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Alternative Treatment Options for Controlling Anthelmintic-Resistant *Haemonchus contortus* Populations in South Dakota Sheep Herds

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ALTERNATIVE TREATMENT OPTIONS FOR CONTROLLING ANTHELMINTIC-RESISTANT *HAEMONCHUS CONTORTUS* POPULATIONS IN SOUTH DAKOTA SHEEP HERDS

BY

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This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy in Biological Sciences degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ABSTRACT

ALTERNATIVE TREATMENT OPTIONS FOR CONTROLLING ANTHELMINTIC-RESISTANT *HAEMONCHUS CONTORTUS* POPULATIONS IN SOUTH DAKOTA SHEEP HERDS

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Haemonchus contortus is a common economically important gastrointestinal nematode that obliges the survival and productivity of small ruminants, especially sheep and goats. Anthelmintic resistance is a primary challenge in ruminant health management programs in preventing and controlling the *H. contortus* populations; therefore, there is a great necessity in finding and developing natural plant products for use as alternatives to commercial anthelmintics for controlling *H. contortus* in pastured sheep and goats. This dissertation has three major aims conducted in three separate studies: the first *in vivo* one was to evaluate the extended effect of a springtime, combined-treatment with 3 anthelmintics having different mechanism of action including moxidectin (Cydectin®), albendazole (Valbazen®), and levamisole (all given orally); this project was also coupled with a rotational grazing program on egg shedding in the United States Northern Great Plains flock of ewes whose *H. contortus* population showed benzimidazole and avermectin resistance. The results of fecal egg counts (FECs) 2 weeks before and after the treatment for 250 ewes showed a 99.99% FEC reduction from 3650 eggs per gram (EPG) to 0.17 EPG. The egg output remained very low until 10 weeks on paddocks. Output peaked on the 16th week at 42.1 EPG. During the following year, mean spring

FEC was only 66.1 EPG and only 5.1 for the fall. Unfortunately, by the spring of 2016 egg output had increased to 1116.0 and remained high in 2017.

The second study was an *in vitro* project aimed to measure the anthelmintic activity of *Melilotus alba* (white sweet clover) on *H. contortus.* The anthelmintic activity of methanol extracts from different sweet clover plant parts (i.e. leaves, stems, pods) were measured using a larval migration assay involving unsheathed third-stage *H. contortus* juveniles (J3). Stems and pods showed no anthelmintic activity, while 97.3% migration inhibition was measured in the leaf-extract at 30 mg/ml. An aqueous extract of the leaves (concentration of 670 mg/ml water) inhibited migration by 98% after 24 hrs., and no motility was observed after 48 hrs. Inhibition was higher at an acidic pH (e.g. pH of 3 and 5) than at a neutral pH (e.g. 7.4). Cytotoxicity of the aqueous leaf-extract was measured with unpolarized bovine ileal epithelial cells at 5 differing concentrations of the extract using an absorbance-based AlamarBlue assay. This assay showed that the extracts were vey toxic (100% cytotoxicity) at high concentrations.

The goal of the third study was to evaluate *M. alba* anthelmintic effect on *H. contortus* under *in vivo* conditions. The experimental yearling ewes naturally and heavily infected with *H. contortus* were fed 0.68 kg of fresh-frozen *M. alba* (white sweet clover) (harvested from local plants in late fall) per day for 2 weeks. FECs were determined daily after the 2 weeks to estimate the post-treatment worm loads. Under these conditions, the results did not show any decrease in egg shedding, no significant effect ($p > 0.05$), within either experimental groups compared to the controls, but it showed an unexpected significant rise in egg output during the treatment period.

The findings of these three studies suggest that the hypobiotic fourth-stage juveniles is the major cause of the rapid massive increase in *H. contortus* in spring; therefore keeping the animals inside the barns for a full year and applying a combined triple treatment while the animals are still inside the barns might help in the eradication of the arresting J4, then the next step is to run FECs next spring, and if the results showed no evidence of haemonchosis, then the animals could be released to the pasture using a rotational grazing system. Since the *M. alba* leaf-extracts showed signs of cytotoxicity to the unpolarized cells, this evidence should be investigated deeply using polarized intestinal cell lines and high doses of leaves should be given to experimental animals. If the future testing of white sweat clover extracts revealed no sign of toxicity, drenches of concentrated leaf-extracts and/or first-year plants containing mostly leaves could be fed to *H. contortus* infected sheep and goats. Such studies are encouraged to better evaluate the usefulness of feeding *M. alba* leaves.

Chapter 1: Introduction

Haemonchus contortus, is one of the gastrointestinal nematodes (GIN), that belongs to the superfamily Trichostrongyloidea, and primarily affecting domestic ruminants, endangering animal welfare and causing serve economic losses (Githigia et al., 2001; Hoste et al., 2005; Jabbar et al., 2006; Troell et al., 2005). Because of its ability to produce massive numbers of eggs depending on blood sucking behavior, these survival practices quickly upsurge adult worm populations in a herd (Besier et al., 2016b). The environmental conditions help the spreading of *H. contortus* globally which support survival of the free-living infectious larvae, third-stage juvenile (J3). Moist and warm soil is considered an optimal media for the survival of *H. contortus* juveniles and therefore, haemonchosis is generally most severe in tropical and subtropical climates. It was found recently that extreme cold climates did not stop the haemonchosis outbreak in ruminant herds including death, in sheep even within 1^{o} of the Arctic Circle (Manninen and Oksanen, 2010). The epidemiological study of Waller et al., 2004 in sothern Sweden revealed that *H. contortus* infective juveniles (J3) are incapable of surviving the cold winters in pastures from that region, but the fecal egg output remained high in the next summer which was an indication that the majority of J3s ingested during mid-summer, July, and early fall remained as hypobiotic fourth-stage larvae (J4) in the abomasal submucosa during winter and those large numbers of ingested J3s resulted in massive adult populations in the spring after lambing. These findings were verified by other studies (Falzon et al., 2014; Grosz et al., 2013) that covered the northern U.S.A. and southern Canada which also showed the inability of *H. contortus* juveniles to survive on pastures in these colder climates. Waller et al., 2004 after their findings projected the

possibility of haemonchosis eradication in their region by treating the flocks with an effective nematocidal prior to their release onto spring pastures. The theory was that haemonchosis outbreaks in extreme cold climates are totally dependent on hypobiotic J4 for winter survival. Waller's possibility was previously proposed by (Campbell and Needham, 1964) and is applied in a recent published study (Besier et al., 2016b). This approach was finally applied by Waller et al., 2006 in a multiyear study employing double ivermectine treatments during Swedish winters to two sheep herds. Their results were impressive because of the susceptibility of *H. contortus* populations to the ivermectin used in the study.

These findings and especially the four-year study of Grosz et al. (2013) encouraged us to further evaluate the possibility of eradicating *H. contortus* populations from the same sheep herd located in a very cold winter climate, in Brookings county South Dakota, a northern region of the U.S.A. Northern Great Plains. Based on the study by Grosz et al. (2013), *H. contortus* was the main species of trichostrongyle and was very resistant to the benzimidazoles and avermectins including the parasite's hypobiotic fourth-stage juveniles which survived albendazole treatment. The anthelmintic resistance of *H. contortus* rested juveniles is believed to be due to the low metabolic rates during hypobiosis (Gatongi et al., 1998; Prichard and Ranjan, 1993). The combination anthelmintic treatments were previously used to improve the nematocidal activity and reduce the anthelmintic resistance (Besier et al., 2016a; Sutherland and Scott, 2010), in addition to decreasing the remaining J4 left in the tissue during early spring and after lambing (Ayalew and Gibbs, 1973; Emery et al., 2016). Rotational grazing was another technique that was used to reduce the transmission of all trichostrongyle nematodes, but

especially for *H. contortus* (Colvin et al., 2008; Jackson et al., 2009). Taking into consideration all the previously used tactics, both tools were used in this study to eliminate *H. contortus* including a triple treatment consisting of different classes of anthelmintics (i.e. an imidazothiazole, benzimidazole and a milbemycin), and a rotational grazing system involving multiple paddocks.

The growing world population even in previously mentioned colder climates increased the demand of animal products. Increased production has been supported by rapid advances in the genomic discipline that have refined animal trait selection; this hasled to genetically improved production animals. However, the desire to increase production and the parasite's inherent survival techniques have also led to increased abuse of anthelmintics; resulting in reduced efficacy due to widespread anthelmintic resistance among many *H. contortus* populations (Rahmann and Seip, 2006). In addition, the increasing public desire for organic food encouraged researchers to find an alternative and safe method to eliminate *H. contortus* infections in small ruminants (Rahmann and Seip, 2006; Sutherland et al., 2013). Some studies have focused mainly on developing organic animal farming and focusing on preventing infections rather than treating them. Methods employed for prevention include reducing animal housing , increasing the grazing period, and minimizing the use of classic anthelmintics (Kijlstra and Eijck, 2006; Sutherland et al., 2013; Thamsborg and Roepstorff, 2003). However, the use of such an approach will be effective only if the pasture and the animal flocks were GIN free, particularly with *H. contortus*. Prevention might be most effective when animals graze on a medicinal plant species with known anthelmintic activities; otherwise these practices

may raise the risk of infection, through the extended exposure of animals to the infectious juveniles (Rahmann and Seip, 2006).

Many published studies have shown the presence of secondary metabolites, rich in bioactive substances, in medicinal plants. Such metabolites can play a role in an animal's physiology and metabolism, and could be parasiticidal (Marcin et al., 2006). These studies included research published in Asia and Africa (Akhtar and Riffat, 1984; Al-shaibani et al., 2009; Githiori et al., 2006; McGawa et al., 2007; Qadir et al., 2010), Australia (Kotze et al., 2009), Europe (Hoste et al., 2006; Manolaraki et al., 2010), North America (Acharya et al., 2014; Fernandez-Salas et al., 2011; Pessoa et al., 2002) and South America (Monteiro et al., 2011; Souza et al., 2008). Several solvents with differing levels of polarity such as dimethyl sulfoxide (DMSO), water, methanol, etc.… were used to extract the secondary products of medicinal plants (Borges and Borges, 2016). DMSO is commonly used to primarily dissolve plant extracts before adding them to water whereas it dissolves both polar and nonpolar compounds. Most *in vitro* studies commonly used nematode eggs or juveniles as an experimental design to measure the anthelmintic activity of secondary products in the screened medicinal plants (Borges and Borges, 2016). These *in vitro* screening techniques primarily used *H. contortus* eggs and juveniles, and if the extracted medicinal plants showed any anthelmintic activity, *in vivo* experiments using small ruminants such as sheep or goats would follow (Lanusse et al., 2016). In the *in vivo* studies, the evaluation of anthelminthic activity of different medicinal plants were mostly measured using the plant extracts and rarely fresh plants. The nematocidal activity of tested medicinal plants can be measured by counting adults worms in the necropsied host after a treatment period; but the reductions in fecal egg

shedding was commonly used in these studies to measure the anthelmintic efficacy because it's an indirect method for measuring parasiticidal effects and most importantly its less expensive (Borges and Borges, 2016; Carvalho et al., 2012; Eguale et al., 2007a, b; Max, 2010; Waghorn et al., 2006).

In the Northern Great Plains of the United States, very few studies revealed the parasiticidal activity of certain medicinal plants such *Melilotus alba* (White Sweet Clover), and one of them was the study of Acharya et al., 2014 who encouraged research to assess the *in vivo* and *in vitro* anthelmintic activity of this plant. Therefore the *in vitro* anthelmintic activity of *M. alba* (White Sweet Clover) methanol and water extracts was measured to determine which portion of *M. alba* contained significant anthelmintic activity on the infective juveniles (J3) of *H. contortus*. White Sweet Clover was selected since it's more appetizing for livestock than the rabbitbrush and sumac species, and also *M. alba* was more abundant than *M. officinalis*. In this study, the experiment model mimicked the techniques used by other scholars who measured the anthelmintic activities of different medicinal plants using *H. contortus* as GIN experimental model (Acharya et al., 2014; Bachaya et al., 2009; Diehl et al., 2004).

Many *in vivo* and *in vitro* studies used the egg hatch assay (EHA) and larval migration assay (LMA) to measure the anthelmintic activity since they are cheap and easy to use (Acharya et al., 2014; Al-shaibani et al., 2009; Coles et al., 2006; Marie-Magdeleine et al., 2010). Therefore, the LMA with *H. contortus* infectious juveniles (J3) were used to measure the anthelmintic activity of methanolic and aqueous extracts of *M. alba* collected from the United States Northern Great Plains. The cytotoxicity test was used to determine the safety of these extracts; therefore, the aqueous leaf extract was

measured with unpolarized bovine ileal epithelial cells at 5 differing concentrations (670, 340, 134, 67, and 17 mg/ml) of the extract using an absorbance-based AlamarBlue assay.

Chapter 2: Lack of Prolonged Benefit for a Triple Combined Anthelmintic Treatment and Rotational Grazing on an Anthelmintic-Resistant Population of *Haemonchus contortus* in Sheep from a North America Cold Winter Climate

2-1. Abstract

Haemonchus contortus is an economically important gastrointestinal nematode that constrains the survival and productivity of sheep and goats. Limiting *H. contortus* populations is a primary challenge in ruminant health management programs because anthelmintic resistant is a growing problem. This study evaluated the extended effect of a springtime, combined-treatment with 3 anthelmintics coupled with a rotational grazing program on egg shedding in a Northern Plains flock of ewes whose *H. contortus* population exhibited benzimidazole and avermectin resistance. Pre-treatment fecal egg counts (FECs) for 250 ewes, analyzed with the McMaster technique, averaged 3650 eggs per gram (EPG). The triple anthelmintic treatment consisted of moxidectin (Cydectin®) at 0.2mg/kg, followed 3 days later with albendzole (Valbazen®) at 7.5mg/kg followed 3 days later with levamisole at 7.5mg/kg (all given orally). Post-treatment FECs were performed 2 weeks after levamisole treatment using 3g samples and a modified Wisconsin sugar floatation technique. Results showed a 99.99% FEC reduction to 0.17 EPG, with 68% of the animals showing no eggs, 27.6% with less than 1 EPG, and 4.4% with 1 to 2 EPG. Animals with greater than 1 EPG were retreated, and ewes (N-248) showing less than 1 EPG were released into the first grazing paddock on June 30, 2014. These ewes were grazed through 10 paddocks averaging 13 acres per paddock until

November 4, 2014 (128 days); ewes rotated through the paddocks 2-3 times. During grazing, 30 fecal samples were collected every-other week. Mean FECs (2 to 18 weeks post-treatment) were as follows: 0.17, 7.55, 0.4, 3.4, 0.94, 9.6, 22.27, 40.06, 42.07, and 30.67 EPG respectively. Fecal egg counts were re-evaluated during the 2015 lambing season for 88 randomly selected ewes from this group. The mean FEC was 80.7 EPG, ranging from 1 to 1000 EPG. PCR analysis verified the presence of *H. contortus* in these samples. Results indicate that while triple treatment and rotational grazing in this region significantly limits egg shedding, some *H. contortus* are capable of surviving under these conditions even in environments where winter pasture survival of juveniles is not possible.

Anthelmintic resistance is a growing problem for *Haemonchus contortus*, even among sheep and goat herds located in winter climates that are lethal to its free-living juvenile stage. Because survival in these climates is entirely dependent on the tissue-dwelling fourth-stage juvenile, researchers have speculated that it may be feasible to aggressively treat and prevent transmission of *H. contortus* enough to eradicate it from herds in these regions. This study evaluated these speculations using a flock of ewes from the Northern Plains with a *H. contortus* population (85% *Haemonchus*. 5% *Teledorsagia* and 10% *Trichostrongylus*) exhibiting benzimidazole and avermectin resistance. Prior to first release into paddocks in the spring of 2014, ewes were given a triple anthelmintic treatment consisting of moxidectin (Cydectin®), followed by albendzole (Valbazen®), followed by levamisole (Prohibit®). Pre- and 2-week post-treatment fecal egg counts (FECs) for 250 ewes showed a 99.99% FEC reduction from 3650 eggs per gram (EPG) to 0.17 EPG. Ewes with less than 1 EPG were grazed 2-3 times through 7 paddocks (mean

5.90 hectares) from June 30 to November 4, 2014. Bi-monthly FECs (N=30) showed egg output remain very low until 10 weeks on paddocks (2 weeks after ewes first rotated onto a previously grazed paddock). Output peaked on the 16th week at 42.1 EPG. During the following year, mean spring FEC was only 66.1 EPG and only 5.1 for the fall; trichostrongyle population diversity was similar the previous spring. Unfortunately, by the spring of 2016 egg output had increased to 1116.0 and remained high in 2017. During the last 2 years, *Teledorsagia* and *Trichostrongylus* accounted for an increased portion of the population. Results indicate that while triple treatment and rotational grazing in this region can limit egg shedding for a couple years, it quickly returns to an equilibrium based upon the ongoing control measures used.

Key Words: gastrointestinal nematode; *Haemonchus contortus*.

2-2. Introduction

Haemonchus contortus is the most dangerous nematodes infecting domestic ruminants due to its blood feeding behavior and its ability to produce massive numbers of eggs which rapidly increase adult worm populations in a herd (Besier et al., 2016b). The global distribution of this species has been influenced significantly by the environmental conditions which support survival of the free-living, third-stage juvenile (J3). Juvenile survival of *H. contortus* is optimal on or in moist and warm vegetation or soil, and therefore, haemonchosis is generally most severe in tropical and subtropical climates. More recently, populations are being found in ruminant herds from cold winter temperate climates that are causing clinical parasitism, including death, in sheep even within 1^{o} of the article circle (Manninen and Oksanen, 2010). An epidemiological study involving two sheep herds from southern Sweden found that J3s are unable to survive during the

cold winters in pastures from this region, but that a high percentage of ingested J3s remained as hypobiotic fourth-stage juveniles (J4) in abomasal tissues throughout the winter and emerge as adults in the spring after lambing (Waller et al., 2004). They also found that by July virtually all of the J3s were remaining as arrested juveniles, and that this "build-up" of juveniles resulted in massive adult populations during the next spring. The inability of *H. contortus* juveniles to survive on pastures in colder climates has been verified even in more southern longitudes such as northern U.S.A. and southern Canada (Falzon et al., 2014; Grosz et al., 2013). Because of the total dependency of the J4s for survival of *H. contortus* populations from this climate, Waller et al. (2004) speculated that it would be possible to eradicate it from herds that were treated with an effective anthelmintic prior to their release onto spring pastures. This possibility has been suggested as early as 1964 (Campbell and Needham, 1964), and is still being expressed in recent publications (Besier et al., 2016b). This goal was apparently met for the two years of a study involving two sheep herds from Sweden in which all of the sheep were treated twice with ivermectin during the winter (Waller et al., 2006). Both *H. contortus* populations in this study were known to be susceptible to ivermectin, and its effectiveness was evaluated in ewes and/or lambs during the spring and autumn for the first year, and during the spring and summer during the second year. The absence of *H. contortus* in ewes and lambs during the first year was determined through fecal egg counts (FEC) combined with egg hatching and juvenile identification on 20 fecal samples per time-period, and also through necropsy observations of abomasal contents for adults and tissue samples for J4s involving 10 lambs. During the second year, the absence of *H. contortus* was only evaluated through the analyses of fecal samples in ewes and lambs.

While no *H. contortus* adults or juveniles were identified even two years after treatment, the low detection methods used in this study may not have allowed a possible re-establishment of a small *Haemonchus* population to be detected within the short duration of the study.

