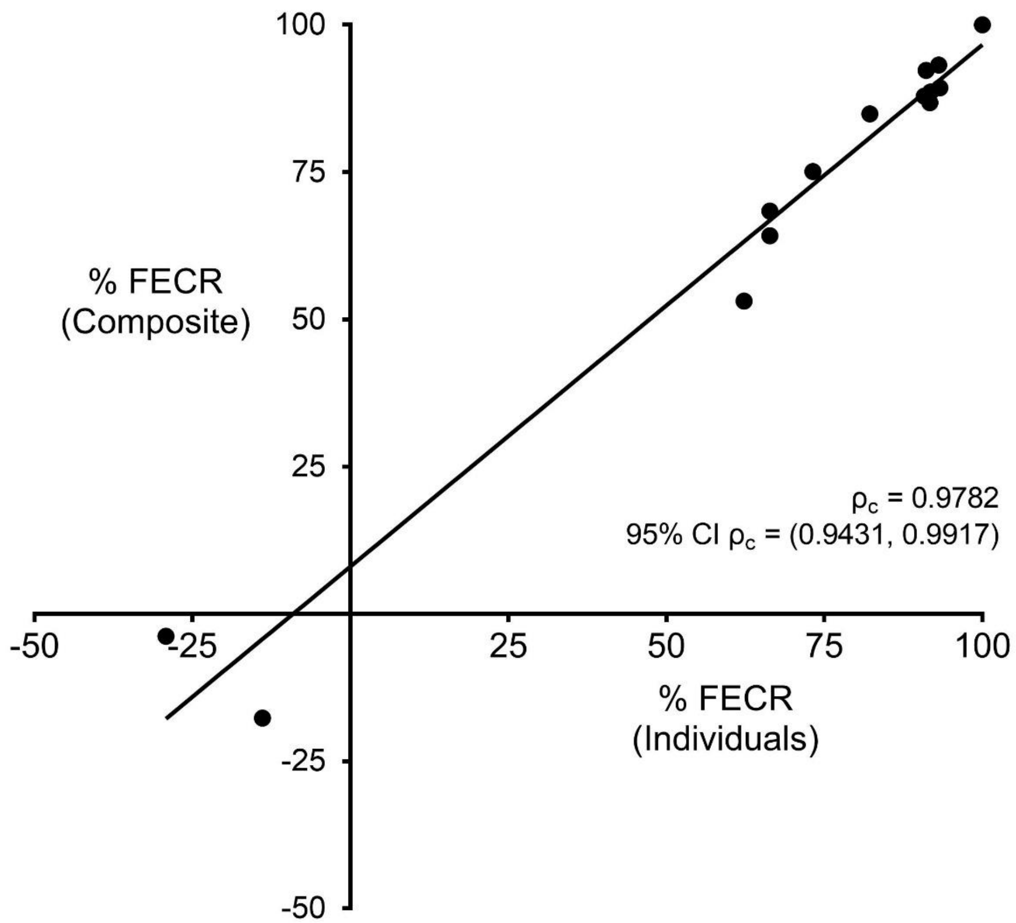

Figure 3.4. Correlation between fecal egg count reduction using individual counts and composite counts. Fecal egg count reduction tests were performed on 14 groups of cattle. Tests which yielded negative fecal egg count reduction were included. ρ_c: Lin's Concordance Correlation Coefficient; 95% CI = 95% Confidence Interval.

Table 3.1. Mean and confidence intervals for fecal egg count reduction using composite or individual samples and mean, standard error of the mean, and range of fecal egg counts using individual samples for 14 groups of cattle.

% Fecal Egg Count Reduction			Fecal Egg Count Using Individual Samples					
Mean		95% Confidence Interval	Pre-Treatment			Post-Treatment		
Composite	Individual	Levecke et al., 2014	Mean	SEM	Range	Mean	SEM	Range
93.2	93.1	(86.9, 97.3)	670.6	141.3	(15, 2100)	46.6	15.6	(0, 195)
84.9	82.2	(53.1, 97.3)	250.9	83.6	(5, 1085)	44.7	25.7	(0, 435)
92.3	91.1	(82.0, 97.1)	545.0	122.2	(0, 1800)	48.2	16.6	(0, 250)
87.9	90.8	(83.9, 95.8)	364.4	87.4	(5, 1080)	33.4	7.4	(0, 90)
88.6	91.8	(82.3, 97.7)	583.9	107.4	(65, 1655)	47.8	19.0	(0, 350)
86.8	91.7	(83.9, 97.0)	500.8	103.3	(25, 1555)	41.3	15.3	(0, 290)
64.2	66.4	(32.8, 88.5)	627.5	162.2	(10, 2370)	210.6	86.1	(0, 1460)
53.1	62.3	(29.9, 84.8)	328.6	77.6	(5, 1285)	123.9	32.8	(0, 485)
75.1	73.2	(41.2, 92.8)	600.3	123.4	(40, 2010)	160.6	62.8	(0, 1110)
89.3	93.3	(82.7, 98.9)	276.5	97.0	(5, 920)	18.5	8.4	(0, 75)
−3.8	−29.2	(−59.0, −2.4)	245.5	31.9	(50, 425)	317.0	51.0	(62.5, 575)
−17.7	−13.9	(−51.9, 18.6)	288.9	25.4	(150, 400)	329.2	51.5	(200, 675)
68.4	66.4	(55.4, 75.9)	82.5	41.9	(0, 480)	27.7	14.5	(0, 144)
100.0	100.0	n/a[a]	193.2	122.7	(0, 1296)	0	0	(0, 0)

[a] The standard deviation is zero. A 95% confidence interval cannot be calculated.

CHAPTER 4

MOTILITY IN THE L3 STAGE IS A POOR PHENOTYPE FOR DETECTING AND MEASURING RESISTANCE TO AVERMECTIN/MILBEMYCIN DRUGS IN GASTROINTESTINAL NEMATODES OF LIVESTOCK [2]

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Abstract

Motility is a commonly used *in vitro* phenotype for assessing anthelmintic activity of candidate compounds, and for detecting anthelmintic resistance in nematodes. Third-stage larvae (L3) of parasitic nematodes are commonly used in motility-based assays because L3 are simple to obtain and can remain viable in storage for extended periods. To improve the measurement of motility of microscopic stages of nematodes, our laboratory developed the Worminator, which quantitatively measures motility of parasites. Using the Worminator, we compared the dose-response characteristics of several avermectin/milbemycin (AM) compounds using L3 from both AM-susceptible and AM-resistant *Cooperia* spp. (abamectin, doramectin, eprinomectin, ivermectin, moxidectin) and *Haemonchus contortus* (eprinomectin, ivermectin, moxidectin). Concentrations tested with the Worminator ranged from 0.156 to 40 μM. Differences in EC50 between AM-susceptible and AM-resistant isolates of *Cooperia* spp. and *Haemonchus contortus* were small, with resistance ratios ranging from 1.00 to 1.34 for *Cooperia* spp., 0.99 to 1.65 for *Haemonchus contortus*. Larval migration inhibition assays were conducted using the same isolates and were equally ineffective for detection of resistance with resistance ratios less than 2.0. These results contrast with those of the Larval Development Assay where we obtained a resistance ratio of 16.48 using the same isolates of *Haemonchus contortus*. Moreover, even at the highest concentration tested (40 μM), 100% inhibition of motility was never achieved and EC50 for Worminator assays were more than 100× higher than peak plasma levels achieved in vivo following treatment. These data demonstrate that dose-response characteristics for inhibition of motility in L3 of gastrointestinal nematodes of livestock do not significantly differ for AM-susceptible

and AM-resistant isolates. These data challenge the suitability of motility as a phenotype for detecting and measuring resistance to AM drugs in gastrointestinal nematodes of livestock.

Introduction

The avermectin/milbemycin (AM) drugs are an important class of anthelmintics used in control of gastrointestinal nematodes (GIN) of livestock. This class of anthelmintics has transformed parasite control, as they are extremely safe while harboring potent broad-spectrum antiparasitic activity. However, resistance to the AMs in many species of important gastrointestinal trichostrongylid nematodes is an increasing problem worldwide and presents a major threat to livestock health and productivity (Kaplan, 2004; Sutherland and Leathwick, 2011; Kaplan and Vidyashankar, 2012).

Effective nematode control programs designed to minimize the development of anthelmintic resistance (AR) should include sensitive methods to detect and monitor AR (Gill et al., 1991; Taylor et al., 2002; Kaplan et al., 2007; Demeler et al., 2010a). The fecal egg count reduction test (FECRT) is currently the preferred method for detection of AR at the farm level, however, the FECRT is labor and cost intensive (Gill et al., 1991). Additionally, the FECRT is rather insensitive, as typically only a single dose level is tested, and can only detect resistance once it is present at relatively high levels (Martin et al., 1989). Alternatively, *in vitro* assays can test multiple anthelmintic compounds and concentrations, providing more detailed information about the level of resistance since it is based on a dose response. *In vitro* assays are considered the most efficient and cost-effective strategy to detect AR (Taylor and Hunt, 1989; Gill et al., 1991; Demeler et al., 2010a).

In vitro assays can measure the effects of anthelmintic compounds on several nematode phenotypes, including development, growth, behavior, and motility (Taylor et al., 2002). Several *in vitro* assays were developed for detecting AR in GIN of sheep including the larval development assay (LDA) (Taylor, 1990a; Gill et al., 1995), larval feeding assay (Álvarez-Sánchez et al., 2005), and larval migration inhibition assay (LMIA) (Wagland et al., 1992; Kotze et al., 2006).

The LDA is the most widely used *in vitro* assay for detecting resistance to the AM drugs, however, the LDA is only well-validated and accurate for detecting AM resistance in *Haemonchus contortus* (*H. contortus*) and *Trichostrongylus colubriformis*. This assay measures the development of nematode eggs to third-stage larvae (L3) in the presence of increasing concentrations of various analogues of anthelmintic compounds (Coles et al., 1988; Gill et al., 1995; Kotze et al., 2014b). The LDA has been evaluated against numerous susceptible and resistant isolates of *H. contortus* and *T. colubriformis* and provides a powerful tool for detection of resistance to the AMs. Additionally, using the analogues ivermectin aglycone and eprinomectin clear discrimination between susceptible and resistant isolates is possible (Gill et al., 1995; Dolinská et al., 2013; Kotze et al., 2014b), including discrimination between ivermectin and moxidectin resistance (Kaplan et al., 2007). The LDA is thought to detect the effects of AM drugs on pharyngeal activity, and it has been suggested, although not proven, that AM affected larvae suffer from starvation even in the presence of an adequate food source due to drug-induced paralysis of the pharyngeal musculature (Geary et al., 1993; Gill et al., 1995). The LDA targets development of eggs, L1, L2, and L3, and thus this assay is able to detect the activity of anthelmintics via many different developmental processes as

compared to assays that only test effects on a single stage such as L3 or eggs. Unfortunately, there is little evidence that the LDA is useful for detecting AR in GIN of cattle. To the best of our knowledge, there has been only a single publication demonstrating positive results for the LDA in detecting AR in GIN of cattle (Demeler et al., 2010b), and this was only for a single species; *Cooperia oncophora*. Since then there have been no further publications demonstrating usefulness of the LDA for detecting AM resistance in GIN of cattle. Furthermore, the usefulness of an assay that can only detect AM resistance in one species of GIN (*C. oncophora*) of

cattle is limited as most populations consist of several different species, and in many of the warmer regions of the world, other species of *Cooperia* are more prevalent. More recent efforts have focused on optimization of the LMIA (Demeler et al., 2012) or alternative systems to measure motility of L3 such as the Worminator (Storey et al., 2014).

The LMIA measures the ability of L3 to migrate through a fine-mesh sieve in the presence of increasing concentrations of an anthelmintic compound (Wagland et al., 1992). This assay has previously been shown to differentiate AM-susceptible and AM-resistant isolates of *C. oncophora* and *H. contortus*, but not *Teladorsagia circumcincta* or *Trichostrongylus colubriformis* (Kotze et al., 2006; Demeler et al., 2010a).The LMIA was evaluated across five laboratories in Europe and shown to exhibit low levels of variability when standardized protocols, techniques, reagents, and well-characterized laboratory isolates were used (Demeler et al., 2010a). Unfortunately, limited data has been published regarding the ability of this assay to detect resistance to AMs in recent field isolates of common GIN of livestock. Areskog et al. (2014) reported the LMIA

produced repeatable dose responses for field isolates of *Cooperia oncophora* and *O. ostertagi*, however, the EC_{50} for ivermectin did not differ between isolates considered as AM-susceptible and AM-resistant according to the FECRT. Further, this assay has rarely been used outside of research purposes and thus its usefulness to farmers as a diagnostic assay has not been realized. Our laboratory examined this assay for discriminating resistant and susceptible isolates of several parasites with little success; with *Dirofilaria immitis* we found no significant differences in the EC_{50} of AM-susceptible and AM-resistant isolates (Evans et al., 2017).

Our inability to detect differences between AM-susceptible and AM-resistant isolates with the LMIA led us to question what phenotype the LMIA is actually measuring and if migration is an appropriate surrogate measurement for motility. The LMIA is essentially a 'black box assay', meaning we can measure the inputs and the outputs of the assay but have no idea of the internal workings. It is unclear what level of correlation exists between motility and migration, or if there is a minimum level of motility required for migration. In the LMIA worms either migrate or do not migrate, and are typically examined at a single time point. Additionally, the LMIA requires optimization for each individual nematode species which presents a challenge for application of the LMIA in assessing resistance in field populations comprised of multiple species.

In an attempt to overcome many of the aforementioned issues, we developed a system to directly quantify motility of microscopic larval stages of GIN. This system, called the Worminator, evaluates anthelmintic activity on larval stages of parasites using computer processing of digital video recordings to quantitatively measure motility of

parasites (Storey et al., 2014). Previously, a micromotility meter was developed by Bennett and Pax (1986) to quantify nematode motility by measuring changes in voltage associated with perturbation of light by nematodes, however, this system did not incorporate magnification by microscopy. Alternatively, the Worminator is comprised of an inverted microscope connected to a video camera, which sends output to a software program that quantifies displacement of pixels between video frames as a measure of motility within a recorded well. Percent inhibition in motility as compared to control wells is then calculated. The ease of use and nature of the Worminator allows readings to be made at multiple time points in the assay, rather than just a single pre-determined end point. Testing of several AM drugs with L3 of *Cooperia* spp. using the Worminator produced repeatable dose response curves (Storey et al., 2014). Based on these accomplishments, we sought to develop an *in vitro* assay for detection of AM-resistance in *Cooperia* spp. using the Worminator with L3 stages. Subsequently, we also evaluated laboratory and field isolates of *Cooperia* spp. and *H. contortus* using both the Worminator and LMIA.

In the present study, we compared the AM dose response characteristics of 8 nematode isolates; *Cooperia* spp. (n=4) and *H. contortus* (n=4), which included both AM-susceptible and AM-resistant isolates. Worminator assays were completed with laboratory isolates of *Cooperia* spp. (abamectin, doramectin, eprinomectin, ivermectin, and moxidectin) and *H. contortus* (eprinomectin, ivermectin, and moxidectin). LMIA were completed with ivermectin for all isolates. In addition, the *H. contortus* isolates were also tested with the LDA. These results were used to evaluate the appropriateness of

motility of the L3 stage as a phenotype to detect resistance to the AM class of anthelmintics.

Materials and Methods

Nematode isolates

Eight isolates of GIN were examined. Details regarding the isolation and characterization of resistance status of each isolate are described by species.

