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Effect of Birdsfoot Trefoil on Exsheathment of *Haemonchus contortus* in Rumen Fistulated Sheep

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EFFECT OF BIRDSFOOT TREFOIL ON EXSHEATHMENT OF *HAEMONCHUS CONTORTUS* IN RUMEN FISTULATED SHEEP

BY

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ABSTRACT:

This study has been developed to address the issue of small ruminant parasite resistance to commercial anthelmintics and to examine the possibility of controlling these parasites using feeds with condensed tannin containing plants. The goal of the research was to determine whether birdsfoot trefoil hay prevents the exsheathment of *Haemonchus contortus* and whether efficacy differs among birdsfoot trefoil cultivars.

During the first phase of research, a method for testing the exsheathment of *H. contortus in vivo* was developed. Various larvae containment capsules were tested to see whether the larvae could escape from the capsules. The most successful capsules were then tested in the rumens of fistulated ewes. Larvae were placed in capsules and suspended in the rumens by cords of various lengths for several different amounts of time. Using the methods developed, it was found that after eight hours in the rumen 82 ± 1% of the larvae were exsheathed.

For the second phase of the research, four rumen fistulated ewes were fed diets of birdsfoot trefoil or a control. Three cultivars of birdsfoot trefoil were fed: Pardee, Empire, and Bruce. These diets were fed to the each of the ewes for 28 days in a Latin 4x4 design. During exsheathment tests, capsules containing third-stage *H. contortus* larvae were placed in the ewes' rumens for 8 hours. They were then examined under a microscope for any changes in exsheathment or motility.

It was found that for all three cultivars, feeding birdsfoot trefoil hay did not affect exsheathment percentages. These results indicate that while further studies should be conducted to confirm these results, research on effectively incorporating condensed tannin containing plants should focus on other life stages of the *H. contortus* parasite.

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PREFACE:

This thesis has been prepared using the Manuscript Format. Chapter I contains a literature review, while chapters II and III each contain a manuscript that will be submitted for publication. Chapter IV covers a summary of future directions that this research should take.

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CHAPTER - I

REVIEW OF LITERATURE

1. Small Ruminant Parasite Problem:

1.1 Economic Impact

Gastrointestinal nematodes are a major economic concern for small ruminant producers across the globe (Nieuwhof & Bishop, 2005; Sackett et al., 2006; Qamar et al., 2011). In the United States, the estimated death loss of sheep due to parasites in 2009 was valued at $2.8 million US dollars (National Agriculture Statistics Service, 2010). A report published by the Meat and Livestock Australia Limited in 2006 estimated that Australia's annual sheep loss due to internal parasites is $283 million US dollars (Sackett et al., 2006). In Great Britain, it is estimated that there is an annual loss of $104 million US dollars due to internal parasites in sheep, $79 million of which is due to reduced growth, and $25 million due to treatment costs (Nieuwhof & Bishop, 2005). As of 2011, the small ruminant herds in Pakistan consisted of about 24.6 million sheep and 52.6 million goats (Qamar et al., 2011). It is estimated that in Pakistan parasite infections in sheep and goats cause a total annual loss of over $2.6 billion US dollars, $1364 million of which is due to parasite associated animal mortality, $1179 million due to reduced milk production, $84 million due to abomasa condemned at slaughter, $0.38 million due to weight loss, and $0.24 million spent on parasite treatments (Qamar et al., 2011).

1.2 Production loss

A review by Charlier et al. (2014) of studies looking at production losses due to internal parasites found that infection could reduce weight gains by 10%-47% and wool production by 0%-21%. They also found that treating parasites could increase milk yield from 9%-40% (Charlier et al., 2014). Parasite infection also causes reduced feed intake and reduced feed efficiency (Coop & Holmes, 1996). An experimental infection of 3000 *Haemonchus contortus* larvae was found to reduce milk production of ewes by 32.6% ($P < 0.01$) (Cobon & O'Sullivan, 1992). Approximately five weeks after an experimental infection of 2000 *H. contortus* larvae, infected lambs gained an average of 0 grams/day for the next 52 days while control lambs gained 98 grams/day; wool growth was also significantly reduced in infected lambs (Cobon & O'Sullivan, 1992).

2. Parasite Resistance to Anthelmintics:

2.1 Resistance in the United States

Anthelmintic resistance is prevalent in the United States (Terrill et al., 2001; Howell et al., 2008; Crook et al., 2016). Forty-six small ruminant farms located in the southern United States, including Puerto Rico and St. Croix, were evaluated for parasite resistance (Howell et al., 2008). It was found that *H. contortus* were resistant to benzimidazole at 98% of the farms, levamisole at 54%, ivermectin at 76%, and moxidectin at 24% (Howell et al., 2008). Thirty-four small ruminant farms from the mid-Atlanic United States were evaluated for anthelmintic resistance (Crook et al., 2016). It was found that *H. contortus* were resistant to benzimidazole at 100% of the farms, levamisole at 24%, ivermectin at 82%, and moxidectin at 47% (Crook et al.,

2016). Two goat farms in Georgia were evaluated for anthelmintic resistance (Terrill et al., 2001). Resistance was found at both farms to ivermectin and levamisole, with one farm additionally having parasitic resistance to benzimidazole (Terrill et al., 2001).

2.2 Global Resistance

Parasite resistance to anthelmintics is a problem for producers all over the globe (Ramos et al., 2002; Howell et al., 2008; Manikkavasagan et al., 2013; Lyndal-Murphy et al., 2014; Chandra et al., 2015). Resistance to benzimidazole in *H. contortus* was examined in 20 locations covering the five regions of Uttar Pradesh, India, and was present in all five regions (Chandra et al., 2015). Another study in southern Queensland, Australia, tested 20 farms and found that there was resistance to levamisole at 42% of the farms and moxidectin at 50% (Lyndal-Murphy et al., 2014). Parasitic infections combined with anthelmintic resistance have been blamed for losses of 10%-50% of weaned lambs in southern Queensland during wet seasons (Lyndal-Murphy et al., 2014). Twenty-seven goat farms in Tamil Nadu (India) were evaluated for parasite resistance to anthelmintics and resistance was found at 81% of the farms to albendazole and 92% for levamisole (Manikkavasagan et al., 2013). An evaluation of the parasite resistance to benzamidizol on eleven farms in Ontario (Canada) found that 91% of the farms had resistant parasites (Barrere et al., 2013). In Santa Catarina (Brazil), sixty-four flocks of sheep were evaluated for anthelmintic resistance (Ramos et al., 2002). Of these flocks, 67% had resistance to ivermectin, 65% to albendanzole, and 15% to levamisole (Ramos et al., 2002). Thus, parasite resistance was highly prevalent in all the locations tested.

3. The gastrointestinal parasite *Haemonchus contortus*:

3.1. Overview

The parasite *Haemonchus contortus* (barber pole worm) is known for being one of the most pathogenic gastrointestinal nematodes (GIN) of small ruminants (Kearney et al., 2016). *H. contortus* do not generally cause diarrhea, but since they feed on the blood of the host, they do cause anemia (Roeber et al., 2013). A study of lambs infected with 10,000 stage-three (L_3) larvae found that onset of anemia began ten days after the infection (Hunter & Mackenzie, 1982). These parasites use a single lancet that extends from their buccal cavity to slice the lining of the abomasum; blood was visible in the mucosal lining seven days after infection (Hunter & Mackenzie, 1982).

3.2 Haemonchus contortus life-cycle

Adults measure approximately 2.5cm and females can lay up to 10,000 eggs per day (Gilleard, 2013, Kearney et al, 2016). After exiting the host via feces, these eggs remain on the pasture while hatching and developing to the infective stage (Roeber et al., 2013). Larvae are identified by five stages during their development into adults, they are referred to as stage-one larvae (L_1) through stage-five larvae (L_5) (Silverman & Patterson, 1960). Larvae are infective once the L_3 stage is reached, but the length of time needed for eggs to hatch and develop to the L_3 stage varies by temperature and moisture (Chaudary et al., 2008). Chaudary et al. (2008) found that, in the subtropical conditions of Pakistan, the number of infective larvae peak on pasture between 15 and 45 days after contamination, with the pastures being mostly clear of infective larvae 90 days post contamination. After ingestion by the host, the L_3 larvae undergo exsheathment and migrate to the abomasum where they develop to maturity in

approximately 18-21 days (Roeber et al., 2013). Silverman and Patterson (1960) found that the rate of larval maturity varied by the age and susceptibility of the host. In young, susceptible lambs, parasites could reach maturity in as few as 12 days, while in older hosts this may take as long as 24 days (Silverman & Patterson, 1960). In resistant animals, the parasites were inhibited at the L_4 or L_5 stages (Silverman & Patterson, 1960). During the L_4 stage, larvae are capable of entering a hypobiotic period in the abomasum of the host, particularly when environmental conditions are not favorable for egg/larva development on pasture (Gatongi et al., 1998; Roeber et al., 2013). Adult *H. contortus* have a short lifespan of only a few months (Roeber et al., 2013). Developing *H. contortus* larvae molt their outer cuticles a total of four

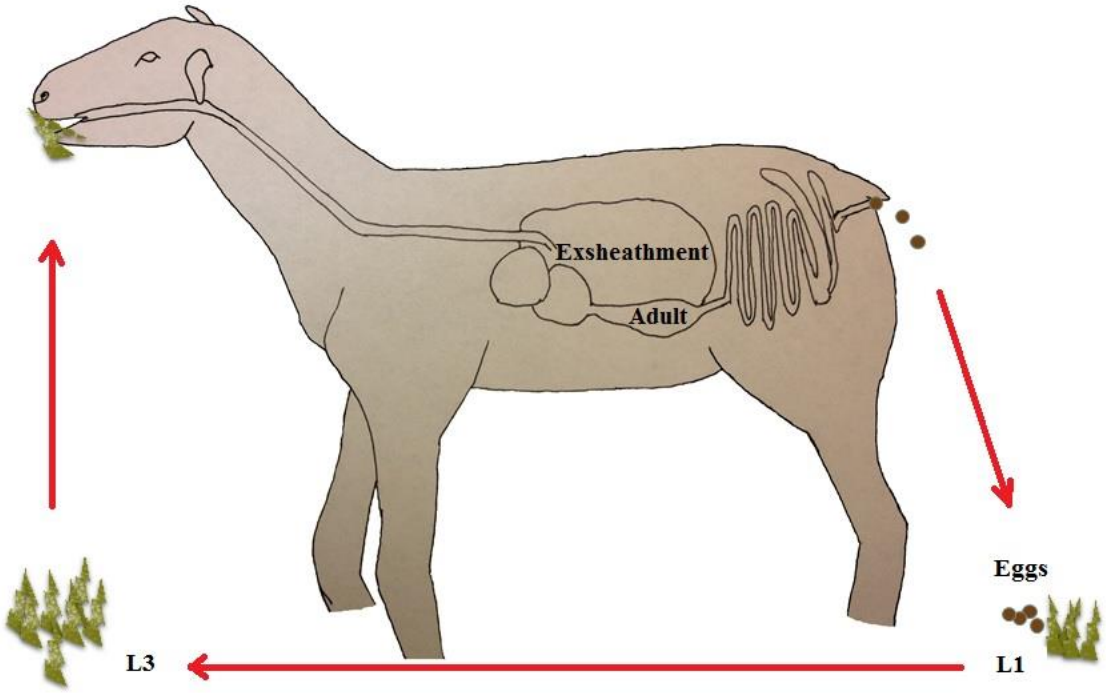

Figure 1: *Haemonchus contortus* life-cycle. Adult parasites live in the abomasum of the host and pass their eggs via the host's feces onto the pasture. Here the eggs hatch and develop from L_1 larvae to L_3 larvae. When pasture containing L3 larvae is consumed these larvae enter the host's rumen and undergo exsheathment. They then migrate to the abomasum and develop into adults.

times (Sommerville, 1957). The second molt, which occurs during the L_3 stage, is generally referred to as exsheathment and is a notable stage because when it occurs the larvae have entered the parasitic portion of their life-cycle (Sommerville, 1957).

3.3. Exsheathment

When infective L_3 *H. contortus* larvae are consumed by a small ruminant, they enter the rumen and exsheathment is triggered (Sommerville, 1957). Sommerville (1957) found that in *H. contortus,* and other species in general, exsheathment was triggered in the gastrointestinal tract just anterior to where that specie's adults reside; these observations were confirmed by Hertzberg (2002) for trichostrongylid species. The cuticle is a transversely striated (Ozerol & Silverman, 1972) protective covering that can shield the larva from digestion by nonspecific proteases during its free-living stages (Fetterer & Rhoads, 1996). While the process of triggering exsheathment is poorly understood, it is thought that the presence of CO_2, which is mediated by carbonic anhydrase, is sensed by chemoreceptors present in the amphids of larvae and triggers the release of noradrenalin which leads to downstream activation of exsheathment (Nikolaou & Gasser, 2006). When exsheathment is triggered, larvae release an exsheathing fluid into the area under the cuticle (Sommerville, 1957; Rogers & Sommerville, 1960). Exsheathing fluid is thought to be released by excretory cells (Wharton, 1991) and is composed of 80% proteins (Ozerol & Silverman, 1969). After the exsheathing fluid is released, a refractile ring forms near the anterior end of the larva, creating a loose cap at the tip of the sheath and allowing the larva to wriggle out (Wharton, 1991).

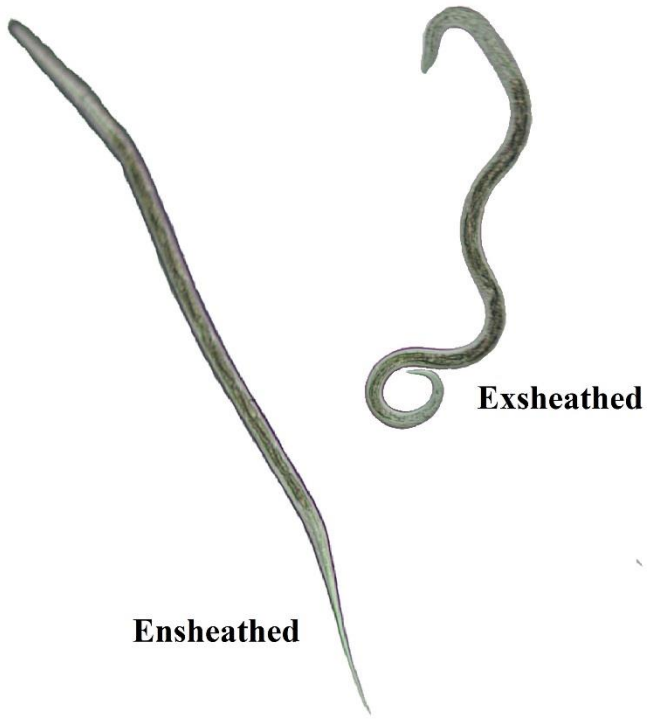

Figure 2: Exsheathment is the shedding of the outer sheath.