To further evaluate the possibility of eradicating *H. contortus* from a herd located in a very cold winter climate, a four-year study was initiated in a group of sheep located in a northern region of the U.S.A. Northern Great Plains that Grosz et al. (2013) previously showed was lethal to J3s on winter pasture. In these sheep, the predominant species of trichostrongyle was *H. contortus*, and as in many current sheep herds, this *H. contortus* population had a significant level of resistance to the avermectins and the benzimidazoles (Grosz et al., 2013). Therefore, a combination treatment consisting of three classes of anthelmintics (i.e. a benzimidazole, a milbemycin and an imidazothiazole) was used in this study in an attempt to eliminate *H. contortus* from each ewe. Combination drench anthelmintic treatments are increasingly being used to improve efficacy, especially once resistance has become a measurable problem (Besier et al., 2016a; Sutherland and Scott, 2010). The combination treatment was used during early spring and after the ewes had lambed to minimize the number of hypobiotic fourth-stage juveniles left in the tissue (Ayalew and Gibbs, 1973; Emery et al., 2016). A previous study involving the same population of *Haemonchus* showed that large numbers of hypobiotic fourth-stage juveniles were able to survive albendazole treatment (Grosz et al., 2013), and this increased resistance of hypobiotic juveniles has been reported in other studies, and thought to result from the low metabolic rates during hypobiosis (Gatongi et al., 1998; Prichard and Ranjan, 1993). To additionally enhance the chance of eradicating

Haemonchus in this herd, ewes were grazed without lambs through a rotational grazing system involving multiple paddocks. Rotational grazing has been an effective management tool for minimizing parasite transmission for all trichostrongyle nematodes, but especially for *H. contortus* (Colvin et al., 2008; Jackson et al., 2009). To provide a high level of sensitivity for detecting *Haemonchus* in the herd post-treatment, a highly sensitive Wisconsin sucrose flotation method was used to harvest eggs combined with a PCR method for identifying *Haemonchus* egg DNA as described by Grosz et al. (2013).

2-3. Material and Methods

2-3.1. Study area and initial herd characteristics

The commercial herd used in this study consisted of 297 cross whiteface composite ewes and is located in east-central South Dakota, USA (N44.5, W96.7). Management and deworming history of this flock have been described previously (Grosz et al., 2013). These ewes were rotationally grazed through 47.25 ha of pasture that were divided into 8 paddocks. A control, untreated group of 44 ewes were kept in a cement feedlot, and fed grass hay and mixed grain. Previous PCR analysis showed that *H. contortus* was virtually the only trichostrongyle species present at detectable levels. The benzimidizole and avermectin resistance status of the worm population in the herd was previous described in the context of their need to overwinter as tissue-dwelling J4s (Grosz et al., 2013). In 2014, lambs were born from March to May.

Grazing season (April 1-September 30) temperatures and precipitation for 2015 and 2016 were recorded for each day from the closest (14km) weather station to the herd at the Oak Lake Field Station outside of White, SD (https://climate.sdstate.edu/tools/et/).

Thirty year (1981-2010) average temperatures and precipitation were recorded (https://climate.sdstate.edu/tools/normals/daily.asp) for each day from Brookings, SD, which is 16 km from the herd. Figure 2-1 shows the daily high and low temperatures for 2015-16 for these two sites, and illustrates how similar the temperatures for these two years were with each other and with the normal values. The mean high and low

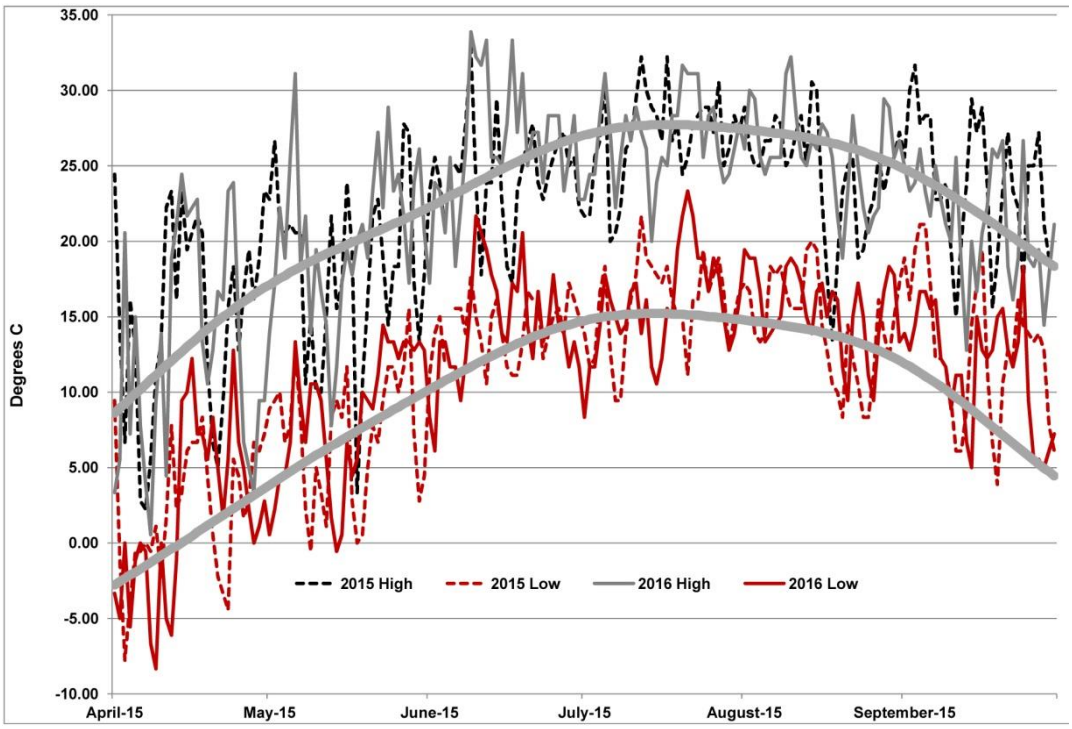

Figure 2.1. Grazing season daily high and low temperatures for 2015 and 2016 at the SDSU Oak Lake Field Station (White, SD) compared to 30 year mean (Normal) daily high (top wide grey line) and low temperatures (bottom wide grey line) from Brookings, SD weather station.

temperature for the 2015 grazing season was 22.1ºC and 11.1ºC respectively, and for 2016 was 22.3ºC and 11.34ºC respectively. Figure 2.2 shows the daily precipitation for 2015-16 along with the 30-year normal values. Precipitation levels for these two years were also very similar to normal values.

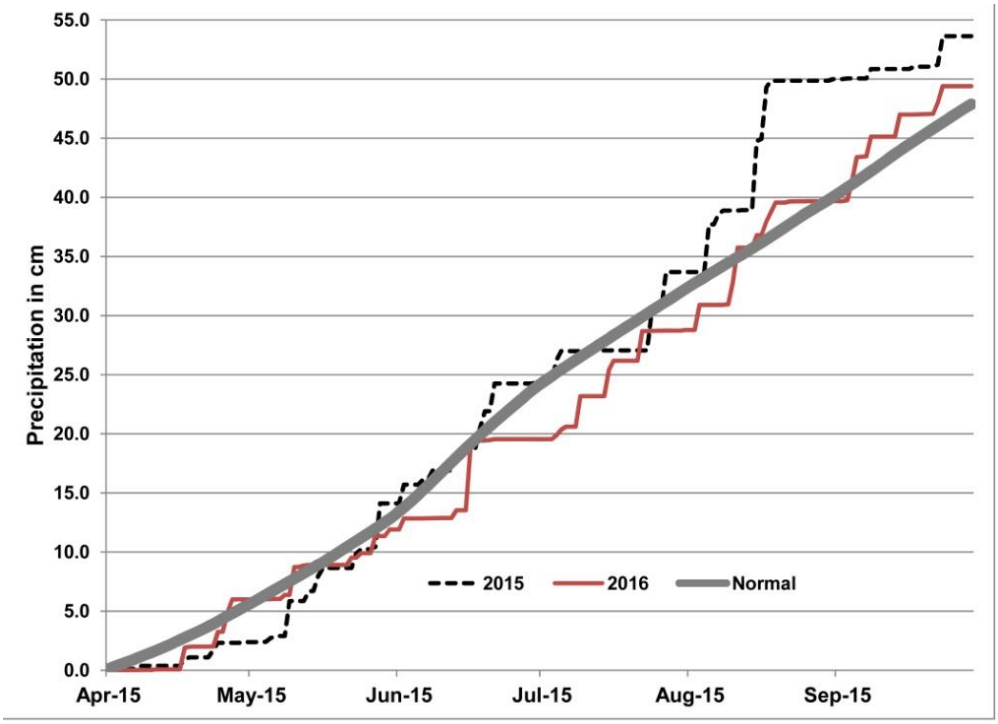

Figure 2.2. Grazing season daily precipitation for 2015 and 2016 at the SDSU Oak Lake Field Station (White, SD) compared to 30 year mean (Normal) precipitation (wide grey line) from Brookings, SD weather station.

2-3.2. Anthelmintic treatments and fecal analyses

Prior to treatment, fecal samples were collected rectally from the 249 ewes. Fecal egg counts (FECs) were performed on 2 g samples using a modified McMaster flotation technique with sensitivity of 50 eggs per gram (Zajac and Conboy, 2012). All FEC results were expressed as eggs per gram of fecal sample (EPG) To assess the levels of aggregation for fecal egg output within the pretreatment samples, the Corrected Moment Estimate of k (CMEK) was calculated according to the formula [CMEK = (mean FEC^2−variance/sample number)/(variance − mean FEC)] (Wilson et al., 2002). From the third week of May until the first week in June, each ewe was treated orally with moxidectin (Cydectin®; dosage of 0.2mg/kg), and then 3 days later with albendazole (Valbazen®; dosage of 7.5mg/kg), again 3 days later with levamisole (Prohibit®; dosage

of 7.5mg/kg). At least 9 days after the final treatment, fecal samples were again collected from 249 ewes, and FECs determined on 3 g samples using a modified Wisconsin sugar flotation technique with a sensitivity of approximately 1 EPG (Cox and Todd, 1962). Any ewe with post-treatment FECs greater than 1 EPG were retreated with moxidectin, and retested. For a few ewes, it was necessary to retreat up to 3 times with moxidectin, and only ewes (N=248) with FECs less than 1 EPG were released onto the pasture**.** Pasture grazing started on June 30.

2-3.3. Pasture rotations and fecal analyses

The 248 ewes with FECs less than 1 EPG (and therefore included in the study) were released into the first grazing paddock on June 30, 2014. This was 3-4 weeks after the end of triple treatment. These ewes grazed through 8 paddocks averaging 5.90 ha per paddock until November 4 (Table 2.1). By the end of the season, ewes had rotated through the 5 main paddocks 2 or 3 times (weeks 1-8, part of 10, 14-18) and 3 minor paddocks once (weeks 9-13). The minor paddocks were primarily used between the second and third rotations in the main paddock because they were being used for hay production at the beginning of the season. Ewes were first rotated back onto a paddock for the second time on August 10 (6 weeks post-release), and for the third time on October 10. The main paddocks (mean area of 4.68 ha) were grazed for an average of 7.21 days per rotation, and the minor paddocks (mean area of 7.94 ha) for 8.33 days. During the 18 weeks, 20-30 random fecal samples were collected from the ground every-other week, and FECs were determined as described previously for the Wisconsin sugar flotation technique. This method was also used on November 17 to determine the FECs from the untreated, feedlot control ewes.

Pad. No.	Area Hectares	1st Rotation Begin-End	Weeks After**	2nd Rotation Begin-End	Weeks After**	3rd Rotation Begin-End	Weeks After**
1	4.56	6/30-7/7	1	8/10-8/18	7	10/15-10/23	16
2	1.36	7/7-7/10	2	8/18-8/21	8	10/10-10/15	15
3	3.63	7/10-7/17	3	8/21-8/26	8	10/23-10/28	17
4	8.07	7/17-8/1	4&5	9/2-9/11	10-11	10/28-11/4	18
5	5.8	8/1-8/10	6	9/2910/10	14		
6	8.88	8/26-9/2	9				
7	5.06	9/11-9/19	11-12				
8	9.89	9/19-9/29	13				

Table 2.1. Rotational grazing time-table for 2014

*Days that the herd was on each paddock
**Approximate number of weeks after ewes were first released onto the first paddock

2-3.4. FECs and pasture rotation for years 2-4

From March 13 to 28 of 2015, fecal samples were collected from 110 randomly selected ewes with new lambs, and FECs were determined using the Wisconsin sugar flotation technique. That year, all ewes were then treated orally with levamisole (Prohibit®; dosage of 7.5mg/kg). On August 9, 2015, fecal samples were collected from 48 ewes while on pasture and FECs were determined as described for the spring. Only 25 fecal samples were analyzed during the 2016 lambing season, and no treatments were given, but FAMCHA scores were determined for all ewes, and any ewes with high scores (i.e. 4s and 5s) and/or poor body condition were not rotated (approximately 100 of the 300 ewes that year) through the pasture. In the 2017 lambing season, 50 FECs were analyzed. FECs were determined for all of these samples using the Wisconsin sugar

flotation technique as described previously. In years 2-4, the pasture rotation scheme was very similar to that described for year 1, but the beginning and ending dates varied slightly based upon grass conditions.

2-3.5. Identification of trichostrongyle genera in selected fecal samples

Trichostrongyle genera were identified in pre- and post-treatment samples with PCR analysis using the DNA isolation protocol, sheep trichostrongyle primers and PCR protocol described by Grosz et al. (2013). The time-periods and the sample numbers tested are shown in Figure 7; samples containing the highest number of eggs for each group were selected for PCR testing. Eggs were isolated from their microscope slides immediately after FECs were performed into 50 ml centrifuge tubes containing 45 ml of distilled water. This material was then centrifuged to pellet the eggs, and the pellet was resuspended in distilled water and stored at -20°C. Eggs were lysed and DNA isolated using a lysing matrix (Lysing Matrix A, MP Biomedicals), a Mini BeadBeater-8 (BioSpec, Bartlesville, OK) and the Qiagen DNeasy Plant Mini DNA extraction kit (Valencia, CA, USA). Eggs were lysed by adding them and 400 µL of the AP1 buffer (from the extraction kit to a lysing matrix microcentrifuge tube and shaking the contents in the bead beater for 3 min. This material was then incubated for 10 minutes at 65°C. DNA was precipitated adding 130 µL of buffer AP2, incubating -20°C for 5 minutes, and centrifuging at 14,000 rpm for 5 min. The material is then added to a QIA shredder Mini Spin Column, centrifuged at 8000 rpm for 1 minute and the flow-through is discarded. The column is washed with 500 µL of buffer AW and centrifuged once at 4000 rpm for 2 minutes and then again at 8000 rpm for 1 minute. DNA was eluted into 100 µL of the elution buffer (Buffer AE), incubated for 5 minutes, centrifuged at FORMAT THE

Table 2.2. Trichostrongyle primers used for genus-level (species listed in parentheses) and species-level identification of trichostrongyle eggs

Genus/Species Primers[Source]	The forward primers are, from 5' to 3'	Base Pair Length
Haemonchus (*contortus*)[H]	For: CATATACATGCAACGTGATGTTATGAA Rev: GCTCAGGTTGCATTATACAAATGATAAA	157
Cooperia (*oncophora*)[Z]	For: TCGATGAAGAGTTTTCGGTGTTC Rev: TTCACGCTCGCTCGTACTTCA	151
Ostertagia leptospicularis[S]	For: ATGAAACTACTACAGTGTGGCTAACA Rev: TTCTTGAACTGAAATGGGAATTATCA	92
Trichostrongylus (*colubriformis*)[Z]	For: CAGGGTCAGTGTCGAATGGTCATTGTCAAATA Rev: CAGGGTCAGTGGTTGCAATACAAATGATAATT	243
Teladorsagia circumcincta[L]	For: CTTAATGATCTCGCCTAGACG Rev: TTATATGTTAGCCACACTGTAGTAG	279
Trichostrongylus axei[B]	For: GATGTTAATGTTGAACGACATTAATATC Rev: GCTAAATGATATGCTTAAGTTCAGC	186
Trichostrongylus colubriformis[B]	For: CACGAATTGCAGACGCTTAG Rev: ACATCATACAGGAACATTAATGTCA	232
Trichostrongylus vitrinus[B]	TrviFd1: ATGTGAACGTGTTGTCACTGTTTA ITS2GR: GCTAAATGATATGCTTAAGTTCAGC	150

Reference source of published primers are shown as a superscript for each genus or species: B=Biset et al., 2014; H=Harmon et al., 2007; L= Learmount et al., 2009; S= von Samson-Himmelsterna and Schnider, 2002; Z=Zarlenga et al., 2001.

8000 rpm for 1 min., and then stored at -20°C.

The primer set used in the genus-level identification of trichostrongyle eggs are shown in Table 2.2, and are from previously published studies (Bisset et al., 2014; Harmon et al., 2007; Learmount et al., 2009; von Samson-Himmelstjerna et al., 2002; Zarlenga et al., 2001). The PCR reaction consisted of 12.5 µL of Promega GoTaq® Green Master Mix (50 U/mL Taq DNA polymerase, 400 µM of dATP, dGTP, dCTP,

dTTP, and 3 mM MgCl2), 1µL of each 0.2µM primer, 1.5 µL genomic DNA and 10 µL Nuclic Acid Free

PCR-H_2O. Amplification were carried out in an Applied Biosystems Veriti ® 96-well thermocycler set at the following conditions for 40 cycles: denaturation at 95°C for 1 min, annealing at 60˚C for 1 min, extension at 72°C for 2 min. PCR products were then separated on a 2% agarose gel containing 5 µL of 15 mg/ml ethidium bromide and run for 90 min at 125V and 125 mA. Bands were visualized with BIORAD ChemiDoc™ XRS+ imaging system (Hercules, CA) and band intensity was visually scored on a 1-4 scale. The 25 samples with the highest FECs in 2017 were further evaluated with primers shown by Bisset et al., (2014) to be specific for the 3 most common species of *Trichostrongylus* (i.e. *T. axei*, T. *colubriformis*, and *T. vitrinus*).

2-4. Result

2-4.1. Pretreatment Fecal Egg Counts

As shown in Figure 2.3, the mean trichostrongyle FEC for the 240 ewes prior to treatment was 3650 EPG, with a high degree of variation (SEM 368.0) ranging from 0 to 49400 EPG. The distribution of eggs among the 2014 samples showed significant aggregation, with 35 (14.6%) ewes showing no evidence of trichostrongyle eggs (EPG of less than 50 EPG), and 4 ewes shedding more than 25000 (Figure 2.4). The group with the highest number of ewes were shedding eggs ranging from 50 to 1000 EPG. The 28 ewes (11.7% of the flock) with egg counts greater than 8000 EPG account for almost half (49.4%) of the total eggs produced in the flock. The 20% (48 ewes) of ewes shedding the highest number of eggs accounted for 65.2% of the total eggs being shed. No ewes were shedding eggs in the 17000 to 24999 EPG range, but 16.1% of the eggs present were

located in 4 of the most highly infected ewes. Egg counts among the 50 samples from 2017 also appeared to be aggregated, but this was more difficult to assess due to the smaller sample size. The CMEK (k) value was 0.406 for 2014 and 0.106 for 2017.