Cooperia spp.

Two laboratory isolates, one AM-resistant and one AM-susceptible, and two AM-resistant field isolates were tested.

Avermectin susceptible *Cooperia* spp. laboratory isolate (TGA-2013)

In June of 2013, feces were collected from a herd of cattle in Thomasville, Georgia, USA where anthelmintics were not routinely used over the past thirty years. Larvae were cultured and included 78% *Cooperia* spp., 10% *Oesophagostomum* spp., 8% *Haemonchus placei*, 3% *Trichostrongylus axei*, and 1% *C. oncophora*. A three-month-old hutch-raised dairy calf presumed to be naïve to GIN was treated with a combination of levamisole and albendazole to remove any worms that might be present. Fourteen days following treatment the calf had a FEC of less than one egg per gram (EPG) and was infected orally with 30,000 L3 from this field population. To purify *Cooperia* spp. From the other species of GIN present in the field sample, we used a strategy based on differential prepatent periods, since *Cooperia* spp. (*C. punctata, C. pectinata*) have the shortest pre-patent period of all species that commonly infect cattle (Leland, 1995). Feces were collected from the calf per rectum from d12-15 post infection. The infection became

patent at d13 post infection and daily coprocultures were established. One hundred L3 were morphologically identified from each culture and 100% of L3 were identified as *Cooperia* spp. A second hutch-raised dairy calf was rendered worm-free with a combination treatment of albendazole and levamisole, confirmed to have a FEC of less than one EPG, and orally infected with 30,000 of these *Cooperia* spp. L3, which we designated as the TGA-2013 isolate. Following patency at d14 post infection, feces were collected periodically over time, cultured, and L3 were stored at 10°C.

The susceptibility of TGA-2013 to ivermectin was first confirmed by treating the original recipient calf (with the mixed species infection) with injectable ivermectin at 0.2 mg/kg (Noromectin®, Norbrook® Inc. USA, Overland Park, Kansas, USA), which yielded 100% reduction in FEC. The susceptibility of the established TGA-2013 *Cooperia* spp. Isolate was then examined in a FECRT using experimentally infected beef calves. Calves (n=3) were treated with a combination of albendazole and levamisole to clear current infections with GIN and moved to concrete pens to prevent transmission of new infections. Prior to infection with the laboratory isolate, FEC performed on the calves yielded negative results (< 1.0 EPG). Ten days post-treatment, each calf was infected orally with 100,000 L3 of the TGA-2013 isolate. FECs were monitored until EPG levels stabilized, which took about 4 weeks. On d31 post-infection each calf was treated with injectable ivermectin at 0.2 mg/kg (Ivomec®, Boehringer Ingelheim, St. Joseph, Missouri, USA). Since only 3 calves were tested, to increase the number of eggs counted and reduce the variability in the FECRT, 12 separate Modified-McMaster FEC with 8 EPG detection sensitivity were performed for each calf both on the day of

treatment and d14 post-treatment. Mean percentage fecal egg count reduction (%FECR) was 98.4%, confirming susceptibility of this isolate to ivermectin.

Avermectin-resistant *Cooperia* spp. laboratory isolate (CGA-2014)

In September of 2014, feces were collected from a herd of cattle in Calhoun, Georgia, USA previously confirmed in 2012 as having AM-resistant *Cooperia*; a FECRT using moxidectin reduced FEC by 39.2% (95% CI: 36.6, 41.7). Feces were cultured, and identification of recovered L3 yielded 72% *Cooperia* spp., 14% *Haemonchus placei*, and 14% *C. oncophora*. A four-month-old hutch-raised dairy calf was treated with a combination of levamisole and albendazole, confirmed to have less than one EPG, and infected orally with 50,000 L3 from this field sample. To purify *Cooperia* spp. from the other GIN present in the field sample, we used the same strategy as described above with feces collected and cultured from d13-16. A second calf confirmed to have less than one EPG was then infected with 40,000 of these *Cooperia* spp. L3, which we designated as the CGA-2014 isolate.

The susceptibility of this isolate to ivermectin was tested using a FECRT in experimentally infected calves using the same methods as described above. Mean %FECR was 47.4%, confirming this isolate as being AM-resistant.

Avermectin-Resistant *Cooperia* field isolate 1 (Comer, Georgia, USA)

This population of parasites was confirmed resistant to topical eprinomectin in 2013 by FECRT, yielding % FECR and 95% CI of 25.9% (5.8, 43.7). Species-specific percent FECR as determined by percentage of L3 recovered in fecal cultures were−153.6% (−222.5,−92.9) and 76.8% (70.5, 82.4) for *Cooperia* spp. and *C. oncophora*, respectively. Fecal samples were obtained from the rectum of 15 calves in

October of 2015, and coprocultures yielded 82% *Cooperia* spp., 8% *Oesophagostomum* spp., 2% *Haemonchus placei*, and 5% *Cooperia oncophora*, and 3% *Ostertagia* spp.

Avermectin-Resistant *Cooperia* field isolate 2 (Colbert, Georgia, USA)

This population of parasites was confirmed resistant to injectable ivermectin at 0.2 mg/kg (Ivomec®, Boehringer Ingelheim Vetmedica Inc., St. Joseph, Missouri, USA) in September of 2015 by FECRT, yielding % FECR and associated 95% CI of −29.2% (−59.0, −2.4). Fecal samples collected from calves (n=11) and cultured prior to treatment included 95% *Cooperia* spp., 4% *Haemonchus placei*, and 1% *Cooperia oncophora*.

Haemonchus contortus

Two laboratory isolates of H. contortus, one AM-resistant and one AM-susceptible, and two AM-resistant field isolates were tested.

Avermectin-susceptible *Haemonchus contortus* laboratory isolate (UGA-SUSC)

This isolate, obtained from Boehringer Ingelheim Vetmedica, Inc., was maintained in the laboratory for many years and had never been exposed to AM drugs. The AM EC_{50} (ivermectin aglycone) and 95% confidence interval for this isolate was 0.91 nM (0.74, 1.13) as determined by DrenchRite® LDA.

Avermectin-resistant *Haemonchus contortus* laboratory isolate (UGA-2004)

History of this isolate was described previously (Williamson et al., 2011). The AM EC_{50} (ivermectin aglycone) and 95% confidence interval for this isolate was 15.00 nM (11.10, 20.25) as determined by DrenchRite® LDA, yielding a resistance ratio of 16.48 as compared to the UGA-SUSC isolate. This EC_{50} is consistent with resistance to ivermectin and susceptibility to moxidectin (Kaplan et al., 2007).

Avermectin-resistant *Haemonchus* field isolate 1 (Athens, Georgia, USA)

In September of 2015, feces were collected from a flock of sheep in Athens, Georgia, USA. L3 included 81% *H. contortus* and 19% *Trichostrongylus colubriformis/Teladorsagia circumcincta*. EC_{50} and 95% confidence interval for *H. contortus* was 19.27 nM (13.56, 27.39) as determined by DrenchRite® LDA, yielding a resistance ratio of 21.18 as compared to the UGA-SUSC isolate. This EC_{50} is consistent with resistance to ivermectin and susceptibility to moxidectin (Kaplan et al., 2007).

Avermectin-resistant *Haemonchus* field isolate 2 (Ebensburg, Pennsylvania, USA)

In August of 2015, feces were collected from a flock of sheep in Ebensberg, Pennsylvania, USA. L3 included 98% *H. contortus* and 2% *Trichostrongylus colubriformis/Teladorsagia circumcincta*. EC_{50} and 95% confidence interval for *H. contortus* was 308.6 nM (16.17, 5887) as determined by DrenchRite® LDA, yielding a resistance ratio of 339.12 as compared to the UGA-SUSC isolate. This EC_{50} is consistent with resistance to both ivermectin and moxidectin (Kaplan et al., 2007).

Worminator assays

Initial stock solutions of 8.0 mM were prepared for each anthelmintic using 100% DMSO as a solvent. Initial stock solutions were then diluted in 100% DMSO to yield 4.0, 1.0, 0.50, 0.25, 0.125, 0.0625, 0.0156, and 0.0 mM working stock solutions for each drug. Working stock solutions were then diluted in deionized water yielding working concentrations of 240, 60, 30, 15, 7.5, 3.75, 0.936, and 0.0 µM in 6% DMSO.

Twenty sheathed L3 were added to each well of a 96-well, nontreated, non-sterile, polystyrene, black with clear flat bottom plate (Corning Costar® #3631, Corning Incorporated, Corning, NY, USA) in 50 µL of deionized water. Drug solution (10 µL)

was then added to wells to produce a final DMSO concentration of 1% and final anthelmintic concentrations of 40, 10, 5, 2.5, 1.25, 0.625, 0.156, and 0.0 µM (negative control). All concentrations were tested in triplicate. After addition of the drug, assay plates were agitated for 5 min using a Mini Shaker (TSZ-S-04, TSZ Scientific LLC, USA), and then incubated for 24 h in the dark at 26 °C. Plates were exposed to fluorescent light for 30 min to stimulate motility of L3 prior to reading (Gill et al., 1991). Worminator readings were taken on each well for 30 s.

Mean movement units (MMU) were measured using the consensus algorithm in WormAssay 1.4.3 for each assay well (Storey et al., 2014). MMU represent the average motility as measured by pixel displacement in an individual assay well over a given period of time. The average MMU for three technical replicates of each drug concentration were used in the statistical analysis and generation of dose response curves. Technical replicates were defined as replicate wells within the same assay plate with larvae derived from the same coproculture and drug solutions made from the same stock solution. The average MMU of the control wells was calculated as the mean of the MMU for three negative control wells per assay plate. The percent inhibition in motility at each concentration as compared to control wells was calculated as previously described by Storey et al. (2014). A higher percentage inhibition in motility indicated a greater effect of the anthelmintic compound. Biological replicates are represented by fecal collections and respective coprocultures setup on a different day from other biological replicates. Fresh stock solutions were prepared for each biological replicate. Results represent the mean of three biological replicates and nine technical replicates.

Larval migration inhibition assays

A 24-well migration plate was prepared as previously described (Evans et al., 2017), with the following modifications. A 28 µm nylon mesh screen (Sefar, Inc., Heiden, Switzerland) was fixed to the base of each migration tube. With the migration tubes in place, 1.0 mL of deionized water with drug concentrations corresponding to those used in the Worminator assays were added to each well. Importantly, the same L3 that were tested in the Worminator assay were used in the LMIA to allow for direct comparison between the two assays. Immediately after completing the Worminator readings, using a dissecting microscope, all 20 L3 in each well of the Worminator incubation plate were collected and gently transferred in a volume of 100 µL into the migration tubes containing the respective drug concentrations. Once all L3 were transferred to the migration tubes, an additional 400 µL of the respective incubation solution was added to the outside of the incubation tube into each well of the migration plate, being careful not to cause turbulence inside. This yielded a final volume of 1.5 mL in each well, and the LMIA plates were then incubated at 26 °C for 24 h to allow L3 to pass through the mesh screens. After 24 h, the migration tubes were gently removed and the non-migrated larvae were washed into empty wells of the assay plate using deionized water. A drop of Lugol's iodine was added to each well to kill the L3 and allow for accurate enumeration of migrated vs non-migrated L3 using an inverted compound microscope. All LMIA assays were completed with ivermectin as this anthelmintic compound yielded the most repeatable dose response curves and best differentiated AM-susceptible and AM-resistant isolates in Worminator assays.

Analysis of dose response

Dose-response curves were generated in GraphPad Prism 7.02 using a variable slope nonlinear regression model (GraphPad Software, La Jolla, California, USA, http://www.graphpad.com/). Drug concentrations were log_{10} transformed prior to analysis. The "log (agonist) vs response variable slope (four parameters) logistic equation" was used to generate EC_{50} values with respective 95% confidence intervals and dose response curves. Controls were plotted as 0.10 μM assigned as the x-value. No constraints were used. Error bars on dose-response curves were displayed as standard error of the mean. The coefficient of determination (R^2) was reported for each isolate. Resistance ratios were calculated as the EC_{50} of the resistant isolate divided by the EC_{50} of the susceptible isolate. For Worminator assays, the average percentage inhibition in motility at the highest concentration of anthelmintic compound tested (MI%) was reported. For LMIAs, the percent inhibition in migration was calculated for each well and corrected migration in control wells.

Results

Worminator assays

Dose response curves for Worminator assays testing the AM-susceptible (TGA-2013) and AM-resistant (CGA-2014) isolates of *Cooperia* spp. with abamectin, doramectin, eprinomectin, ivermectin, or moxidectin are displayed in Fig. 4.1. The coefficient of determination (R^2) ranged between 0.92 and 0.98 (Table 4.1) for all anthelmintic compounds tested against *Cooperia* spp., indicating good fit of the data with the dose-response algorithm with all compounds tested. Ivermectin yielded the highest coefficient of determination, meaning observed outcomes were best replicated by the

model when ivermectin was tested as compared to other anthelmintics. Inhibition of motility as compared to control wells at the highest concentration tested (40 μM) rarely exceeded 90%, indicating that the AM drugs do not completely inhibit motility of L3 even following incubation in very high concentrations. For both laboratory isolates of *Cooperia* spp., abamectin was the most potent compound and moxidectin was the least potent compound tested. Overall, EC_{50} were consistently between 2.0 and 6.0 μM. Resistance ratios ranged between 1.00 and 1.34 for the five AM compounds tested with AM-susceptible (TGA-2013) and AM-resistant (CGA-2014) laboratory isolates of *Cooperia* spp. Importantly, there were no significant differences in EC_{50} between the AM-susceptible and AM-resistant isolates of *Cooperia* spp. for any AM analog tested (Table 4.1).

Dose response curves for Worminator assays testing the AM-susceptible (UGA-SUSC) and AM-resistant (UGA-2004) laboratory isolates of *H. contortus* with eprinomectin, ivermectin, or moxidectin are displayed in Fig. 4.2. Repeatable dose response curves as evidenced by high R^2 values were generated, and ivermectin yielded the most repeatable dose response curves for *H. contortus* as previously identified with *Cooperia* spp. (Table 4.1). Resistance ratios ranged between 0.99 and 1.65. Ivermectin yielded the highest resistance ratio (1.65) and the highest coefficient of determination in the Worminator assay and therefore was used for subsequent LMIA assays with field isolates of *Cooperia* spp. and *H. contortus*. As per results with *Cooperia* spp., motility was not completely inhibited at even the highest concentration (40 μM). Interestingly, motility of the AM-resistant *H. contortus* (UGA-2004) isolate was elevated as compared to control wells when tested with concentrations of ivermectin and moxidectin between

0.156 and 2.5 μM. This phenotype was not identified with the AM susceptible *H. contortus* isolate (UGA-SUSC). The EC50 for ivermectin was higher for UGA-2004 as compared to UGA-SUSC, with non-overlapping 95% CI, indicating that despite having a small resistance ratio, we were able to discriminate the two isolates using ivermectin (Table 4.1). However, no difference was identified in the EC_{50} of AM-susceptible and AM-resistant *H. contortus* with eprinomectin or moxidectin (Table 4.1).

Dose response curves for inhibition in motility (Worminator) and inhibition in migration (LMIA) of *Cooperia* and *Haemonchus* laboratory and field isolates following incubation with ivermectin are displayed in Fig. 4.3. For *Cooperia* spp., the Worminator yielded repeatable dose response curves, however resistance ratios ranged between 0.61 and 1.18. The LMIA yielded resistance ratios of 1.94 and 1.12 for CGA-2014 and AM Resistant *Cooperia* Field Isolate 1, respectively (Table 4.2). The AM Resistant *Cooperia* Field Isolate 2 yielded poor dose-response characteristics for the LMIA, leading to a low coefficient of determination, and inability to calculate a CI for the EC_{50}. Thus, the EC_{50} and resistance ratio calculated for this isolate were not reliable (Table 4.2).