4. Anthelmintic plants:

4.1 Lespedeza cuneata

Consumption of several condensed tannin (CT) containing plants has been found to reduce gastrointestinal nematode burdens in small ruminants (Hoste, 2006; Shaik et al., 2006). One such plant, *Lespedeza cuneata* (sericea lespedeza), has been extensively researched and found to have anti-parasitic effects (Lange et al., 2006; Shaik et al., 2006; Terrill et al., 2007; Joshi et al., 2011; Gujja et al., 2013). Sericea lespedeza is a legume that is native to east Asia and was introduced to the United States for its potential uses including use as a hay for livestock (Ohlenbush et al., 2007). In general, feeding trials have shown that consumption of sericea lespedeza reduces fecal egg counts by greater than 50%, while reduction in adult worm counts are inconsistent (Table 1). Sericea lespedeza hay was fed to Boer goats with GIN

infections for 6 weeks, and by the final week, fecal egg counts dropped by 88% compared to control animals (Shaik et al., 2006). The adult abomasal worm count of *H. contortus* was also reduced by 62-77% (male-female) (Shaik et al., 2006). Joshi et al. (2011) fed sericea lespedeza leaf meal to young male goats for up to 63 days. A 23% non-significant reduction in adult worms was found after 63 days compared to control animals, and while fecal egg counts were significantly reduced to approximately 90% lower than control animals (Joshi et al., 2011). Ewe lambs were fed sericea lespedeza hay for 49 days and this diet was associated with 67-86% lower fecal egg counts than those of control animals (Lange et al., 2006). Worm counts from the treatment groups were lower than the control, but the difference was not statistically significant (Lange et al., 2006). Terrill et al. (2007) fed young male goats a diet of pelleted sericea lespedeza for 4 weeks. Compared to control animals a 70% reduction in the fecal egg count for the experimental diet was found, as well as a 75% reduction in adult worm burdens (Terrill et al., 2007). Young male goats were fed a sericea lespedeza leaf meal pellet for 11 weeks as a supplement to grazing, and fecal egg counts as well as combined abomasal and small intestine worm counts were both significantly lower for goats on the experimental diets than those on a control diet (Gujja et al., 2013).

4.2 Lotus corniculatus

Another legume that has been tested for anthelmintic properties is *Lotus corniculatus* (birdsfoot trefoil) (Marley et al., 2003; Heckendorn et al., 2007). Birdsfoot trefoil can outproduce alfalfa in poor-quality soils (Hall & Cherney, 1993). Generally, it was found the birdsfoot trefoil reduced adult abomasal worm counts, but

fecal egg count reductions were not consistent (Table 2). Marley et al. (2003) had male lambs grazing on birdsfoot trefoil cultivar (cv.) Leo for 35 days, and although there was no significant difference for the fecal egg counts at the end of the study, there were significantly fewer adult worms found in the abomasa of lambs on the birdsfoot trefoil diet than those on the control diet. Birdsfoot trefoil cv. Odenwälder was grazed by lambs for 17 days and, compared to the control animals, was associated with a 58% lower *H. contortus* fecal egg counts, but no significant difference was found in worm counts (Heckendorn et al., 2007). For two consecutive years, ewes and their lambs grazed on pastures of perennial ryegrass/white clover or birdsfoot trefoil cv. Grasslands Goldie for 86 days and 91 days respectively (Ramirez-Restrepo et al., 2004). Fecal egg counts of the ewes consuming birdsfoot trefoil were significantly lower than those on the control pasture both years (Ramirez-Restrepo et al., 2004). Fecal egg counts of the lambs were lower for the birdsfoot trefoil groups for most of the study, however, they increased to approximately equal or exceeded the control groups near weaning (Ramirez-Restrepo et al., 2004). One-hundred twenty lambs were grazed for 95 days on either birdsfoot trefoil cv. Grasslands Goldie or perennial ryegrass/white clover with each group further split to equal groups of regularly dewormed lambs and "trigger-drenched" lambs; ivermectin was used for deworming (Ramirez-Restrepo et al., 2005a). Trigger-drenched groups were dewormed when mean fecal egg counts reached 1000 eggs/gram for either group (Ramirez-Restrepo et al., 2005a). For the trigger-drenched groups, the lambs grazing on birdsfoot trefoil actually had significantly higher fecal egg counts on day 49 (Ramirez-Restrepo et al., 2005a). While trigger-drenched lambs grazing birdsfoot trefoil had significantly lower

abomasal worm counts for *H. contortus* than control lambs, they had higher abomasal worm counts of both *Teladosargia circumcincta* and *Trichostrongylus axei* (Ramirez-Restrepo et al., 2005a).

4.3 Onobrychis viciifolia

Onobrychis viciifolia (sainfoin) is a legume that is palatable to sheep and has first cut yields that are comparable to alfalfa (Tilleyet al., 2008). In general, fecal egg counts were reduced by consumption of sainfoin, but adult worm counts were not reduced (Table 3). Lambs grazing sainfoin cv. Visnovsky for 17 days had a 57% difference in fecal egg counts compared to the control group, but no significant change in worm counts (Heckendorn et al., 2007). Sainfoin hay was fed to lambs for 56 days with a trickle infection of *Trichostrongylus colubriformis* being given after the first two weeks of sainfoin consumption and continuing throughout the study (Rios-De Alvarez et al., 2008). Lambs consuming sainfoin had lower fecal egg counts than those on the control diet, but no significance was found between the post-trial worm counts (Rios-De Alvarez et al., 2008). Lactating dairy goats living on pasture were brought indoors and fed sainfoin hay or a control hay for periods of 10 days each month; fecal egg counts were lower for does consuming sainfoin hay (Hoste et al., 2005). Cull goats living on pasture were fed sainfoin hay or ryegrass (control) hay for seven days each month (Paolini et al., 2005a). Fecal egg counts of the sainfoin group were significantly lower after 6 weeks and 8 weeks of the study ($P < 0.05$ and $P < 0.001$ respectively); around week 8 two goats from the control group died and five more were dewormed due to low packed cell volumes while no animals from the sainfoin group required treatment (Paolini et al., 2005a). Total worm counts for both

groups were not significantly different (Paolini et al., 2005a). Young Alpine goats were fed sainfoin hay for 9 days, and on days 4, 5, and 6 they received trickle infections of *H. contortus* larvae (Paolini et al., 2005b). The goats were slaughtered and although there were lower total worm counts for the control group, the difference was not statistically significant (Paolini et al., 2005b).

4.4 Other Plant Species

Male lambs grazed *Cichorium intybus* (chicory) for 35 days and worm counts showed that there were fewer adult abomasal worms infecting lambs on the experimental diet than those on the control diet ($P < 0.001$), but no significant difference was found between the final fecal egg counts (Marley et al., 2003). Chicory cv. Grasslands Puna was also grazed by lambs for 17 days and although no significant difference was found for the worm count, a 69% difference in *H. contortus* fecal egg count was found (Heckendorn et al., 2007). In another study, heather (61% *Calluna vulgaris* L.; 25% *Erica Umbellata* L.; 12% *Erica cinerea* L.) was offered free choice to goats every three days for five months and these goats had lower fecal egg counts ($P < 0.001$) and no deaths, while in the control group the two goats with the highest fecal egg counts died during the study (Frutos et al., 2008). Ram lambs grazing on sulla (*Hedysarum coronarium*) for 28 days had lower ($P < 0.05$) egg counts than the control group (Niezen et al., 1995).

5. Condensed tannins:

5.1 Structure

The anthelmintic properties of plants are primarily attributed to the plant's condensed tannin content (Hoste et al., 2006). The term condensed tannin

Table 1

Summary of studies feeding *Lespedeza cuneata*

Infection	Treatment	Treatment Length	FEC Reduction	Worm Count Reduction	Condensed Tannin	Animals	Study Size	Reference
Prior natural infection & concurrent *H. contortus* trickle infection	Hay	6 Weeks	88%	Adult Abomasal 60.6%	22.4%	6-8 Month Boer Buck Kids	20	Shaik et al., 2006
Concurrent infection with 5000 *H. contortus* larvae	Ground Leaf Meal	5 Weeks	---	Abomasal and SI: 33.3%	---	8-10 Month Buck Kids	10	Joshi et al., 2011
Prior infection with 5000 *H. contortus* larvae	Ground Leaf Meal	4 Weeks	90%	Abomasal and SI: NS	---	8-10 Month Buck Kids	25	Joshi et al., 2011
Prior natural infection & concurrent *H. contortus/T. colubriformis* trickle infection	Hay	7 Weeks	77-86%	Abomasal: NS	22.4%	4 Month Ewe Lambs	12	Lange et al., 2006
Concurrent *H. contortus/T. colubriformis* trickle infection	Hay	7 Weeks	67-82%	Abomasal: NS	22.4%	4 Month Ewe Lambs	12	Lange et al., 2006
Prior natural infection	Ground Hay	4 Weeks	54%	Adult Abomasal *H. contortus*: 38%	6.4%	5-6 Month Kiko x Spanish Buck Kids	12	Terrill et al., 2007

Prior natural infection	Pelleted	4 Weeks	70%	Adult Abomasal *H. contortus*: 75%	6.5%	5-6 Month Kiko x Spanish Buck Kids	12	Terrill et al., 2007
Prior & concurrent natural infection	75% Pellet	11 Weeks	84%	Abomasal and SI: NS	5.7%	Spanish Buck Kids	20	Gujja et al, 2013
Prior & concurrent natural infection	95% Pellet	11 Weeks	94%	Abomasal and SI: 32%	5.7%	Spanish Buck Kids	20	Gujja et al, 2013

FEC and worm count reductions represent differences in findings for experimentally fed animals compared to findings for control animals. Condensed tannin contents are measured as a % of Dry matter. Abbreviations: NS = Not Significant, SI = Small Intestine, FEC = Fecal Egg Count

Table 2

Summary of studies feeding *Lotus corniculatus*

Infection	Treatment	Treatment Length	FEC Reduction	Worm Count Reduction	Condensed Tannin	Animals	Study Size	Reference
Natural Infection	Pasture cv. Leo	5 Weeks	NS	Adult Abomasal: 62%	---	5 Month Male Llyen Lambs	48	Marley et al., 2003
Prior infection: 7000 *H. contortus* & 15,000 *Cooperia curticei*	Fresh Fodder (68% BFT) cv. Odenwälder	2.4 Weeks	58% (*H. contortus* only)	Adult Abomasal & SI: NS	1.5%	4-5 Month Lambs	12	Heckendorn et al., 2007
Natural Infection	Grazing cv. Grasslands Goldie	12.3 Weeks	lower (P = 0.06)	---	2.5%	Romney Ewes	50	Ramirez-Restrepo et al., 2004
Natural Infection	Grazing cv. Grasslands Goldie	13 Weeks	lower (P < 0.001)	---	2.5%	Romney Ewes	50	Ramirez-Restrepo et al., 2004
Natural Infection	Grazing cv. Grasslands Goldie	12.3 Weeks	NS	---	2.5%	Romney Lambs	~100	Ramirez-Restrepo et al., 2004

Natural Infection	Grazing cv. Grasslands Goldie	13 Weeks	Higher ($P < 0.01$)	---	2.5%	Romney Lambs	~100	Ramirez-Restrepo et al., 2004
Concurrent Natural Infection (regularly dewormed)	Grazing cv. Grasslands Goldie	13.6 Weeks	NS	Abomasal *H. contortus*: 43%	4.0%	Suffolk x Romney Male Lambs	60	Ramirez-Restrepo et al., 2005a
Prior & Concurrent Natural Infection (Trigger dewormed)	Grazing cv. Grasslands Goldie	13.6 Weeks	Higher day 49 ($P < 0.001$)	Abomasal: 49%	3.1%	Suffolk x Romney Male Lambs	60	Ramirez-Restrepo et al., 2005a

FEC and worm count reductions represent differences in findings for experimentally fed animals compared to findings for control animals. Condensed tannin contents are measured as a % of Dry matter. Abbreviations: BFT = Birdsfoot trefoil, cv. = cultivar, SI = small intestine, NS = not significant, FEC = fecal egg count

Table 3

Summary of studies feeding *Onobrychis viciifolia*

Infection	Treatment	Treatment Length	FEC Reduction	Worm Count Reduction	Condensed Tannin	Animals	Study Size	Reference
Prior infection: 7000 *H. contortus* & 15,000 *Cooperia curticei*	Fresh Fodder (61% sainfoin) cv. Visnovsky	2.4 Weeks	57%	Adult Abomasal & SI: NS	2.6%	4-5 Month Lambs	12	Heckendorn et al, 2007
Concurrent 12,000 *T. colubriformis* trickle infection	Hay	7 Weeks	52%	Adult & Juvenile SI: NS	Tannin 2.0%	4 Month Texel x Scottish Greyface Lambs	16	Rios-De Alvarez et al., 2008
Prior & concurrent natural infection	Hay (10 days/month)	~32 Weeks	Lower ($P < 0.05$)	---	2.5%	Lactating Dairy Goats	120	Hoste et al., 2005
Prior & concurrent natural infection	Hay (7 days/month)	12 Weeks	(Week 8) 66%	NS	2.7%	Over 2 Year Goats	18	Paolini et al., 2005a
Concurrent *H. contortus* trickle infection	Hay	1.3 Weeks	---	All Stages: NS	3.2%	5 Month Alpine Kids	14	Paolini et al., 2005b

FEC and worm count reductions represent differences in findings for experimentally fed animals compared to findings for control animals. Condensed tannin contents are measured as a % of Dry matter. Abbreviations: cv. = cultivar, SI = small intestine, NS = not significant, FEC = fecal egg count