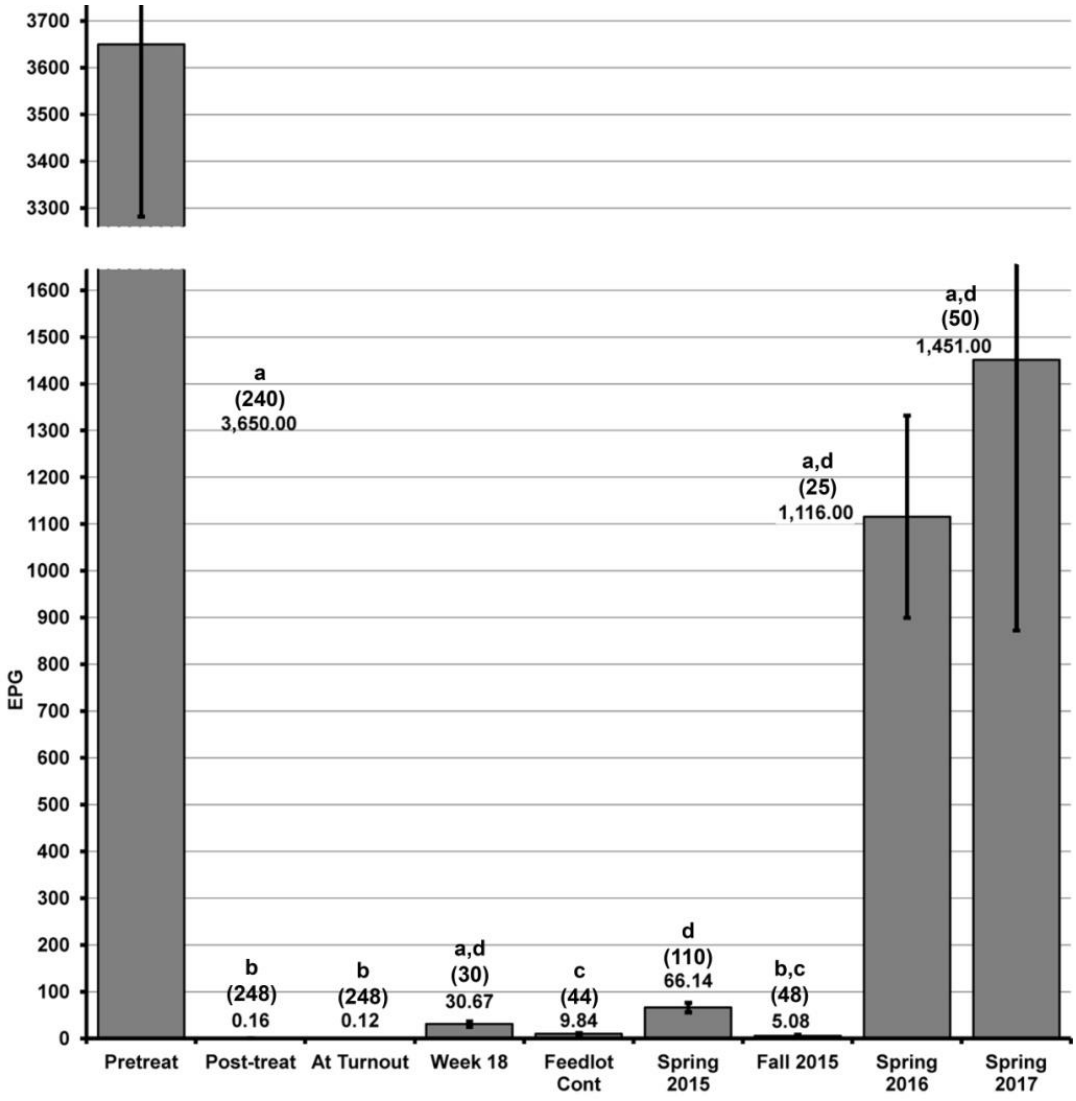

Figure 2.3. Mean trichostrongyle eggs per gram (EPG) fecal egg counts during various time periods in the 2014 and 2015 grazing seasons and the 2016-2017 spring lambing periods. Error bars are the standard errors for each mean. Numbers above each bar represent the mean; numbers in parentheses are the sample size. Bars with the same letter are not statistically different.

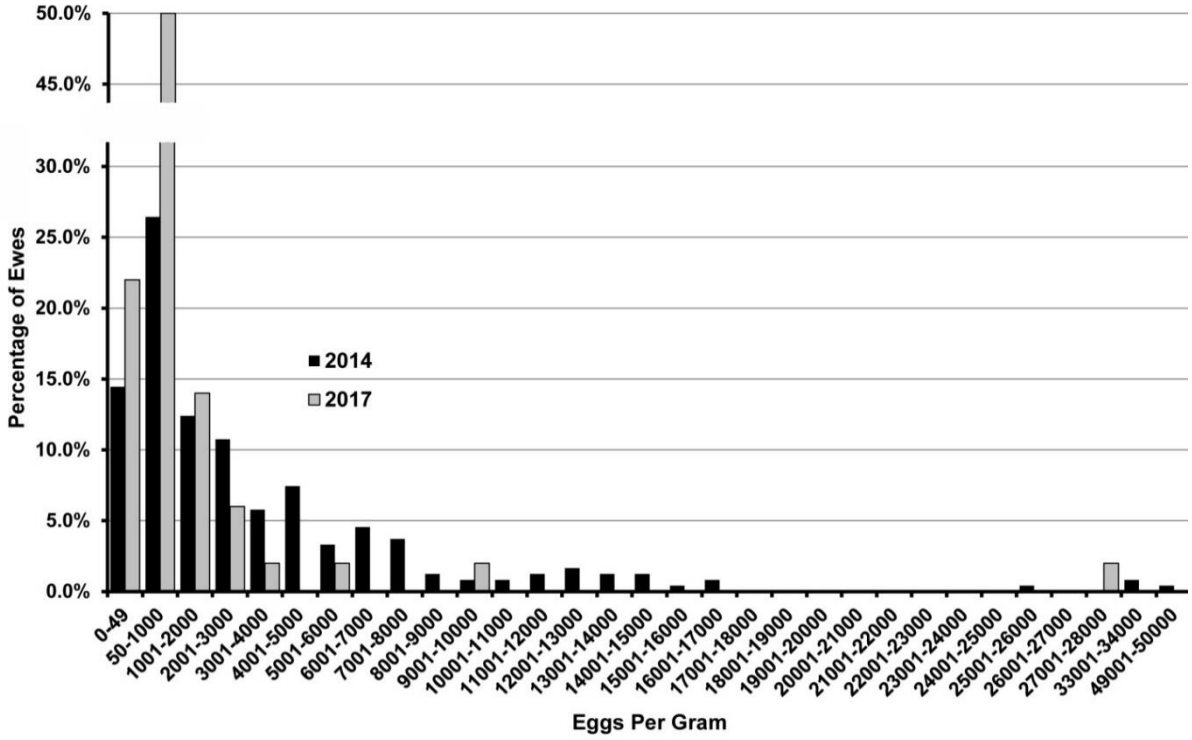

Figure 2.4. Aggregation of trichostrongyle fecal egg counts from 240 pre-treated ewes in 2014 and from 50 ewes tested in 2017. Each bar represents the number of ewes containing that range of fecal egg counts.

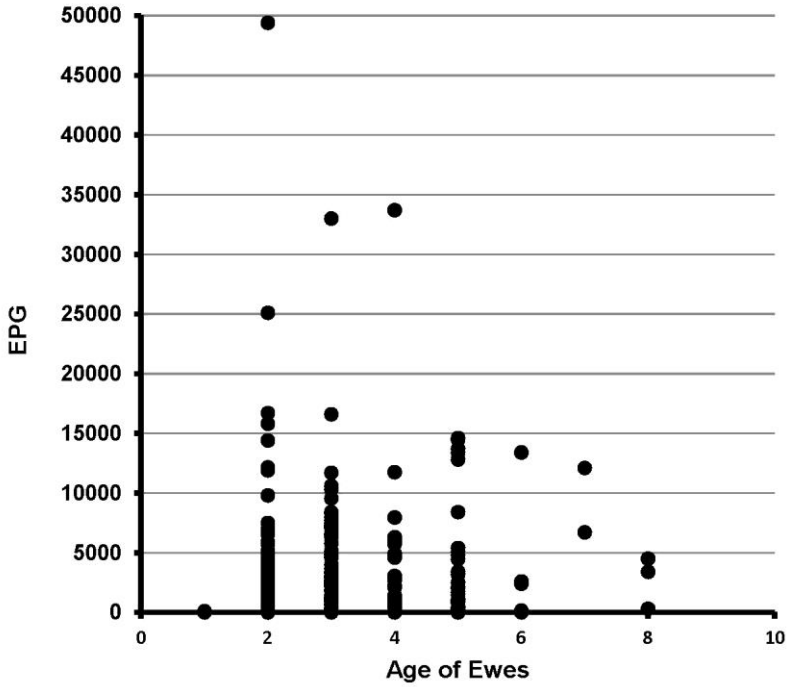

Figure 2.5. Effect of age on individual fecal egg counts; Scatterplot graph. Each closed circle represents the fecal egg count for 1 ewe in eggs per gram.

The ewe age-related distribution of FECs (Figure 2.5) showed that low egg counts occurred in all age groups. Six out of the 7 ewes with FECs greater than 15,000 EPG were found in the 2 and 3-year-old ewes, but this group contained 58.8% of all ewes. Only 2 of the 13 ewes 6 years and older showed less than 100 EPG, and 2 were shedding more than 10000 EPG. Mean egg output in the 2-year-old ewes were numerically higher than the other age groups, but was only statistically higher than that of the 5 year-old ewes (Figure 2-6). Two-year-old ewes accounted for 33.43% of all the eggs counted during the study, and ewes less than 4 years of age accounted for 65.45% of all the eggs.

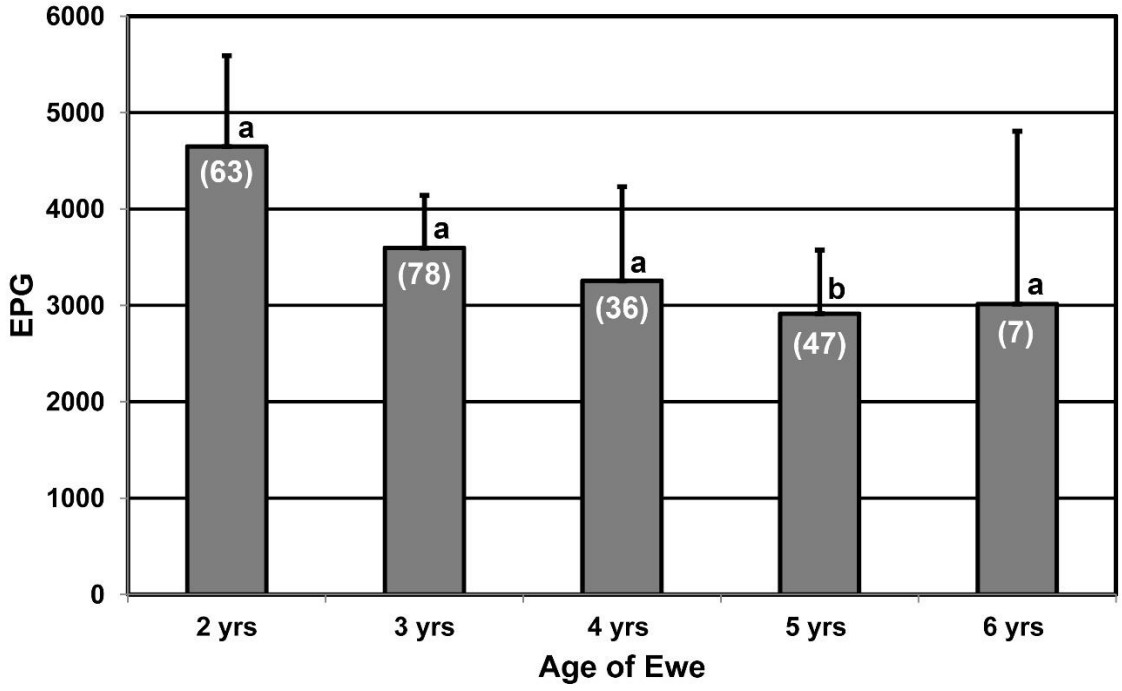

Figure 2.6. Effect of age on mean fecal egg counts; Bar graph. The numbers in parentheses represent the sample number for each of the age groups. Bars with the same letter are not statistically different.

2-4.2. First year post-treatment fecal egg counts

Triple treatment resulted in a 99.99% reduction in mean FEC, down to 0.16 EPG (SEM=0.02) among the 249 ewes (Figure 2.3), with 68% showing no eggs, 27.6%, containing less than 1 EPG, and 4.4% with 1-1.67 EPG. To better evaluate the

effectiveness of this triple treatment, a second data evaluation was conducted after eliminating ewes with pretreatment FECs less 500 EPG. Among these 160 paired pre- and post-treatment samples, the mean egg reduction was 99.99% (SEM = 0.0013, 95% confidence interval 99.99-100), ranging from 100% to 99.87%. A comparison of these reductions against the pre-treatment FEC showed that higher than 99.97% reductions were achieved among all samples with pre-treatment FECs greater than 5,000 EPG (Figure 2.7). After retreatment and retesting of the 11 ewes with FECs greater than 1 EPG, 4 samples contained even more eggs than in the previous FEC, with 1 sample containing 70.0 EPG. The other 3 samples dropped below 1 EPG after the third treatment, but a fourth ewe still did not meet the threshold, and was excluded from study. Therefore, the FEC among the ewes that were allowed into the paddocks was 0.121 EPG (Figure 2.3).

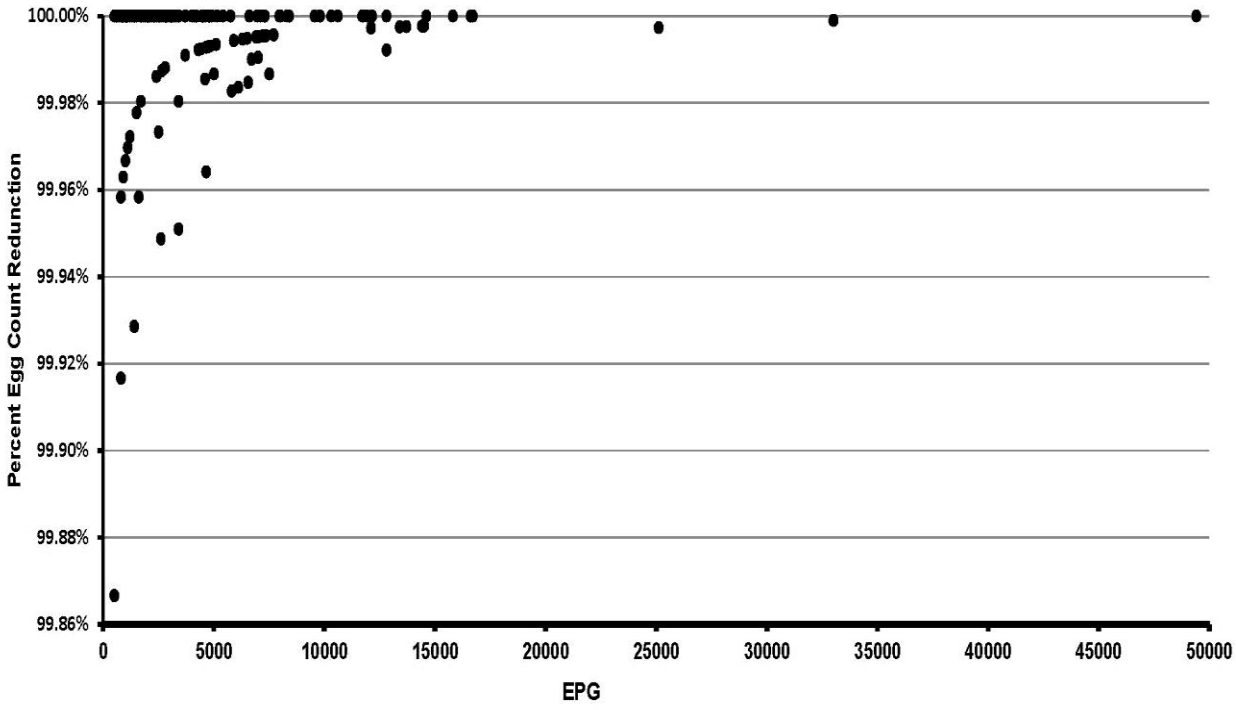

Figure 2.7. Effect of pretreatment egg count on the post-treatment percent egg count reduction

Two weeks after the ewes were released into the first paddock (approximately 5-6 weeks post-treatment), 18 of the 20 fecal samples tested had 0 EPG, one had 1 EPG, and one animal had 150 EPG; this single high value pushed the mean FEC to 7.55 EPG (Figure 2.8). The FECs for the week 4 collection ranged from 0 to 5 EPG, with 30 out of the 34 samples showing no eggs. The FECs and number of samples with FECs above 1 EPG remained low among samples collected 6 and 8 weeks after release into the paddocks. By the eighth week, the ewes had rotated back into the first paddock for about 1 week, and by the tenth week, the mean FEC had increased to almost 10 EPG. Among the 30 samples collected during this tenth week, 6 had FECs greater than 10 EPG and 22 above 1 EPG. By this period, ewes had grazed through 3 paddocks a second time, and it is likely that larval transmission was occurring into the entire flock. FECs continued to increase within the collected samples until after the fourteen week. By week 14, FECs ranged from 2.0 to 205.0 EPG, with 23 out of the 30 samples having FECs greater than 10, and all of the samples were above 1 EPG (Figure 2.8). By the end of the grazing season (November 4), the mean FEC had dropped to 30.67 EPG, and 3 of the samples had FECs less than 1 EPG. The final mean FEC was less than one-tenth the egg-output of the pretreated ewes during that spring (Figure 2.3). During the last 4 weeks of the first grazing season (October 10-November 4), 16 of the 90 (17.8%) samples had more than 50 EPG (Figure 2.8).

Among the 30 fecal samples collected on November 17 from the feedlot control ewes, the mean FEC was 9.84 (SEM = 2.29) EPG, ranging from 0 to 65.0 EPG (Figure 2.3). This was approximately one-third the egg output measured in the pastured ewes at the end of the grazing season. Eleven of the 30 samples contain less than 1 EPG, and 9 samples contain more than 20 EPG.

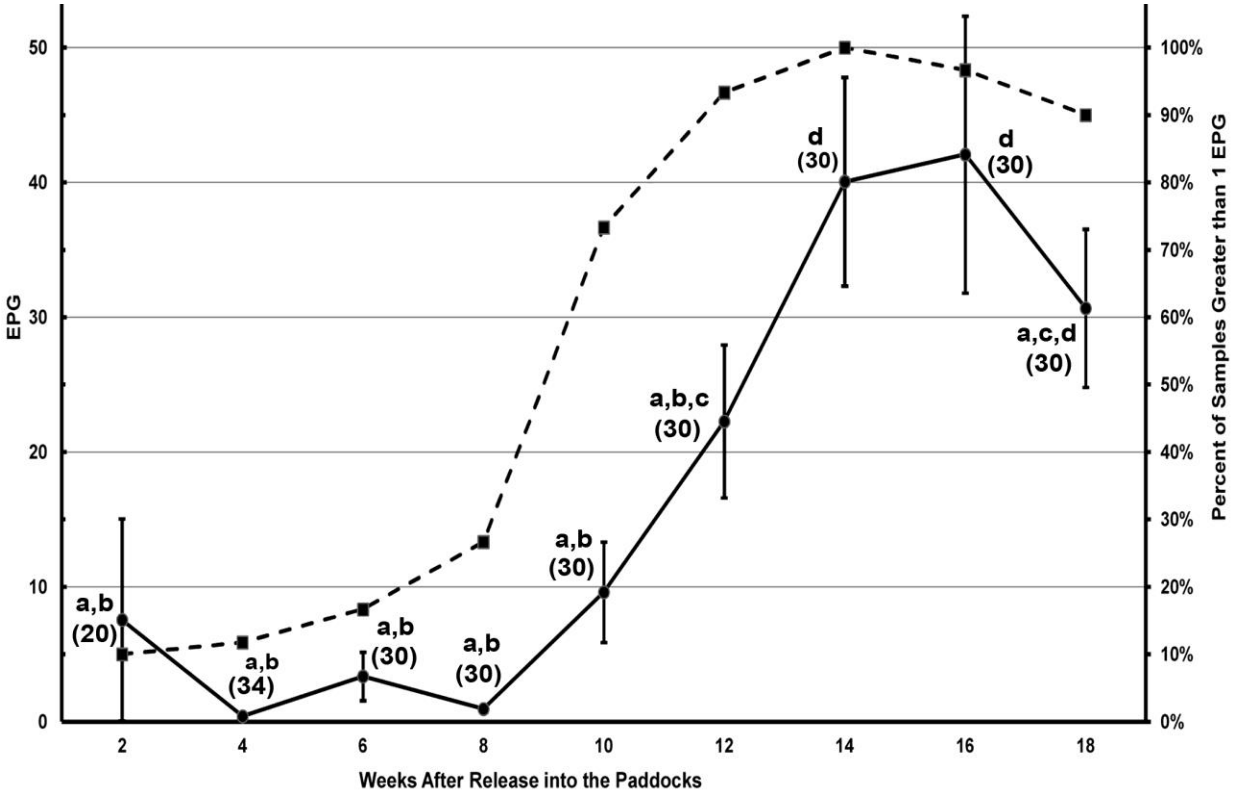

Figure 2.8. Fecal egg counts weeks after releasing treated ewes onto the paddocks. Solid line is the mean FECs for each time-period; dashed line is the percentage of samples with FECs above 1 EPG. Sample size is shown in parentheses for each time-period. Data points with the same letter are not statistically different.