For *H. contortus*, EC_{50} determined by the Worminator were not different between susceptible and resistant isolates, yielding resistance ratios of 1.32 and 1.02 for the Resistant Field Isolates 1 and 2, respectively. The LMIA yielded poor dose-response characteristics for two isolates of *H. contortus*, making accurate comparisons of these to other isolates difficult (Fig. 4.3, Table 4.2).

Discussion and Conclusion

The present study evaluated motility of L3 as a phenotype for detection of resistance to AM drugs using the Worminator, a system that directly quantifies motility of microscopic stages of nematodes. Motility is a commonly-used phenotype for the assessment of anthelmintic activity. Both qualitative (Gill et al., 1991) and quantitative (Bennett and Pax, 1986) measurements of motility have been described in the literature. A number of systems have been developed to quantitatively measure motility and test anthelmintic activity in nematodes (Bennett and Pax, 1986; Smout et al., 2010) (Marcellino et al., 2012; Storey et al., 2014; Nutting et al., 2015). However, few studies have evaluated quantitative motility to detect AM resistance in GIN of livestock (Demeler et al., 2010a; Demeler et al., 2010b; Dolinská et al., 2016). In this study, we tested four isolates each of *Cooperia* spp. and *H. contortus* to investigate whether motility of L3 would provide a useful phenotype for detecting resistance to AM anthelmintics. To the best of our knowledge, this is the first report of quantitative evaluation of motility for detection of AM resistance in two important species of GIN of livestock using recently established, well-characterized laboratory and field isolates with several AM analogues.

In the present work, resistance ratios were less than 2.0 between AM-susceptible and AM-resistant laboratory isolates of *Cooperia* spp. and *H. contortus* for all anthelmintic compounds tested with the Worminator (Table 4.1). Using field isolates, resistance ratios were also less than 2.0 and confidence intervals overlapped for EC_{50} of all AM-susceptible and AM-resistant field isolates tested with the Worminator. Confidence intervals overlapped between AM-susceptible and AM-resistant laboratory

isolates of *Cooperia* spp. and *H. contortus* for all drug and isolate combinations tested, except for ivermectin with *H. contortus* which yielded a resistance ratio of 1.65 (Table 4.1). Smout et al. (2010) reported similar results with a resistance ratio of 1.11 and overlapping 95% confidence intervals for the AM-resistant *H. contortus* Wallangra isolate and AM-susceptible *H. contortus* Kirby isolate using an alternative motility assay, xCELLigence (Roche). Since ivermectin was the only AM analog tested that differentiated AM-susceptible and AM-resistant *H. contortus*, even with a low resistance ratio of 1.65, we chose to use this analog in subsequent assays to evaluate field isolates of *Cooperia* spp. and *H. contortus* in Worminator and all LMIA tests (Fig. 4.3, Table 4.2). Further, LDA resistance ratios for the *H. contortus* isolates were 16.48, 21.18, and 339.12 for the AM-resistant laboratory isolate, field isolate 1, and field isolate 2, respectively, as compared to the AM-susceptible laboratory isolate. Thus, the LDA yielded resistance ratios at least 10 times higher than those generated from the Worminator assays. These results are consistent with Raza et al. (2015) who reported resistance ratios for the AM-resistant Wallangra isolate as compared to the AM-susceptible Kirby isolate of 20.74 and 2.82 for the LDA and LMIA, respectively.

The LMIA yielded a resistance ratio of 1.94 for AM-susceptible and AM-resistant laboratory isolates of *Cooperia* spp. Furthermore, resistance ratios for field isolates of *Cooperia* spp. and *H. contortus* were both less than 1.5. Though, these field isolates were comprised of multiple species rather than a monoculture of *Cooperia* spp., the majority of the L3 were *Cooperia* spp. so it is unlikely that the other species had a major impact on the results of the assay. Reports that the LMIA detected differences in susceptible and resistant *H. contortus* but not *T. circumcincta* or *T. colubriformis* suggest that the LMIA

may not be appropriate for field isolates comprised of multiple species (Kotze et al., 2006). It is also possible that migration of worms in the LMIA is not only a function of motility, but also involves sensory functions of L3, which has been previously reported as a potentially important aspect of AM resistance in *H. contortus* (Urdaneta-Marquez et al., 2014). If this is true, AM drugs may impair the ability of AM-susceptible L3 to migrate through a fine mesh sieve in the LMIA, independent of their level of motility. In contrast, if AM-resistant L3 retain sensory capabilities they may retain their ability to migrate even when their motility is impaired.

Several interesting phenotypes were identified for all isolates and AM compounds evaluated with the Worminator. First, complete inhibition of motility was not achieved even at the highest concentrations (40 µM) of anthelmintics tested suggesting that AM compounds do not completely inhibit motility of sheathed L3 *in vitro*. Due to solubility limitations of the AM drugs, it is not possible to test concentrations greater than 40 µM. It appears that the AMs paralyze the central portion of L3 and the head and tail remain slightly motile, demonstrating a slight jerking motion or a very sluggish motility. Previously, Geary et al. (1993) described this phenotype in adults of *H. contortus* as ivermectin paralyzed the mid-body region but heads and tails maintained motility. Folz et al. (1987) reported less than 70% reduction in motility of L3 of *H. contortus* following incubation with greater than 100 µM ivermectin. Dolinská et al. (2016) also reported 49.7–97.8% maximum percent reduction in L3 motility of six isolates of *H. contortus* following incubation with ivermectin. The dose related levels of inhibition in motility reported in Dolinská et al. (2016) and Folz et al. (1987) should be interpreted with caution as concentrations of ivermectin greater than 100 µM were tested, which are above

the solubility limit of this compound. Thus, the actual amount of drug the worms were in contact with cannot be accurately inferred. Additionally, Dolinská et al. (2016) tested 10-fold dilutions of ivermectin and eprinomectin and did not report 95% confidence intervals for calculated EC_{50} and EC_{99}. Even with these limitations, it is still clear that AM compounds do not completely inhibit motility of L3 *in vitro*.

Interestingly, the concentration of AM required to inhibit the motility of L3 by 50% is more than 100-fold greater than the peak plasma concentration achieved in cattle following subcutaneous administration of 200 μg/kg of doramectin, ivermectin, or moxidectin (Lanusse et al., 1997). In contrast, the concentration of AM required to inhibit larval development from the egg in AM-susceptible *H. contortus* is at least 1,000 times less than that required to inhibit L3 motility. For example, the EC_{50} for the avermectin-susceptible *H. contortus* (UGA-SUSC) in the LDA was 0.82 nM while the EC_{50} in the Worminator was 2.60 μM. Gill et al. (1995) also described this phenotype, as the EC_{50} of the McMaster isolate of *H. contortus* was 1.0 nM in the LDA and 300 nM in an L3 motility assay (Gill et al., 1991). The concentration of ivermectin required to inhibit pharyngeal pumping and development of adult *H. contortus in vitro* are 10–100 fold lower than the concentration required to inhibit motility of L3 *in vitro* (Geary et al., 1993). To inhibit motility of L3 of *H. contortus*, the LMIA required 500-fold the concentration of ivermectin to inhibit development in the LDA (Demeler et al., 2013). This discrepancy in the concentration of anthelmintic compound required for activity suggests that the mechanism of action, expression of drug targets, penetration of drug into the tissues of the worm, and/or drug detoxification and drug efflux mechanisms may differ among life stages of GIN. For example, L3 do not feed and thus inhibition of

pharyngeal pumping will not occur; consequently, paralysis in this life stage would only result from effects on the musculature of body wall (Gill et al., 1995). The reduced sensitivity of sheathed L3 raises questions about the suitability of L3 as a stage for detecting activity of potential anthelmintic compounds. An alternative explanation for the discrepancy in concentrations *in vivo* and *in vitro* may be that subtle motility effects of the AMs such as those associated with less than 50% inhibition in motility may be relevant to parasite expulsion and thus EC_{50} values may be less important than perceived in the above discussion (Kotze et al., 2012b). Rather than quantitatively evaluating motility, Gill et al. (1991) qualitatively evaluated the motility of L3 following incubation with AM compounds and defined motile larvae as those with 'normal sinusoidal thrashing motility' and non-motile larvae as those moving in a 'restricted manner'. Gill et al. (1991) obtained resistance ratios from 2.7 to 8.7 with ivermectin for known resistant isolates, further suggesting that subtle changes in L3 motility characterized as 'restrictive' motility may be more important than reduction in quantitative motility by 50% as compared to control wells.

As previously reported by Gill et al. (1991), we observed an increase in motility of L3 upon exposure to fluorescent light, which may be associated with natural response to sunlight. L3 of *H. contortus* exhibited reduced motility following incubation in the absence of light at 25 °C, but exposure to light stimulated rapid sinusoidal motility for 10–15 min before returning to a low motility state by 40–60 min post exposure. In the present study, preliminary experiments demonstrated that following incubation for 24 h in the absence of light at 26°C, exposure to fluorescent light for 30 min achieved optimum motility of L3 in control wells.

In reference to the suitability of the L3 stage of GIN for detection of AM resistance, our results clearly indicate that motility of sheathed L3 is not an appropriate phenotype for this purpose in *Cooperia* spp. and *H. contortus*. Future research efforts should evaluate alternative life stages including the L4, which may be more appropriate for detection of AM resistance. Specifically, the L4 as a feeding parasitic stage may more accurately reflect the adult in vivo drug-parasite interaction and therefore express a more similar resistance profile. Until appropriate and validated molecular diagnostic markers are available for detecting resistance to AM drugs, efforts should continue to focus on the development and optimization of *in vitro* assays for detection of AR.

Acknowledgements

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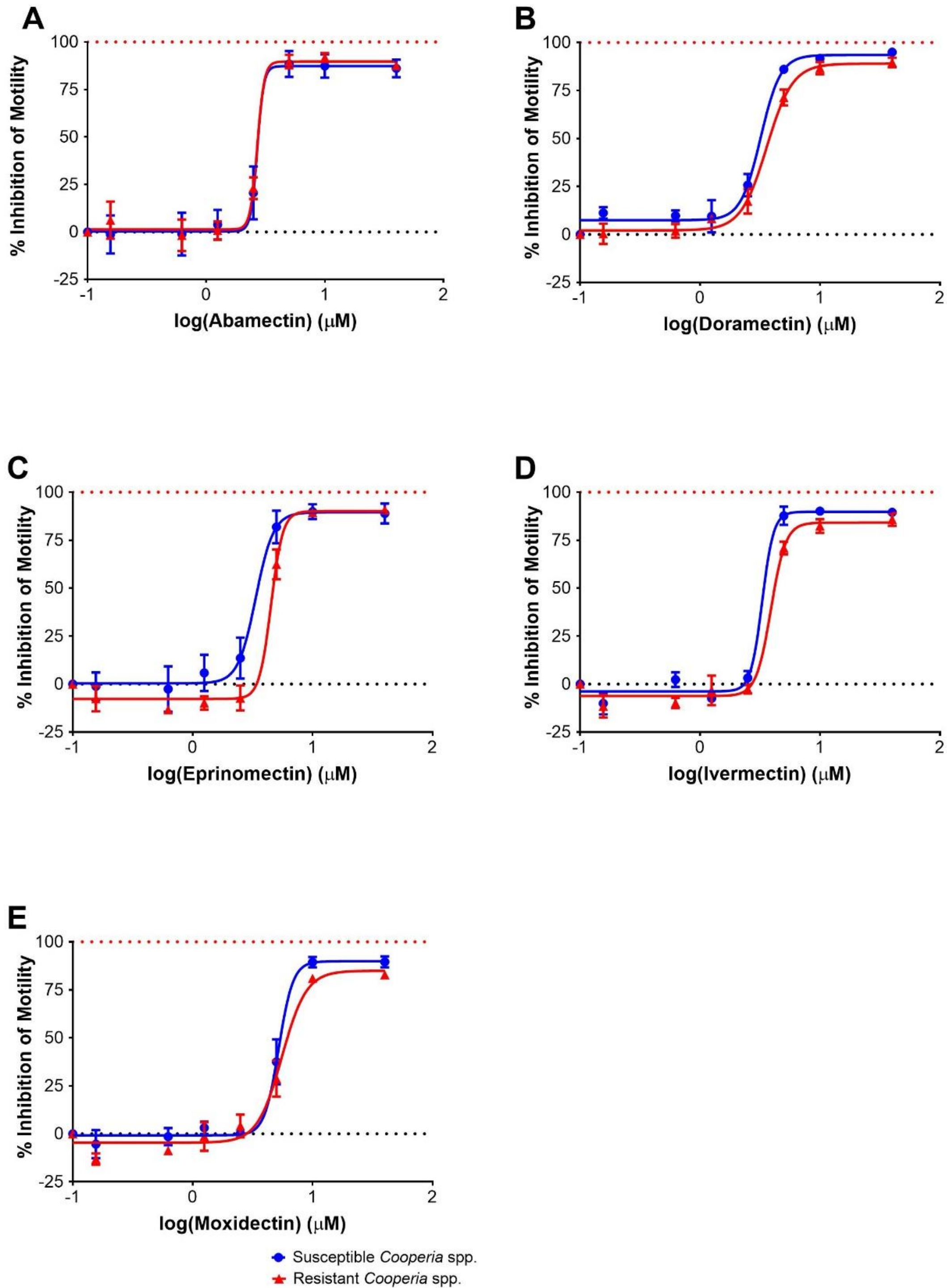

Figure 4.1. Worminator assay dose response curves for *Cooperia* spp. Dose response for inhibition of motility of third-stage larvae of avermectin/milbemycin-susceptible (TGA-2013) and avermectin/milbemycin-resistant (CGA-2014) *Cooperia* spp. following

incubation with anthelmintic compounds (A: Abamectin, B: Doramectin, C: Eprinomectin, D: Ivermectin, E: Moxidectin). Circles (blue) represent the susceptible isolate and triangles (red) represent the resistant isolate.