Table 4

Summary of studies feeding other condensed tannin containing plants

Infection	Treatment	Treatment Length	FEC Reduction	Worm Count Reduction	Condensed Tannin	Animals	Study Size	Reference
Natural Infection	Grazing chicory (cv. Grasslands Puna)	5 Weeks	NS	Abomasal Adult & L4: 51%	---	5 Month Male Llyen Lambs	48	Marley et al., 2003
Prior infection: 7000 *H. contortus* & 15,000 *C. curticei*	Fresh Fodder 84% chicory (cv. Grasslands Puna)	2.4 Weeks	69% *H. contortus* only	Adult Abomasal & SI: NS	0.3%	4-5 Month Lambs	12	Heckendorn et al., 2007
Natural Infection	Fresh Cut Heather (Every 3 days)	18 Weeks	Lower ($P < 0.001$)	---	6.4% Tannins	Lactating Cashmere Goats	48	Frutos et al., 2008
Natural Infection	Grazing Sulla	4 Weeks	Lower ($P < 0.05$)	---	12.1%	5 Month Romney Lambs	90	Niezen et al., 1995

FEC and worm count reductions represent differences in findings for experimentally fed animals compared to findings for control animals. Condensed tannin contents are measured as a % of Dry matter. Abbreviations: cv. = cultivar, SI = small intestine, NS = not significant, FEC = fecal egg count

(proanthocyanidin) is used to refer to polymers composed of flavan-3-ol sub-units (Reed, 1995). The condensed tannin contents of plants vary in concentration and structure, and it is hypothesized that both of these factors contribute to the level of anthelmintic efficacy (Quijada et al., 2015). The most abundant flavan-3-al sub-units found in condensed tannins are procyanidins and prodelphinidins (Figure 3) (Reed, 1995). The ratio of procyanidin to prodelphinidin has been proposed as a potential factor related to the efficacy of condensed tannins, with Quijada et al. (2015) finding this ratio to show a non-significant trend in efficacy in an *in vitro* exsheathment assay. When purified monomers of either procyanidin or prodelphinidin were tested against an *in vitro* exsheathment inhibition assay, the prodelphinidins were found to be more efficacious (Brunet & Hoste, 2006). For their *in vitro* tests, the catechin and epicatechin forms of procyanidins did not inhibit exsheathment; the gallocatechin form of prodelphinidin showed total inhibition of exsheathment, but the epigallocatechin form did not inhibit exsheathment (Brunet & Hoste, 2006). Two additional structural features that are being examined is the length of the polymer, which is measured by its molecular weight, as well as the stereochemistry of the flavan-3-ol sub-units (Figure 1) (Quijada et al., 2015). An *in vitro* larval migration study by Naumann et al. (2014) looked specifically at average molecular weights of the condensed tannin containing plants and found that it had a slight correlation with efficacy, but probably is not the only factor involved.

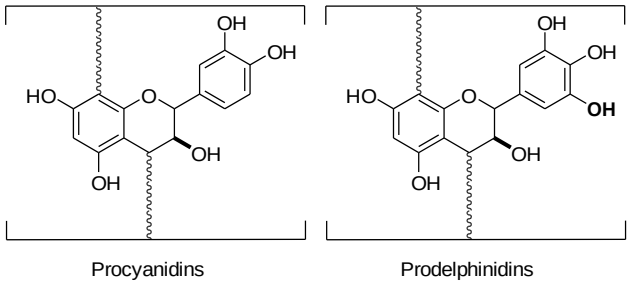

Figure 3: *General structure of two common forms of condensed tannins. Bold bonds represent the cis/trans determining bond.*

5.2 Measuring condensed tannin concentration

The concentration of condensed tannins can be determined using 4-(dimethylamino)cinnamaldehyde method (DMAC) (Neilson et al., 2016). Since condensed tannins have binding properties, the concentration measurements can be broken down into several categories: extractable, protein bound, fiber bound, and total condensed tannins (Naumann et at., 2014). Protein bound condensed tannins are thought to be the most biologically relevant to anthelmintic properties (Naumann et al., 2014). These measurements can be determined using the butanol-HCL method, where, following a series of extractions, the concentration is determined by a measure of light absorbance at a wavelength of 550nm (Naumann et al., 2014). Condensed tannin concentration can also be measured using UV spectra (Azuhnwi et al., 2013). The molecular weight of condensed tannins can be determined using Gel Permeation Chromatography and can be reported as either an average or a range (Huang et al., 2010). Percentages of cis or trans stereochemistry, as well as ratios of procyanidin to prodelphinidin, can also be determined by forms of chromatography (Quijada et al., 2015). Another method that can be used to determine structural characteristics of condensed tannins is matrix assisted laser desorption/ionization and time of flight mass spectral analysis (MALDI-TOF) (Krueger et al., 2000).

5.3 Changes in condensed tannin content over the life-cycle of a plant

Condensed tannin content can vary over the course of the plant's life; a single cultivar of birdsfoot trefoil had higher condensed tannin content in 2-year vs 1-year-old plants (Hedqvist et al., 2000). Sainfoin was also found to vary in condensed tannin concentration, % cis versus % trans ratios, and prodelphinidin to procyanidin ratios

over two harvests 42 days apart (Azuhnwi et al., 2013). Birdsfoot trefoil was examined for changes in condensed tannin content and it was found that for both cultivars tested the concentration was significantly lower in the fall than in the spring and summer (Gebrehiwot et al., 2002).

5.4 Variations between cultivars

Hedqvist et al. (2000) measured the variation in condensed tannins content of seven cultivars of birdsfoot trefoil and found that they varied extensively. The concentration of the condensed tannins ranged from 0.3%-1.0%, and the ratios of prodelphinidin to procyanidin ranged from 16:84 to 33:67 (Hedqvist et al., 2000). Six cultivars of sainfoin was also found to vary significantly in condensed tannin concentration, cis/trans ratios, prodelphinidin to procyanidin ratios, and molecular weight (Azuhnwi et al., 2013). For birdsfoot trefoil, cv. ARS-2620 was found to have 60%-70% more condensed tannins than cv. Norcen (Gebrehiwot et al., 2002).

5.5 Proposed Mechanism of Action

The effects of anthelmintic plants were first attributed to the broad category of plant secondary metabolites (Athanasiadou & Kyriazakis, 2004) and the search for a more specific cause of efficacy has led to the condensed tannins (Quijada et al., 2015). Although no consensus has been reached as to the mechanism of action for the condensed tannins, there have been several hypotheses suggested (Hoste et al., 2006; Cedillo et al., 2015). The possible mechanisms can be divided into two categories: a direct mode of action, where the condensed tannins act directly on the parasite, and an indirect mode of action, where the condensed tannins increase immune response by the host (Hoste et al., 2006). This higher immune response may be due to the ability of

condensed tannins to increase the amount of protein bypassing the rumen, which allows increased uptake by the host in the small intestine (Hoste et al., 2006). For example, a feeding trial by Rios-De Alvarez et al. (2008) found that feeding sainfoin to lambs with *T. colubriformis* infections resulted in increased levels of Pan T cells, eosinophils, and mast cells in the lambs' intestinal tissue. One proposed mechanism of action, where the condensed tannins act directly on the parasite, stems from evidence that the condensed tannins may be binding to the cuticle of the parasites, which may interfere with feeding, or other physiologic processes (Hoste et al., 2006). Another proposed mechanism is that the condensed tannins may be binding to enzymes secreted by the parasite and preventing their utilization by the parasite (Hoste et al., 2006). The mechanism of action specifically relating to inhibition of exsheathment also remains unknown (Alonso-Diaz et al., 2008). Most exsheathment inhibition testing has been performed *in vitro,* where an indirect mode of action is not feasible, so the inhibition seen in these assays can be attributed to a direct effect of either condensed tannins or other compounds on the larvae. It has been hypothesized that the condensed tannins may act directly on the sheath of larvae (Williams et al., 2014), however, incubating L_3 *H. contortus* in sainfoin extract for three hours prior to electron microscopy was not associated with any visible change to the sheath (Brunet et al., 2011). Incubation in sainfoin was associated with an internal accumulation of vesicles in L_3 *H. contortus* larvae, rupturing of the hypodermis in *Trichostrongylus colubriformis* larvae, and intracellular disorganization in both (Brunet et al., 2011). It is possible that these inner changes negatively affect the exsheathing mechanism in larvae.

5.6 Other benefits of birdsfoot trefoil consumption

Other benefits of small ruminants consuming birdsfoot trefoil have been found, and these benefits may be due to condensed tannins. Lambs grazing birdsfoot trefoil cv. Grasslands Goldie were found to have higher levels of carcass weight gain per day than control groups (Ramirez-Restrepo et al., 2004; Ramirez-Restrepo et al., 2005a). Lambs also had higher clean-fleece weights and longer staple lengths than those on the control pastures (Ramirez-Restrepo et al., 2004). Ramirez-Restrepo et al. (2005b) also found that there was an increase in reproductive efficiency for ewes grazing birdsfoot trefoil during the breeding season, which may be due to the condensed tannins increasing protein availability.

6. *In vitro* assays and Exsheathment:

6.1 In vitro assays

There are several *in vitro* assays that are used to screen for potential anthelmintic plants. These assays include the larval exsheathment inhibition assay, larval migration inhibition assay, egg hatching assay, larval development assay, and adult motility inhibition assay (Bachaya et al., 2009; Alonso-Diaz et al., 2011; Moreno-Gonzalo et al., 2013). These methods can be used to screen large amounts of potentially anthelmintic plants to determine which plants might warrant further examination (Mengistu et al., 2016). *In vitro* assays are also useful for testing differing isolated types of condensed tannins (Brunet & Hoste, 2006).

6.2. In vitro exsheathment assays

The exsheathment inhibition assay has been widely used for testing the *in vitro* anthelmintic effects of plants as shown in Table 5 (Bahuaud et al., 2006; Brunet et al.,

2007; Alonso-Diaz et al., 2008; Azando et al., 2011; Alonso-Diaz et al., 2011; Oliveira et al., 2011a; Oliveira et al., 2011b; von Son-de Fornex et al., 2012; Moreno-Gonzalo et al., 2013; Mengistu et al., 2016). For the exsheathment inhibition assay, the larvae are placed in the chosen concentration of leaf/plant extract for a period of three hours prior to being artificially exsheathed using sodium hypochlorite and sodium chloride (Mengistu et al., 2016).

Another less harsh method of artificially inducing exsheathment, developed by Conder and Johnson (1996), bubbles CO_2 gas into larvae in an Earl's Balanced Salts solution. This method has less of a negative impact on the viability and infectivity of the larvae (Conder & Johnson, 1996).

6.3. In vivo exsheathment assays

Exsheathment of *H. contortus* larvae *in vivo* has only been attempted a few times and is done by placing L_3 larvae into a porous container and placing it into the rumen of a fistulated sheep (Sommerville, 1957; Hertzberg et al., 2002; Brunet et al., 2007) (Table 6). Sommerville (1957) used a "Cellophane dialysis sac" (p. 19) to contain the larvae and defined exsheathed larvae as those that had a refractile ring. *H. contortus* were found to exsheath in the rumen, and exsheathment was examined at several time points up to 5.3 hours, at which point 85% had exsheathed (Sommerville, 1957). Sommerville (1957) also reported that some lower levels of exsheathment were observed and not included in the data. Hertzberg et al. (2002) placed larvae in 5 μm mesh bags each closed with a cord and suspended them approximately 25 cm deep in the rumen. They found that larvae were 90% exsheathed after 1 hour (Hertzberg et al., 2002). Brunet et al. (2007) fed sainfoin or a control to fistulated sheep and compared

exsheathment rates between the different diets. Larvae were placed in a microtube capped with a Nunc™ Cell Culture Insert which was then placed in a 50 μm mesh bag and suspended 20 cm deep in the rumen (Brunet et al., 2007). After 2.7 hours the control larvae averaged about 78% exsheathed while the larvae from the sheep fed sainfoin averaged just over 30% exsheathed (Brunet et al., 2007).

7. Summary and Conclusion:

Due to their growing resistance to anthelmintics, gastrointestinal nematodes are a major concern for small ruminant producers. Current research has shown that feeding small ruminants condensed tannin containing plants may offer a potential alternative method for controlling these parasites. However, there are several areas of research that are lacking. Various cultivars of condensed tannin containing plants have different levels of condensed tannins, and it is still necessary to determine which cultivars are most efficacious. Similarly, determining which structural varieties of condensed tannins are most efficacious would provide a much more efficient way of identifying and producing efficacious plants. It also needs to be determined what other secondary compounds are involved. Finally, determining the stages of parasites affected by condensed tannin containing plants will allow the most effective incorporation of these plants into the diets of small ruminants.