2-4.4. FECs for Years 2-4

During the 2015 lambing season (March 13-28), FECs were measured for 110 randomly selected ewes. All of the ewes were excreting at least 1 EPG and the mean FEC was 66.14 (SEM = 10.26) EPG (Figure 2.3), ranging from 1 to 1000 EPG. The spring

2015 mean FEC was approximately twice that of the FEC at the end of the 2014 grazing season (Figure 2.3). The CMEK (k) value was 0.371, and so even though FECs during this collection period were significantly lower than pretreatment values for 2014, the level of aggregation was similar. The mean FEC for the 2016 lambing season increased significantly over the 2015 season, up to 1116.0 EPG (SEM = 216.20) for the 25 samples. During the 2017 spring season, a firm re-establishment of the trichostrongyle population was confirmed at a level similar to that of 2016, but significantly below the pretreatment value of 2014.

2-4.5. PCR Identification of trichostrongyle genera present

Throughout the study, PCR results involve genus-level primers showed the presence of *Haemonchus* in 88 of the 90 (97.8%) fecal samples tested. *Teladorsagia* and *Trichostrongylus* were found in 26.7% and 50.0% of the tested samples respectively, but *Cooperia* and *Ostertagia* were not detected. The 90 tested samples were among the most heavily infected samples from each time period, ranging from 12 to 33,700 eggs/sample. The overall sensitivity of the PCR assay was illustrated by its detection of *Haemonchus*, *Teladorsagia* and *Trichostrongylus* all in 1 sample containing only 12 trichostrongyle eggs; the specificity of the assay was also illustrated by the detection of only *Haemonchus* among 7 pretreatment samples containing a total of 98,200 eggs. Among the tested samples, prevalence for *Haemonchus* remained stable throughout the study period, with 1 sample testing negative in the spring of 2014 and 1 in spring of 2016 (Figure 2.9). Half of the samples from the 2 fall collections were infected with *Teladorsagia*, but it was less commonly found during the spring collections, particularly

after 2014. In contrast, *Trichostrongylus* prevalence appeared to significantly increase after the first year, infecting 88% of the ewes by the final year of the study (Figure 2.9).

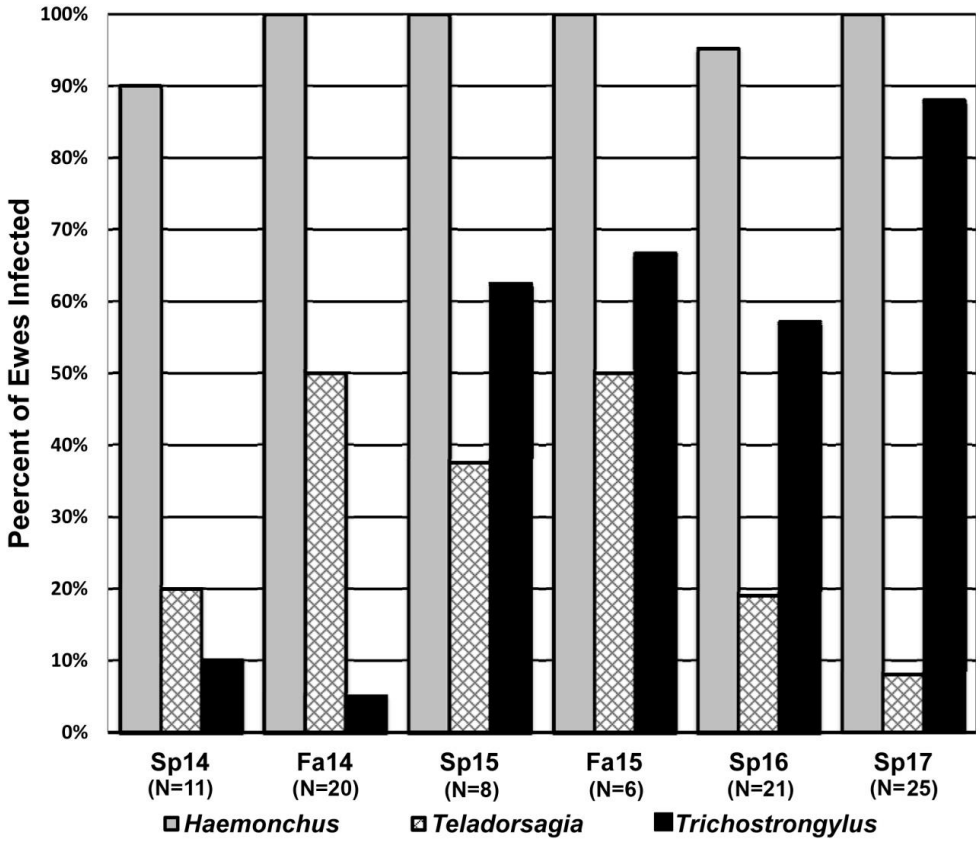

Figure 2.9. Prevalence of *Haemonchus* sp., *Teladorsagia* sp. and *Trichostrongylus* spp. eggs based upon PCR analysis for eggs recovered during each biyearly collection period. The Sp14 collection was taken prior to treatment.

A similar trend was noted when the intensity of the bands were included in the analysis and each genus expressed as a percentage of the total band intensity. Prior to treatment, *Haemonchus* accounted for 85% of the band intensities within the group, and *Teladorsagia* and *Trichostrongylus* accounted for 5 and 10% respectively (Figure 2.10). At the end of the first grazing season, band intensity for *Haemonchus* remained stable at 84%, but *Trichostrongylus* had almost disappeared among the eggs (5% of the band intensities). *Trichostrongylus* was again present among the eggs samples by the

following spring, and increased each collection period, accounting for 44% of the intensities by the end of the study. *Teladorsagia* represented roughly 15% of the DNA intensities during the fall collections and about 5% during all of the spring collections except during 2017, when it dropped to 1%. *Haemonchus* accounted for more than 80% of the DNA recovered from eggs until after the spring of 2015; this dropped to about 55% by 2017 (Figure 2.10).

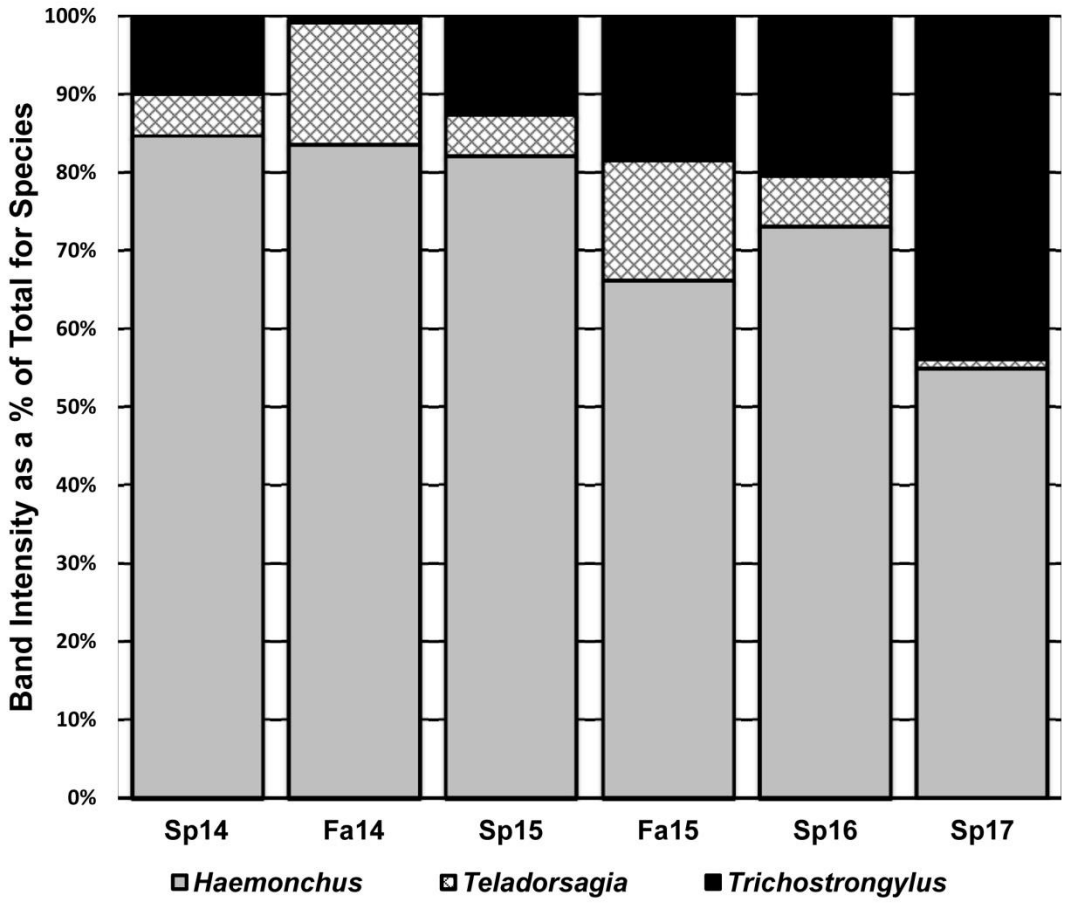

Figure 2-10. Relative band intensities for *Haemonchus* sp., *Teladorsagia* sp. and *Trichostrongylus* spp. egg DNA based upon PCR analysis for eggs recovered during each biyearly collection period. The Sp14 collection was taken prior to treatment during the spring of 2014.

Additional PCR testing of the 25 DNA samples from 2017 with 3 primer pairs specific for the 3 most common species of *Trichostrongylus* showed the presence of very

dim *T. axei* bands for 2 of the samples, but also shown bright *T. colubriformis* bands for 22 samples. The same 3 samples that were negative for *T. colubriformis* were also negative for *Trichostrongylus* with the genus-level primers. *Trichostrongylus vitrinus* was not identified in any of the 25 samples.

2-5. Discussion

There have been very few published studies involving sheep parasites from the North American Northern Great Plains. A ten-year study from 1944-1954 was conducted in sheep herds from northwestern South Dakota in response to chronic dysentery problems among lambs from those herds (Harshfield, 1955). Even with the relatively cold winters and dry summers characteristic to this region, *H. contortus* was found in most of the sheep and lambs necropsied during the study. *Trichostrongylus* spp. was the other nematode identified in most of the animals, but they also frequently found *Teladorsagia circumcincta*, *Nematodirus* spp., and *Chabertia ovina* within the animals. Though not in the Northern Great Plains, an extensive sheep-parasite survey was conducted among 31 farms in 2006 and 32 in 2007 at approximately the same longitude as the northwestern South Dakota 1944-1954 study and our present study, but in southeastern Ontario and southwestern Quebec (Mederos et al., 2010). In 14 lambs from 7 of the Ontario farms, 36.4% of the adult trichostrongyles were *Trichostrongylus axei*, 38.3% were *Trichostrongylus colubriformis*, 6.1% were *H. contortus*, 5.8% were *T. circumcincta*, 2.50% were *Cooperia* spp. 0.16% and *Chabertia ovina*; *Trichostrongylus vitrines*, *Oesophagostomum columbianum* were collectively less than 0.5% of the total (Mederos et al., 2010).

This species diversity was not seen in a 2007-2010 study (Grosz et al., 2013) involving the same flock of ewes used in the present study. At that time in the flock, PCR analysis of trichostrongyle eggs from 63 tested ewes showed that all contained *Haemonchus* sp., and only 1 ewe (1.6%) was positive for any trichostrongyle genera other than *Haemonchus* sp.; this ewe contained *Teladorsagia* sp. in addition to *Haemonchus*. Prior to this 2007-2010 study, an aggressive deworming system had been used on this flock over many years (Grosz et al., 2013). This and a growing anthelmintic resistance within the *Haemonchus* population was likely responsible for the virtual lack of any other trichostrongyle species *Haemonchus* sp. After 2010, lambs were no longer grazed with ewes, and a rotational grazing system was initiated which decreased the need for regular anthelmintic use, enabling them to be used primarily for treating ewes showing symptoms of haemonchosis. By the present 2014 study, both *Trichostrongylus* and *Teladorsagia* had emerged back into the flock, accounting for a combined 15% of the PCR products in ewes prior to triple treatment.

During the first year of the South Dakota study, the *Trichostrongylus* population appeared to be more significantly diminished by the combination treatment and rotational grazing system used during the study, but it rebounded effectively after that. By the end of the study period, 100% of the ewes were still infected with *Haemonchus* sp., but almost 90% also contained *T. colubriformis* with a few containing *T. axei*. Though not verified, the *Teladorsagia* species identified with PCR in this study was almost certainly *T. circumcincta* based upon other studies from this region (Mederos et al., 2010); likewise, the *Haemonchus* species was almost certainly *H. contortus.* During 2014 and 2015, *Teladorsagia circumcincta* were much more prevalent during the fall season than

during the spring. During the final 2 years, samples were only collected during the spring, so the fall versus spring comparison for *T. circumcincta* could not be made, however, the spring percentage of *T. circumcincta* remained low and very low respectively during these spring seasons. By the end of the study, *T. axei* appeared to account for about half of the collected eggs, and *H. contortus* accounted for the other half.

In colder climates where lambing is generally scheduled to occur during the spring season, egg production by sheep trichostrongyles is highest during the spring due to the combined effects of a seasonal and periparturient release of hypobiotic juveniles to develop into egg-producing adults (Gibbs, 1986). Prior to the spring treatment, more than 85% of the ewes in the present study were excreting trichostrongyle eggs, and their pretreatment mean FECs was very high; about three times higher than the spring mean FECs from the 31 ewe flocks used in an Ontario and Quebec study (Mederos et al., 2010). However, it is likely that some of the individual flocks in the Canadian study would have mean FECs similar to the South Dakota herd. As with most parasites systems, the trichostrongyles in the present study were over-dispersed within members of the flock, such that about 65% of the eggs were being excreted by about 20% of the ewes. This is slightly less than many human parasites, in which less than 20% of the population typically contains 80% of the worms (Wilson et al., 2002). A common approach to measuring the level of population aggregation within a group of hosts is to calculate the corrected moment estimate of *k* (CMEK). The distribution of most parasite populations are best described by the negative binomial distribution where CMEK is roughly one or less (Wilson et al., 2002). In the present study *k* was significantly below one, and lower

than the *k*-values reported thus far for sheep (Barger et al., 1984; Sreter et al., 1994), for goats (Rinaldi et al., 2009), and for bison (Eljaki et al., 2016). Because of this high level of aggregation in the flock used for this study, the elimination of trichostrongyles among the 12% most heavily infected ewes would cut egg contamination in the pasture by approximately half. Several studies have shown that younger ewes generally contain more trichostrongyles than the older ewes (Colditz et al., 1996; Good et al., 2006; Selemon, 2018). This trend seemed to also exist in the present study, though the differences in FECs were only statistically significant between the 2-year-old and 5-year-old ewes. In this flock, treatment of only the 2 and 3-year-ewes would have diminished pasture contamination by over 65%.

Treating sheep and goats with a combination of anthelmintics with differing modes of action has become a valuable tool for combating the growing levels of anthelmintic resistance present in many populations of ruminant trichostrongyles throughout the world (Bartram et al., 2012; Lanusse et al., 2018). However, even the combination treatments are not always effective if the level of resistance for each of the anthelmintics is too high (Kaplan and Vidyashankar, 2012). The triple-treatment approach used in the present study, consisting of commercially available products representing 3 different classes of compounds (i.e. benzimidazole, milbemycin and imidazothiazole compounds), provided a very effective 99.99% reduction in mean FEC, down to where only 1 in 6 ewes would be excreting a single trichostrongyle egg from a population of *Haemonchus* that had shown some resistance to albendazole and doramectin (Grosz et al., 2013). This reduction was comparable to some combination studies and much higher than many of the other studies, depending on the level of resistance and the anthelmintic combinations being

used (Baker et al., 2012; Holsback et al., 2016; Leathwick et al., 2012; Miller and Craig, 1996). Following the first triple treatment, 238 out of the 249 ewes had FECs below 1 EPG, and the remaining 11 ewes were retreated and retested a few weeks after the initial treatment. Surprisingly, the FECs for three of these ewes were higher than results from the first testing, and one was considerably higher. A third triple treatment reduced the FECs to below one EPG for the first three ewes, but didn't for the fourth ewe, which was not included in the study. The post-treatment eggs could have come from adults that were not killed by the treatment, but they also might have come from adults that had survived treatment as hypobiotic fourth-stage juveniles, and then matured to fertile adults before the ewes were retested.

During the first rotation through the first five paddocks, the mean FECs remained below 5 EPG, except for the first sampling period which was two weeks after release into the first paddock and approximately 5-6 weeks post-treatment. For this first period, one of the samples contained 150 EPG, while the other 19 samples were very low (1 EPG or lower). Even while rotating the second time in the first two paddocks, the EPG remained very low and the number of ewes excreting more than one EPG remained below 30%. These observations are consistent with a previous finding that *H. contortus* third-stage juveniles are not able to survive during South Dakota winters while on pasture, and that at least some J4s were more resistant to anthelmintic treatment than the adult stage (Grosz et al., 2013). Two weeks after the ewes started their second rotation through the paddocks, egg shedding increased linearly for the next four weeks, and plateaued during the first week in October (Week 14). At this point, all of the samples contained at least one trichostrongyle egg, and the mean FEC had reached the minimum detection limit of

50 EPG used in the Sweden eradication study by Waller et al. (2006). During these four weeks, ewes were grazing through some paddocks for the first time and also other paddocks for the second time. For the remainder of the grazing season, ewes grazed through paddocks a third time, and yet FECs remained similar through this time, the likely result of ingested juveniles entering hypobiosis in the ewes instead of progressing to the adult stage (Capitini et al., 1990). By the end of the first grazing season, it is very likely that all of the ewes had become infected with the trichostrongyles, and almost 18% of them surpassed the minimum detection limit of 50 EPG used in the Sweden eradication study by Waller et al. (2006). These South Dakota results are, therefore, not consistent with the Sweden study, and the most significant difference between the two studies is likely the level of resistance among the population of *H. contortus* in the South Dakota herd. While the triple treatment effectively collapsed the adult population, enough more highly resistant arrested J4s must have survived treatment such that the population of *H. contortus* was able to get re-established by the end of the season.