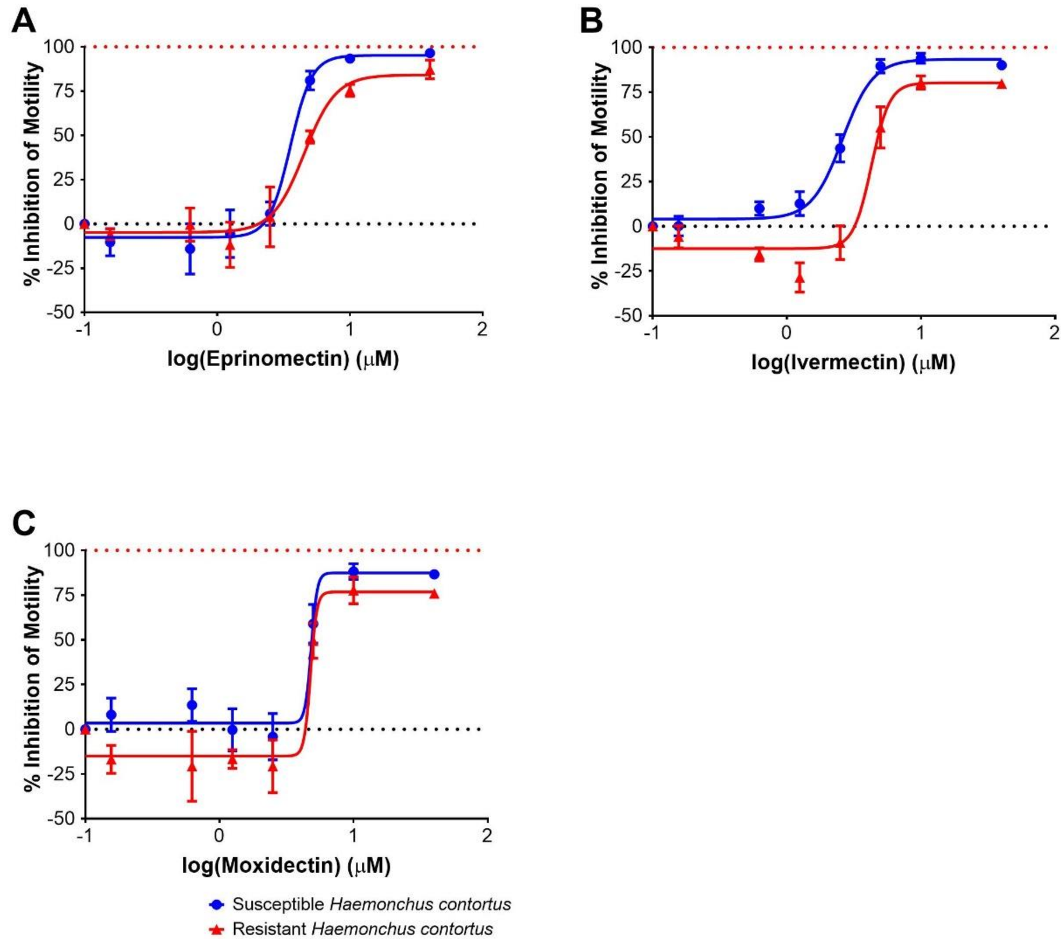

Figure 4.2. Worminator assay dose response curves for *Haemonchus contortus*. Dose response for inhibition of motility of third-stage larvae of avermectin/milbemycin-susceptible (UGA-SUSC) and avermectin/milbemycin-resistant *H. contortus* (UGA-2004) following incubation with anthelmintic compounds (A: Eprinomectin, B: Ivermectin, C: Moxidectin). Circles (blue) represent the susceptible isolate and triangles (red) represent the resistant isolate.

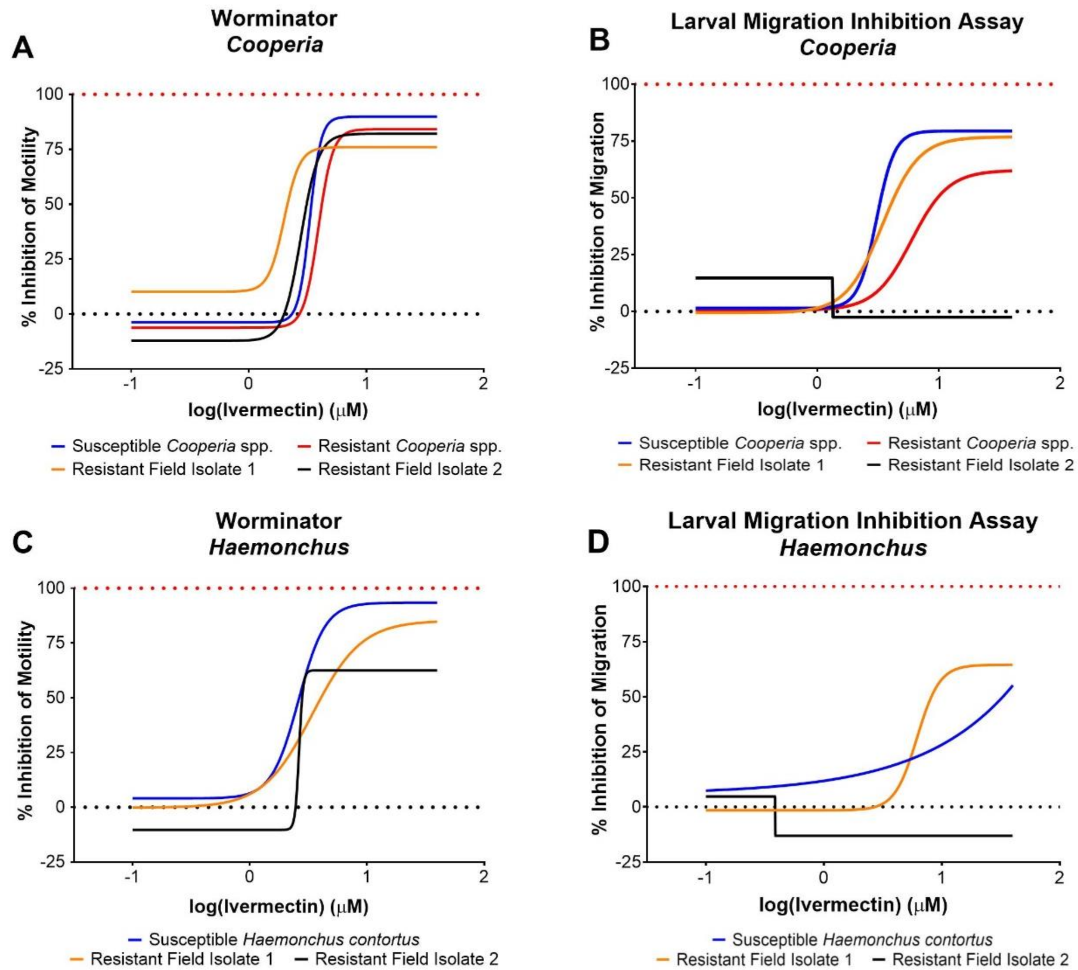

Figure 4.3. Dose response curves for inhibition of motility or inhibition of migration using the Worminator or Larval Migration Inhibition Assay, respectively, for third-stage larvae of *Cooperia* spp. and *Haemonchus contortus* following incubation with ivermectin. Four isolates of *Cooperia* spp. were tested including an AM-susceptible laboratory isolate (TGA-2013), AM-resistant laboratory isolate (CGA- 2014), and two AM-resistant field isolates. Three isolates of *Haemonchus contortus* were tested including an AM-susceptible laboratory isolate (UGA-SUSC) and two resistant field isolates.

Table 4.1. EC_{50} (μM), 95% confidence intervals (CI), inhibition of motility at highest concentration tested (MI%), and resistance ratios (RR) for avermectin/milbemycin susceptible (TGA-2013) and resistant (CGA-2014) *Cooperia* spp. and susceptible (UGA-SUSC) and resistant (UGA-2004) *Haemonchus contortus* in the Worminator assay.

	Susceptible *Cooperia* spp.			Resistant *Cooperia* spp.				Susceptible *H. contortus*			Resistant *H. contortus*			
Anthelmintic Compound	EC_{50} (CI)	R^2	MI(%)	EC_{50} (CI)	R^2	MI(%)	RR	EC_{50} (CI)	R^2	MI(%)	EC_{50} (CI)	R^2	MI(%)	RR
Abamectin	2.70[a]	0.92	87.29	2.70[a]	0.96	87.63	1.00							
Doramectin	3.21 (2.81, 3.67)	0.97	94.83	3.60 (3.20, 4.05)	0.98	89.91	1.12							
Eprinomectin	3.36 (2.64, 4.28)	0.93	89.09	4.50 (3.19, 6.37)	0.97	90.73	1.34	3.56 (2.89, 4.37)	0.94	97.36	4.52 (3.54, 5.77)	0.89	95.29	1.27
Ivermectin	3.30 (2.62, 4.15)	0.98	89.61	3.90 (3.30, 4.62)	0.97	86.05	1.18	2.60 (2.28, 2.96)	0.97	97.20	4.28 (3.30, 5.54)	0.92	87.74	1.65
Moxidectin	5.22 (4.45, 6.13)	0.96	89.77	5.55 (4.75, 6.49)	0.95	82.75	1.06	4.83[a]	0.88	96.53	4.78[a]	0.87	92.13	0.99

[a] The 95% confidence interval was very wide.

Table 4.2. Inhibition of motility (Worminator) versus inhibition of migration (Larval Migration Inhibition Assay) of third-stage larvae of avermectin/milbemycin susceptible and resistant laboratory and field isolates of *Cooperia* spp. and *Haemonchus contortus* following incubation with ivermectin.

		Inhibition of Motility			Inhibition of Migration		
Species	Isolate	EC_{50} (CI)	R^2	RR	EC_{50} (CI)	R^2	RR
Cooperia spp	Susceptible	3.30 (2.62, 4.15)	0.98		3.07 (2.63, 3.59)	0.97	
	Resistant	3.90 (3.30, 4.62)	0.97	1.18	5.97 (4.41, 8.08)	0.88	1.94
	Resistant Field 1	2.00 (1.02, 3.92)	0.95	0.61	3.44 (3.21, 3.68)	1.00	1.12
	Resistant Field 2	2.77 (1.30, 5.92)	0.90	0.84	1.34[a]	0.54	0.44
Haemonchus contortus	Susceptible	2.60 (2.28, 2.96)	0.97		7.77 × 105[a]	0.44	
	Resistant Field 1	3.42 (2.18, 5.36)	0.85	1.32	6.17 (5.09, 7.48)	0.94	0.00
	Resistant Field 2	2.64[a]	0.96	1.02	0.39[a]	0.26	0.00

[a] The 95% confidence interval was very wide.

CHAPTER 5

EVALUATION OF CHANGES IN DRUG SUSCEPTIBILITY AND POPULATION GENETIC STRUCTURE IN *HAEMONCHUS CONTORTUS* FOLLOWING WORM REPLACEMENT AS A MEANS TO REVERSE THE IMPACT OF MULTIPLE-ANTHELMINTIC RESITANCE ON A SHEEP FARM[3]

[3] George, M.M., Vatta, A.F., Howell, S.B., Storey, B.E., McCoy, C.J., Wolstenholme, A.J., Redman, E.M., Gilleard, J.S., and Kaplan, R.M. In preparation for submission to the International Journal for Parasitology: Drugs and Drug Resistance.

Abstract

A population of *Haemonchus contortus* that was highly resistant to benzimidazoles and avermectin/milbemycins and, which contained a subpopulation that was resistant to levamisole was replaced with a susceptible laboratory isolate of *H. contortus* in a flock of sheep. The anthelmintic susceptibility and population genetics of the newly established worm population were subsequently evaluated for 3.5 years using *in vivo*, *in vitro*, and molecular methods. In the immediate post-replacement period, successful replacement of the resistant population with a susceptible population was confirmed using phenotypic and genotypic measurements; larval development assay EC_{50} indicated full anthelmintic susceptibility; albendazole treatment yielded 98.7% fecal egg count reduction; pyrosequence genotyping of single nucleotide polymorphisms in positions 167 and 200 of the isotype-1 beta tubulin gene were present at 0.00 and 1.67%, respectively; microsatellite genotyping of the replaced population indicated the background haplotype was similar to the susceptible laboratory isolate; and haplotypes of the isotype-1 beta tubulin gene were similar to the susceptible laboratory isolate. In an effort to sustain the anthelmintic susceptibility of the new worm population, targeted selective treatment was implemented using albendazole. Surprisingly, within 1.5 years post-replacement, the population had reverted to a resistant phenotype. Resistance to albendazole, ivermectin, and moxidectin was confirmed via fecal egg count reduction test, larval development assay, and pyrosequence genotyping. Targeted selective treatment was then carried out using levamisole. However, within one year, resistance was detected to levamisole. Population genetic analysis using a panel of 9 neutral polymorphic microsatellite markers demonstrated there was a gradual change in the

genetic structure of the population until the final population was similar to the initial resistant population. This work demonstrates the power of combining molecular, *in vitro*, and *in vivo* assays to study phenotypic and genotypic changes in a field population of nematodes, enabling improved insights into the epidemiology of anthelmintic resistance.

Introduction

The highly pathogenic parasitic nematode *Haemonchus contortus* has a remarkably high propensity to develop drug resistance (Gilleard and Devaney, 2013). Anthelmintic resistance to multiple classes of drugs is a serious problem on small ruminant farms across the globe (Howell et al., 2008; Torres-Acosta et al., 2012; Playford et al., 2014), providing a significant challenge to effective parasite control (Kaplan and Vidyashankar, 2012). To slow the development of anthelmintic resistance, Leathwick and Besier (2014) suggest producers implement refugia-based control strategies, use highly-effective combinations of anthelmintics, and prevent the introduction of resistant nematodes. Though implementation of these strategies will slow the development of resistance, the prevalence of farms with multiple-anthelmintic resistance and total anthelmintic failure is already high and is increasing (Martínez-Valladares et al., 2013; Herrera-Manzanilla et al., 2017; Howell et al., 2017). Thus, the aforementioned strategies to slow the development of resistance are no longer useful or practical on many of these properties (Leathwick et al., 2012), leaving few options for chemical-based control. Hence, replacement of a resistant population with a susceptible population (Leathwick et al., 2015) may be an attractive strategy for properties where the parasite population is already resistant to multiple classes of anthelmintics. Replacement with a susceptible population of gastrointestinal nematodes would allow producers to reclaim the use of

classes of anthelmintics that were previously ineffective. If then used in conjunction with sustainable integrated parasite management (sIPM) strategies (van Wyk et al., 2006; Leathwick and Besier, 2014), this approach could serve as a long-term solution for these farms by maintaining the effectiveness of the anthelmintics into the future.

Replacement of a resistant population of gastrointestinal nematodes with a susceptible population is a strategy designed to replace resistant alleles with susceptible alleles (van Wyk and Van Schalkwyk, 1990). Replacement was previously tested in gastrointestinal nematodes of sheep with various methodologies for introduction of susceptible parasites (van Wyk and Van Schalkwyk, 1990; Bird et al., 2001; Aumont, 2002; Sissay et al., 2006; Moussavou-Boussougou et al., 2007; Muchiut et al., 2018). The percent fecal egg count reduction (FECR) measured soon after introduction of susceptible parasites can be used to assess the 'success' of initial level of replacement. Level of drug efficacy following replacement varied widely among these studies depending on the strategy used to reduce the resistant population, the methodology to introduce the susceptible population, the season the study was conducted, and the period of time following replacement that FECR was evaluated (Muchiut et al., 2018). Previously published evaluations of replacement measured changes in susceptibility at points in time that were less than 18 months from replacement (van Wyk and Van Schalkwyk, 1990; Bird et al., 2001; Aumont, 2002; Sissay et al., 2006; Moussavou-Boussougou et al., 2007; Muchiut et al., 2018), which at most represented two grazing seasons. Eighteen months likely is not a sufficient period in most cases to determine if the observed short-term changes in susceptibility are sustainable long-term. To the best of our knowledge, the present study is the first to evaluate the resistance status for a longer-term; we maintained

surveillance for 3.5 years post-replacement. Longer-term evaluation is necessary to determine if the worm replacement strategy tested was sustainable under the experimental and field conditions used.

In this study we used multiple phenotypic and genotypic approaches to examine changes in the drug susceptibility and population genetics of the replaced *H. contortus* population. For measuring the resistance phenotype, we used both *in vivo* (FECR) and *in vitro* (Larval Development Assay) measures, and for the resistance genotype for benzimidazoles we used pyrosequencing of the β-tubulin gene (Samson-Himmelstjerna et al., 2009b). In addition, we evaluated changes in the genetics of the nematode population during the course of the replacement period under study using microsatellite genotyping (Redman et al., 2008b; Redman et al., 2015) and examination of β-tubulin haplotypes to measure changes in the population structure. This strategy allowed us to not only monitor the changes in drug resistance over time, but also to gain deeper insights into what was happening at the population genetic level.