Table 5

Summary of *in vitro* exsheathment inhibition results post exposure to condensed tannin extract

Plant Species	Concentration condensed tannin extract (μg/mL)	% Inhibition	Reference
Acacia etbaica	150-1200	14.9%-98.8%	Mengistu et al., 2016
Acacia gaumeri	75-1200	66.3%-94.3%	Alonzo-Diaz et al., 2011
Acacia pennatula	1200	97.2%	Alonzo-Diaz et al., 2008
Anadenanthera colubrina	300	100.0%	Oliveira et al.,2011a
Arachis pintoi	1200	100.0%	von Son-de Fornex et al., 2012
Brosimum alicastrum	75-1200	NS-97.5%	Alonzo-Diaz et al., 2011
Cadaba farinosa	150-1200	1.3%-36.6%	Mengistu et al., 2016
Calluna vulgaris	150-1200	31.6%-100.0%	Moreno-Gonzalo et al., 2013
Capparis tomentosa	150-1200	8.2%-100.0%	Mengistu et al., 2016
Castanea sativa	600	100.0%	Bahuaud et al., 2006
Cratylia argentea (cv. Yacapani)	1200	100.0%	von Son-de Fornex et al., 2012
Cratylia argentea (cv. 22386)	1200	100.0%	von Son-de Fornex et al., 2012
Cratylia argentea (cv. Veranera)	1200	100.0%	von Son-de Fornex et al., 2012
Dichrostachys cinerea	150-1200	66.3%-100.0%	Mengistu et al., 2016

Dodonaea angustifolia	150-1200	29.8%-100.0%	Mengistu et al., 2016
Erica cinerea	150-1200	15.8%-100.0%	Moreno-Gonzalo et al., 2013
Erica erigena	600	17.6%	Bahuaud et al., 2006
Erica umbellata	150-1200	49.5%-100.0%	Moreno-Gonzalo et al., 2013
Euclea racemosa	150-1200	73.7%-100.0%	Mengistu et al., 2016
Gliricidia sepium	1200	20.8%	von Son-de Fornex et al., 2012
Havardia albicans	75-1200	89.6%-98.1%	Alonzo-Diaz et al., 2011
Leucaena leucocephala	75-1200	NS-91.0%	Alonzo-Diaz et al., 2011
Leucaena leucocephala	300	100.0%	Oliveira et al.,2011a
Leucaena leucocephala	1200	89.4%	Alonzo-Diaz et al., 2008
Lysiloma latisiliquum	1200	95.0%	Alonzo-Diaz et al., 2008
Maerua angolensis	150-1200	8.9%-100.0%	Mengistu et al., 2016
Maytenus senegalensis	150-1200	63.1%-100.0%	Mengistu et al., 2016
Mimosa tenuiflora	300	100.0%	Oliveira et al.,2011a
Myracrodruon urundeuva	0.31	100.0%	Oliveira et al.,2011b
Newbouldia laevis	300-1200	41.9%-94.8%	Azondo et al., 2011
Onobrychis viciifolia (sainfoin)	150-1200	NS-86.7%	Brunet et al., 2007
Pinus sylvestris	600	0.0%	Bahuaud et al., 2006
Piscidia piscipula	1200	95.2%	Alonzo-Diaz et al., 2008

Rhus natalensis	150-1200	39.7%-100.0%	Mengistu et al., 2016
Sarothamnus scoparius	600	NS	Bahuaud et al., 2006
Senna singueana	150-1200	3.1%-100.0%	Mengistu et al., 2016
Zanthoxylum zanthoxyloïdes	300-1200	87.9%-99.0%	Azondo et al., 2011

*% Inhibition = (Control % - Treatment %)/Control % *100*

NS = Not Statistically Significant

Table 6

Summary of *in vivo* exsheathment studies

Diet	Inhibition	Hours in Rumen	Exsheathed Control	Depth in Rumen	Time of Insertion	Larval Containment	Reference
---	---	5.3	85%	---	---	Cellophane dialysis sac	Sommerville, 1957
---	---	1	90%	25 cm	1 hr Pre-Feeding	5 µm mesh bag	Hertzberg et al., 2002
100% Sainfoin	59%	2.7	78%	20 cm	1 hr Post-Feeding	Microtube w/Nunc™ Cell Culture Insert	Brunet et al., 2007
75% Sainfoin	38%	2.7	78%	20 cm	1 hr Post-Feeding	Microtube w/Nunc™ Cell Culture Insert	Brunet et al., 2007
25% Sainfoin	NS	2.7	78%	20 cm	1 hr Post-Feeding	Microtube w/Nunc™ Cell Culture Insert	Brunet et al., 2007

Abbreviation: NS = Not Significant

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CHAPTER – II

Development of a procedure for *in vivo* ruminal exsheathment of *Haemonchus contortus* L_3 larvae

To be submitted as a short communication to Veterinary Parasitology

Development of a procedure for *in vivo* ruminal exsheathment of *Haemonchus contortus* L_3 larvae

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Highlights:

● A reproducible method for *in vivo* rumen exsheathment of *Haemonchus contortus* was developed for use in fistulated sheep.

● Larvae were 82 ± 1% exsheathed after 8 hours.

● Over 190 capsules were tested with minimal infection of the fistulated sheep.

Abstract:

The goal of this research was to develop a method for the *in vivo* testing of potential *H. contortus* exsheathment inhibitors without causing infection of the host. A containment capsule for larvae using a 3.8 cm piece of Tygon® tubing (ID 9.5 mm, OD 14.3 mm) capped at each end with an 8 μm Nunc™ Cell Culture Insert was designed and suspended 20 cm deep in the rumen of fistulated ewes. Each capsule contained approximately 2000 L_3 ensheathed larvae. Some batches of L_3 larvae were found to not exsheath well, and use of these batches was discontinued. Using the methods described in this paper, placing the larvae capsules in the rumens for eight hours resulted in exsheathment rates of 82 ± 1%. During the testing of these capsules no significant infection of the ewes occurred. This method can be used for much more extensive exsheathment testing as detailed explanations of previous methods are not available.

Keywords: exsheathment, ecdysis, *Haemonchus contortus*, barber pole worm, *in vivo,* strongylid

1. Introduction:

Parasitic infections of livestock are a significant concern due to their global economic impact caused by heavy production loss and death of the hosts (Roeber et al., 2013). *Haemonchus contortus*, a blood feeding parasite that can cause severe anemia in the host, is the most pathogenic parasite of small ruminants (Qamar et al., 2011; Kearney et al., 2016). *Haemonchus contortus* parasites have a life-cycle that requires several conditions to be met. A single adult female parasite residing in the abomasum can lay up to ten thousand eggs in a single day, which must then exit the host via feces and remain undisturbed on the pasture until they have developed to third-stage infective larvae (L_3) (Kearney et al., 2016). In order to successfully infect a host, the L_3 larvae must be ingested and undergo a critical exsheathment stage in the rumen (Sommerville, 1957; Roeber et al., 2013).

The potential for preventing *H. contortus* infections through inhibition of exsheathment has been widely explored through *in vitro* testing (Brunet et al., 2007; Oliveira et al., 2011; Azando et al., 2011; Alonzo-Diaz et al., 2011; von Son-de Fornex et al., 2012). Very few studies have reported *in vivo* exsheathment testing of *H. contortus* (Sommerville, 1957; Hertzberg et al., 2002; Brunet et al., 2007), and only one has examined potential exsheathment inhibition (Brunet et al., 2007). This may, in part, be due to the lack of an established validated procedure for conducting *in vivo* testing of *H. contortus* exsheathment in rumen fistulated sheep. Reported studies have varied in their time to exsheathment (Sommerville, 1957; Hertzberg et al., 2002; Brunet et al., 2007) as well as recovery of L_3 from exsheathment containers (Brunet et al., 2007).

An effort was made to reproduce published methods for *in vivo* exsheathment (Sommerville, 1957; Hertzberg et al., 2002; Brunet et al., 2007), and we were unable to replicate their results. Difficulties in reproducing the *in vivo* protocols utilized in previous studies (Sommerville, 1957; Hertzberg et al., 2002; Brunet et al., 2007) included an inability to procure the supplies used, finding that a large percentage of larvae escaped and infected the animals, and capsules not providing a sufficient amount of flow of the rumen contents into the capsule to cause consistent exsheathment of the larvae. Due to the difficulty and cost of maintaining fistulated sheep, an ideal larvae capsule will prevent larvae from escaping and thereby infecting the sheep. This would allow continuous testing in a small number of sheep without pauses to treat the sheep with anthelmintics and then having to wait for any residual chemicals to clear their system. Because of the high numbers of capsules potentially being used in *in vivo* experiments, even a small percentage of escaped larvae could lead to significant infections. The objective of this study, therefore, was to develop an *in vivo* exsheathment system that would: **A)** reproducibly exsheath *H. contortus* L_3 larvae *in vivo* and **B)** minimize parasitic infections in the fistulated animals.

2. Methods:

2.1. Experimental design:

Four rumen fistulated Dorset cross ewes were used for *in vivo* exsheathment tests of *H. contortus* L_3 larvae. All procedures used in this study were approved by the University of Rhode Island's Institutional Animal Care and Use Committee (IACUC). Potential capsules for containing larvae in the rumens were first tested in water to ensure their ability to contain the majority of larvae placed in them for an extended

period of time. Successful containment capsules were then tested *in vivo* in the rumens of the fistulated ewes to determine if the flow through the capsule membrane was sufficient to trigger exsheathment of the larvae. The percent exsheathment of the larvae was extensively tested for multiple rumen exposure times to develop a standard for the expected exsheathment of *H. contortus* L_3 larvae.

2.2. Animals:

Four Dorset cross ewes born the spring of 2012 and 2013 were used for these experiments. The ewes were housed at Peckham Farm (University of Rhode Island) and monitored weekly for potential *H. contortus* infection through measurements of fecal egg counts. Fecal samples were examined using the modified McMaster technique (Whitlock, 1948; Zajac & Conboy, 2012).

2.3. Rumen Cannula Placement:

In the spring of 2015, rumen cannula (8C, Bar Diamond™, Inc., Parma, ID) were placed into a fistula that was created by surgically opening the rumen wall in each of four ewes (Tufts Ambulatory Service, Woodstock, CT). Surgery was done using a paravertebral block, and needle pricks were used to ensure sufficient anesthetic was used. A portion of the ewe's skin was removed and the abdominal muscles were incised to allow access to the rumen. The rumen wall was then also incised and sewn to the cut edge of the skin. The incision area was cleaned, and the cannula was inserted. Post-surgery pain medications were administered for a minimum of five days. The surgical area was cleaned daily for the first week and as needed thereafter using a modification of a previously established procedure (Penn State, 2011).

2.4. Larvae

Haemonchus contortus L_3 larvae used in these experiments were either obtained directly from Dr. Anne Zajac (Virginia Maryland College of Veterinary Medicine, Blacksburg, Virginia), or cultured from the manure of donor lambs that had been infected with larvae obtained from Dr. Zajac. Larvae were isolated from manure using the Baermann Technique (Todd et al., 1970). Each batch of larvae was under four months at the time of usage, with day zero defined as the day of Baermann collection. During initial testing, some batches of larvae were found to not exsheath well using the *in vivo* methods described. These batches were eliminated from further study. Batches that were found to exsheath well (≥ 80%) were used in future experiments and were called 'pre-tested' batches. For exsheathment tests, approximately 2000 ensheathed L_3 larvae were pipetted into each containment capsule.

2.5. Larval Containment Capsules:

2.5.1. Nalgene™ Capsules:

This containment capsule was made by capping each end of a short piece of flexible Tygon® tubing with an inner diameter of 9.5 mm and an outer diameter of 22.2 mm (Fisher Scientific, Hampton, NH) (Figure 1A). Caps were made by cutting the center out of the tops to 5 mL Nalgene™ LDPE vials (#6250-0005, Fisher Scientific, Hampton, NH). On the inner side of the top, the hole was then covered with 5 µm CellMicroSieves™ membrane (N5R, BioDesign Inc., Carmel, NY) and glued in place. Various glues were tested including Silicone (Momentive Performance Materials Inc., Waterford, NY) and Loctite® Stik'N Seal (Henkel Corporation, Westlake, OH).

2.5.2. Metal Capsules:

Another containment capsule that was used for exsheathment testing was made out of a brass metal hose union (HU22-12MHX P, Brass Craft®, Novi, MI) capped at each end by a female hose swivel barbed adaptor (HU126-6-12X P, Brass Craft®, Novi, MI) with the barbed swivel removed and replaced with a 5 µm CellMicroSieves™ piece of membrane (N5R, BioDesign Inc., Carmel, NY) (Figure 1B). Post-exsheathment larvae were placed in 15 mL falcon tubes (Globe Scientific Inc., Paramus, NJ) and centrifuged at 1000 RPM for three minutes, and the top supernatant was pipetted off so that the larvae was suspended in less than 2 mL of liquid for easier microscopic examination.

2.5.3. Nunc™ Capsules:

Finally, a capsule using the same Nunc™ Cell Culture Inserts as used by Brunet et al. (2007) was developed. While Brunet et al. (2007) used a "microtube (1 cm diameter x 3 cm long) closed" (p. 1255) with a single Nunc™ Cell Culture Insert, for this study the capsules were made by inserting a Nunc™ Cell Culture Insert (140629, Thermo Scientific, Waltham, MA), which has an 8.0 µm membrane, into each end of a 3.8 cm long piece of flexible Tygon® tubing with an inner diameter of 9.5 mm and an outer diameter of 14.3 mm (Fisher Scientific, Hampton, NH) (Figure 2C). In order to ensure a firm seal, the 'Nunc™ top' must be inserted far enough so that at least 3/4ths of the insert is covered by the tubing. Softening the tubing in warm water may be necessary for full insertion of the top into the tubing. Since the outer diameter of the Nunc™ top (OD: 12 mm; ID: 11 mm) is larger than the inner diameter of the Tygon® tubing (9.5 mm), a firm seal is made. The key attribute of the Nunc™ tops is that they provide an

8 µm membrane that is already sealed to plastic. After inserting the first Nunc™ top, the larvae are pipetted into the capsule and the tube is sealed with the second Nunc™ top. The air pocket created by sealing the tube with the second Nunc™ top is removed by submerging the capsule in water and inserting a 25 G or smaller needle with a syringe through the tubing into the air pocket and drawing back on the syringe. Removing the larvae post-exsheathment testing required cutting the Tygon® tubing in several locations around one Nunc™ top and removing the Nunc™ top.

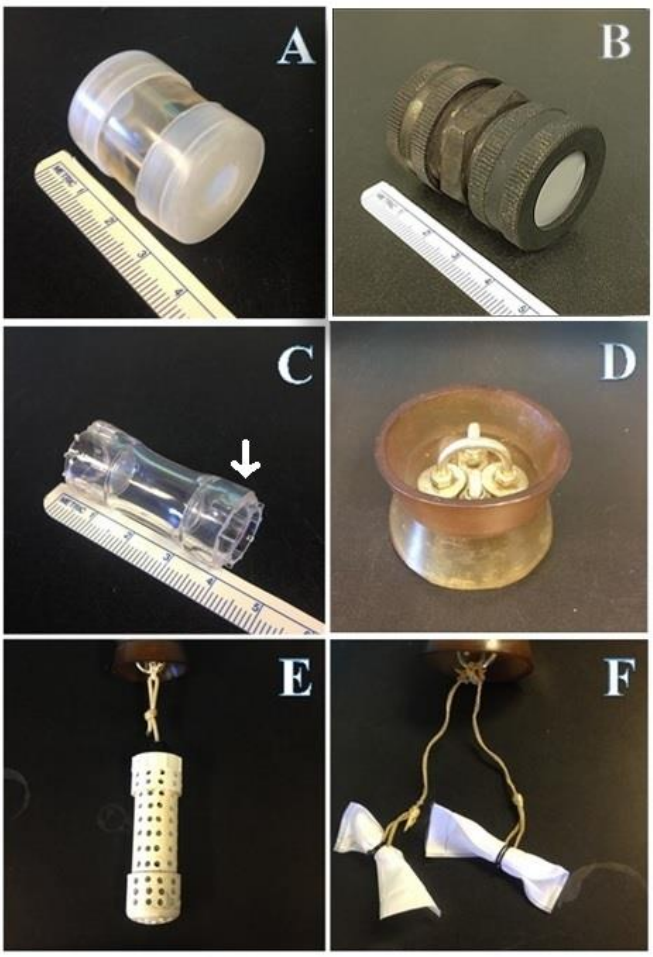

Figure 1. Larval containment and suspension system for exsheathment of *Haemonchus contortus in vivo*. Nalgene™ Capsules were made by capping each end of a short piece of flexible Tygon® tubing (ID 3/8 in, OD 7/8 in) (Figure 1A). Metal capsules were made with a metal hose union (Brass Craft®) capped at each end by a female hose swivel barbed adaptor (Brass Craft®) (Figure 1B). Nunc™ capsules were assembled by capping each end of Tygon® tubing (ID 3/8 in, OD 9/16 in) with an Nunc™ top (white arrow) (Figure 1C). A cannula stopper with a U-bolt fixed to it was used for exsheathment testing (Bar Diamond™, Inc.) (Figure 1D). Two methods of suspending capsules in the rumen were used (Figure 1E; Figure 1F).