During the spring 2015 lambing season, FECs were about twice as high as they were at the end of the fall 2014 grazing season even though they were only one-tenth that of the pretreatment counts during the spring 2014 lambing season. Because of these significant FECs, all of these ewes were again treated with levamisole prior to turn-out that year. With the additional treatment and continued rotational grazing, FECs during that fall were the lowest values of the study. Yet in spite of this very low fall egg shedding among the ewes at the end of the grazing season, egg-shedding increased by more than 220 fold by the next spring lambing season, and were similarly high during the spring 2017 lambing season. It is difficult to explain the apparent massive population

expansion from Fall 2015 to spring 2016. It is possible that FECs from the fall of 2015 significantly underestimated the actual population levels of *H. contortus* because a much higher percentage of incoming J3s didn't mature to adults (and hence a low FEC during that time), but rather remained as arrested juveniles to come out during the spring 2016 lambing season. Regardless of the biological explanation for this rapid massive increase, the results demonstrate how quickly *H. contortus* populations can rebound even within a cold winter temperate climate after an effective collapse of the adult population and with a robust rotational grazing system.

Chapter 3: Anthelmintic Activity of *Melilotus alba* (White Sweet Clover) on *Haemonchus contortus* Infective Juveniles and its Cytotoxicity on Bovine Ileal Epithelial Cells

3-1. Abstract

There is growing interest in finding natural plant products for use as alternatives to commercial anthelmintics for controlling *Haemonchous contortus* in pastured sheep and goats. A previous study revealed that methanol extracts from *Melilotus alba* (white sweet clover) possessed anthelmintic activity in egg-hatch and larval migration assays involving *H. contortus.* However, no anthelmintic activity was identified when freshly harvested, mature second-year *M. alba* plants (predominantly stems and pods) were fed to yearling ewes naturally infected with *H. contortus*. To understand these conflicting results, the anthelmintic activity of methanol extracts from different sweet clover plant parts (i.e. leaves, stems, pods) were measured using a larval migration assay involving unsheathed third-stage *H. contortus* larvae. Stems and pods showed no anthelmintic activity, while 97.3% migration inhibition was measured in the leaf extract at 30 mg/ml. An aqueous extract of the leaves (concentration of 670mg/ml water) inhibited migration by 98% after 24 hrs, and no motility was observed after 48 hrs. Inhibition was higher at an acidic pH (e.g. pH of 3 and 5) than at a neutral pH (e.g. 7.4). Cytotoxicity of the aqueous leaf extract was measured with unpolarized bovine ileal epithelial cells at 5 differing concentrations (670, 340, 134, 67, and 17 mg/ml) of the extract using an absorbance-based AlamarBlue assay. After 48 hr treatments, extract concentrations higher than 134 mg/ml were 100% lethal to cells; 11.4% of the cells died at 17 mg/ml. These *in vitro*

studies indicated that *M. alba* leaves contain one or more compounds with anthelmintic activity that is soluble in methanol and water, but that these compounds are also cytotoxic to unpolarized intestinal cells. Future *in vivo* studies should focus on young first-year plants that are composed primarily of leaves, but ewes should also be carefully monitored for any toxicity problems.

3-2. Introduction

Haemonchus contortus is one of the gastrointestinal nematodes (GIN) negatively affecting the profitability of the small ruminant industry (Githigia et al., 2001; Hoste et al., 2005; Jabbar et al., 2006). The problem of anthelmintic resistance among the commercial anthelmintics used to control *H. contortus* is discouraging the use of these compounds globally (Jackson and Coop, 2000). The residual chemical compounds left in the tissues from these anthelmintics is another concerning factor among consumers who are looking for organic products free of any residues from these synthetic drugs. These factors are encouraging researchers worldwide to find alternative natural anthelmintics to control GIN infestation (Waller and Thamsborg, 2004).

The presence of secondary metabolites, rich in bioactive substances, that can have active roles in an animal's metabolism and physiology, was an inspiring factor for many researchers to study the parasiticidal activity in medicinal plants (Marcin et al., 2006). Many published studies have revealed the presence of certain metabolites with anthelmintic activities including studies published in Asia and Africa (Akhtar and Riffat, 1984; Al-shaibani et al., 2009; Githiori et al., 2006; McGawa et al., 2007; Qadir et al., 2010), Australia (Kotze et al., 2009), Europe (Hoste et al., 2006; Manolaraki et al., 2010), North America (Acharya et al., 2014; Fernandez-Salas et al., 2011; Pessoa et al., 2002)

and South America (Monteiro et al., 2011; Souza et al., 2008). The secondary products in these studies were extracted and concentrated using solvents with differing levels of polarity (Borges and Borges, 2016). Benzene, hexane, pentane, and toluene are nonpolar solvents; while acetone, acetonitrile, ethanol, isopropanol, methanol, methyl ethyl ketone, n-propanol and water are examples of polar solvents. Dimethyl sulfoxide (DMSO) is an important polar solvent which dissolves both polar and nonpolar compounds and is miscible in a wide range of organic solvents as well as water; therefore, DMSO is widely used to initially dissolve plant extracts before adding them to another solvent, particularly for aqueous solutions.

Nematode juveniles or eggs exposed to the compounds in the medicinal plants have been used in *in vitro* experimental systems to screen for nematocidal efficacy within the secondary products of these medicinal plants (Borges and Borges, 2016). Once *in vitro* screening tools recognize parasiticidal activity within these extracted products, then *in vivo* studies involving infected hosts are used to evaluate their anthelmintic activities under more natural conditions. Many of the *in vitro* screening studies involve *H. contortus* eggs and/or third-stage juveniles (J3s), while many of the *in vivo* studies involve *H. contortus* adults in sheep or goats due to this parasite's prevalence, its economic importance, and its growing levels of anthelmintic resistance (Lanusse et al., 2016). Some of these *in vivo* studies have used fresh plants to evaluate anthelminthic activity, but most studies have used concentrated extracts from the plants. Some studies have tested the anthelmintic efficacy of these secondary products by counting adult worms in the necropsied host after a treatment period; however, a fecal egg count reduction test (FECRT) is more commonly used because it's a less expensive, indirect

method for measuring anthelmintic effects (Borges and Borges, 2016; Carvalho et al., 2012; Eguale et al., 2007a, b; Heckendorn et al., 2007; Max, 2010; Waghorn et al., 2006).

Recently, Acharya et al. (2014) used an egg hatch assay (EHA) and a larval migration assay (LMA) involving *H. contortus* to screen for anthelmintic activity in methanol extracts from 40 plants that are native or naturalized within the U.S.A. Northern Great Plains. Of the 40 methanolic extracts screened, 7 (*Chrysothamnus viscidiflorus*, *Ericameria nauseosa*, *Liatris punctata*, *Melilotus alba*, *Melilotus officinalis*, *Perideridia gairdneri*, and *Sanguinaria canadensis*) showed significant egg-hatch inhibition. These 7 plant extracts also showed inhibitory activity in the LMA, with *E. nauseosa* and *R. aromatica*. showing the highest activity. Even though these two species had higher activity, *Melilotus alba* was chosen for the present study because it is much more palatable to livestock than *Chrysothamnus viscidiflorus*, *Ericameria nauseosa*, *Liatris punctata*, *Perideridia gairdneri*, and *Sanguinaria canadensis* brush , and also *M. alba* was more abundant in the study area than *M. officinalis*.

For the present study, the *in vitro* anthelmintic activity of *Melilotus alba* (White Sweet Clover) methanol and water extracts were compared to determine if the active component(s) in the methanol extracts were also soluble in the type of aqueous condition present in the ruminant digestive system.

The larval migration assay (LMA) and egg hatch assay (EHA) are cheap and easy to use, and both have been used by many researchers to measure the nematocidal activity in *in vitro* assays (Acharya et al., 2014; Al-shaibani et al., 2009; Coles et al., 2006; Marie-Magdeleine et al., 2010). The experiment model used in this study followed the LMA

techniques used in similar studies (Acharya et al., 2014; Bachaya et al., 2009; Diehl et al., 2004).

In order to determine the safety of using the aqueous extract of *M. alba*, its cytotoxicity effects were evaluated with unpolarized bovine ileal epithelial cells at 5 differing concentrations (670, 340, 134, 67, and 17 mg/ml) of the extract using an absorbance-based AlamarBlue assay.

3-3. Materials and Methods

3-3.1. Harvesting Haemonchus contortus infective juveniles (J3s)

In this study, fecal samples from naturally-infected yearling ewes that were heavily infected with *H. contortus* were harvested from a farm, containing approximately 1000 head, in the north-eastern portion of Brookings County, South Dakota (Acharya et al., 2014). The specimens were collected during August to October of 2013, and the fecal samples, containing high numbers of *H. contortus* eggs, were cultured for 7 to 10 days at 27°C before the J3s were collected and stored in 15-20 ml of tap water, where kept in Petri dishes at 4°C until used (less than 5 days).

3-3.2. Plant material collection

Sweet clover plants were collected from different areas in South Dakota including two farms in the southern and northern parts of Brookings County from August to September 2013. The plants were kept intact in 4 liter plastic bags and stored at -20°C until further use.

3-3.3. Preparation and Dilution of the Sweet Clover Extracts

The methanol extracts were prepared by adding 5 grams each of frozen sweet clover leaves, pods and stems to 50 ml methanol, homogenizing for 30 seconds, and then

incubating the homogenate in a covered 100 ml glass container on a shaker for 48 hrs. at room temperature. The extracts were collected in 50 ml conical centrifuge tubes and concentrated in a rotary evaporator at 40°C under reduced pressure for 10 minutes; and the concentrated extracts were covered by a perforated parafilm and stored at -20°C prior to lyophilization. The concentrate was lyophilized over-night in a Virtis AdVantage 2.0 BenchTop Freeze Dryer/Lyophilizer. Different concentrations of sweet clover (1, 2.5, 5, 10, 20, 30 and 40 mg/ml) were used in the LMA. The extracted powder was dissolved in a phosphate buffer saline (PBS, 0.05 M, pH 7.2) with and without 0.5% dimethylsulphoxide (DMSO). In addition, the frozen sweet clover extracted powder was dissolved in saline (pH 3 and 5) with and without 0.5% DMSO. DMSO was used since it is a polar aprotic solvent whereas both polar and nonpolar compounds were easily dissolved in it and it was miscible in different organic solvents as well as water.

The water extracts were collected by grinding 60 grams of frozen sweet clover leaves (SCL) that had been harvested from 140 gm of whole sweet clover plants, and gradually adding it into 90 ml of distilled water. Thereafter, the mixture was transferred to four 50 ml centrifuge tubes and centrifuged (30 min at 300 × g) at 4° C. The supernatant (670 mg/ml, pH 5.94) was then transferred to 2 ml tubes and kept at -20°C until use. Three different concentrations (67, 335 and 670 mg/ml) were examined using saline as the diluent.

3-3.4. Juvenile migration and motility assays

The infective stage (J3s) of *H. contortus* were collected after culturing eggs for 7 to 10 days (Rabel et al., 1994). Stored J3s were exsheathed with sodium hypochlorite (1%) for 15-18 minutes and then washed 3 times in PBS for 3 min via 300 x g

centrifugation conditions (Demeler et al., 2010). After the exsheathment, 200 µl of the larval suspension containing 40-100 L3s along with 200 µl sweet clover extract at the different concentrations were incubated for 24 hrs. in 48 well plates at 27°C. After the 24 hrs. incubation, the mixtures were pipetted into 96 well plates fitted with sieves (MilliporeSigma™ MultiScreen™-Mesh Filter Plates with 96-Well Receiver Plates (Cat. Num. MANMN2010, 20µm, Burlington, Massachusetts), and then incubated for another 24 hrs. at 27°C. After the incubation, the sieve plate was removed and the number of L3s that passed the sieves were counted in each well.

Motility of J3s was also measured after the first 24 hr incubation period, and the percent-inhibition was determined using the following formula: inhibition (%) = (A−B)/(A) × 100. where, A = % of J3s that migrated in the negative control, and B = % of J3s that migrated in different concentrations of extracts.

5) Cytotoxicity:

5.1) Plant preparation:

3-3.5 *Cytotoxicity Evaluation*

The previously water-extracted *M. alba* (White Sweet Clover) leaves (670 mg/ml) were used to evaluate the cytotoxicity of this extract. The extract-containing sample was prepared by lyophilizing 7.5 ml of the SCLE (containing 670 mg/ml) and diluting the resultant powder into Dulbecco's Modified Eagle Medium (DMEM) media containing Fetal Bovine Serum (FBS) to make a stock solution of 1340 mg/ml SCLE. The newly made solution was filtered (with a 20 µm sterilizing filter), and then five different concentrations (670, 340, 134, 67, and 17 mg/ml) were used immediately for the study.

Normal bovine ileal epithelial cells (BIECs) were used in the cytotoxicity evaluation of *Melilotus alba* leaf-extract. These cells were grown in *IPEC-J2* culture media consisting of DMEM supplemented with 200mM L-Glutamine, epidermal growth factor (1000x), ITS (1000x), Antibiotics (100x) in a 5% CO_2, 95% humidity and 37 °C incubator.

Cell viability was measured with Alarmable™ Cell Viability Assay kit (Thermo Scientific) according to the manufacturer's recommended protocol, For this alamarBlue assay, viability of the mammalian cell lines was measured through the use of a sensitive, reliable and rapid colorimetric/fluorometric growth indicator consisting of resozurin which is blue in color when in the oxidized state. It will be reduced to a fluorescent dye (resofurin) having a red color when it accepts electrons from $FADH_2$ and NADH in the electron transport chain. This change can be easily identified by measuring either changes in absorbance or fluorescence in a spectrophotometer.

For the assay, the culture media was removed from BIECs growing in T-25 flask, and the cells were washed once with 5 ml phosphate buffer saline (PBS) and then trypsinized through the addition of 0.5 ml Trypsin-EDTA and with an incubation for 10-15 min at 37 °C temperature and 5% CO_2 to detach the cells from the flask. After incubation, the detached cells were washed with 5 ml of DMEM media not containing FBS, and cell suspension was centrifuged for 5 min at 300 × g. The resultant pellet of cells was re-suspended in 5 ml complete media (containing FBS). Viability of the cells was measured through the addition of 40 µl tryptan blue to 40 µl of cell suspension. Tryptan blue penetrates the cytoplasmic membrane of dead cells and stains the cytoplasmic organelles blue which then can be seen and counted in a haemocytometer.

The measured viability was 93.5%, and these cells could be used because viability was greater than 90%. Total cells were then counted and diluted such that 10,000 cells in 200 µl media could be seeded in each of the 96 wells for the plate. These cells were then incubated at 37 ℃ temperature and 5% CO_2 for 18 hr. to allow the cells to attach to the bottom of each well. After the incubation, cells were washed with 200 µl PBS to remove any dead cells, and then 200 µl of the extract at the five different concentrations (670, 340, 134, 67, and 17 mg/ml) were transferred to each well in triplicate for each concentration, and incubated for another 48 hrs. at 37 ℃ temperature and 5% CO2. After the incubation, the media was removed from each well, and washed 3 times with 200 µl PBS. Complete media (180 µl) was then added to each well, except that of the positive control wells which received 200 µl of 70 % ethanol, and the cells were incubated for another 10 min at previously mentioned conditions. The two positive control wells were washed and 180 µl of complete media was added to those two wells. Finally, 20 µl of alamarBlue reagent was added to all wells in an amount equal to 10% of the well volume, and the plate was incubated for another 4 hrs before the fluorescence was measured in each well at an excitation wavelength of 528 nm and emission wavelength at 620 nm in a BioTek Synergy 2 Multi-detection Microplate Reader. Readings from the blank were subtracted from the experimental readings to get the actual readings of the viable cells.

3-3.6 Statistical analyses

Microsoft Excel was utilized to create the bar graphs by plotting the mean of each group supported by its standard error. The groups data of each experiment was then analyzed for their statistical differences using PAlentological STatistics (PAST) Version 3.23 Øyvind Hammer, Natural History Museum University of Oslo 1999-1919 (Hammer

et al., 2001) (https://folk.uio.no/ohammer/past/). Analysis Of Variance (ANOVA) was used to test the equality of the means among the groups, statistically significant results obtained by ANOVA was followed by multiple comparison analysis tests using Tukey's HSD (honestly significant difference) test. The ED50 values of the extracts was calculated using APA AAT Bioquest, Inc. (2019, April 03). Quest Graph™ ED50 Calculator.". Retrieved from https://www.aatbio.com/tools/ed50-calculator

3-4. Results

3-4.1. Effect of white sweet clover on the migration of *H. contortus* juveniles:

3.4.1.1. Effect of leaves, stems and pods on the migration of *H. contortus* J3s

Sweet clover plants were found to consist of 45% stems, 33% pods and 11% leaves by weight. Extracts from each of these three components caused different levels of larval migration inhibition; the mean larval migration inhibition for these extracts of the *M. alba* ranged from 5.57 - 97.26% (Figure 3.1). The three portion extracts showed different anthelmintic activity whereas the leaf-extract inhibited 97.26%, the stem-extract 19.46%, while the pods-extract had the lowest effect with 5.57% of the J3 passing through the sieves at 50 mg/ml. Based on the percentage of migration inhibition with 50 mg/ml the most effective portion of the white sweat clover was the leaf-extract which had highly significant effect ($p= 0.005$) compared to the negative control and also had significant effect ($p= 0.019$) compared to the pods extract, but was not significant ($p>0.05$) to the stems extract, while the stems and pods extracts showed no significant effect ($p>0.05$) compared to the negative control. The three different portions of *M. alba* were extracted using the methanol. The ED50 value obtained for *M. alba* leaf-extract was 8.28 mg/ml. Levamisole inhibited 100% larval migration at 0.01 g/ml.

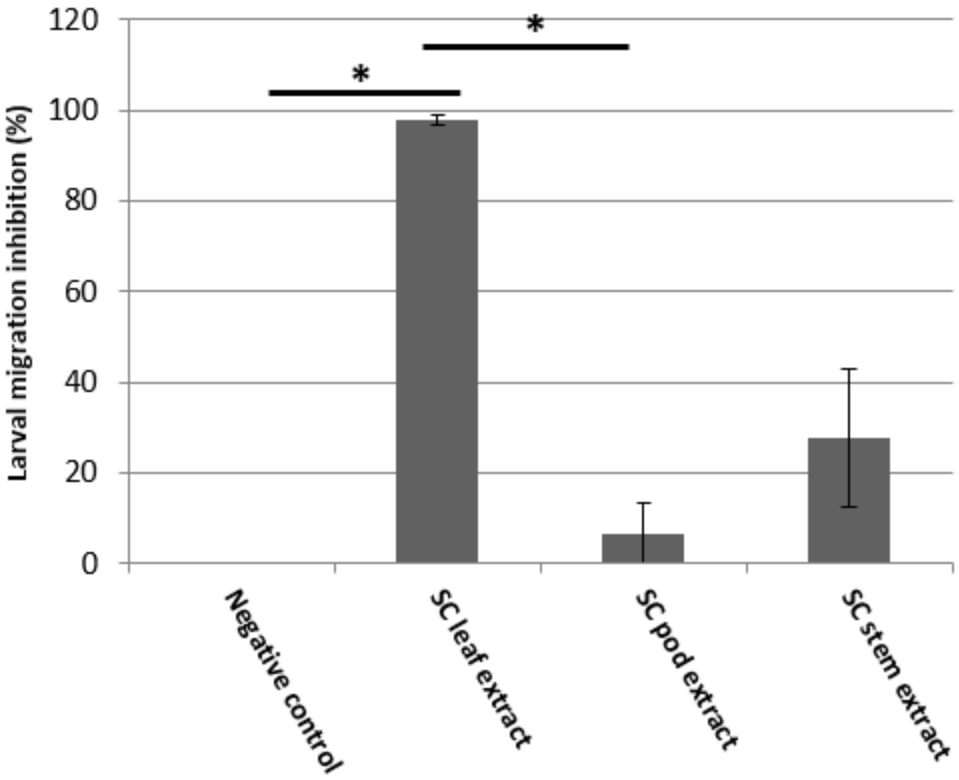

Figure 3.1 Effect of white sweet clover stems, leaves and pods on *H. contortus* larval migration. Inhibition rate was significantly different in leaf-extract compared to pods. * = p≤0.5

3-4.1.2. Concentration effect for methanol extract from sweet clover leaves on H. contortus exsheathed 3rd stage juvenile migration

The inhibition of larval migration mean percent of the five-different concentrations of *M. alba* methanol extracts is shown in (Figure 3.2). The mean percent inhibition of larval migration of the four different concentrations of leaf-extracts ranged from 23.77% to 97.3%. The percentage of larval migration inhibition, was similar, 21.4% and 23.77%, in both negative control and the 1 mg/ml diluent respectively while the inhibition was increasing with increasing the extract concentration whereas the highest inhibition rate, 97.3%, was at 30 mg/ml while the other three different concentrations (5 mg/ml, 10 mg/ml, and 20 mg/ml) revealed 28%, 73.9%, and 87.7% larval inhibition respectively.