Materials and Methods

Experimental design

The timeline of the study is detailed in Table 5.1. In Spring of 2011, the University of Georgia's sheep flock (Location 1) experienced an outbreak of severe haemonchosis. On the 3rd of August 2011, lambs (n=14) and mature sheep (n=28) were removed from contaminated pastures and housed in pens with concrete floors until September 23, 2011. Sheep were treated orally with a combination of levamisole (Prohibit®, Agri Laboratories, Ltd., St. Joseph, Missouri, USA, 8 mg/kg) and albendazole (Valbazen®, Pfizer Animal Health, Parsippany, New Jersey, USA) on the

3rd,4th and 5th of August 2011. The initial dose of albendazole was 15 mg/kg, with subsequent doses at 7.5 mg/kg. The FECR and associated 95% confidence interval measured on August 15, 2011 was 100.0% (99.1, 100.0). Approximately four weeks after treatment (September 2, 2011), mean fecal egg count (FEC) had increased to 363 eggs per gram (EPG). Sheep were then re-treated using the same 3-day regimen on September 21, 2011 which reduced mean FEC by 95% to 18 EPG. Two days later on September 23, 2011, each sheep was infected orally with 5,000 third-stage larvae (L3) of UGA-SUSC (George et al., 2018), a laboratory isolate of *H. contortus* that is fully susceptible to avermectin/milbemycins, levamisole, and benzimidazoles. Approximately one week later (September 27, 2011), sheep were moved to a new property at the UGA Double Bridges Farm (Location 2), that did not previously graze livestock. Prior to the introduction of the sheep, grass washings were conducted according to the methods of Hansen and Perry (1994) to estimate the number of L3 per kg of dry matter present in each of the four paddocks. Relatively low numbers of L3 were recovered (Appendix B), likely the result of white-tailed deer that are common in this area. Grazing of paddocks commenced with the least contaminated paddocks to allow time for the L3 on the more heavily contaminated paddocks to die. At the new property, sheep grazed Max Q fescue grass. Approximately four weeks after being introduced to the new pastures (October 25, 2011), the sheep were evaluated using FAMACHA© (van Wyk and Bath, 2002); none of the adult sheep were treated but all 14 lambs were administered albendazole (7.5 mg/kg). Additional treatments with albendazole (7.5 mg/kg) based on FAMACHA© scores of 4 or 5 were administered to 10 sheep on May 3, 2012, and to 27 sheep based on FAMACHA© scores of 3, 4, or 5 on July 7, 2012.

Fecal egg count reduction tests

A fecal egg count reduction test (FECRT) was performed using a label dose of albendazole (7.5 mg/kg) in Fall 2011. In Spring 2013, a FECRT was conducted using label doses of albendazole (7.5 mg/kg), ivermectin (Ivermectin Sheep Drench, Durvet, Inc., Blue Springs, Missouri, USA, 200 µg/kg), levamisole (8 mg/kg), and moxidectin (Cydectin®, Boehringer Ingelheim, St. Joseph, Missouri, USA, 200 µg/kg). In Spring 2014, a FECRT was conducted with levamisole (8 mg/kg), moxidectin (200 µg/kg), and a combination of levamisole (8 mg/kg) and moxidectin (200 µg/kg). In Spring 2015, a FECRT was conducted with levamisole (8 mg/kg), moxidectin (200 µg/kg), a combination of levamisole (8 mg/kg) and moxidectin (200 µg/kg), and a combination of albendazole (7.5 mg/kg), levamisole (8 mg/kg), and moxidectin (200 µg/kg). All anthelmintics were administered orally.

FEC were performed using a modified-McMaster technique with a 25 or 8 EPG limit of detection. Homogenization of the sample and sodium nitrate solution, slide preparation, and counting were completed as previously described (Noel et al., 2017).

The mean FECR and associated 95% confidence interval were calculated using the web interface of the eggCounts package modified for individual efficacy (http://shiny.math.uzh.ch/user/furrer/shinyas/shiny-eggCounts/) (Wang et al., 2018).

Larval coproculture

Coprocultures were prepared on each sampling date by combining approximately 10.0 g of feces per individual. Vermiculite and water were mixed with the pooled feces and incubated at room temperature (approximately 25°C) for 10-14 days. L3 were recovered with the Baermann technique (Dinaburg, 1942) and phenotypically identified.

Greater than 98% of L3 were identified as *H. contortus* at all collections. L3 from 9 collections were stored at -20°C in water until molecular experiments were completed (Table 5.1).

Larval development assay

DrenchRite® Larval Development Assays were performed to evaluate the *in vitro* resistance levels of the *H. contortus* populations throughout the course of the study. The assay was performed in accordance with the manufacturer's guidelines (DrenchRite, Microbial Screening Technologies, Armidale, New South Wales, Australia) with minor modifications as described previously (Kaplan et al., 2007; Howell et al., 2008). This assay evaluates the development of eggs to third-stage larvae in the presence of increasing concentrations of thiabendazole, levamisole, and ivermectin aglycone to provide a measure of susceptibility/resistance to benzimidazoles, imidizothiazoles, and avermectins/milbemycins, respectively.

Drug concentrations were $\log_{10}$ transformed prior to analysis. For each well, the percent development to L3 was calculated after correction for development in control wells. Dose response analysis was performed with GraphPad Prism 7.02 (GraphPad Software, La Jolla, California, USA, http://www.graphpad.com) using a variable slope nonlinear regression model. The "log (inhibitor) vs response variable slope (four parameters) logistic equation" was used to generate effective concentration 50 (EC_{50}) values with respective 95% confidence intervals. The EC_{95} was also calculated for levamisole and ivermectin aglycone. The x-value for controls were plotted as 0.10 nM for ivermectin, 0.01 µM for levamisole, and 0.003 µM for thiabendazole. The coefficient of determination (R^2) was reported for each sample. Resistance ratios were calculated as the

EC_{50} or EC_{95} of the sample divided by the EC_{50} or EC_{95} of the susceptible isolate (UGA-SUSC). Results for levamisole were also analysed using a 2-population model in Fit logit (Waller et al., 1985; Dobson et al., 1987) to determine if the population contained a more highly resistant subpopulation and the level of resistance of this subpopulation.

Microsatellite genotyping and population genetic analysis

A panel of 9 neutral microsatellite loci (Appendix C, (Otsen et al., 2000; Redman et al., 2008b; Redman et al., 2012; Redman et al., 2015)) were used to genetically differentiate the population at nine time points throughout the study (Table 5.1). Duplicate lysate pools were used as template for PCR amplification. For each lysate, 100 third-stage larvae were mixed in 100 µL of nuclease free water (Catalog No. W3513, Sigma, Los Angeles, California, USA), 300 µL of DirectPCR (Tail) Lysis Buffer (Catalog No. 101-T, Viagen Biotech, St. Louis, Missouri, USA), and 10 µL Proteinase K (Catalog No. P8107S, New England BioLabs, Inc., Ipswich, Massachusetts, USA) and incubated for 12h at 55°C, 1h at 90°C, and cooled to 4°C. PCR amplification was conducted in duplicate to yield a total of four amplicons per time point for each microsatellite. PCR were performed in 10 µL reactions containing 5 µL JumpStart REDTaq ReadyMix Reaction Mix (Catalog No. P0982-800RXN, Sigma, Los Angeles, California, USA), 1 µL of neat lysate, 0.5 µM of each oligonucleotide primer (Integrated DNA Technologies, Skokie, Illinois, USA), and 3 µL of nuclease free water (Catalog No. W3513, Sigma, Los Angeles, California, USA).Thermocycling conditions were 94°C for 2 minutes, 40 cycles of 90°C for 15 seconds, 54°C for 30 seconds, and 72°C for 1 minute, and a final extension step of 72°C for 7 minutes. Amplicons were diluted 1:40 in ultrapure water.

Fragment analysis was performed by capillary electrophoresis using an ABI 3730xl sequencer (Applied Biosystems, ThermoFisher Scientific, South San Francisco, California, USA). The internal size standard GeneScan™ 500 ROX™ (Applied Biosystems, ThermoFisher Scientific, South San Francisco, California, USA) was used. Chromatograms were analyzed in Geneious R8 8.0.2 (Biomatters, Ltd., Auckland, New Zealand).

Peak height was used as an estimation of allele frequency (Redman et al., 2008b; Redman et al., 2012). The relative percentage of total peak height was calculated for each allele as the height of a single allele divided by the sum of height for all called alleles multiplied by 100. A population of 100 individuals was modeled according to the calculated allele frequency and the principles of Hardy-Weinberg equilibrium. A pairwise population matrix of Nei genetic distance was generated and principle coordinate analysis was performed in GenAlEx version 6.501 (Peakall and Smouse, 2006, 2012).

Pyrosequence genotyping of single nucleotide polymorphisms associated with benzimidazole resistance in the isotype-1 β-tubulin gene

A 385 bp fragment of the isotype-1 β-tubulin gene containing the position 167 and 200 codons was amplified as previously described (Samson-Himmelstjerna et al., 2009a) in duplicate from two independent lysates of 100 L3 at nine time points throughout the study. Briefly, the forward primer HcPy2PCR For: 5'- GAC GCA TTC ACT TGG AGG AG -3') and reverse biotinylated primer HcPy2PCR Rev: 5'Biotin-CAT AGG TTG GAT TTG TGA GTT -3') were used to amplify a 385 bp region of the isotype-1 β-tubulin gene (Samson-Himmelstjerna et al., 2009a). PCR were completed in 50 μL of total volume using the iProof™ High-Fidelity DNA Polymerase Kit (Catalog

No. 172-5302, Bio-Rad Laboratories, Inc., Hercules, California, USA) comprised of the following: 10 µL 5x iProof Buffer (1x Final), 1 µL 10 mM dNTP mix (200 µM final concentration), 2.5µL forward primer (0.5 µM final concentration), 2.5 µL reverse primer (0.5 µM final concentration), 2.0 µL DNA neat lysate, 31.5 µL nuclease free water, and 0.5µL iProof DNA Polymerase (0.02 U/µL). PCR conditions consisted of an initial denaturation step at 98°C for 30 seconds; 35 cycles of the following: denaturation at 98°C for 10 seconds, annealing at 43°C for 30 seconds, extension at 72°C 1 minute; and a final extension of 72°C for 7 minutes. A 2% agarose gel was used to visualize and confirm presence of amplicons of 385 bp in size.

Next, the relative frequency of the F167Y and F200Y SNPs isotype-1 β-tubulin gene were determined using the allele quantification assay available via pyrosequence genotyping (Pyromark Q24, Qiagen, Hilden, Germany). Previously published sequencing primers were used to sequence the F167Y and F200Y mutations: Hc167PySeq1 (5' – ATA GAA TTA TGG CTT CGT -3') and Hc200PySeq1 (5' –TAG AGA ACA CCG ATG AAA CAT-3') (Samson-Himmelstjerna et al., 2009a). The dispensation order was set at A/TCTCCGTTGTT for the P167 and A/TCTGTATTGAC for the P200. Relative peak heights were used as an approximation of allele frequency for pooled samples as previously validated in pools of adult *H. contortus* (Chaudhry et al., 2015). Plasmids were prepared containing a 385 bp fragment of the isotype-1 β-tubulin gene and with either the wild-type (susceptible) T**T**C sequence or the resistant T**A**C sequence at both the 167 and 200 codon positions. A standard curve was then generated using varying ratios (0:10, 1:9, 3:7, 5:5, 7:3, 9:1, 10:0) of the two plasmids containing the susceptible and resistant genotypes. Measured allele frequencies were corrected based on this

standard curve.

Sequencing of isotype-1 β-tubulin haplotypes

A 385 bp fragment of the isotype-1 β-tubulin gene encompassing the region containing the three potentially resistance-associated SNPs (F167Y, F200Y, E198A) was PCR amplified from lysates of 100 L3 collected at six time points throughout the study (Table 5.1). The forward primer Hc BTUB For: 5'– GAC GCA TTC ACT TGG AGG AG -3' and reverse primer Hc BTUB Rev: 5'- CAT AGG TTG GAT TTG TGA GTT -3' were used to amplify the 385 bp fragment (Samson-Himmelstjerna et al., 2009a). PCR were completed in 50 µL of total volume using the iProof™ High-Fidelity DNA Polymerase Kit (Catalog No. 172-5302, Bio-Rad Laboratories, Inc., Hercules, California, USA) comprised of the following: 10 µL 5x iProof buffer (1x final dilution), 1 µL 10 mM dNTP mix (200 µM final concentration), 2.5µL forward primer (0.5 µM final concentration), 2.5 µL reverse primer (0.5 µM final concentration), 2.0 µL DNA neat lysate, 31.5 µL nuclease free water, and 0.5µL iProof DNA Polymerase (0.02 U/µL). PCR conditions consisted of an initial denaturation step at 98°C for 30 seconds; 35 cycles of the following: denaturation at 98°C for 10 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds; and a final extension of 72°C for 10 minutes. Amplicons were visualized on a 2% agarose gel and cloned directly from purified PCR product (QIAquick® PCR Purification Kit, Catalog No. 28106, Lot No. 145017272, Qiagen, Hilden, Germany). Purified PCR products were cloned using the Zero Blunt® TOPO® PCR Cloning Kit (Catalog No. 450245, Lot No. 1697045, Invitrogen™ by Life Technologies™, Carlsbad, California, USA). Twenty clones per time point (n=6) were selected and plasmids were purified (GenElute Plasmid Mini Prep Kit, Catalog No.

PLN350-1KT, Lot No. SLBK4190, Sigma, St. Louis, Missouri, USA). Purified plasmid DNA was sequenced using conventional Sanger sequencing in both forward and reverse orientations to create a consensus sequence for each clone.

Bioinformatic and phylogenetic analysis of isotype-1 β-tubulin haplotypes

A ClustalW alignment of 120 sequences (20 from 6 time points as listed in Table 1) was created in Geneious R8 8.0.2 (Biomatters Ltd., Auckland, New Zealand). A split network (Huson and Bryant, 2006) was generated based on genetic distance using the NeighborNet method (Bryant and Moulton, 2004). Branches with bootstrap support thresholds above 70% were displayed.

Results

Larval development assay

Prior to replacement, LDA data indicated that the resident population of *H. contortus* at location 1 was highly resistant to both benzimidazole and macrocyclic lactone drugs and susceptible to levamisole; resistance ratios using EC_{50} were 491.00, 0.76, 54.60 for thiabendazole, levamisole, and ivermectin aglycone, respectively (Table 5.2).

EC_{50} values for thiabendazole indicated that replacement was successful; the post-replacement value was very similar to the susceptible isolate used for replacement (Table 5.2). However, by 1.5 years post-replacement the population reverted to a very high level of resistance that was maintained throughout the study.

According to the EC_{50}, the population was susceptible to levamisole prior to replacement (location1). However, a subpopulation of *H. contortus* that represented 32% of the population prior to replacement was resistant to levamisole as indicated by the

EC_{95} resistance ratio of 1.40 (Table 5.2). Following replacement, EC_{95} was reduced by greater than 50% as compared to pre-replacement levels and similar to the susceptible isolate, indicating successful replacement of the majority of the resistant subpopulation. Yet, 1.5 years following replacement the overall population was resistant to levamisole and there was a highly resistant subpopulation representing 24% of the total population, despite there being no treatments with levamisole administered other than those used in performance of the FECRTs.