2.6. Larval Escape Tests:

Potential capsules were tested for the ability of larvae to escape by placing a capsule containing larvae into small (various sizes based on the capsule of interest) containers filled with tap water. These were then placed in a Daisy incubator (ANKOM Technology, Macedon, NY) set at 37 ºC with the rotation function on (1.1 RPM). This not only simulated the temperature of the rumen, but also the movement. The capsules were left overnight and the liquid in the container but exterior to the containment capsules, was examined microscopically to determine if any larvae had escaped from the capsule, and the number of larvae that escaped was quantified.

2.7. Suspension of exsheathment capsules in Rumen:

For secure attachment of capsules, a cannula stopper with a U-bolt permanently fixed to it was used during exsheathment testing (Bar Diamond™, Inc., Parma, ID) (Figure 1D). Two methods were used for attachment of larvae capsules to the U-bolt.

For the first method, capsules were placed inside a short piece of capped PVC pipe (polyvinyl chloride) that contained numerous holes to allow ruminal fluid flow into and out of the PVC (Figure 1E). These were modeled after PVC containers used for holding digestion bags in rumen fistulated animals (#3T, Bar Diamond™, Inc., Parma, ID). During exsheathments, this container was placed inside the rumen and attached to the U-bolt securely by a cord. Metal capsules were primarily tested using this method.

A second method of attachment was also used (Figure 1F). Each capsule was contained within a 5x10 cm ANKOM heat-sealed 50 µm concentrate bag (R510, ANKOM Technology, Macedon, NY) to prevent clogging of the capsule membranes with large particles (Brunet et al., 2007). In order to suspend the larvae capsules

beneath the fiber mat of the rumen, cords with loops at the bottom were fixed to the U-bolt so that the distance from the U-bolt to the bottom of the loop in the cord was 20 cm, which is similar to distances used by Hertzberg et al. (2002, 25 cm) and Brunet et al. (2007, 20 cm). Each capsule was then attached to its own cord (Hertzberg et al., 2002). An easy and secure attachment was achieved by using two small Zip ties and wrapping them around the capsule and through the loop in the cord. During testing of Nunc™ capsules this method was generally used.

2.8. Timing of Capsule Placement:

It has been observed that the time since a host feeds can affect the ability of rumen fluid to cause the *in vitro* exsheathment of *H. contortus* (Whitlock et al., 1959). Thus, it is important to establish a consistent exsheathment testing protocol relative to feeding time. Hertzberg et al. (2002) report feeding approximately 1 hour after insertion of larvae, while Brunet et al. (2007) report feeding 1 hour prior to insertion of larvae. Although initially exsheathment was tested at multiple timepoints relative to feeding, for most experiments conducted by this lab, sheep were fed just after larvae capsule insertion. This made placement of the capsules in the rumen easier as the rumens were not as full.

2.9. Length of Larval Exposure to Rumen:

Capsules with larvae were left in the rumens of the fistulated sheep for between 1.5 hours and 12 hours. As the goal was to determine the length of time that was required for larvae to consistently exsheath in high numbers, differing lengths of time were tested more or less extensively depending on results obtained. The timepoints tested included 3, 6, 8, 9, and 12 hrs.

2.10. Exsheathment and Motility Determination:

After removal from the rumen, the larvae were moved to non-membranous containers and examined under a microscope for exsheathment and motility. A larva was considered motile if movement occurred within five seconds of viewing it (Skantar et al., 2005) and exsheathed only if it had completely exited the cuticle.

3. Results:

3.1. Nalgene™ Capsules:

When tested for larval escapes, Nalgene™ capsules showed inconsistent results. The results ranged from zero larvae escaping to numbers of larvae escaping that were too numerous to count. Because of the potential for high numbers of larvae escaping, these capsules were not tested *in vivo*.

3.2. Metal Capsules:

The metal capsules averaged 5 ± 2 larvae (0.3%) escaping per test, which was considered acceptable, and they were then used for *in vivo* testing. Metal capsules were tested for exsheathment percentages after 3, 6, 9, and 12 hours of rumen exposure (Figure 2). The percent exsheathment (mean ± SEM) for these timepoints were 66 ± 2%, 81 ± 2%, 80 ± 5%, and 88 ± 2%, respectively. Tarnishing of the brass was observed, and since the brass capsule was not inert, its use was discontinued.

3.3. Nunc™ Capsules:

The Nunc™ topped capsules averaged 3 ± 2 larvae (0.2%) escaping per test. After the transition to Nunc™ capsules was made from metal capsules, exsheathment was most extensively examined after 6 and 8 hours of rumen exposure (Figure 2). The mean percent exsheathment found at these two timepoints were 73 ± 4% and 77 ± 1%,

respectively. The most variable factor observed was the larvae themselves. Some batches of larvae had much better exsheathment than others. By pre-testing batches of larvae to determine which had the highest exsheathment percentages, and using only those batches that exsheathed well, the variation in the percent exsheathment was greatly reduced. Twelve tests were run with two capsules per ewe using only pre-tested larvae with a total of 96 capsules being tested (Figure 2). The larvae from these tests had average exsheathments of 82 ± 1%.

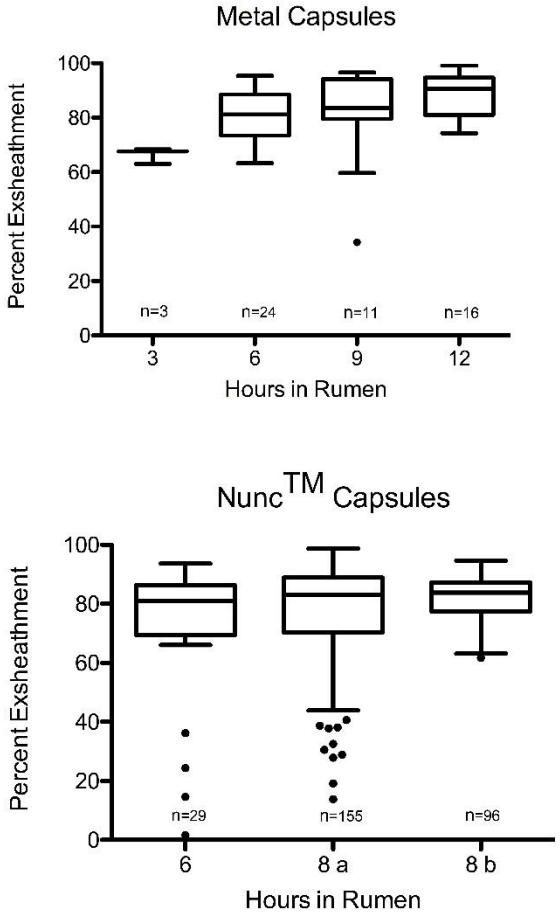

Figure 2. Exsheathment percentages for two types of capsules. Tukey boxplots show the median exsheathment percentage represented by the middle horizontal line, the first and third quartiles by the boxes, and the data within 3/2 of the interquartile range shown by the whiskers. Metal capsules: Exsheathment percentages were examined at four timepoints. Nunc™ capsules: Results from exsheathment percentages examined at two timepoints. The eight hour timepoint is further split into 8a and 8b. While 8a represents all of the applicable exsheathments measured at eight hours, 8b shows only the exsheathments that were completed using the final methods including pre-testing the larvae.

3.4. Fecal Egg Counts:

During the period of Nunc™ top capsule testing, fecal samples were taken weekly, and fecal egg counts were performed. The average fecal egg count for the four ewes during the testing was 25 ± 4 eggs per gram. The average for the eight weeks prior to the testing had been 25 ± 7 eggs per gram.

4. Discussion:

Testing of three containment capsules for the *in vivo* exsheathment of *H. contortus* showed that the Nunc™ capsules were most suited to these tests. This was determined through the measurement of larval escapes and exsheathment percentages attained in fistulated animals.

The Nalgene™ capsules did not fulfill the requirement for sufficiently containing the larvae and thus were not tested *in vivo* to see if larvae exsheathment was attained. While the metal capsules described in this paper fulfilled both of these requirements, due to the observed tarnishing of the brass these capsules were discontinued. It was also hypothesized that the size and weight of the metal capsules combined with the PVC suspension chamber (500g total) would allow only minimal movement of the capsules within the rumen and therefore might not accurately represent *in vivo* larval experience. Thus, neither of these capsules are recommended for *in vivo* exsheathment testing.

While the containment bags used for *in vivo* exsheathments by Sommerville (1957) and Hertzberg et al. (2002) would have a larger membranous surface area, the Nunc™ capsules described here have twice the membrane surface area of those described by Brunet et al. (2007). The successful exsheathment of the larvae indicated

that sufficient flow of ruminal fluid into the capsules was achieved. The goal of minimizing the parasitic infection of the fistulated animals was also achieved as evidenced by the fecal egg counts remaining low throughout the. Thus, the Nunc^{TM} capsule successfully fulfilled the requirements described and are recommended for use in future *in vivo* exsheathment tests.

The *in vivo* exsheathment results of 82% exsheathed after 8 hours most closely agrees with the findings of Sommerville (1957) who reported that 85% of *H. contortus* L_3 larvae were exsheathed after 5.3 hours. Brunet et al. (2007) was able to obtain exsheathment of 78% after only 2.7 hours, but no other study has replicated the findings of Hertzberg et al. (2002) who reported 90% of the larvae were exsheathed in 1 hour. These discrepancies between studies emphasize the importance of further *in vivo* exsheathment studies.

It was found that results with only pre-tested batches of larvae had more consistently successful exsheathments. The finding that exsheathment was not consistent between different larvae batches may explain the comments of Sommerville (1957) who reported finding that in regards to *in vivo* exsheathment (referred to here as ecdysis) "Occasionally slower rates of ecdysis than those recorded here were observed, particularly with *H. contortus*" (p. 21). Identifying the variable that causes some batches of larvae to not exsheath well could prove useful not only to researchers by making their *in vivo* results more consistent, but could itself be considered as a potential control for parasite infection.

5. Conclusion:

This procedure opens the way for increased *in vivo* testing of *H. contortus*

exsheathment, and using these methods, potential exsheathment inhibitors that have high efficacy *in vitro* can more readily be evaluated *in vivo*.

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CHAPTER – III

To be submitted to Veterinary Parasitology

Effect of birdsfoot trefoil hay on *in vivo* exsheathment of *Haemonchus contortus*

Effect of birdsfoot trefoil hay on *in vivo* exsheathment of *Haemonchus contortus*

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Highlights:

- Birdsfoot trefoil hay did not inhibit exsheathment of *Haemonchus contortus.*
- Contrary to previous *in vitro* studies, condensed tannin plants may not inhibit exsheathment of *Haemonchus contortus in vivo.*

Abstract:

Although extensive research has been done on the inhibition of exsheathment of *Haemonchus contortus* by *in vitro* exposure to the extract of condensed tannin containing plants, only one study has previously attempted to replicate this process *in vivo* and it was found that consumption of sainfoin slowed the exsheathment rate. For this study, four rumen fistulated ewes were fed three cultivars of birdsfoot trefoil (*Lotus corniculatus*, with condensed tannin concentrations of 5.3 (Bruce), 2.6 (Empire), and 8.4 (Pardee) mg/g for each cultivar) hay or an alfalfa/grass hay control in a Latin 4x4 design. The effect of consumption of birdsfoot trefoil on the exsheathment of *H. contortus* larvae *in vivo* was evaluated. For each exsheathment test, two capsules with 2000 ensheathed L_3 larvae were placed in the rumen of each ewe for eight hours. Larval containment capsules were made by capping each end of a short piece of Tygon® tubing (ID 9.5 mm, OD 14.3 mm) with an 8 µm Nunc™ Cell Culture Insert. Larval exsheathment and motility were examined pre and post rumen exposure. Three exsheathment tests were run per diet cycle. No significant difference was found between the exsheathment for the different cultivars of birdsfoot trefoil and the control diet. These results highlight the importance of further *in vivo* testing since *in vitro* results may not be indicative of *in vivo* efficacy.

Keywords: exsheathment, ecdysis, *Haemonchus contortus*, barber pole worm

1. Introduction:

Internal parasites are a detriment to the health of small ruminants and place a major economic burden on small ruminant producers worldwide (Nieuwhof & Bishop, 2005; Sackett et al., 2006; Qamar et al., 2011). The main parasites include *Teladorsagia circumcincta*, several *Trichostrongylus* species, and *Haemonchus contortus* (Roeber et al., 2013). *Haemonchus contortus* is found globally and is the most pathogenic gastrointestinal nematode (GIN) of small ruminants (Qamar et al., 2011; Roeber et al., 2013; Kearney et al., 2016). This parasite feeds on the blood of its host, and infections can cause anemia, reduced production of wool, milk, and meat, and in severe cases may lead to the death of the host (Roeber et al., 2013; Preston et al., 2014). While commercial anthelmintics are commonly used for the control of internal parasites in small ruminants, *H. contortus* have become increasingly resistant to all of the commercially available anthelmintics (Howell et al., 2008; Gilleard, 2013). Parasite resistance to anthelmintics in small ruminants has impacted Australia since the 1980s, and more recently it has become a global problem (Waller et al., 1995; Manikkavasagan et al., 2013; Lyndal-Murphy et al., 2014; Chandra et al., 2015). In the United States, studies have found parasite resistance to benzimidazole at 98-100% of the farms, levamisole at 24-54%, ivermectin at 76-82%, and moxidectin at 24%-47% (Howell et al., 2008; Crook et al., 2016). Thus, alternative options for the control of gastrointestinal parasites are needed.