Based on the percentage of larval migration inhibition with 30 mg/ml the most effective concentration of the white sweat clover leaf-extract which had very significant effect (3.87E-06) compared to the negative control, followed by 20 mg/ml and 10 mg/ml

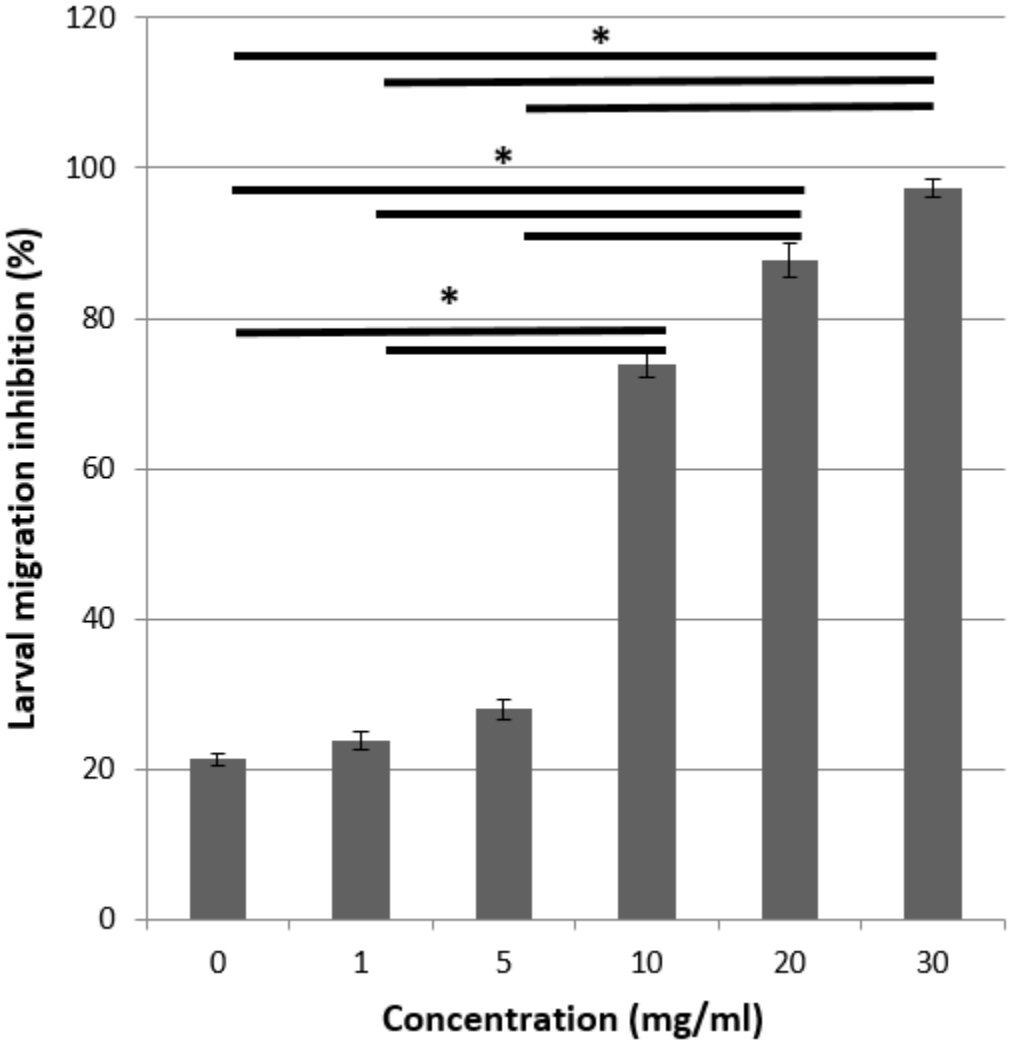

Figure 3.2 Effect of five different concentrations of *Melilotus alba* methanol extracts (diluted in 0.5% DMSO) on the *H. contortus* 3rd stage larval migration rate. The higher concentrations showed significant effect compared to the negative control. * = p≤0.5

whereas both of them showed also a highly significant effect, p= 0.0002276 and 0.007536, respectively compared to the negative control, while both 5 mg/ml and 1

mg/ml had no significant effect (p >0.05). The ED50 value obtained for *M. alba* leaf-extract was 8.28 mg/ml. Levamisole inhibited 100% larval migration at 10 mg/ml.

3-4.1.3. Effect of pH of methanol extract of white sweet clover leaves on H. contortus exsheathed juvenile migration

The results showed that the larval migration inhibition of the 30 mg/ml white sweet clover extracted in methanol ranged from 45.64 to 81.51% when diluted in DMSO and 46.37 to 72.48% without DMSO (Figure 3.3). Adding the DMSO provided no significant

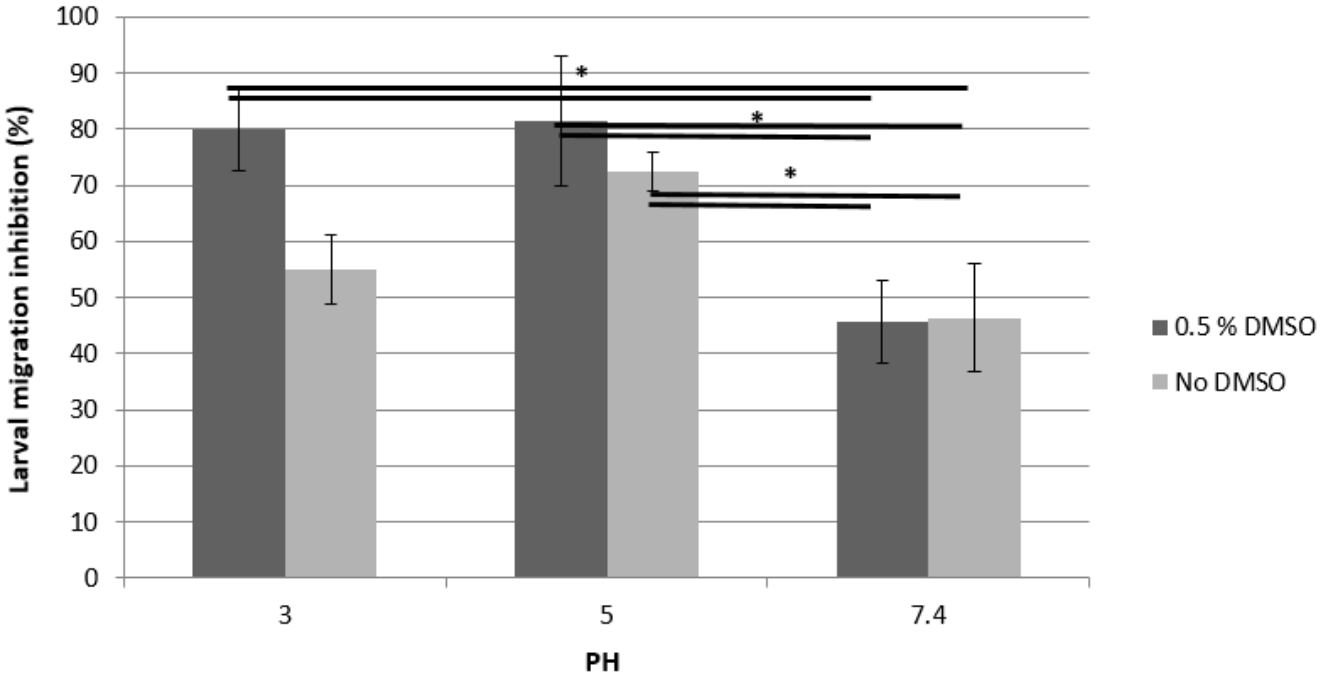

Figure 3.3 Effect of pH of *Melilotus alba* methanol extracts (diluted in 0.5% DMSO and not) on the *H. contortus* 3rd stage larval migration/mortality rate. * = p≤0.5

larval migration inhibition (p>0.05) at the three tested pH 3, 5 and 7.4, 80.1%, 81.51% and 45.64% respectively, compared to 55.03%, 72.48% and 46.37% extracts without DMSO also at pH 3, 5 and 7.4 respectively. At pH 3 there was significant difference

($p<0.05$) between the extract with DMSO, 80.1%, and the one without the DMSO, 55.03%. The pH seems to play an important role in larval migration activity at the lower concentrations (3 and 5) which increases the inhibition significantly compared to the extracts with and without the DMSO, whereas the extract with DMSO at pH 3 showed significant different ($p=0.01776$ and $p=0.02178$) compared to DMSO and without DMSO extracts at pH 7.4 respectively, but it was not significant ($p>0.05$) compared to the DMSO and no DMSO extracts at pH 5. While both extracts at pH 5 also showed significant difference, the DMSO extracts revealed ($p=0.01776$ and 0.02178) compared to DMSO and without DMSO extracts at pH 7.4 respectively, and no DMSO extracts revealed ($p= 0.03895$ and 0.04678) compared to DMSO and without DMSO extracts at pH 7.4 respectively. Levamisole inhibited 100% larval migration at 0.01 g/ml.

3-4.1.4. Effect of water extract of white sweet clover leaves on the migration of *H. contortus* exsheathed 3rd stage juveniles

The inhibition of larval migration mean percent of the three different concentrations of *M. alba* water extracts is shown in (Figure 3.4). The mean percent inhibition of leaf-extracts ranged from 7.07% to 97.42%. The percentage of larval migration inhibition at 67 mg/ml was less than the negative control (14.36%), while the larval inhibition was increasing with increasing the extract concentration whereas the highest inhibition rate, 97.42% was at 670 mg/ml while the other different concentration 335 mg/ml revealed 49.29% larval inhibition. Based on the percentage of larval migration inhibition, the 670 mg/ml, the most effective concentration of the white sweat clover leaf-extract, had very significant effect ($p=2.24E-09$) compared to the negative control, followed by 335 mg/ml

showed also a significant effect (p= 0.0006497) compared to the negative control, while 67 mg/ml had no significant effect (p>0.05). The ED50 value obtained for *M. alba* leaf-extract was 345 mg/ml. Levamisole inhibited 100% larval migration at 10 mg/ml.

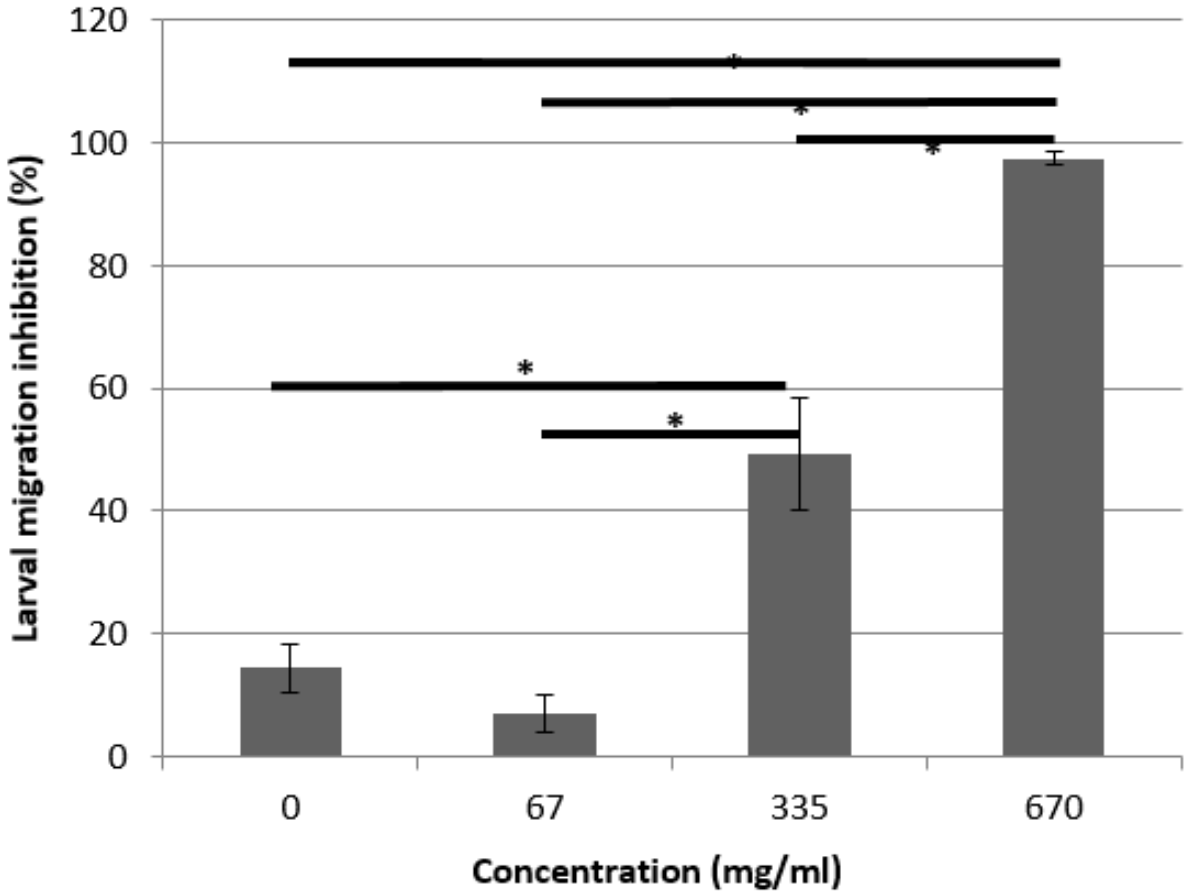

Figure 3.4 Effect of different concentrations of *Melilotus alba* water extracts on the *H. contortus* 3rd stage larval migration. The higher concentrations showed significant effect compared to the negative control. * = p≤0.5

3-4.2. The effect of SCL different concentrations extracted in water on non polarized BIECs cytotoxicity

The AlamarBlue assay was used to measure the cytotoxic activity of *M. alba* leaf-water-extracts on BIECs. After 48 hrs. treatment the cytotoxicity mean results ranged

from 11 to 100% whereas the effect was very toxic at the higher concentrations with 100% cytotoxicity at 1340, 670 and 340 mg/ml, while the cytotoxicity results were 97% and 72% at 134 and 67 mg/ml of SCL water extract. The lowest concentration of M. alba leaf- water-extract, 17 mg/ml, killed 11% of the cultured cells (Figure 3.5). Extract used concentration response curve on the viability of BIECs with ED50 value 54 mg/ml *M. alba* leaf-aqueous-extract. The inhibition cell growth was not monitored in the negative control well while the cytotoxic effect was 100% in the observed positive control well. Results from the AlamarBlue assay were expressed as the viable cells percentage in treated wells compared to the untreated controls.

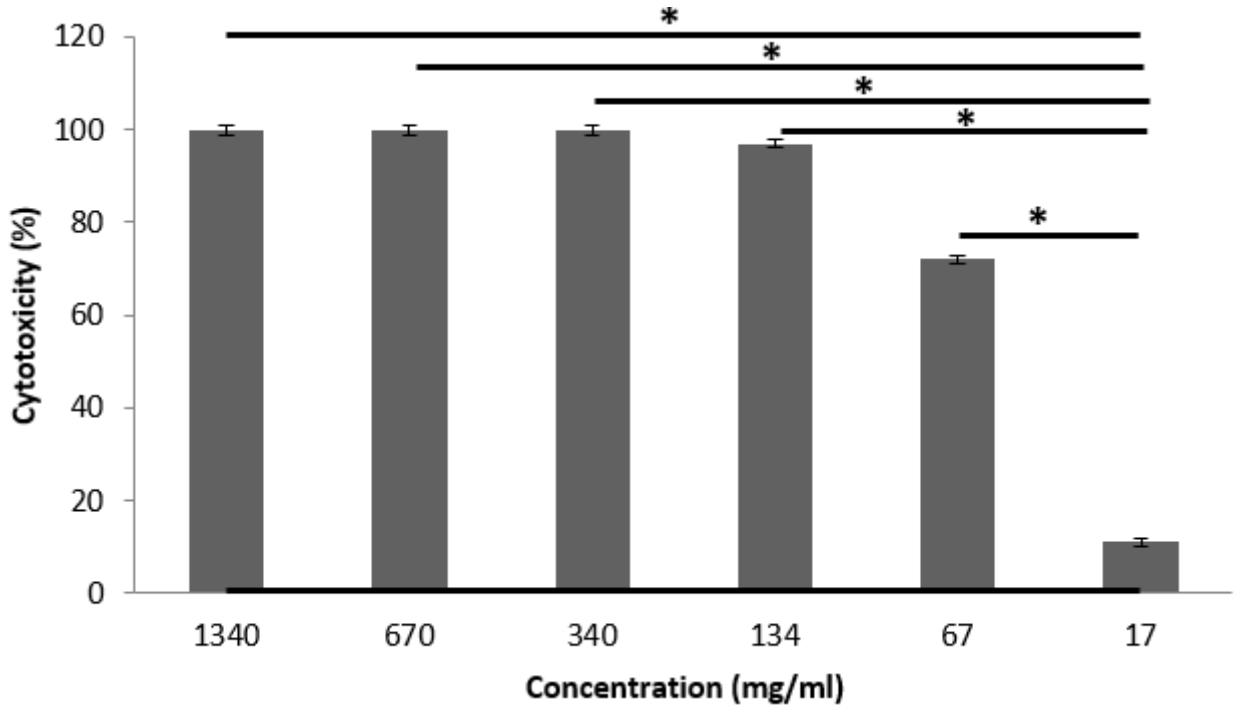

Figure 3.5 Percentage cytotoxicity of BIECs with different concentrations of *M. alba* leaf-water-extract in AlamarBlue assay. Cells were incubated with six different concentrations (1340 – 17 mg/ml) of the extract. * = p≤0.5

Discussion

The results from this study were very compatible to a previous study involving the *in vitro* effects of *M. alba* methanol extracts on *H. contortus* eggs-hatching and juvenile migration (Acharya et al., 2014). The present study found at the highest concentrations of *M. alba* leaves both the methanol and aqueous extracts inhibited migration by 97 % compared to an inhibition of 19.46% and 5.57% J3 migration inhibition using methanol stems and pods extracts respectively. So, the negative effect of *M. alba in vivo* study on fecal egg output could be due to an inadequate amount of leaves being used in the feeding experiment which were confirmed by this *in vitro* study findings whereas the inhibitory rate was significantly higher in leaf-extract compared to stems and pods. This finding was also studied by other researchers (Diehl et al., 2004; Nikolic and Zlatkovic, 2010) who proposed that the amount of plants secondary metabolites determines the inhibitory activity of plant extracts and thus the presence of these metabolites can differ depending on the growth stage of the plant, type of plant tissues, and the growing conditions of the plant when collected. All these findings explained the negative results of the *in vivo* study, Chapter 4, whereas most of the fresh plant material given to the ewes contained stems of second-year plants. Several previous studies (Ketzis et al., 2002; Barrau et al., 2005; Macedo et al., 2009) revealed the anthelmintic activities of different secondary metabolites such as flavonoids (non-tannins) and alkaloids found in the medicinal plants. The presence of these essential oils, flavonoids (non-tannins) and alkaloids were found in different medicinal plants including *M. alba, M. officinalis, E. nauseosa L. punctata, S. canadensis C. viscidiflorus, P. gairdneri,* and *E. nauseosa* (Wagner et al., 1973; Giannasi and Chuang, 1976; Nicollier and Thompson, 1982; Carr et al., 1986; Hegerhorst et al.,

1987; Mead et al., 1992; Stevens et al., 1999; Sutiashvili and Alaniya, 1999; Newton et al., 2002; Ahmed et al., 2006; Hegazy et al., 2007; Tabanca et al., 2007; Anwer et al., 2008). This study showed that the highest anthelmintic activity was found in the *M. alba* leaf-extracts which might be due to the presence of tannins which was identified in the leaves of other plants including *A. uva-ursi, G. viscosissimum, L. corniculatus, R. glabra* and *R. aromatica* (Buziashvili et al., 1973; Bate-smith 1980; Lowther et al., 1987; Saxena et al., 1994; Marshall et al., 2008; Min et al., 2008; Pegg et al., 2008; Amarowicz et al., 2009); while other researchers (Molan et al., 2003; Paolini et al., 2003; Hoste et al., 2006; Ekbote et al., 2010) mentioned the parasiticidal efficacy of tannins.