For ivermectin aglycone, EC_{50} and EC_{95} values were greatly reduced following replacement, showing a reduction in resistance ratio of more than 15 and 100 times for the EC_{50} and EC_{95}, respectively. However, these values were still two to three times higher than the susceptible isolate used for replacement (Table 5.2). These differences from the susceptible isolate are rather small, and the values are still well within the susceptible range established for avermectin drugs in this assay (Crook et al., 2016). The most likely explanation is a combination of inter-assay variability and the fact that replacement was not absolute. Similar to other drugs, by 1.5 years following replacement, LDA indicated a high level of resistance to the macrocyclic lactone class. Additionally, there was a highly resistant subpopulation that continued to increase in level of resistance throughout the 3.5 years following replacement. These changes occurred despite there being no treatments with avermectin/milbemycin drugs administered other than those used in performance of the FECRTs.

Fecal egg count reduction test

Treatment with albendazole administered seven weeks following replacement reduced FECs by 98.7% (Table 5.3), indicating that replacement was successful, which

was consistent with results of the LDA. However, by the time efficacy of albendazole was tested again 1.5 years later, the population was highly resistant demonstrating a FECR of only 52.5%. This change in FECR occurred despite the sheep only being treated selectively on two occasions with albendazole, with less than 30% of the sheep receiving a treatment. Furthermore, the sheep were not treated with an avermectin/milbemycin during those 1.5 years, however, FECR indicated substantial resistance to both ivermectin and moxidectin which was again consistent with results of the LDA. In contrast, the population initially remained susceptible to levamisole. Interestingly, one year later (two and a half years following replacement), results of the FECRT indicated resistance to levamisole, as well as to moxidectin and a combination of both levamisole and moxidectin. Resistance to these same three treatments and a combination of albendazole, moxidectin, and levamisole was present 3.5 years following replacement (Table 5.3).

Allele frequency of β-tubulin gene single nucleotide polymorphisms

Prior to replacement, 100.00% and 23.71% of the population of *H. contortus* contained the F200Y (T**T**C>T**A**C) and F167Y (T**T**C>T**A**C) resistant polymorphisms in the isotype 1 *β*-tubulin gene, respectively (Table 5.4). The susceptible isolate (UGA-SUSC) was comprised almost entirely of the susceptible genotype at both the position 200 and 167 loci (Table 5.4). The frequency of resistant genotypes was reduced following replacement to 1.67% for the F200Y mutation and 0.00% for the F167Y mutation. Over the following months, the frequency of the F200Y resistant polymorphism increased steadily to 18.09% and 33.40% at 2.5- and 4-months following replacement, respectively. Fourteen months following replacement the F167Y mutation increased in frequency to 4.15% which then remained at a similar level throughout the

duration of the study. However, the F200Y mutation continued to increase in frequency to near fixation by 2.5 years following replacement.

Microsatellite genotyping

A panel of 9 neutral, polymorphic microsatellite loci were used to describe the genetic relationships between the samples over the course of the study. All of the microsatellites were polymorphic with the total number of alleles per marker varying between two (Hcms40) and fourteen (Hcms25). The principle coordinate analysis was highly informative as it explained 88.33% of variability in the data, 76.32% of variability explained by the x-axis and 12.01% of variability explained by the y-axis (Figure 5.1). The resistant population present prior to replacement was the most genetically distant from the susceptible laboratory isolate. For all nine markers, the allele frequencies were similar for the susceptible laboratory isolate and the immediate post-replacement population (1.5 months post-replacement), indicating that these were genetically similar and that replacement was successful. However, over time the population became increasingly similar to the resistant population present prior to replacement. This is visually displayed by a shift to the right of the data points in the principle coordinate analysis over time following replacement (Figure 5.1).

Frequency of isotype-1 β-tubulin haplotypes

Twenty-three haplotypes of the isotype-1 β-tubulin gene were identified (Figure 5.2, Appendix D). Thirteen of the 23 haplotypes contained the susceptible T**T**C genotype at the position 200 and 167 loci and 10 haplotypes contained the resistant T**A**C genotype at the position 200 and/or 167 loci. Overall, following replacement, the number of haplotypes present initially increased and then decreased.

The susceptible isolate was comprised of 5 haplotypes, all of which had the T**T**C genotype indicating that all the haplotypes were susceptible to the benzimidazoles. The most common haplotypes in the susceptible isolate were 8 and 9 which were present at 55% and 20%, respectively. The initial replacement population (1.5 months post-replacement), had a haplotype pattern that was very similar to the susceptible laboratory isolate with haplotypes 8 and 9 again being the most common and present at 36.8% and 31.6%, respectively. However, one new haplotype was identified that was not present in the susceptible isolate, and one other haplotype that was initially identified in the susceptible isolate that then disappeared. These two haplotypes were identified at very low frequencies in each respective population.

Six haplotypes were present in the initial resistant population (location 1), and all of these carried the resistant T**A**C genotype at the position 200 and/or 167 loci. Two of these haplotypes, 14 and 18 were most common, present at 47.8% and 30.4%, respectively.

In the replaced population, the percentage of resistant haplotypes increased from 0.00% at 1.5 months following replacement to 32.0% at 4 months and 65.1% at 1.5 years following replacement, with haplotype 14 being present at 0.0%, 24.0%, and 47.8% at these time points, respectively.

At the final time point where haplotypes were measured (2.5 years post-replacement), the population was comprised of 4 resistant haplotypes. Eighty-five percent of the sample was comprised of a single resistant haplotype (14), which was also the most common haplotype in the initial resistant population. Haplotypes 8 and 9, the most common susceptible haplotypes in the pre-replacement susceptible laboratory isolate

(UGA-SUSC), decreased continuously over the four month and 1.5 year time points, and were completely absent 2.5 years after replacement.

Relatedness of isotype-1 β-tubulin haplotypes

A split network diagram was used to describe the relatedness of the 9 haplotypes that were observed at more than one time point throughout the sampling period (Figure 5.3). To provide a visual display of changes in the frequency of each haplotype over time, the percentage of the population comprised of each haplotype at the 6 time points tested are displayed in bar graphs (Figure 5.3).

The nine haplotypes segregated into three clusters (Figure 5.3). Haplotypes 8 and 9, which both contain the susceptible T**T**C genotype at the position 200 and 167 loci, were phylogenetically very similar and were present in highest frequencies in the susceptible isolate and immediately following replacement. However, the other two clusters in Figure 5.3 contain both susceptible and resistant genotypes.

Although resistant haplotype 18 which contains both the F167Y and F200Y resistance associate polymorphisms was the second most common haplotype prior to replacement (location 1), haplotype 18 decreased in frequency from 1.5 to 2.5 years following replacement. The two haplotypes which occurred at multiple time points in the study and contained the F167Y mutation, haplotypes 18 and 19, were present prior to replacement but decreased over time following replacement. However, the only resistant haplotype (F200Y) that occurred at multiple time points in the study and did not contain the F167Y mutation, haplotype 14, increased in frequency towards fixation following replacement.

Discussion and conclusions

The present study evaluated changes in drug susceptibility and population genetic structure following replacement of a multiple-anthelmintic resistant population of *H. contortus* with a susceptible laboratory isolate on a sheep farm. While the strategy of replacement has previously been tested as a methodology to replace resistant alleles with susceptible alleles in gastrointestinal nematodes of sheep (van Wyk and Van Schalkwyk, 1990; Bird et al., 2001; Aumont, 2002; Sissay et al., 2006; Moussavou-Boussougou et al., 2007; Muchiut et al., 2018), this is the first time this strategy has been evaluated for a period greater than 18 months following replacement. Additionally, this study is the first to use both population genetics analyses and measurement of molecular markers for resistance following replacement. Combining these data with *in vivo* and *in vitro* phenotypic data enabled us to not only precisely measure the changes in drug susceptibility over time, but also explain these changes at the population genetic level.

We show using multiple phenotypic and genetic measures that the initial resistant population was successfully replaced with a susceptible laboratory isolate. Resistance ratios based on LDA EC_{50} were reduced from 491.00 to 1.18 for thiabendazole and from 54.60 to 2.96 for ivermectin aglycone (Table 5.2). Treatment with albendazole reduced FEC by 98.7% (Table 5.3) and the frequency of the F200Y SNP associated with benzimidazole resistance was reduced from 100.00% to 1.67% (Table 5.4). Aumont (2002), Bird et al. (2001), and Sissay et al. (2006) also reported reversion to susceptibility based on FECRT results, however egg reduction was the only measurement used, which is rather insensitive (Martin et al., 1989), and follow-up was conducted for only a short period of time following replacement. Using controlled efficacy tests, van Wyk and Van

Schalkwyk (1990) reported reversion to susceptibility in three out of five test paddocks following introduction of a susceptible isolate. Using both controlled efficacy tests and genetic markers of benzimidazole resistance, Moussavou-Boussougou et al. (2007) also reported reversion to susceptibility, however follow-up was only conducted 4 months following replacement.

It is important to appreciate that replacement is relative, not absolute, and is based on the principle of dilution. We were unable to completely remove the initial resistant population with the treatment regimen used; this result should be expected most often as replacing a highly multiple-anthelmintic resistant population is challenging. Despite reducing the mean initial FEC by greater than 99%, sheep still had a mean FEC of 18 eggs per gram following two rounds of treatment. Moussavou-Boussougou et al. (2007) reported approximately 20% of benzimidazole-resistance alleles in the post-replacement population which is more than 10 times the level detected in the present study. These data indicate that our protocol was much more successful in reducing and diluting the benzimidazole-resistant alleles in the population. It is likely that most of the few remaining nematodes shedding these eggs were highly resistant to benzimidazoles, and possibly also to levamisole, the two drugs used for treatment. Consequently, despite replacing the majority of the resistant *H. contortus* population, the sheep remained infected with a small number of highly-resistant worms.

After confirming that we had successfully replaced the resistant population, we treated only with albendazole, and treatments were applied infrequently, and selectively using FAMACHA (van Wyk and Bath, 2002). Thus, we expected resistance to benzimidazoles to develop slowly over time, and we aimed to monitor the dynamics of

changes in susceptibility by making both phenotypic and genotypic measurements. However, surprisingly, when we retested the population using FECRT 1.5 years post-replacement, we found that there was reversion to resistance not only to albendazole, but also to ivermectin, and moxidectin (Tables 5.2, 5.3, and 5.4). We know from previous experiments that the initial multi-drug resistant population on the sheep farm (location 1) was highly resistant to both ivermectin and moxidectin. Given this prior knowledge, and the new data, it is highly likely that the 'resistant survivors' carried from location 1 were highly resistant to both ivermectin and moxidectin, since reversion to resistance occurred despite no treatments being administered with these drugs. We hypothesized that the small population of 'resistant survivors' rapidly increased in relative frequency following the move to location 2 for reasons unrelated to treatment, leading to the rapid reversion to resistance of the overall population. This led us to examine the population genetics in greater detail in order to gain deeper insights into why this rapid reversion occurred.

The primary resistant isotype-1 β-tubulin haplotype prior to replacement (haplotype 14) was also the most common resistant haplotype following development of resistance at time points of 4 months, 1.5 years, and 2.5 years (Appendix D). These results provide strong genetic evidence the initial 'resistant survivors' increased in frequency over time following replacement. The big question is why this occurred when the population was under such low drug-selection pressure.

Though it is not possible to prove without performing a new large and detailed study, it seems very likely that field-fitness of UGA-SUSC, which was a laboratory isolate, was impaired, and did not compete well with the resistant sub-population. This reduced level of fitness in UGA-SUSC may be associated with the methodology used to

cycle laboratory isolates. UGA-SUSC was cryopreserved for many years prior to being passaged through lambs or kids. We received the isolate after passage had been performed for several generations, and we do not have information on the number of L3 that were used to establish the infection in the initial passage. Subsequent passages at the University of Georgia were made with only a few thousand L3 that developed in coproculture under highly controlled laboratory conditions. Under these conditions, each passage creates a genetic bottleneck and can further reduce the diversity of the isolate. This contrasts greatly to a field situation where L3 must develop in a sometimes hostile and changing environment, thousands of worms are infecting numerous animals in a pasture environment, tens to hundreds of millions of eggs are shed onto the pasture each day, and transmission with new parasites is constantly occurring in a dynamic fashion. Thus, one would expect field isolates to be superior in fitness and diversity under natural environmental conditions as compared to laboratory isolates. Specifically, the resistant population remaining prior to replacement evolved under natural environmental conditions in Athens, Georgia, and thus would be expected to have much greater environmental fitness than a lab population that was maintained under highly controlled and optimal conditions.

Five haplotypes of the isotype 1 β-tubulin gene were identified in the susceptible laboratory isolate (UGA-SUSC) and six haplotypes were identified in the population prior to replacement. Redman et al. (2015) surveyed 7 seven sheep farms in the UK with *H. contortus* and reported between one to eight haplotypes in this gene per farm. Thus, the number of haplotypes identified in UGA-SUSC and prior to replacement are consistent with those previously reported in other *H. contortus* field populations. As

would be expected, the number of haplotypes identified in the isotype 1 β-tubulin gene was highest at 4 months and 1.5 years following replacement, as 'resistant survivors' and UGA-SUSC worms mated and reproduced under minimal drug selection pressure. However, the relatedness of these haplotypes is also important to describe the diversity in the isotype 1 β-tubulin gene throughout the course of replacement. Haplotypes 8 and 9 were present at 55.0% and 20.0%, respectively, in UGA-SUSC and clustered together in the network analysis (Figure 5.3), suggesting these haplotypes were closely related and the laboratory isolate lacked diversity. However, the 'resistant survivors' contained haplotypes 14 and 18 at 47.8% and 30.4%, respectively. Haplotypes 14 and 18 were more distantly related on the network analysis (Figure 5.3) suggesting that this field population was more diverse.

Interestingly, resistant haplotypes 18 and 19 that both contain the F167Y mutation decreased over time following replacement, however, resistant haplotype 14 that did not contain the F167Y mutation, increased in frequency towards fixation following replacement. These results suggest that these haplotypes varied in levels of fitness and may suggest that haplotypes that contain the F167Y mutation are less fit than those that do not contain the mutation. The F200Y resistance polymorphism is more common than the F167Y mutation in many populations and countries (Chaudhry et al., 2015).

It is quite likely that single nucleotide polymorphisms that occurred only once in the entire data set of 120 sequences of the isotype 1 β-tubulin gene are artifacts due to polymerase induced errors, while single nucleotide polymorphisms appearing more than once in the data set are likely to be real polymorphisms (Redman et al., 2015). Thus, bioinformatic filtering of the isotype-1 β-tubulin sequences to remove these polymerase

chain reaction mutations from the dataset is required to allow for more accurate interpretation of these data.

In the present study, successful replacement of a resistant population of *H. contortus* with a susceptible population did not achieve long-term success. However, we feel that a flaw in our methodology, rather than a flawed strategy was responsible. In the course of our genetic analyses performed following completion of the field phase of the study, we demonstrated that UGA-SUSC lacked genetic diversity. Additionally, we did not 'field-adapt' the isolate prior to using it to infect the sheep. Given our results, it seems quite plausible that UGA-SUSC was "unfit" for survival in the natural world, and was not able to adequately survive and reproduce in the environmental conditions in Athens, Georgia. We believe that parasite replacement remains a viable strategy, but that great care is needed in the methodology used. If we were to attempt parasite replacement again, we would first field-adapt the susceptible parasite population over a lengthy period so the parasites would undergo natural environmental selection over several life-cycles of infection, and be required to survive and reproduce under natural field conditions of the local climate. In conclusion, the present study demonstrates the experimental power of combining molecular, *in vitro*, and *in vivo* assays to describe phenotypic and genotypic changes in field populations of nematodes. Only by understanding the changes occurring on the population genetic level, can we truly understand the impact that our control strategies have on parasite populations.