A variety of condensed tannin containing plants have been tested for potential anti-parasitic properties with several being found to affect fecal egg counts or worm burden counts (Hoste, 2006). Sericea lespedeza (*Lespedeza cuneata*) has been well

documented as a plant with anthelmintic properties (Shaik et al., 2006; Terrill et al., 2007; Joshi et al., 2011; Gujja et al, 2013). Feeding sericea lespedeza hay to buck kids was associated with 88% lower fecal egg counts and 61% lower abomasal worm burdens than animals receiving a control diet (Shaik et al., 2006). Unfortunately, sericea lespedeza's area of adaptation does not include the northeastern United States (Ohlenbush et al., 2007). Unlike sericea lespedeza, birdsfoot trefoil (*Lotus corniculatus*) has a large area of adaption that includes the northeastern United States (Steiner, 1999). Feeding a fresh fodder of birdsfoot trefoil (BFT) cultivar (cv.) Odenwalder to lambs with established worm burdens for 17 days was found to reduce fecal egg counts by 63% compared to control animals (Heckendorn et al., 2007). The fodder contained approximately 68% BFT and had a condensed tannin content of 15g/kg (Heckendorn et al., 2007). Lambs grazing birdsfoot trefoil cv. Leo for 35 days were also found to have less total adult helminths in their abomasum and intestines than lambs on a control diet (Marley et al., 2003).

Although the mechanism of action that causes some condensed tannin containing plants to have anthelmintic properties has not yet been determined (Cedillo et al., 2015), the level of efficacy that these plants exhibit is thought to be related to the structure of the condensed tannins present (Quijada et al., 2015). One such structural component is the ratio of prodelphinidins to procyanidins (Brunet & Hoste, 2006; Quijada et al., 2015). An *in vitro* exsheathment inhibition test showed that purified prodelphinidins were more effective at preventing larval exsheathment than procyanidins (Brunet & Hoste, 2006). Other structural features that are being examined as potential indicators of efficacy include the stereochemistry of the

condensed tannin sub-units and the molecular weight of the condensed tannins (Quijada et al., 2015). Different cultivars of condensed tannin plants, including birdsfoot trefoil, often have varying condensed tannin contents (Hedqvist et al., 2000; Azuhnwi et al., 2013).

During *H. contortus* infection, third-stage (L_3) larvae are consumed by the host, and while the larvae are in the rumen, exsheathment is triggered (Sommerville, 1957). Exsheathment is the process by which larvae shed their outer protective cuticle. *In vitro* testing of certain condensed tannin containing forages have found that these plants are capable of reducing the percentage of *H. contortus* larvae successfully completing the exsheathment stage, and this assay is used to screen for potential anthelmintic plants (Brunet et al., 2007; Alonzo-Diaz et al., 2011; Oliveira et al., 2011; von Son-de Fornex et al., 2012; Moreno-Gonzalo et al., 2013; Mengistu et al., 2016). Only one previous study has attempted to show a similar result *in vivo* (Brunet et al., 2007). That experiment was done using fistulated sheep and found that feeding increasing amounts of the condensed tannin containing plant sainfoin (*Onobrychis viciifolia*) slowed the exsheathment process (Brunet et al., 2007). The study fed sainfoin as a fresh chopped forage (Brunet et al., 2007), and no similar studies have been reported using other anthelmintic plants or diets in the form of hay. In order to effectively incorporate tannin-containing plants into the diets of small ruminants, a clearer understanding of the effect of these plants on *in vivo* exsheathment of larvae is needed to determine if *in vitro* techniques of exsheathment are accurately reflecting what occurs *in vivo*.

The purpose of this study is to evaluate the *in vivo* ability of birdsfoot trefoil hay to

prevent exsheathment of *H. contortus,* as well as determine the difference in efficacy between three cultivars of birdsfoot trefoil. In an *in vitro* test, freeze-dried birdsfoot trefoil was found to reduce the exsheathment of the cattle parasites *Cooperia oncophora* and *Ostertagia ostertagi* (Novobilsky et al., 2011). In our laboratory, Barone et al. (2016) found that, during *in vitro* tests, aqueous extracts of varying cultivars of freeze-dried birdsfoot trefoil reduced the percent exsheathment of *H. contortus* larvae. Based on these results, three commercially available cultivars representing a broad range of efficacies were chosen for *in vivo* testing.

2. Methods:

2.1. Experimental Design:

Four ruminally fistulated 3-4 year old Dorset cross ewes were fed three cultivars of birdsfoot trefoil hay (Pardee, Empire, and Bruce) and a control hay of alfalfa/grass in a Latin 4x4 design (Table 1). This design was used to evaluate the ability of varying cultivars of birdsfoot trefoil to prevent the *in vivo* exsheathment of *H. contortus* L_3 larvae. The ewes consumed each new diet for a minimum of 20 days prior to an eight-day testing period of exsheathment rate (Figure 1). Larval populations used for these exsheathment tests were selected by pre-testing the exsheathment rate in the control ewe, and only batches that exsheathed well ($\geq$ 80%) were used. During the testing period, three exsheathment tests were run during the eight-day experimental period for each of the four diet cycles. Two thousand L_3 larvae, contained in porous capsules, were placed into the rumen of the fistulated ewes for a period of eight hours. Pre- and post-experimental percent exsheathment and percent motility of the larvae was determined by microscopic examination. Two capsules were placed in each ewe per

exsheathment test. This resulted in a total of 24 exsheathment measurements for each diet. The ewes' overall health was monitored throughout the study by daily visual inspections and weekly measurements of body weight, body condition, FAMACHA© scores, packed cell volume, and fecal egg counts. Rumen pH measurements were also taken prior to each *in vivo* exsheathment test. This study was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Rhode Island (AN12-11-008).

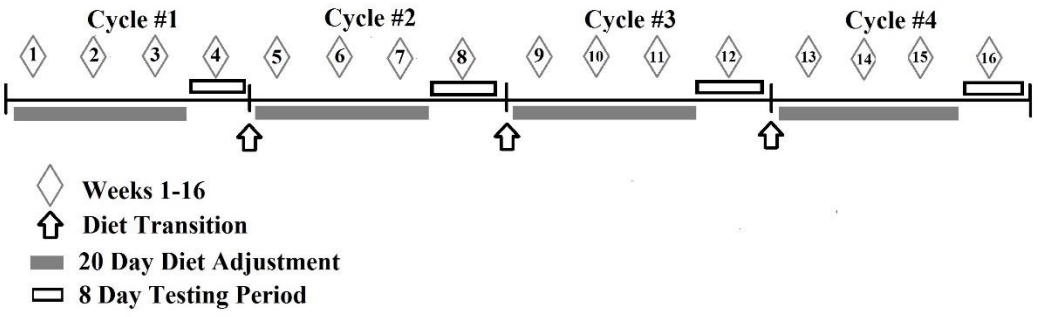
Table 1

Latin 4x4 design. For every cycle, each ewe consumed a different diet.

	Ewe 1206	Ewe 1301	Ewe 1308	Ewe 1314
Cycle #1	Alfalfa/Grass	BFT - Bruce	BFT - Empire	BFT - Pardee
Cycle #2	BFT - Bruce	BFT - Empire	BFT - Pardee	Alfalfa/Grass
Cycle #3	BFT - Empire	BFT - Pardee	Alfalfa/Grass	BFT - Bruce
Cycle #4	BFT - Pardee	Alfalfa/Grass	BFT - Bruce	BFT - Empire

Figure 1

The timeline of feed transitions and testing periods.



2.2. Ewes:

The Dorset cross ewes, born during the springs of 2013 and 2014, had rumen cannulas (Model 8C, Bar Diamond™, Inc., Parma, ID) inserted into surgically created rumen fistulas in the spring of 2015 by Tuft's Ambulatory Service (Woodstock, CT). This was done as a standing surgery using a paravertebral block. A circular incision equivalent to the inner diameter of the cannula was made through the skin and the skin was removed. The abdominal muscles were incised and part of the rumen was drawn out and an incision was made. The cut wall of the rumen was sewn to the cut edge of the skin, and the cannula was inserted. Post-operative care included daily cleaning of the surgical area for the first week, and cleaning as needed thereafter using a modification of the previously established procedure by Penn State (2011). During the study, the ewes were housed individually in indoor 8'x8' pens at Peckham Farm (University of Rhode Island).

2.3. Birdsfoot Trefoil Hay and Control Hay:

Birdsfoot trefoil was planted at Peckham Farm (University of Rhode Island) in May of 2014. The field was seeded at a rate of 20 lbs/acre with inoculated birdsfoot trefoil seed. Cultivar Pardee seeds were purchased from Seedway (Shoreham, VT), Empire from Ernst Conservation Seeds (Meadville, PA), and Bruce from Welter Seed & Honey Co. (Onslow, IA). The birdsfoot trefoil was hayed in July of 2015 and stored for one year prior to the feeding trial. The fields were managed organically, and the hay was sprayed with PRESERVOR™ hay and crop treatment (IBA Inc., Millbury, MA) prior to being baled as round bales. Prior to hay production, % BFT biomass was determined by cultivar. For each cultivar, three random quadrants were measured by

the use of a hoop and clipped at approximately 4 inches above the soil to equal the hay mowing height. Samples were separated as BFT or other, and allowed to air dry. Dried samples were weighed and percent BFT was determined. Empire was found to have 63 ± 4% (mean ± SEM) BFT, Pardee 65 ± 5% BFT, and Bruce 70 ± 9% BFT (Ferguson, unpublished). An alfalfa and grass hay mix was purchased from an outside source and fed as the control hay (Premium Alfalfa/Grass Grab & Go®, Standlee Hay Co. Inc., Kimberly, ID; Grass Hay, Pleasant View Farms, Somers, CT).

2.4. Diet:

Throughout the study, ewes were provided with free choice water and minerals. The control diet contained a mix of alfalfa and grass hay and was purchased from an outside source. When transitioning between the control hay and birdsfoot trefoil hay, on day 1 of a new diet, ewes were fed 25% of the new diet and 75% of the previous diet. The percentage of the new diet was increased by 25% each day until 100% of the feed was the new diet. Diets were formulated to meet or exceed dietary requirements (National Research Council, 2007). Nutrient analysis of all feedstuffs was conducted by Dairy One (Ithaca, NY). For each cycle, a 16% protein sheep pellet (Central Connecticut Co-op, Manchester, CT; Blue Seal, Muscatine, IA) was fed equally to the ewes on each of the four diets. This pelleted grain was fed at a rate of 68 g/day during cycles one and two, but was gradually increased to 454 g/day for cycles three and four.

2.5. Larvae:

Haemonchus contortus larvae used in the exsheathment trials were provided by Dr. Anne Zajac (Virginia Maryland College of Veterinary Medicine, Blacksburg, Virginia) or were recovered from fecal cultures from donor lambs artificially infected with *H.*

contortus larvae provided by Dr. Zajac. The Baermann Technique (Todd et. al, 1970) was used to recover the larvae from the fecal samples incubated at room temperature for eight days. After incubation, the manure was placed in cheese cloth and suspended in a funnel with a short piece of tubing attached to the stem. A clamp was affixed to the end of the tubing. The funnel was filled with water covering the fecal matter. The larvae migrated out of the manure and were collected at the bottom of the clamped tube. The tube was then clamped above the larvae, and the lower clamp was removed, allowing the larvae suspended in only a small amount of water to be collected. Larvae were considered to be age zero on the day of collection and were under three months of age at the time of use. After collection from the cultures, larvae were stored at 4°C and adjusted to room temperature for 20-24 hours prior to placement in the rumen. In order to maximize exsheathment rates, batches of larvae were selected by testing their exsheathment rates in the control animal prior to the study's exsheathment tests.

2.6. Exsheathment:

The exsheathment method used for this study was developed in this laboratory (Lonngren et al., 2017). For each ewe, approximately 2,000 ensheathed L_3 larvae were placed in each of two containment capsules. Containment capsules were made by capping each end of a small 3.8 cm piece Tygon® tubing (ID 9.5, OD 14.3 mm, Fisher Scientific, Hampton, NH) with an 8 µm Nunc™ Cell Culture Insert (#140629, Thermo Scientific, Waltham, MA) (Figure 2A). The capsules were each placed in a 50 µm heat-sealed concentrate bag (R510, ANKOM Technology, Macedon, NY) and suspended in the rumen of the ewe by a 20 cm cord (Figure 2B). The capsules were placed in the rumen of each ewe immediately prior to the morning feeding and

removed after eight hours. After removal from the rumen, the larvae in each capsule were transferred to a 2 mL capsule and a minimum of 150 larvae were examined for motility and exsheathment. Only motile larvae were included in exsheathment calculations. Motility was defined by movement within 5 seconds of viewing (Skantar et al., 2005). Larvae were defined as exsheathed if they were entirely free of their cuticle. Percentages of exsheathment were adjusted based on pre-experiment larval exsheathment. This was accomplished by using the following formulas.

$$\% \text{ Exsheathed} = \frac{\#\text{Exsheathed} - y}{\text{Total} - y} \times 100\%$$

Where:

y = (Pre-Experiment %Exsheathed) x (Total Larvae Counted)

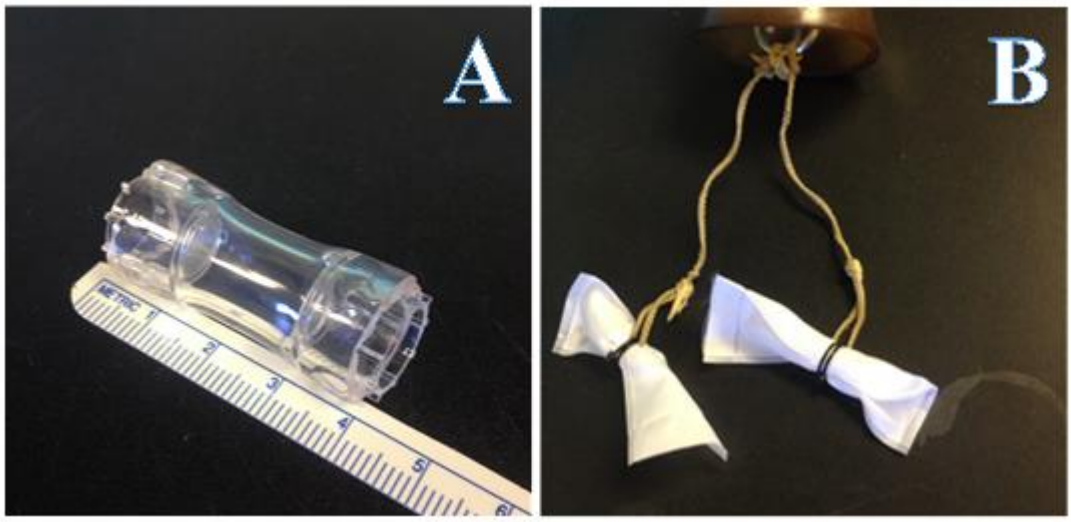


Figure 2. Larval containment system for *in vivo* exsheathment of *Haemonchus contortus*. Larval containment capsules composed of Tygon® tubing (ID 9.5 mm, OD 14.3 mm) capped on each end with 8 µm Nunc™ Cell Culture Insert (Figure 2A). Capsules were each placed in a 50 µm heat-sealed concentrate bag and suspended on a 20 cm cord attached to a U bolt on the inner edge of the cannula plug (Figure 2B).