In this study, *M. alba* leaf-methanol-extracts were also tested at higher concertation, 30 mg/ml, using DMSO as a diluent and without DMSO and the anthelmintic activity was measured at three different pH levels (3, 5, and 7.4), whereas the results revealed higher larval migration inhibition at lower pH (3 and 5), while adding DMSO did not affect the inhibition. Acharya et al. (2014) also studied the effect of DMSO used in extracts of eight plants (*C. viscidiflorus, E. nauseosa, G. viscosissimum, L. punctata, M. alba, M. officinalis, P. gairdneri,* an *R. aromatica*) at their highest concentration (50 mg/ml), and MOPS used in extracts of six plants (*C. viscidiflorus, E. nauseosa, L. punctata, M. alba, M. officinalis,* and *P. gairdneri*), the results revealed 100% inhibition in the DMSO and MOPS at their highest concentration (50 mg/ml). This study finding was compatible with Acharya et al. (2014), but they suggested that the pH of the diluents has negative effect on the secondary metabolites like essential oils, alkaloids and flavonoids which was opposite to our findings whereas the highest J3 migration inhibition was at pH 3 and 5. The relationship between the pH level and anthelmintic

activity might be due to complex formation between certain tannins and proteins at lower pH. This theory was proved before by Kallithraka et al. (1997) who found that the protein binding capacity of tannins reached the peak when the wine pH is 3.2-3.8. Acharya et al. (2014), suggested that the inhibition of *H. contortus* egg-hatching at lower pH using the DMSO were probably associated with the presence of tannin metabolites in plant extracts. Marie-Magdeleine et al. (2009) observed that some plant metabolites have similar chemical structures of anthelmintic compounds that affect the nematode nervous system. This finding could explain the nematocidal activity of *M. alba* leaf-extracts which might possess secondary metabolites affecting the parasite nervous system.

The *in vitro* cytotoxicity results of the *M. alba* leaf-aqueous-extracts revealed significant BIECs toxicity at the highest concentrations. These findings were compatible with many previous studies which also reported that some drugs were not toxic *in vivo* but they were toxic *in vitro* (Campbell et al., 1983; Foreyt, 1993; Geary, 2005; Molinari et al. 2009) and that ivermectin was safe to use as anthelmintic for livestock treatment but it caused toxicity to CHOK1 cells *in vitro*. The study of Lanbeck and Paulsen, 1995 showed that cefuroxime, dicloxacillin and erythromycin caused cytotoxicity to three different cell lines *in vitro*. Many other studies investigated the effect of certain chemical compounds that were safe to use *in vivo* but they were toxic *in vitro* experiments, like Acharya et al. (2014) reported that *E. nauseosa* and *R. aromatica* were toxic to IPEC-J2 cells. *M. alba* is considered a livestock feed and its hay is also used to feed these animals. So, this cytotoxicity findings suggest that the *M. alba* extract might have cytotoxicity if the animal consumes large doses of leaves or leaf-extracts. Therefore, further studies should be focused on *in vitro* experiments by measuring the cytotoxicity of *M. alba*

leaves extracts using polarized BIECs; and running more *in vivo* experiments by giving live animal drenches of these extracts to better analyze any adverse effects of the plant. Also, we suggest a deep investigation about the main chemical compound found in the *M. alba* that produces the parasiticidal efficacy, which may be directly beneficial in controlling the anthelmintic resistance.

Chapter 4: *In Vivo* Anthelmintic Activity of *Melilotus alba* (White Sweet Clover) from the United States Northern Great Plains on *Haemonchus contortus* Nematodes in Naturally Infected Lambs

4-1. Abstract

Anthelmintic resistance in the gastrointestinal nematode, *Haemonchous contortus*, is a growing health problem for commercially-raised sheep and goats, and so alternatives to the commonly-used anthelmintics are needed. A previous study showed that methanol extracts from *Melilotus alba* sweet clover possessed anthelmintic activity to *H. contortus* infective juveniles under *in vitro* conditions. For this reason, the anthelmintic activity of *M. alba* was evaluated under *in vivo* conditions in 18 yearling ewes naturally infected with *H. contortus*. Heavily-infected ewes were selected and divided into 2 experimental groups and 1 control group. One of the experimental groups was fed 0.68 kg of fresh-frozen *M. alba* sweet clover (harvested from local plants in late fall) per day for 2 weeks. This group was also given a mixed Garrison/sweet clover hay *ad lib*. The second group received only Garrison/sweet clover hay, while the control group received only Garrison hay. *Haemonchus* fecal-egg- counts were determined daily after the 2 weeks to estimate the post-treatment worm loads. Under these conditions, the results did not show any decrease in egg shedding, no significant effect ($p > 0.05$), within either experimental group compared to the controls, but it showed an unexpected significant increase in egg output, more than 4500 EPG, in the third day of the experiment, and stayed significantly higher in the treated ewes even 10 days after the end of treatment.

Key Words: *Haemonchous contortus*; anthelmintics; *Melilotus alba*; *Haemonchus* fecal-egg- count

4-2. Introduction

Haemonchous contortus is one of the gastrointestinal nematodes (GIN) primarily affecting small ruminants, endangering animal welfare and causing server economic losses (Troell et al., 2005). In regions where livestock productions is common, anthelmintics that had provided great strategies for GIN control in the past are no longer as effective, and there is a need to find alternative treatment strategies in order to maintain current levels of livestock productivity (Kaplan and Vidyashankar, 2012).

Researcher's interest in medicinal plants has been due to the presence of secondary metabolites, rich in bioactive substances, that can have active roles in an animal's metabolism and physiology (Marcin et al., 2006). Some of these metabolites have shown anthelmintic activities in published studies involving plants from African and Asian countries (Akhtar and Riffat, 1984; Al-shaibani et al., 2009; Githiori et al., 2006; McGawa et al., 2007; Qadir et al., 2010), Australia (Kotze et al., 2009), Europe (Hordegena et al., 2006; Hoste et al., 2006; Manolaraki et al., 2010; Marie-Magdeleine et al., 2010; Waller et al., 2001), North America (Acharya et al., 2014; Fernandez-Salas et al., 2011; Pessoa et al., 2002) and South America (Monteiro et al., 2011; Souza et al., 2008). For most of these studies, solvents with differing levels of polarity are used to extract and concentrate the secondary products (Borges and Borges, 2016). Examples of nonpolar solvents include pentane, hexane, benzene, and toluene; examples of polar solvents include water, ethanol, methanol, acetone, methyl ethyl ketone, isopropanol, n-propanol, and acetonitrile. Dimethyl sulfoxide (DMSO) is also an

important polar solvent, but it dissolves both polar and nonpolar compounds and is miscible in a wide range of organic solvents as well as water; therefore, DMSO is commonly used to initially dissolve plant extracts before they are added to another solvent, especially water. For convenience, these extracted secondary products are usually screened initially for anthelmintic activity using nematode juveniles or eggs exposed to the compounds in an *in vitro* experimental system (Borges and Borges, 2016). Once *in vitro* screening tools identify anthelmintic activity within these extracted products, then their activities are further tested with *in vivo* studies involving infected hosts. Because of its economic importance, its prevalence, and it's growing level of anthelmintic resistance, many of the *in vitro* screening studies involve *H. contortus* eggs and juveniles, and many of the *in vivo* studies involve *H. contortus* adults in sheep or goats (Lanusse et al., 2016). These *in vivo* studies have generally used the medicinal plant extracts, but occasionally have used fresh plants to evaluate anthelminthic activity; this activity can be measured by counting adults worms in the necropsied host after a treatment period, but most often studies measure reductions in fecal egg shedding as a less expensive, indirect method for measuring anthelmintic effects (Borges and Borges, 2016; Carvalho et al., 2012; Eguale et al., 2007a, b; Max, 2010; Waghorn et al., 2006).

Acharya et al. (2014) evaluated the *in vitro* anthelmintic effect of methanol extracts from 40 Northern Plains plants using egg hatch and larval migration assays and identified five plants with significant anthelmintic activity (Figure 4-1) including: *Ericameria nauseosa* (rubber rabbitbrush), *Rhus aromatica* (fragrant sumac), *Chrysothamnus viscidiflorus* (green rabbitbrush), *Melilotus officinalis* (yellow sweet clover), and *Melilotus alba* (white sweet clover). This *in vitro* activity was further was further

evaluated in Chapter 3 of this dissertation to determine which portion of *M. alba* contained significant activity, and if an aqueous extract of this species also contained anthelmintic activity. *Melilotus alba* was selected for further studies because sweet clover is much more palatable for livestock than the rabbitbrush and sumac species, and because *M. alba* was more plentiful than *M. officinalis*.

Based upon the encouraging results from the sweet clover *in vitro* studies, the present study was performed to evaluate the *in vivo* effects of *M. alba* consumption on FECs in lambs naturally infected with *H. contortus*. In this study, the experimental animals were fed whole fresh white sweet clover plants containing leaves, pods and minor stems in an approach that was similar to an *in vivo* study involving three freshly harvested tanniferous forages (*Cichorium intybus*, *Lotus cornicultus*, and *Onobrychis viciifolia*) that were fed to *H. contortus*- and *Cooperia* curticei- infected lambs for 17 consecutive days and FECs were measure every other day for the duration of the study (Heckendorn et al., 2007).

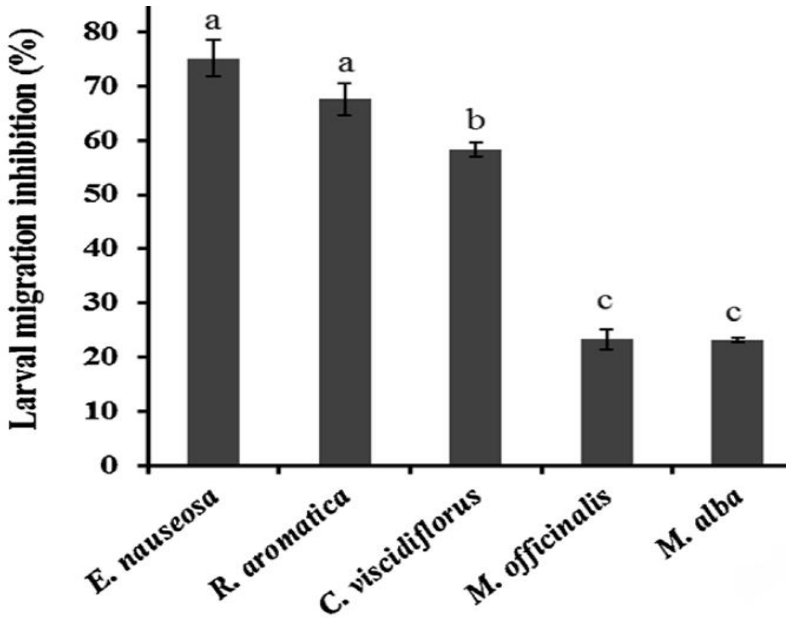

Figure 4.1. Effect of five different medicinal plants on *Haemonchus contortus* larval migration/mortality from Acharya et al. (2014).

4-3. Materials and Methods

4-3.1. Experimental protocol

In this study, 18 yearling ewes were selected for the study from a farm, containing approximately 1000 ewes and lambs located in north-eastern Brookings County, South Dakota. These ewes were naturally infected with *H. contortus* which was the primary trichostrongyle nematode identified in this flock according to previous studies (Acharya et al., 2014; Grosz et al., 2013). This population of *H. contortus* has a history of anthelmintc resistance to the avermectins and benzimidazoles (Grosz et al., 2013). Prior to the study, the ewes had been grazed on grass pastures from May until the end of August. During that time, fecal samples were collected on a monthly basis, and FECs per gram of feces (EPG) were counted randomly for approximately 50 ewes using a modified McMaster technique; and out of these ewes, 18 yearling ewes containing the highest numbers of trichostrongyle eggs (generally 800 EPGs and higher) were selected for the study.

The selected ewes were blocked according to EPGs and divided into two experimental groups and one control group and housed in three separate pens (6 animals per pen) in a covered barn with a concrete floor and one open side. The experiment was performed on September 5th- 27th, 2013.

4-3.2. Experimental diets

White Sweet Clover (*M. alba*) was collected from the farm containing the ewes used in the study and from grassland areas associated with the South Dakota State University Oak Lake Field Station, which is located 20 km east of the sheep farm, and chopped,

weighed into 0.5-1 kg aliquots and frozen at -20ºC in plastic bags. Plants were harvested during late summer (July and August) of 2013 when most were in full flower, and major stems from each plant were not included in the aliquots. Sweat clover hay containing about 5-10% sweet clover, and other 90-95% different kinds of grasses (predominantly Garrison hay) were mowed and bailed.

The "sweet clover" experimental group was fed 0.68 kg of the fresh-frozen sweet clover per day for nine days starting on September 10, 2013, the ewes were not easily eating the WSC stems and pods and therefore the experimental WSC feed was mixed with previously made molasses containing sugar and rice flour. This group was also given a mixed Garrison/sweet clover hay *ad lib*. The "clover hay" experimental group received only Garrison/sweet clover hay, while the "hay" control group received only Garrison hay *ad lib*. All the animals in the study groups were given 0.5 kg of mixed grain a day and free access to the water.

4-3.3. Sampling procedures and analysis

Fresh fecal samples were collected from the rectum of all ewes daily for the first 16 days of the experiment and every two days for the last week of the confinement trial, placed in a sealable plastic bag, sealed and stored at 4ºC. Trichostrongyle fecal-egg-counts were determined using a modified McMaster technique within 2 weeks of collection to estimate the pre- and post-treatment as an estimation of adult worm loads. In this technique, 1 gram of fecal sample was weighed in a small disposable cup, 14 ml of saturated sodium nitrate solution (specific gravity 1.26) was added as a flotation solution to the feces, and the feces allowed to soak for 10 – 15 minutes before mixing well with a tongue depressor. Then, the mixture was passed through a sieve funnel with a sieve pore

size of 100 µm into a 50 ml centrifuge tube. Immediately after vortexing the tube, about 2 ml of the suspension was pipetted to a McMaster slide filling both champers. After waiting for 1-2 minutes to allow eggs to float to the top of the slide, the eggs inside of grid areas of the two champers were counted using an Olympus BX2 microscope at low power (10X objective), and multiplied by 50 to calculate the EPG.

The mean EPG for each group for each day were calculated along with their standard errors and graphed in Figure 4-2. For an analysis of treatment effects, the sampling time-periods were divided into 3 different phases: the 6-day pretreatment phase (Sept. 5-10), the 8-day treatment phase (Sept. 11-18) and the 9-day post-treatment phase (Sept. 19-27). Differences between the mean EPGs for each group and at different sampling periods were statistically identified using the Mann-Whitney pairwise test in PAlentological STatistics (PAST) Version 3.23 Øyvind Hammer, Natural History Museum University of Oslo 1999-1919 (Hammer et al., 2001)(https://folk.uio.no/ohammer/past/).

4-4. Results

The anthelmintic activity of fresh-frozen white sweet clover was assessed by utilizing the FECRT approach on 6 *H. contortus*-infected animals as shown in Figure 4.2. The effects of sweet-clover treatment were not only compared to the pretreatment data, it was also compared with a control group that was feed Garrison hay during the 23 days of the study. The mean EPG for the 20 sampling days in the hay control group was 1,967.9 EPG (SD 638.3), ranging from 1066.7 to 3225.0 EPG. The EPGs for these sampling days for the control group varied somewhat, and of the 19 day-to-day comparisons for the 20 days, only 14 were significantly different from one of the other days, and these divergent time-points were associated with the upper peaks and lower valleys shown in Figure 4.2.

Within this control group, the mean FEC during the treatment days (days 9/5 through 9/10) was significantly higher ($p=0.049$) than during the pretreatment days (day 9/11 through 9/19), but they were similar ($p>0.05$) to the post-treatment samples (day 9/20 through 9/27). The pretreatment mean FECs for all three groups were not statistically different ($p>0.05$) from each other, and this validated the blocking choices made during the assigning of the yearling ewes into the 3 groups.

During the treatment phase, the mean FECs for all of the groups increased significantly over the combined pretreatment groups. The mean count for the clover treatment group was significantly higher than that of the hay control group for both the treatment ($p=0.02$) and post-treatment phases ($p<0.05$), but not higher than the clover hay group for either phases ($p>0.05$). In fact, during the treatment and post-treatment phases, the mean daily FECs for the clover treatment group were all numerically higher than that of the other two groups for all of the sampling time-points (Figure 4.2). The mean FECs for the clover hay group was not statistically different from that of the hay control group for either the treatment or the post-treatment phases ($p>0.05$).

Collectively, the results showed that the groups of animals fed fresh-frozen sweet clover significantly increased trichostrongyle egg output (per gram of feces) over that of the control hay group, but not the clover hay group. This increase provides clear evidence that the sweet-clover exerted no anthelmintic effect on the trichostrongyles in the clover treatment group. Daily FECs varied throughout the study for each of the groups, and while the degree of this variation differed for each day among the groups, the variation pattern was very similar within each group (Figure 4.2). This increase in the

concentration of eggs continued during the study even after treatment ended.