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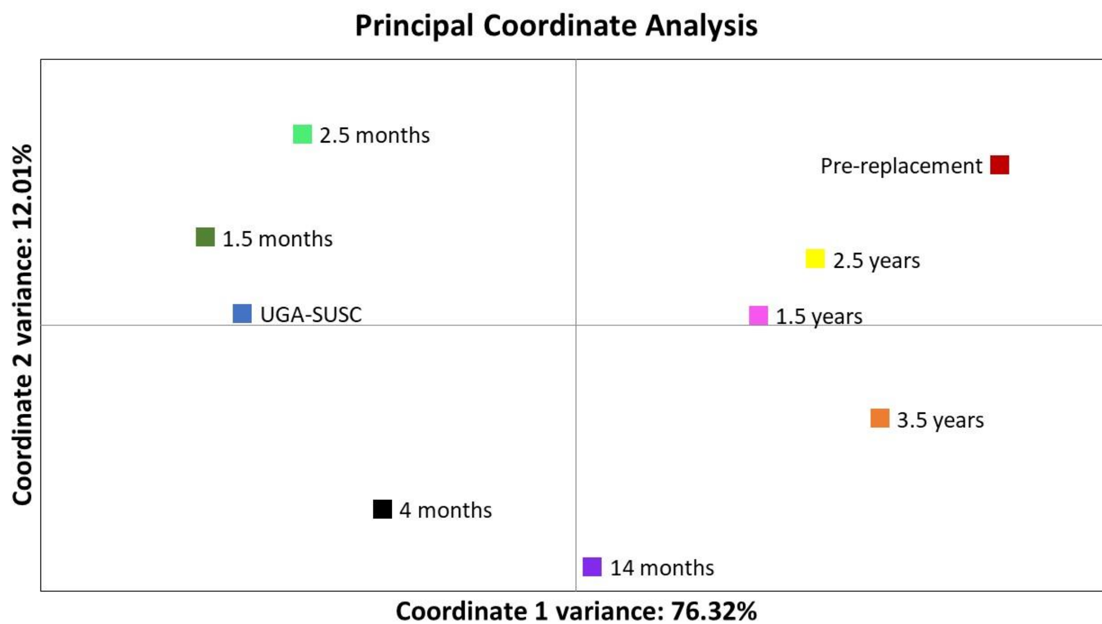

Figure 5.1. Principle coordinate analysis of microsatellite genotypes. Principle coordinate analysis was performed using GenAlEx 6.501 (Peakall and Smouse, 2006, 2012). A panel of 9 neutral polymorphic microsatellite loci were used to genotype duplicate pools of 100 third-stage larvae at 9 time points throughout the study. Time post-replacement is indicated next to each data point. Peak heights were used as an approximation of allele frequency. The percentage of variation explained by the first two coordinates is shown on the X and Y axes of the graph.

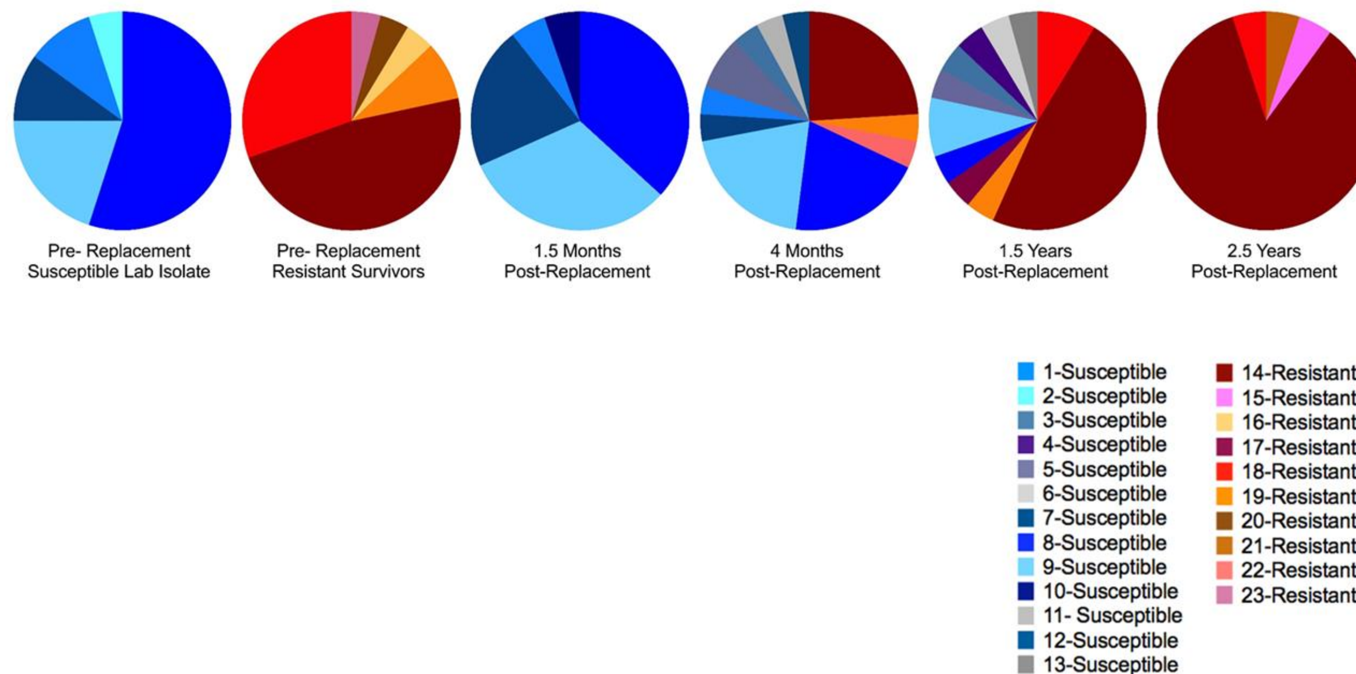

Figure 5.2. Relative frequency of isotype-1 β-tubulin haplotypes. At 6 time points throughout the study, a region of the isotype-1 β-tubulin gene was PCR amplified from a pool of 100 third-stage larvae, cloned, and 20 clones were sequenced per time point. The relative haplotype frequency at each time point is demonstrated in a pie chart. Haplotypes can be tracked over time as haplotypes are labeled by color. Blue/gray haplotypes are susceptible at both P167 and P200. Red/brown haplotypes are resistant at P167 and/or P200.

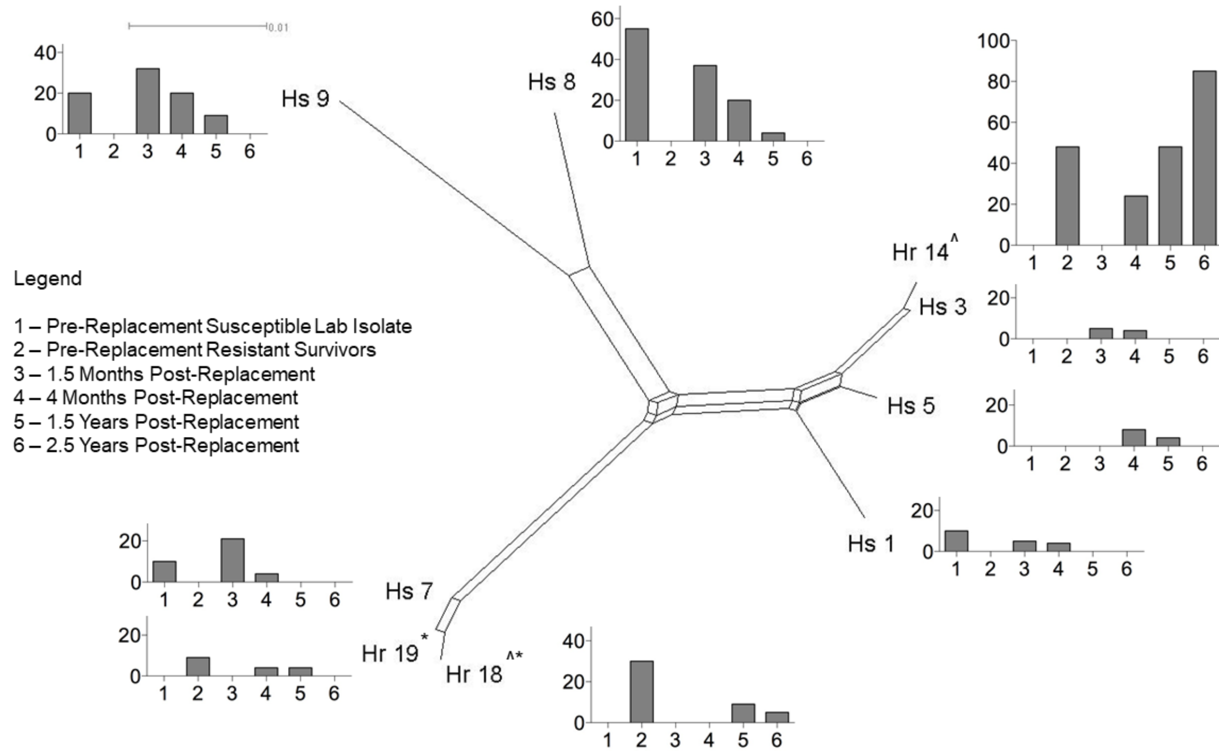

Figure 5.3. Network analysis of sequences of isotype-1 β-tubulin sequences. A split network was generated with the neighbor-net method of SplitsTree4 to display the relationships between 9 haplotypes that were observed more than once throughout the study over 6 time points. For each haplotype, haplotype frequency (%) per time point is shown on the y axis and time point is shown on the x axis. The six time points can be tracked across the tree as time points are labeled by number. * indicates the F167Y mutation was present. ^ indicates the F200Y mutation was present.

Table 5.1. Timeline of experiment with dates of fecal collections (FECAL), fecal egg count reduction tests (FECRT), larval development assays (LDA), microsatellite genotyping (MSAT), pyrosequence genotyping (PYRO), and sequencing of isotype-1 β-tubulin haplotypes (HAPLO).

Time	Description	FECAL	FECRT	LDA	MSAT	PYRO	HAPLO
Spring 2011	Clinical haemonchosis in flock of sheep						
3 August 2011	Move flock from contaminated pasture to concrete Drench sheep with levamisole[a] and albendazole[b]	√	√	√	√	√	√
4 August 2011	Drench sheep with levamisole[a] and albendazole[c]						
5 August 2011	Drench sheep with levamisole[a] and albendazole[c]						
12 September 2011	Drench sheep with levamisole[a] and albendazole[b]						
13 September 2011	Drench sheep with levamisole[a] and albendazole[b]						
14 September 2011	Drench sheep with levamisole[a] and albendazole[c]						
21 September 2011	Fecal collection for FECRT	√					
23 September 2011	Infect each sheep with 5,000 susceptible L3			√	√	√	√
27 September 2011	Move sheep to clean pasture						
25 October 2011	Targeted selective treatment (14 of 42 sheep) with albendazole[c] using FAMACHA©						
10 November 2011	1.5 months post-replacement	√	√	√	√	√	√
13 December 2011	2.5 months post-replacement	√			√	√	
1 February 2012	4 months post-replacement	√			√	√	√
3 May 2012	Targeted selective treatment (10 of 44 sheep) with albendazole[c] using FAMACHA©	√					
12 July 2012	Targeted selective treatment (27 of 46 sheep) with albendazole[c] using FAMACHA©	√					
4 December 2012	14 months post-replacement	√		√	√	√	√
2 April 2013	1.5 years post-replacement	√	√	√	√	√	
14 May 2014	2.5 years post-replacement	√	√	√	√	√	√
5 May 2015	3.5 years post-replacement	√	√	√	√	√	

[a] 8 mg/kg levamisole
[b] 15 mg/kg albendazole
[c] 7.5 mg/kg albendazole

Table 5.2. Larval development assay results

Time post-replacement	Thiabendazole			Levamisole					Ivermectin aglycone				
	EC_{50} (μM)	Resistance ratio[a]	R^2	EC_{50} (μM)	Resistance ratio[a]	EC_{95} (μM)	Resistance ratio[b]	R^2	EC_{50} (nM)	Resistance ratio[a]	EC_{95} (μM)	Resistance ratio[b]	R^2
UGA-SUSC	0.03	-	0.92	0.93	-	3.87	-	0.96	0.90	-	2.77	-	0.89
Pre-replacement	12.28	491.00	0.86	0.71	0.76	5.40	1.40	0.90	49.10	54.60	645.43	233.01	0.94
1.5 months	0.03	1.18	1.00	0.94	1.01	2.14	0.55	0.97	2.66	2.96	6.09	2.20	0.98
1.5 years	54.91	2,195.52	0.78	1.89	2.02	6.57	1.70	0.96	18.44	20.50	88.45	31.93	0.99
2.5 years	28.61	1,143.94	0.78	2.73	2.92	38.53	9.96	0.93	26.12	29.04	108.03	39.00	0.97
3.5 years	17.85	713.71	0.85	2.87	3.07	14.69	3.80	0.82	15.99	17.78	488.51	176.36	0.93

[a] Resistance ratio was calculated as EC_{50} of tested isolate divided by EC_{50} of UGA-SUSC.

[b] Resistance ratio was calculated as EC_{95} of tested isolate divided by EC_{95} of UGA-SUSC.

Table 5.3. Mean percent fecal egg count reduction and associated 95% confidence intervals[a,b]

Time post-replacement[c]	Albendazole	Ivermectin	Levamisole	Moxidectin	Moxidectin + Levamisole	Albendazole + Moxidectin + Levamisole
1.5 months	98.7 (97.0, 99.4)					
1.5 years	52.5 (33.1, 77.7)	55.1 (36.8, 78.5)	98.8 (96.7, 99.8)	60.7 (48.8, 70.3)		
2.5 years	19.3 (10.0, 39.1)		31.4 (14.8, 52.6)	28.6 (13.3, 50.2)	42.7 (16.4, 67.5)	
3.5 years			23.2 (12.8, 42.9)	22.1 (10.3, 40.6)	62.1 (36.9, 84.8)	67.5 (49.1, 80.4)

[a] Fecal egg count reduction tests were performed using a modified-McMaster technique using 25 or 8 EPG detection.

[b] Mean fecal egg count reduction and 95% confidence interval were calculated using the eggCounts package for individual efficacy.

[c] All drugs and drug combinations were not tested at each time point.

Table 5.4. Allele frequency of single nucleotide polymorphisms in the isotype-1 β-tubulin gene associated with benzimidazole resistance

Time post-replacement	Percent resistant genotype at position 200 (%TAC)	Percent resistant genotype at position 167 (%TAC)
UGA-SUSC	0.28	0.00
Pre-replacement	100.00	23.71
1.5 months	1.67	0.00
2.5 months	18.09	0.00
4 months	33.40	0.00
14 months	69.68	4.15
1.5 years	84.06	5.85
2.5 years	95.19	4.52
3.5 years	96.03	4.71

CHAPTER 6

CONCLUSIONS

This body of work contributed valuable new techniques and insights to improve the diagnosis and control of anthelmintic resistance in gastrointestinal nematodes of livestock. A practical and cost-efficient diagnostic test for anthelmintic resistance in gastrointestinal nematodes of cattle was developed and validated, presenting a major advance in diagnostic testing for resistance. The phenotype of motility was critically evaluated and determined to be inappropriate for detection of resistance to avermectin/milbemycins in third-stage larvae of gastrointestinal nematodes of livestock, representing a significant addition to our knowledge of how the avermectin/milbemycins affect third-stage larvae. Finally, we combined *in vivo, in vitro*, and molecular methodologies to describe changes in a field population of *H. contortus* which yielded a plethora of knowledge regarding how these techniques can be combined to study field populations and better understand the results of future experiments. The outcomes of each specific aim are discussed in more detail below.