2.7 Rumen pH

The rumen pH of each ewe was taken immediately prior to every exsheathment test. A sample of rumen fluid was taken from deep in the rumen, and pH was measured

within 15 minutes using an accumet^{TM} portable pH meter and automatic temperature compensation (ATC) electrode (AP115, Fisher Scientific, Hampton, NH). The meter was calibrated per manufacturer instructions with buffers of pH 4 and pH 7 prior to each use.

2.8. Condensed Tannin analysis:

The condensed tannin concentration was determined using the 4-(dimethylamino)cinnamaldehyde method (DMAC) by the Reed Research Group (Cardiovascular, Mucosal Immunology, and Phytochemistry Research Cores, University of Wisconsin, Madison, WI). Extracts were prepared by addition of proanthocyanidin extraction solution, and absorption readings were taken at 640 nm and measured by a Thermo Scientific Varioskan Flash plate reader (Hudson, NH) (Feliciano et al., 2012; Krueger et al., 2016). Analyses were performed on each cultivar of birdsfoot trefoil using pure freeze-dried samples. Results were adjusted to reflect the % BFT biomass of each cultivar's hay.

2.9. Statistics:

Data was analyzed with R (R Core Team, 2016, Vienna, Austria) using an additive effects model appropriate for Latin 4x4 designs (Montgomery, 2013). The experimental model used was $\gamma_{ijk} = \mu + T_{i+}\alpha_j + \beta_k + \varepsilon_{ijk}$ where μ is the overall mean % exsheathment, T_i is the treatment effect from feeding birdsfoot trefoil ($i = 1, 2, 3, 4$), α_j is the effect of the ewes ($j = 1, 2, 3, 4$), β_k is the effect of Latin square rows ($k = 1, 2, 3, 4$), and ε_{ijk} is the error. Differences were considered biologically relevant at $P < 0.05$. Analysis of food intake, pH, and bodyweight data were completed using the same model as exsheathment data, but the response variables were daily feed intake,

pH, and percent change in weight from the pre-study baseline, respectively. Although the feed intake data was not normally distributed, the analysis was justified due to the large sample size (Ghasemi & Zahediasl, 2012).

3. Results:

3.1. Diet and Ewes:

The results of the forage analysis are reported in Table 2. The control diet was altered to a higher ratio of grass hay to alfalfa hay after the first cycle to better match the protein levels of the BFT diets. The percentage of dry matter consumed for each diet is reported in Table 3. After the first two cycles, the ewes were allowed free choice access to treatment and control hay, and their grain intake was increased (68 to 454 g/day) in order to maintain body condition, as there was a decrease in percent bodyweight during the cycles 1 and 2 (Figure 3). Table 5 shows the comparison of average daily nutrient intake to National Research Council (NRC) requirements. The ewes' average change in percent bodyweight by cycle was negative for cycles 1 and 2, and positive for cycles 3 and 4 (Table 4). Fecal egg counts were low throughout the study with the highest egg count at only 150 eggs per gram.

Table 2

Comparison of the nutritional content of the forages and grain fed during study.

	Control (C1)	Control (C2-4)	BFT Bruce	BFT Empire	BFT Pardee	Grain
DM	90.8	92.4	92.0	91.9	91.1	89.2
% CP	19.9	12.7	12.3	11.4	12.4	18.9
% TDN	56	58	51	50	51	74
ME (Mcal/kg)	2.16	2.16	1.87	1.82	1.85	3.00

C1 = cycle 1 of the study, C2-4 = cycle 2 through cycle 4, % DM = % Dry Matter, % CP = % Crude Protein (Dry Matter), % TDN = % Total Digestible Nutrients (Dry Matter), ME = Metabolizable Energy.

Table 3

Daily dry matter intake by diet and cycle.

	Control (kg/day)	BFT Bruce (kg/day)	BFT Empire (kg/day)	BFT Pardee (kg/day)	Cycle* (kg/day)
Cycle #1	1.64 ± 0.00	1.66 ± 0.00	1.65 ± 0.00	1.56 ± 0.03	1.66 ± 0.01^{a}
Cycle #2	1.65 ± 0.02	1.47 ± 0.05	1.66 ± 0.00	1.63 ± 0.00	1.60 ± 0.01^{a}
Cycle #3	1.88 ± 0.05	2.07 ± 0.04	1.87 ± 0.05	2.53 ± 0.06	2.09 ± 0.04^{b}
Cycle #4	2.24 ± 0.08	2.00 ± 0.04	2.38 ± 0.05	2.01 ± 0.05	2.02 ± 0.05^{b}
Diet*	1.89 ± 0.03^{ab}	1.80 ± 0.03^{a}	1.89 ± 0.03^{b}	1.93 ± 0.04^{b}	

*Means within a single row or column with differing superscripts vary significantly ($P < 0.05$).

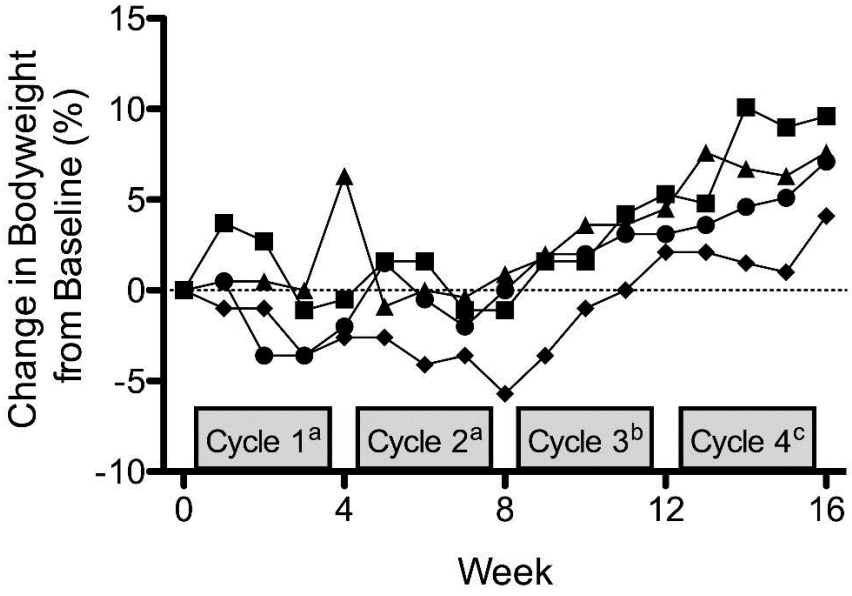


Figure 3. Percent change in ewes' weight from a pre-study baseline over time. Ewes shown are 1206 (●), 1301 (■), 1308 (♦), and 1314 (▲). Ewes' weights increased after diets were changed to free choice. Cycles with differing superscripts vary significantly ($P < 0.05$).

Table 4

Percent change in ewes' weight from pre-study baseline

	Ewe 1206 (%)	Ewe 1301 (%)	Ewe 1308 (%)	Ewe 1314 (%)	Cycle* (%)
Cycle #1	-2.2 ± 1.0	1.2 ± 1.2	-2.1 ± 0.6	1.8 ± 1.5	-0.3 ± 0.7^{a}
Cycle #2	-0.3 ± 0.7	0.3 ± 0.8	-4.0 ± 0.4	-0.1 ± 0.4	-1.0 ± 0.5^{a}
Cycle #3	2.6 ± 0.3	3.2 ± 0.9	-0.6 ± 1.2	3.4 ± 0.6	2.1 ± 0.6^{b}
Cycle #4	5.1 ± 0.8	8.4 ± 1.2	2.2 ± 0.7	7.0 ± 0.3	5.7 ± 0.7^{c}
Ewe*	1.3 ± 0.8^{a}	3.3 ± 0.9^{b}	-1.1 ± 0.7^{c}	3.0 ± 0.8^{b}	

*Means within a single row or column with differing superscripts vary significantly ($P < 0.05$).

Table 5

Comparison of average daily nutrient intake to NRC requirements.

Cycle	Diet	DM (kg/day)	CP (kg/day)	TDN (kg/day)	ME (Mcal/day)
#1	Control	1.55	0.308	0.88	3.40
	BFT Bruce	1.59	0.200	0.82	3.04
	BFT Empire	1.58	0.184	0.80	2.94
	BFT Pardee	***1.48***	0.188	***0.77***	***2.81***
#2	Control	1.59	0.204	0.92	3.47
	BFT Bruce	***1.41***	0.178	***0.73***	***2.71***
	BFT Empire	1.59	0.186	***0.81***	2.96
	BFT Pardee	1.55	0.196	***0.80***	2.94
#3	Control	2.14	0.296	1.30	4.92
	BFT Bruce	2.31	0.310	1.27	4.77
	BFT Empire	2.12	0.272	1.16	4.34
	BFT Pardee	2.71	0.362	1.47	5.47
#4	Control	2.48	0.338	1.49	5.69
	BFT Bruce	2.24	0.303	1.24	4.65
	BFT Empire	2.59	0.326	1.39	5.20
	BFT Pardee	2.23	0.303	1.23	4.60
NRC requirements	100 kg Ewe	1.54	0.116	0.82	2.94

Bold and italicized numbers represent consumption under nutrient requirements. DM = Dry Matter, CP = Crude Protein, TDN = Total Digestible Nutrients, ME = Metabolizable Energy. Nutrient differences during transitions between diets were ignored.

3.2. Exsheathment:

There was no difference in percent exsheathment between the three cultivars of birdsfoot trefoil and the control diet (P = 0.29), and no difference in percent exsheathment between the cultivars (Figure 4). On average, the motility was similar pre and post exsheathment. Pre-experiment motility was 94 ± 1%.

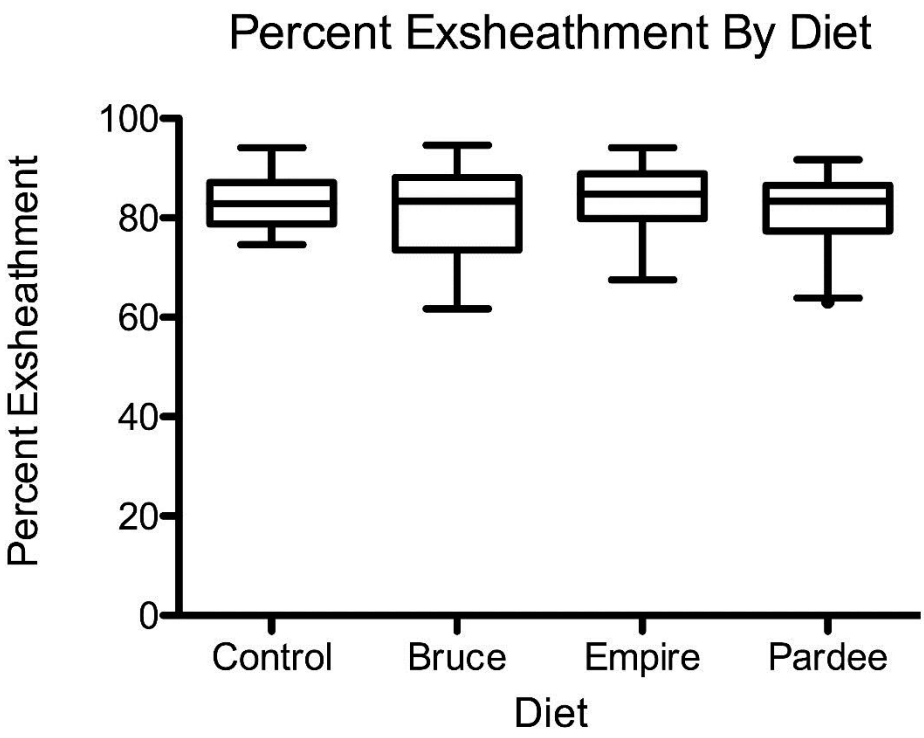


Figure 4. Percent *in vivo* exsheathment for each diet. Data is shown as Tukey's boxplots with the middle horizontal line representing the median, each box representing the first or third quartile of the data, and each whisker representing the data within 1.5 times the interquartile range. There was no difference between diets (P = 0.29).

3.3. Rumen pH:

Rumen pH is reported by diet and cycle (Table 6). Rumen pH was higher when BFT Empire was consumed compared to the control (6.60 ± 0.04 vs 6.37 ± 0.10). Rumen pH did not differ between the control diet and BFT Bruce or BFT Pardee. There was also no difference in rumen pH between the cultivars of birdsfoot trefoil.

Table 6

Comparison of pH for each diet and cycle.