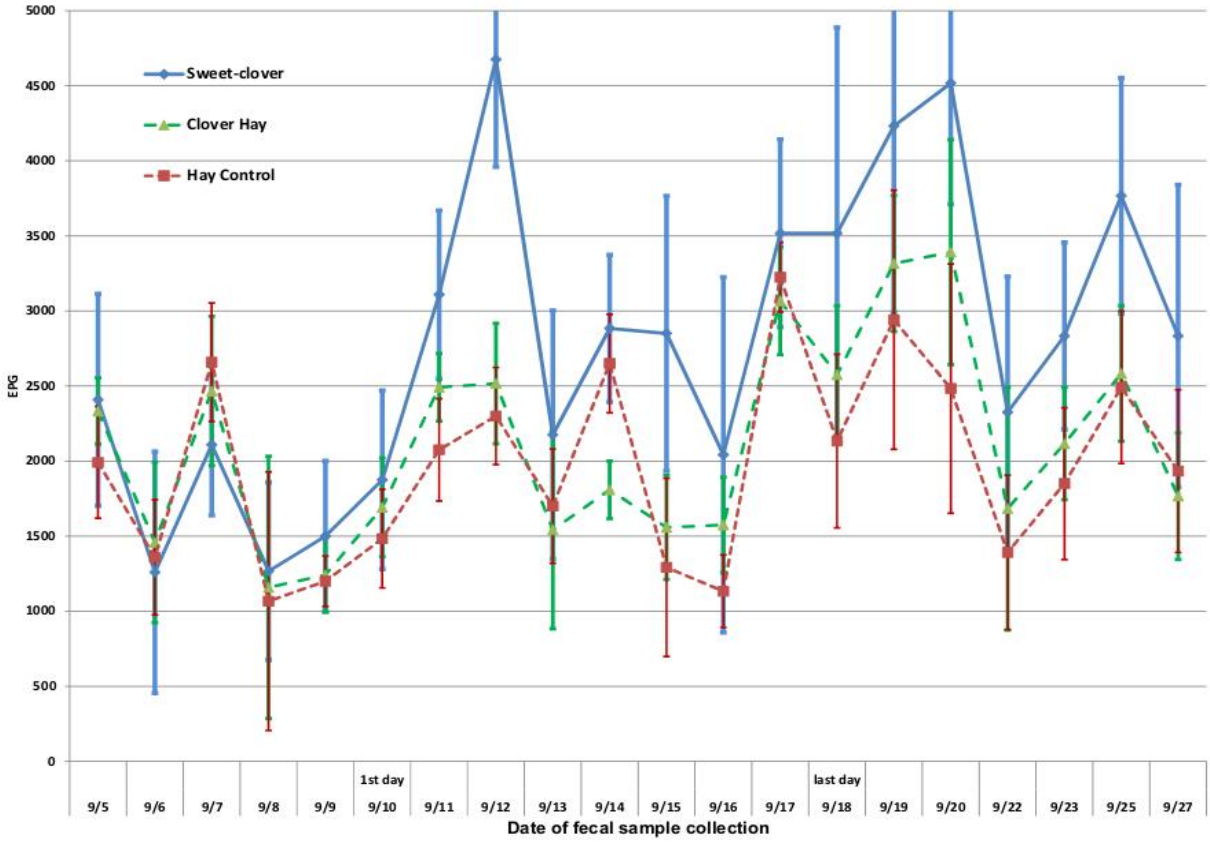

Figure 4.2. Effect of white sweet clover on fecal egg count of *Haemonchus contortus* naturally infected ewes among the fresh-frozen sweet-clover experimental group (sweet-clover), the clover/Garrison hay experimental group (clover-hay) and the Garrison hay control group (hay control) during the 3 phases of the study: the pretreatment phase (Sept. 5-10), the treatment phase (Sept. 11-18) and the post-treatment phase (Sept. 19-27).

4-5. Discussion

This study is a unique *in vivo* experiment that used fresh *M. alba* leaves, pods and small stems as an experimental diet in an attempt to decrease the number of adult worms (shedding trichostrongyle eggs) present in naturally infected young ewes. In contrast, most of the other *in vivo* studies have used plants extracts as experimental treatment material, and not a whole fresh plant diet (Khan et al., 2006; Eguale et al., 2007; Max, 2010; Carvalho et al., 2012).

The results of this study showed no decrease in the *H. contortus* population, but instead it showed an unexpected significant rise in egg output which reached its peak, more than 4500 EPG, in the third day of the experiment, and stayed significantly higher in the treated ewes even 10 days after the end of treatment. The explanation of these findings could be due to the animals not eating adequate amounts of feed since they did not like the taste of sweat clover and/or it might be the sweat clover contained some chemicals that played a role in lowering the intestinal motility. Both possible theories could cause constipation which usually decreased the amount of water in the fecal material causing increase egg concentration per gram of feces. So, the results of this *in vivo* study was completely opposite to the results of Heckendorn et al. (2007) which showed a significant reduction in fecal egg shedding of *H. contortus* by 89%, 63% and 63% compared to chicory, birdsfoot trefoil and sainfoin respectively. That study brings a third possible explanation for the differences, in that the amount of medicinal plants used in Heckendrorn's study was 6 – 9 kg a day per each lamb which was 10 to 15 times higher than the amount of *M. alba* used in this *in vivo* study (0.68 kg/animal a day).

Based on the study of Acharya et al. (2014), *M. alba* leaf-extracts in both DMSO & MOPS at higher concentration revealed a very significant effect ($p < 0.05$) on *H. contortus* eggs hatching. Acharya's findings were very compatible with our *in vitro* study, chapter 3, whereas *M. alba* leaf-aqueous and methanol-extracts at the highest concentration both showed 97% mortality of the infectious larvae, L3, of *H. contortus* which was measured by using the larval migration assay, compared to 16% and 9.6% L3 mortalities using methanol stems and pods extracts respectively. So, the mortality rate was significantly different in leaf-extract compared to stems and pods, and these findings

explain the negative effect of *M. alba in vivo* study on fecal egg output whereas most of the fresh plant material given to the ewes contained stems of second-year plants and this may have resulted in an inadequate amount of leaves being used in the feeding experiment. Therefore, to better understand the effect of *M. alba in vivo*, different portions should be fed separately, i.e. the experimental animals should be divided into four experimental groups: first group fed just pods, second group fed stems, third group fed leaves and the fourth one is a control group. In addition, further *in vivo* studies involving higher quantities of first-year plants, are encouraged to better evaluate the usefulness of feeding *M. alba* leaves.

Chapter 5: Final Discussion

This dissertation focused exclusively on *H. contortus* treatment and prevention issues in sheep from the Northern Great Plains of the United States. The first study (Chapter 2) primarily evaluated the use of three commercial anthelmintics to eliminate *H. contortus* adults from a commercial herd of ewes that were naturally infected with a population of benzimidazole- and avermectin-resistant worms, and then determine if the population could be kept low with an aggressive rotational grazing system. The second study (Chapter 3) continued an *in vitro* evaluation of the anthelmintic and cytotoxic activities of *M. alba* extracts that had shown promise in an earlier study. The third study (Chapter 4) evaluated the therapeutic value of fresh *M. alba* plants used as a daily feed to decrease egg-shedding in ewes that were naturally infected with *H. contortus*.

In the North American Northern Great Plains, only a few studies have been published involving sheep parasites. From 1944-1954, a study was conducted to determine the cause behind chronic signs of dysentery in sheep herds from northwestern South Dakota (Harshfield, 1955). Despite the extreme weather conditions in the Northern Great Plains, *H. contortus* was the primary parasite found in most of the sheep and lambs necropsied during the study. Other species were also identified within these animals including: *Trichostrongylus* spp., *Teladorsagia circumcincta, Chabertia ovina* and *Nematodirus*. Another extensive study was conducted in 2006 and 2007 just east of the Northern Great Plains involving more than 30 farms from southwestern Quebec and southeastern Ontario (Mederos et al., 2010). The Mederos et al. (2010) study showed that among lambs necropsied from 7 of the Ontario farms, 36.4% of the adult trichostrongyles were *Trichostrongylus axei*, 38.3% were *Trichostrongylus colubriformis*, 6.1% were *H.*

contortus, 5.8% were *T. circumcincta,* 2.50% were *Cooperia spp.* 0.16% and *Chabertia ovina; Trichostrongylus vitrines, Oesophagostomum columbianum* were collectively less than 0.5% of the total. Grosz et al. (2013) showed no specises diversity in the same farm that used in our triple treatment study, whereas, PCR results revealed that among the 63 tested ewes, all contained *Haemonchus* sp., and only 1 ewe (1.6%) was positive for any trichostrongyle genera other than *Haemonchus* sp.; this ewe had *Teladorsagia* sp. in addition to *Haemonchus*. Prior to the Grosz et al. (2013) study, an aggressive deworming system had been used on this flock over many years. Based on these facts, increasing resistance of *H. contortus* to different commercial nematocidals was apparently the cause for the virtual lack of any other trichostrongyle species. After 2010, they integrated more preventative measures to their worm-control strategies which allowed them to decrease their treatment interventions. For the preventative measures, they no longer allowed the lambs to graze with the ewes, and they started a rotational grazing system which decrease the need for regular anthelmintic use, enabling them to be used primarily for treating ewes showing symptoms of haemonchosis. In our 2014 triple treatment study, fecal samples were collected prior to treatment, and PCR results revealed both *Trichostrongylus* and *Teladorsagia* had emerged back into the flock forming 15% of the ewes analyzed.

The first year of our triple combination study, the *Trichostrongylus* population were significantly reduced by the three anthelmintics and rotational grazing system, but it rebounded effectively after that. By the end of the study period, 100% of the ewes were still infected with *Haemonchus* sp., but almost 90% also contained *T. colubriformis* with

a few containing *T. axei*. By the end of the study, *H. contortus* accounted for 50% of the eggs collected and *T. axei* appeared to account for most of the other half.

In colder climates, egg output of sheep trichostrongyles reached their peak during spring, simultaneously when lambing is generally scheduled to occur. The egg production increase is possibly due to the combined effects of a seasonal and periparturient release of hypobiotic juveniles to develop into egg-producing adults (Gibbs, 1986). In this study, about 85% of the ewes were shedding high numbers of trichostrongyle eggs before the spring treatment. Their pretreatment mean FEC was three times higher than the spring mean FEC from the 31 ewe flocks used in an Ontario and Quebec study (Mederos et al., 2010). Some of the individual flocks in the Canadian study had mean FECs similar to the South Dakota herd.

Because of the global growing concern of anthelmintic resistance, combinations of parasiticides with differing modes of action are thought to be the most effective way to control worm problems among anthelmintic-resistance populations of ruminant trichostrongyles (Bartram et al., 2012; Lanusse et al., 2018). However, such practices might not always be effective if the level of resistance for each of the anthelmintics is too high (Kaplan and Vidyashankar, 2012). In our study, a combination of three different classes of commercial anthelmintic (i.e. imidazothiazole, benzimidazole, and milbemycin compounds), were used, and provided an impressive 99.99% reduction in mean FEC. The reduction results of this study were similar to some combination studies and much higher than many of the other studies, depending on the parasiticidal combinations being used and the level of anthelmintic resistance (Baker et al., 2012; Holsback et al., 2016; Leathwick et al., 2012; Miller and Craig, 1996). The triple treatment method effectively

reduced the EPG to as low as < 1 EPG in about 95% of the treated ewes. The 5% of the treated ewes that did not respond to the initial triple-treatment were subjected to a second round of treatment, yet, no improvement were achieved, and marked increase in FECs occurred when compared to results from the initial triple-treatment. A third try of the triple treatment system was effective in reducing the FECs to less than 1 EPG in three out of the four ewes that did not respond to the second treatment. The non-responding ewes could be attributed to genetic variations in anthelmintic resistance among the worm population within specific ewes, and/or variations in the maturation of the more anthelmintic-resistant hypobiotic fourth-stage juveniles which may have remained as L4s during the first triple-anthelmintic treatment.

The experiment animals had an average EPG below 5 EPG during the first rotation period, however, at least one ewe was shedding high numbers of eggs as illustrated in the sample collected during the first two weeks that had 150 EPG, while the other 19 samples had as low or less than 1 EPG. The second rotation of these animals in the first and second paddocks had the same low EPG results (most ewes had as low or less than 1 EPG while less than 30% had a little higher EPG). These results are consistent with suggestions by Grosz et al. (2013), who described the unfavorable weather impact on the *H. contortus* 3rd stage juveniles leading to their death, and that the higher infection could be attributed to the survival of the J4s; these J4s could have higher anthelmintic resistance compared to the adult stage larvae.

In the second rotation through the paddocks and a couple weeks after that, egg output increased linearly for the next four weeks, and plateaued during the first week in October (Week 14). At this point, all of the samples contained at least one trichostrongyle egg,

and the mean FEC had reached the minimum detection limit of 50 EPG used in the Sweden eradication study by Waller et al. (2006). Ewes were grazing through some paddocks, in these four weeks, for the first time and other paddocks for the second time. FECs stayed similar through the third time of grazing through paddocks, which might be due to the ingested juveniles entering the hypobiotic stage in the ewes instead of maturing to the adult stage (Capitini et al., 1990). About 18% of the grazed ewes exceeded the minimum detection limit of 50 EPG used in the Sweden eradication study by Waller et al. (2006) by the end of the first grazing season and most likely all of the ewes had become infected with the trichostrongyles and it's an indication that the *H. contortus* population has more anthelmintic resistance in the South Dakota herd than the Sweden herd. Therefore, the highly resistant hypobiotic J4s were able to survive the triple treatment and re-establish themselves by the end of the season. During the next spring, FECs were about double that of the preceding fall. In the 2016 and 2017 lambing season, egg output elevated by more than 220 fold. This study indicated that maturation of arrested J4s to the adult stage are a major cause of the rapid and extensive increase in *H. contortus* egg output among the ewes during the spring lambing season. Therefore, treatment with multiple anthelmintics of different chemical classes combined with a rotational grazing system was not enough to eliminate *H. contortus* from the herd of ewes. It also appears that it is necessary to prevent grazing until all of the hypobiotic J4s have emerged and eliminated by treating the adults before the ewes can safely be allowed out onto the pastures. This may require that the ewes avoid grazing during the spring and summer grazing season, and only allowed out onto a pasture until fall, and only after the FECs are

0 EPGs. Even then a rotational grazing system may be necessary to avoid the buildup of infective juvenile once grazing starts.

The *in vitro* results from the present *M. alba* leaf-extract study were very consistent with results from the Acharya et al. (2014) study, whereas the *M. alba* leaf-methanol-and aqueous-extracts showed 97% J3 migration inhibition for *H. contortus* compared to 19.46% and 5.57% inhibition from methanol stems and pods extracts respectively. Several studies have reported the presence of different secondary metabolites such as flavonoids (non-tannins) and alkaloids found in the medicinal plants that possess anthelmintic activities (Barrau et al., 2005; Ketzis et al., 2002; Macedo et al., 2009). These secondary metabolites were found in different medicinal plants including *C. viscidiflorus, E. nauseosa L. punctata, M. alba, M. officinalis, P. gairdneri,* and *S. canadensis* (Ahmed et al., 2006; Anwer et al., 2008; Carr et al., 1986; Giannasi and Chuang, 1976; Hegazy et al., 2007; Hegerhorst et al., 1987; Mead et al., 1992; Newton et al., 2002; Nicollier and Thompson, 1982; Stevens et al., 1999; Sutiashvili and Alaniya, 1999; Tabanca et al., 2007; Wagner et al., 1973). In our *in vitro* study, *M. alba* leaf-extracts showed the highest parasiticidal efficacy which might be due to the presence of tannins which was identified in the leaves of other plants including *A. uva-ursi, G. viscosissimum, L. corniculatus, R. glabra* and *R. aromatica* (Amarowicz et al., 2009; Bate-smith, 1980; Buziashvili et al., 1973; Lowther et al., 1987; Marshall et al., 2008; Min et al., 2008; Pegg et al., 2008; Saxena et al., 1994); while other researchers (Ekbote et al., 2010; Hoste et al., 2006; Molan et al., 2000; Paolini et al., 2004) mentioned the tannins was responsible for anthelmintic activity. In addition, *M. alba* leaf-methanol-extracts at 30 mg/ml were tested with and without DMSO and without DMSO, and at

three different pH levels (3, 5, and 7.4). The results revealed that J3 migration was inhibited more at a lower pH (3 and 5), and that the presence of DMSO in the treatment solution did not affect the level of inhibition. These results were consistent with the Acharya et al. (2014) findings who studied the effect of DMSO used in extracts from eight plants (*C. viscidiflorus, E. nauseosa, G. viscosissimum, L. punctata, M. alba, M. officinalis, P. gairdneri,* an *R. aromatica*) at their highest concentration (50 mg/ml), and also MOPS used in the extracts from six plants (*C. viscidiflorus*, *E. nauseosa*, *L. punctata, M. alba*, *M. officinalis,* and *P. gairdneri*). Their results with *M. alba* were very compatible with our results which showed 100% inhibition in the DMSO (at a low pH) and MOPS (at a neutral pH) at 50 mg/ml. Anthelmintic activity at an acidic (3.2-3.8) pH can result from complex formations between proteins and certain tannins (Kallithraka et al., 1997). Acharya et al. (2014) speculated that at a lower pH (with DMSO), the inhibition of *H. contortus* egg-hatching might be related to the presence of tannin metabolites within some of the tested plant extracts. Some plant metabolites have chemical structures similar to anthelmintic compounds that affect the nematode nervous system (Marie-Magdeleine et al., 2010); therefore, the nematocidal efficacy of *M. alba* leaf-extracts might be due to the presence of secondary metabolites that affect the parasite nervous system.

The results of the *in vivo* study showed no reduction in the *H. contortus* egg output, but it showed an unpredicted significant increase in fecal egg shedding, more than 4500 EPG, in the third day of the experiment, and stayed significantly higher in the treated ewes even 10 days after the end of treatment. The suggestion of these findings is that the animals did not get an adequate amount of white sweat clover because the plant taste was

undesirable for animals and/or the plant has some chemical compounds that played a role in lowering the intestinal motility. Therefore, both of these possible concepts resulted in constipation and lead to increased egg concentration in the feces. This finding was completely opposite to the results of Heckendorn et al. (2007), a study that showed a significant decrease in egg output of *H. contortus* by 89%, 63% and 63% when fed chicory, birdsfoot trefoil and sainfoin respectively. Therefore, the reason behind the difference between Heckendrorn's results and our *in vivo* results might be due to the amount of fresh plants fed, which was 6 – 9 kg a day per each lamb in Heckendrorn's study and was about 10 to 15 times higher than the amount of *M. alba* used in our study (0.68 kg/animal a day).

Although, the *in vitro* studies indicated that *M. alba* leaves possessed compound(s) with nematocidal activity that is/are soluble in methanol and water, but these compounds were cytotoxic to unpolarized intestinal cells (BIEC). The *in vitro* cytotoxicity of certain drugs and medicinal plants was reported by many researchers. For example, several studies have reported that ivermectin was safe to use as an anthelmintic for livestock treatment but it caused toxicity to CHOK1 cells *in vitro* (Campbell et al., 1983; Foreyt, 1993; Geary, 2005; Molinari et al., 2009). A study by Lanbeck and Paulsen showed that cefuroxime, dicloxacillin and erythromycin caused cytotoxicity to three different cell lines *in vitro* (Lanbeck and Paulsen, 1995). Acharya et al. (2014) reported that *E. nauseosa* and *R. aromatica* were toxic to *IPEC-J2* cells.

Both yellow and white sweet clover have been planted into pastures to add fertility to the soil and to help provide feed for grazing livestock feed (Goplen, 1980). There have not been any reports of toxicity from the fresh sweet clover plants, but improperly cured

hay from sweet clover causes a bleeding condition known as sweet clover disease, resulting from the formation of dicoumarol in the hay during microbial spoilage. Our findings that the *M. alba* extracts are toxic to unpolarized BIECs may not represent a significant problem for animals because only the luminal portion of the gastrointestinal cells would be exposed to the cytotoxic compounds in the natural intestine. It is still possible that the polarized cells may be more resistance than the unpolarized cells when located in this orientation in the gut. Based on this possibility, the cytotoxicity of *M. alba* leaf-extracts should be investigated using polarized BIECs. In addition, live animal pathology models (i.e. rodents and then sheep) should be evaluated following oral dosing with the extract material to better analyze any potentially beneficial and/or adverse effects from consumption of the whole plant or concentrated extract. Also, we suggest further research studies to determine the main chemical compounds found in the *M. alba* extract and their anthelmintic effects and mechanisms.

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