Specific aim 1: The results of this study provide strong evidence that composite sampling of fecal samples in beef calves is a scientifically valid approach for evaluating anthelmintic efficacy and detecting resistance. FECRT were performed on 14 groups of cattle using both individual and composite FEC methods. To measure how well the results of composite sampling reproduce those of individual sampling, Lin's Concordance Correlation Coefficient was utilized to describe both the linear relationship

between methods and the slope and y-intercept of the line relating the data sets. There was little difference between the approaches with a concordance correlation of 0.99 in mean FEC found between methods. Mean FEC based on individual counts ranged between 0 and 670.6 eggs per gram of feces, indicating that the results of this study are applicable to a wide range of FEC levels. Standard error of the mean FEC and range of FEC were reported for each group prior to and following treatment to describe the variability of the data set. Concordance correlation was greater than 0.95 for drug efficacy between individual and composite sampling methods, demonstrating composite sampling is appropriate to evaluate drug efficacy.

Notably, for all groups tested the efficacy calculated by composite sampling was within the 95% confidence interval for efficacy calculated using individual sampling. The use of composite samples was shown to reduce the number of FEC required by 79%. These data demonstrate that pooling fecal samples from a group of cattle and then performing repeated FEC on that composite sample yields results very similar to performing individual FEC on those same animals, while substantially reducing the cost of performing a FECRT as compared to individual fecal samples. Furthermore, we have developed suggested methods for using composite samples in a FECRT, provided a cost comparison for this methodology, and described potential issues associated with the use of composite samples that must be considered.

Further work should be completed to determine the proposed requirement of counting 140 eggs under the microscope prior to treatment. Mathematical modelling of populations with various resistance levels and distributions of fecal egg counts must be conducted to determine if this threshold of 140 eggs is sufficient or excessive. Much time

can be wasted by conducting too many fecal egg counts, however, an erroneous result may be the alternative problem if too few fecal egg counts are completed. This work will require the collaboration between parasitologists and statisticians who are familiar with over-dispersed non-parametric data such as fecal egg counts.

A comparison of groups of various numbers of animals to determine if composite sampling is appropriate for groups ranging from 2-50 animals would be highly beneficial. It is possible that composite sampling may not be appropriate for small numbers of animals and thus this remains to be tested.

Most importantly, future work should focus on outreach and extension of this methodology to parasitology labs throughout the world, veterinarians, and livestock producers. Composite sampling in cattle has been well-validated. However, an increase in diagnostic testing for anthelmintic resistance among livestock producers will remain unrealized if the findings of this study are not extended to stakeholders.

Specific aim 2: Our results clearly demonstrate that motility of third-stage larvae of gastrointestinal nematodes of livestock is not an appropriate *in vitro* phenotype for detection of resistance to the avermectin/milbemycin class of anthelmintics. Using the Worminator, we compared the dose-response characteristics of several avermectin/milbemycin compounds using L3 from both avermectin/milbemycin-susceptible and resistant *Cooperia* spp. (abamectin, doramectin, eprinomectin, ivermectin, moxidectin) and *H. contortus* (eprinomectin, ivermectin, moxidectin). Concentrations tested with the Worminator ranged from 0.156 to 40 μM. Differences in EC_{50} between avermectin/milbemycin-susceptible and resistant isolates of *Cooperia* spp. and *H. contortus* were small, with resistance ratios ranging from 1.00 to 1.34 for

Cooperia spp., 0.99 to 1.65 for *H. contortus*. Larval migration inhibition assays were conducted using the same isolates and were equally ineffective for detection of resistance with resistance ratios less than 2.0. These results contrast with those of the Larval Development Assay where we obtained a resistance ratio of 16.48 using the same isolates of *H. contortus*. Moreover, even at the highest concentration tested (40 μM), 100% inhibition of motility was never achieved and EC_{50} for Worminator assays were more than 100× higher than peak plasma levels achieved *in vivo* following treatment. These data demonstrate that dose-response characteristics for inhibition of motility in L3 of gastrointestinal nematodes of livestock do not significantly differ for avermectin/milbemycin-susceptible and resistant isolates. These data challenge the suitability of motility as a phenotype for detecting and measuring resistance to avermectin/milbemycin drugs in gastrointestinal nematodes of livestock.

Future research efforts should evaluate alternative life stages including the L4, which may be more appropriate for detection of avermectin/milbemycin resistance. Specifically, the L4 as a feeding parasitic stage may more accurately reflect the adult in vivo drug-parasite interaction and therefore express a more similar resistance profile. This will require substantial investment as culture L3 to L4 *in vitro* is a complex process which requires technical expertise and validated protocols. Fourth-stage larvae are vulnerable outside of the host and require specific conditions for survival *in vitro*. Until appropriate and validated molecular diagnostic markers are available for detecting resistance to avermectin/milbemycin drugs, efforts should continue to focus on the development and optimization of *in vitro* assays for detection of anthelmintic resistance.

Specific aim 3: A population of *H. contortus* that was highly resistant to benzimidazoles and avermectin/milbemycins and, which contained a subpopulation that was resistant to levamisole was replaced with a susceptible laboratory isolate of *H. contortus* in a flock of sheep. In the immediate post-replacement period, successful replacement of the resistant population with a susceptible population was confirmed using phenotypic and genotypic measurements; larval development assay EC_{50} indicated full anthelmintic susceptibility; albendazole treatment yielded 98.7% fecal egg count reduction; pyrosequence genotyping of single nucleotide polymorphisms in positions 167 and 200 of the isotype-1 beta tubulin gene were present at 0.00 and 1.67%, respectively; microsatellite genotyping of the replaced population indicated the background haplotype was similar to the susceptible laboratory isolate; and haplotypes of the isotype-1 beta tubulin gene were similar to the susceptible laboratory isolate. In an effort to sustain the anthelmintic susceptibility of the new worm population, targeted selective treatment was implemented using albendazole. Surprisingly, within 1.5 years post-replacement, the population had reverted to a resistant phenotype. Resistance to albendazole, ivermectin, and moxidectin was confirmed via FECRT, larval development assay, and pyrosequence genotyping. Targeted selective treatment was then carried out using levamisole. However, within one year, resistance was detected to levamisole. Population genetic analysis using a panel of 9 neutral polymorphic microsatellite markers demonstrated there was a gradual change in the genetic structure of the population until the final population was similar to the initial resistant population. This work demonstrates the power of combining molecular, *in vitro*, and *in vivo* assays to study phenotypic and genotypic changes in a field population of nematodes, enabling improved insights into the epidemiology of anthelmintic resistance.

Although replacement of resistant alleles with susceptible alleles was not an effective long-term strategy for control of a multiple-anthelmintic resistant population of *H. contortus*, alternatives in methodology of replacement may be more effective long-term. The susceptible isolate (UGA-SUSC) was maintained in the laboratory for many years and never exposed to avermectin/milbemycin drugs. This isolate lacked diversity and the fitness of this isolate under field conditions was not tested prior to replacement.

For future experiments aimed to introduce laboratory isolates to the field, adapting these isolates to the field over time prior to the start of the experiment is recommended. It is possible that UGA-SUSC was not able to survive and reproduce in the environmental conditions in Athens, Georgia. The use of seeder lambs to lace paddocks with the isolate of interest may be more effective to adapt an isolate to field conditions. Moussavou-Boussougou et al. (2007) used seeder lambs to introduce an isolate of *Teladorsagia circumcincta.* Subsequently, tracer lambs could be used to determine the infectivity of the paddock prior to introduction of the experimental flock which may provide an indication of the ability of the laboratory isolate to reproduce under field conditions in the environment tested. Seeder lambs should lace the paddock with the isolate of interest, but experimental sheep should also be infected with the isolate of interest. It is important to increase the ratio of susceptible parasites to resistant parasites. The use of both seeder lambs and experimental sheep to introduce susceptible parasites will contribute to the total number of susceptible parasites introduced.

A highly effective anthelmintic should be used to reduce the resident population of resistant parasites prior to replacement. This will further increase the ratio of susceptible to resistant parasites. Unfortunately, the initial resistant population contained

a sub-population that were resistant to levamisole and the authors had no alternative approved anthelmintic option to remove these parasites. In an ideal setting, a fully effective anthelmintic that was not previously used in the population should be used to remove as many resistant nematodes as possible.

Moussavou-Boussougou et al. (2007) suggested that treatment with benzimidazoles in a population of *Teladorsagia circumcincta* that was resistant to benzimidazoles prior to replacement should be avoided for a minimum of two years following substitution. This period of time should allow for genetic drift and for the nematode population to shed any remaining resistant alleles. It is important to minimise the use of anthelmintics for as long as possible following replacement. Non-anthelmintic strategies for parasite control (Leathwick and Besier, 2014; Muchiut et al., 2018; van Wyk et al., 2006) to avoid the necessity of anthelmintic treatment for a minimum of 1 year following replacement should be implemented. Unfortunately in the present study, a subset of lambs (n=14) required treatment 4 weeks following introduction to the new location. The use of copper oxide wire particles or tannin-containing plants such as *Sericea lespedeza* for treatment may have reduced the drug selection pressure placed on the population.

In summary, this dissertation yields valuable and practical approaches to the diagnosis and control of anthelmintic resistance in gastrointestinal nematodes of livestock. These developments provide solutions for livestock producers to test for anthemintic resistance on their farms. The findings of this dissertation clarified much confusion in the field of *in vitro* assays for detection of resistance to avermectin/milbemycins by clearly demonstrating that motility of third-stage larvae is a

poor phenotype for detection of resistance to avermectin/milbemycins. Finally, we developed and tested an intervention strategy for multiple-anthelmintic resistance in a flock of sheep and used a range of powerful methodologies to describe the biology and population genetics throughout the study. This is the first time these methods have been combined to study an intervention strategy for multiple-drug resistance in a field population of gastrointestinal nematodes and sets the framework for future field studies to employ these valuable tools to describe changes in population genetics and anthelmintic resistance.

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APPENDICES

A. R Code for calculation of percent fecal egg count reduction and associated 95% confidence interval according to gamma method

The code for calculation of percent fecal egg count reduction and associated 95% confidence interval according to the gamma method (Levecke et al., 2015) is presented below with commentary included after #.

R Code

```
data = read.csv ("data.csv", header = T, as.is = T) #data sheet named data.csv
attach (data) #attach csv file named data.csv
1-mean(Post)/mean(Pre) #calculation of % FECR
ERR <- 1-mean(Post)/mean(Pre)
term1 <- (mean(Post)/mean(Pre))**2
term2 <- var(Post)/mean(Post)**2
term3 <- var(Pre)/mean(Pre)**2
term4 <- - 2*cor(Pre,Post)*sqrt(var(Post))*sqrt(var(Pre))/(mean(Pre)*mean(Post))
VAR <-  term1*(term2+term3+term4)
var <- VAR / length(Pre)
a <- ((1-ERR)**2)/var
b <- var/(1-ERR)
1-c(qgamma(0.975,shape = a, scale = b), qgamma(0.025,shape = a, scale = b))
#calculation of 95% confidence interval of %FECR according to gamma method
```

B. Third-stage larvae in grass samples from four paddocks

	Number of third-stage larvae per kg of dry matter		
Paddock	*Haemonchus* spp.	*Trichostrongylus* spp.	*Oesophagostomum* spp.
1	0	0	0
2	66	6	0
3	1723	0	8
4	291	0	0

C. Microsatellites for population genetics analysis

Microsatellite	Type	Allele size range	Primer sequences (5' → 3')	Tag	Source
Hcms22co3	8mer	210-250	F: GAGCTTCATTGAGAGAATGGAATT R: GTTTCTTAGGTCCTCATATACGATCAACTAA	NED	Redman et al., 2008
Hcms25	Dinucleotide	186-228	F: ACAGGAGTTATGAATTTCCGG R: GTTTCTTGCTTCAGTTTGAATTGCTTCCC	FAM	Otsen et al., 2000
Hcms27	Dinucleotide	258-358	F: ACATAAATCTAGGTAGGGTAGG R: GTTTCTTACAGAAGAACGATCAGAATCTC	FAM	Otsen et al., 2000
Hcms36	Dinucleotide	150-154	F: GCATAGCGGCAAGGACGTATGG R: GTTTCTTCATGACGTACTCTGGTTGTTCG	HEX	Otsen et al., 2000
Hcms40	Dinucleotide	285-297	F: TCGATAGTTGTCACTTCCAA R: GTTTCTTTCGAATCCTGAGTCTACCGT	FAM	Otsen et al., 2000
Hcms8a20	Tetranucleotide	148-248	F: CAAACTTGACCCGACCTCTC R: GTTTCTTAGCGCGTTGCACAAAACATT	FAM	Redman et al., 2008
Hcms2884	8mer	82-98	F: TCGGCTGCTTTCATAGAC R: GGTATCGACCAAGATTCAG	HEX	Redman et al., 2015
Hcms3561	Trinucleotide	263-287	F: CCTACATGTCTCCCATATGTC R: TTAGCGAAGTAATAGCGTGCC	HEX	Redman et al., 2012
Hcms22193	6mer	147-222	F: ATCCACTTTCACTCCTATATCA R: GTGTGCGTGTATCTGTTG	HEX	Redman et al., 2015

D. Relative frequency of isotype-1 β-tubulin haplotypes[a]

			Time post-replacement			
Haplotype	UGA-SUSC	Pre-replacement	1.5 months	4 months	1.5 years	2.5 years
1	10.0	0.0	5.3	4.0	0.0	0.0
2	5.0	0.0	0.0	0.0	0.0	0.0
3	0.0	0.0	0.0	4.0	4.3	0.0
4	0.0	0.0	0.0	0.0	4.3	0.0
5	0.0	0.0	0.0	8.0	4.3	0.0
6	0.0	0.0	0.0	0.0	4.3	0.0
7	10.0	0.0	21.1	4.0	0.0	0.0
8	55.0	0.0	36.8	20.0	4.3	0.0
9	20.0	0.0	31.6	20.0	8.7	0.0
10	0.0	0.0	5.3	0.0	0.0	0.0
11	0.0	0.0	0.0	4.0	0.0	0.0
12	0.0	0.0	0.0	4.0	0.0	0.0
13	0.0	0.0	0.0	0.0	4.3	0.0
14	0.0	47.8	0.0	24.0	47.8	85.0
15	0.0	0.0	0.0	0.0	0.0	5.0
16	0.0	4.3	0.0	0.0	0.0	0.0
17	0.0	0.0	0.0	0.0	4.3	0.0
18	0.0	30.4	0.0	0.0	8.7	5.0
19	0.0	8.7	0.0	4.0	4.3	0.0
20	0.0	4.3	0.0	0.0	0.0	0.0
21	0.0	0.0	0.0	0.0	0.0	5.0
22	0.0	0.0	0.0	4.0	0.0	0.0
23	0.0	4.3	0.0	0.0	0.0	0.0

[a] Haplotypes 1-13 are susceptible at both P167 and P200. Haplotypes 14-23 are resistant at 167 and/or P200.