	Control (pH)	Bruce (pH)	Empire (pH)	Pardee (pH)	Cycle* (pH)
Cycle #1	6.91 ± 0.03	6.71 ± 0.04	6.60 ± 0.03	6.36 ± 0.09	6.65 ± 0.06[a]
Cycle #2	6.03 ± 0.10	6.37 ± 0.03	6.76 ± 0.11	6.52 ± 0.09	6.42 ± 0.09[b]
Cycle #3	6.24 ± 0.02	6.25 ± 0.01	6.58 ± 0.02	6.60 ± 0.04	6.42 ± 0.05[b]
Cycle #4	6.41 ± 0.02	6.41 ± 0.06	6.44 ± 0.05	6.48 ± 0.01	6.41 ± 0.03[b]
Diet*	6.37 ± 0.10[a]	6.44 ± 0.05[ab]	6.60 ± 0.04[b]	6.49 ± 0.04[ab]	

*Means within a single row or column with differing superscripts vary significantly ($P < 0.05$).

3.4. Condensed Tannin analysis:

The adjusted condensed tannin content of each cultivar of birdsfoot trefoil was 5.3 mg/g, 2.6 mg/g, and 8.4 mg/g for Pardee, Empire, and Bruce, respectively.

4. Discussions:

This study found that *in vivo* exsheathment of *H. contortus* was not affected by consumption of birdsfoot trefoil hay, and there were no differences in exsheathment between the cultivars fed. An additional finding was that the rumen pH of the ewes consuming Empire was higher than those consuming the control diet of alfalfa/grass.

Although a difference was found between the ruminal pH of ewes consuming birdsfoot trefoil cv. Empire and the control diet, these pH values were still within the normal range for sheep and are likely not biologically relevant (Dehority & Tirabasso, 2001). No significant difference in exsheathment was found between the control diet and the three cultivars of birdsfoot trefoil. Thus, feeding birdsfoot trefoil hay to ewes did not inhibit the exsheathment of L_3 *H. contortus* placed in their rumens, despite

Barone et al. (2016) finding that the cultivars Empire and Pardee greatly inhibited exsheathment *in vitro*. The study by Barone et al. (2016) used a 25 mg/mL aqueous extract of freeze dried birdsfoot trefoil and exsheathment was inhibited by over 75% for the cultivars Pardee and Empire, but was not inhibited for Bruce. Exsheathment of *H. contortus* has been extensively researched *in vitro,* and is used as a method for screening potential anthelmintic plants (Oliveira et al., 2011; von Son-de Fornex et al., 2012; Moreno-Gonzalo et al., 2013; Mengistu et al., 2016).

Prevention of *in vivo* exsheathment by the feeding of condensed tannin containing plants was examined in a study by Brunet et al. (2007), and it was found that in animals consuming 100% sainfoin diets, *H. contortus* larvae exposed to ruminal fluids for 2.7 hours had 59% lower exsheathment than animals on control diets. Other than the difference in the condensed tannin containing plants that were fed (sainfoin vs birdsfoot trefoil), there are several other variations between the studies that could explain the contrasting results. Brunet et al (2007) fed fresh sainfoin while the birdsfoot trefoil was fed as hay. Condensed tannin content of the birdsfoot trefoil may have been altered by the hay drying process or the extended storage if low levels of fermentation occurred. A previous study found that the condensed tannin containing forage sericea lespedeza had a lower condensed tannin content as hay (15.3%) than as a fresh forage (19.9%) (Puchala et al., 2012). Terrill et al. (1990) found that freeze drying was the preservation method that best maintained the condensed tannins found in fresh plants. Since the condensed tannin content of the hay fed during this study was calculated based on freeze-dried samples, the actual concentration may have been lower. The condensed tannin content of the birdsfoot trefoil fed during this study was

calculated to be between 0.3% and 0.8%, while the tannin content of the sainfoin fed by Brunet et al. (2007) was 3.9%.

Additionally, while the forage fed by Brunet et al. (2007) was reported to be 100% sainfoin, the birdsfoot trefoil hay used in this study was found to be between 63-70% birdsfoot trefoil due to the organic management of the hay fields (Ferguson, unpublished). Thus, there were lower concentrations of condensed tannins in the birdsfoot trefoil hay than in the sainfoin. However, the longest exposure to rumen fluid that the larvae experienced in the tests by Brunet et al. (2007) was 2.7 hours and because of that, their results may only indicate a delay in exsheathment and not exsheathment inhibition. In which case, these delayed larvae may have exsheathed if further rumen exposure had occurred. Based on the findings of Lonngren et al. (2017), exsheathment percentages continue to rise after three hours of rumen exposure.

Condensed tannin concentration is likely not the only factor involved in anthelmintic efficacy. Condensed tannins are polymers of flavan-3-ols and can exist in many forms (Reed, 1995). Some of the structural differences of condensed tannins that have been proposed as potentially related to anthelmintic efficacy include the ratio of procyanidins to prodelphinidins, molecular weight, and stereochemistry (Molan et al., 2003; Brunet & Hoste, 2006; Naumann et al., 2014). Brunet & Hoste (2006) found that monomers of prodelphinidins were more effective at inhibiting *in vitro* larval exsheathment than procyanidins. An *in vitro* larval migration inhibition experiment found that the molecular weight of condensed tannins has a slight correlation (R^2 = 0.34, P = 0.05) to efficacy (Naumann et al., 2014). The effect of stereochemistry was investigated by Molan et al. (2003) by comparing monomers of condensed tannins

through an *in vitro* egg hatch assay. Significance due to stereochemistry was only found when testing flavon-3-ol gallate derivatives (Molan et al., 2003). It has also been suggested that other secondary compounds besides condensed tannins may be involved in inhibiting exsheathment (Mengistu et al., 2016). An *in vitro* exsheathment study of ten east African plant species found that *Maerua angolensis,* which does not contain condensed tannins, inhibited *H. contortus* exsheathment. Thus, without further research to determine the effects of structural differences on anthelmintic efficacy, lower vs. higher condensed tannin content alone does not provide sufficient information to draw a conclusion as to why there was a difference in results between this *in vivo* exsheathment study and the one conducted by Brunet et al. (2007).

Although *in vivo* exsheathment trials have been limited, if exsheathment is inhibited by anthelmintic plants, then it would be expected that in feeding trials where infections are given to animals already consuming an anthelmintic plant, experimental animals would have significantly lower established worm burdens than control animals. However, this is often not the case. Feeding sainfoin (*Onobrychis viciifolia*) to small ruminants has been associated with a decrease in fecal egg counts (Hoste et al., 2005; Paolini et al., 2005a; Heckendorn et al., 2007; Rios-De Alvarez et al., 2008). However, Paolini et al. (2005b) found by worm burden counts that feeding sainfoin to young goats did not prevent L_3 *H. contortus* from developing and establishing themselves in the abomasa of the goats. This indicates that exsheathment was not inhibited. Small ruminant feeding trials with sericea lespedeza (*Lespedeza cuneata*) have found this plant to be associated with anti-parasitic effects (Lange et al., 2006; Shaik et al., 2006; Terrill et al., 2007; Joshi et al., 2011; Gujja et al., 2013). However, a

study by Lange et al. (2006) found that when sericea lespedeza hay was fed during a trickle infection of *H. contortus*, while the fecal egg counts of the ewe lambs consuming the experimental diet were approximately 67%-82% lower than the control, the adult abomasal worm count was not significantly different between diets. This is again indicating that exsheathment was not inhibited and implies that the anthelmintic effects must be occurring during a different stage of the *H. contortus* life-cycle.

In order to determine whether the discrepancy in results between this study and the study by Brunet et al. (2007) was due to a difference in anthelmintic properties between the two experimental diets, or due to the shorter ruminal incubation period used by Brunet et al., further *in vivo* exsheathment studies should be conducted.

5. Conclusion:

Feeding birdsfoot trefoil hay to ewes did not significantly affect the exsheathment of *H. contortus* L_3 larvae placed in their rumens for 8 hours. Based on the results of this and previous studies, it is likely that despite efficacy *in vitro,* anthelmintic plants may not prevent *H. contortus* exsheathment *in vivo.* Instead, anthelmintic plants are likely acting upon a different stage in the life-cycle of *H. contortus*. However, in order to confirm these results further *in vivo* experiments should be conducted with a variety of anthelmintic plants.

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CHAPTER - IV

FUTURE DIRECTIONS

Introduction:

The goal of this research was to determine whether birdsfoot trefoil inhibits the *in vivo* exsheathment of *Haemonchus contortus* L_3 larvae and if some cultivars of birdsfoot trefoil have higher efficacy than others. During the first phase of this research, a containment capsule for the *in vivo* exsheathment of *H. contortus* was designed and tested, and a method for testing larval exsheathment rates in rumen fistulated sheep was established. One difficulty that presented itself was frequent batches of larvae that did not exsheath well. For the purpose of these experiments, this difficulty was overcome by discarding these batches of larvae. Although preliminary testing was done to try to determine a potential difference between batches of larvae that exsheathed well and those that didn't, no clear answer was found. In the future, testing should be done to determine if there is an environmental factor involved in the growth and storage of these larvae that is contributing to this issue. For the second phase of the research, a feeding trial was completed using four rumenally fistulated ewes fed three cultivars of birdsfoot trefoil hay and a control hay in a Latin 4x4 design. There was no difference observed between the exsheathments of *H. contortus* L_3 larvae in ewes consuming any of the cultivars of birdsfoot trefoil and those consuming the control hay. However, future testing will need to be completed in order to determine if these results can be generalized to other anthelmintic plants, or to fresh birdsfoot trefoil. These tests can examine variabilities such as fresh plants versus

dried, other cultivars or species, and higher percentages of the forage in the sheep's diet. These possibilities for further testing are outlined below.

Larval Variability:

Larval exsheathment variability by batch was observed throughout the *in vivo* exsheathment testing. While some batches of larvae exsheathed well, others did not. There are several variables in the culturing of the larvae that can be explored. In this lab, *H. contortus* eggs are collected from donor lambs and suspended over a dish of water by cheese cloth for eight days at room temperature. It can be tested to see if more closely controlling the temperature and humidity of the developing larvae during this period has an effect on their exsheathment. A second factor that can be tested is whether larvae cultured from newly infected donor lambs exsheath more reliably than those from lambs with older infections, where the adult parasites are nearing the end of their life-cycle. It is possible that the offspring of the older parasites have less vigor and don't exsheath as well. Another possibility includes seasonal changes affecting the worm populations in the donor lambs, and while this would be harder to control, it can be noted during other exsheathment tests.

Weed Control:

The birdsfoot trefoil hay that was fed during this exsheathment study was between 63-70% birdsfoot trefoil (Ferguson, unpublished), while the rest was weeds. This high number of weeds present was due to the goal of organically managing the birdsfoot trefoil hay plots. However, in future exsheathment testing if weed control was used on the plants during their growth, the higher concentration of the anthelmintic plants may show some level of exsheathment inhibition.

Grazing:

The birdsfoot trefoil that was fed during the feeding trial was hay that had been stored for approximately one year. Another variable that could be tested for is the potential for changes in the condensed tannins during the haying process and storage. In order to remove the hay drying factor, a feeding trial can be done with the animals grazing birdsfoot trefoil prior to *in vivo* exsheathment tests. Alternatively, plants could be clipped and fed to the animals daily as a fresh fodder.

Other Cultivars or Species:

While three cultivars of birdsfoot trefoil hay were not found to inhibit *in vivo* exsheathment, there are other cultivars that could be tested. These cultivars in this study were chosen from six commercially available cultivars to represent a broad range of inhibition efficacies based on *in vitro* exsheathment tests. However, non-commercial cultivars with different condensed tannin content may be found to have efficacy. Similarly, other anthelmintic plants can be examined for *in vivo* exsheathment inhibition.

Other Parasitic Stages:

During future *in vivo* exsheathment feeding trials in fistulated sheep, simultaneously, lambs with established worm burdens can be fed equivalent test and control diets and monitored for anthelmintic efficacy. This would allow a comparison to be made between equivalent diets towards L_3 larvae undergoing exsheathment in fistulated ewes and adult worm populations in lambs. If the exsheathment in fistulated animals is not inhibited, but adult populations in lambs are affected by the plants fed, it will substantiate a claim that exsheathment is not inhibited by anthelmintic plants.

Conclusion:

Further testing is needed to determine if exsheathment of *H. contortus* L_3 larvae can be inhibited by anthelmintic plants. Anthelmintic plants have many factors involved in their production that can cause them to have varying efficacies. A single study can simultaneously examine several of the factors discussed to determine if exsheathment inhibition occurs. This further research is warranted as it will indicate if future research should continue to examine exsheathment, or if it should focus on other life stages of the parasite. Ultimately, understanding the stages of the parasites affected by these plants will allow producers to feed these plants at the most effective times.

APENDIX 1

Rumen Fistula Surgery and Maintenance:

Surgery:

During the spring of 2015 four ewes underwent the following rumen fistulation surgery which was approved by the University of Rhode Island's Institutional Animal Care and Use Committee (IACUC) (AN12-11-008). The ewes were transported to Tuft's Ambulatory Service (Woodstock, CT). The surgery was done with the animals standing and a paravertebral block was used. Needle pricks ensured that the area was sufficiently anesthetized. The area of the paralumbar fossa between the 13th rib, hipbone, transverse process, and the ventral edge of the flank was clipped and cleaned for surgery. A round incision the size of the inner diameter of the cannula was made through the skin of the ewe, and the skin was removed. The abdominal muscles beneath, external oblique, internal oblique, and transverse abdominus, were cut through to gain access to the peritoneal cavity and the rumen. The rumen was then partially removed through the opening and an incision was made into it. Topical penicillin was placed on the muscle layer, and the cut edges of the rumen were then sewn to the cut edges of skin. The incision area was cleaned and the cannula was inserted prior to the animals being transported back to Peckham Farm at the University of Rhode Island.

Maintenance of Rumen Fistulas:

The surgical site was maintained by daily cleaning for the first week, and cleaning as needed thereafter. The general procedure for cleaning the fistulas of the ewes is as follows. The rumen fistula should be cleaned when a buildup of rumen fluid is

observable, and frequently enough that fly larvae do not become present in the buildup. Generally, during the summer fly season this requires cleaning the fistulas three times a week. This can be reduced during the winter to once a week or less during the coldest weeks.

APENDIX 2

Procedure for Capsule Escape Test:

Supplies:

- Capsule to be tested
- L_3 *Haemonchus contortus* larvae
- Daisy incubator (ANKOM Technology, Macedon, NY)
- Water tight container just large enough to hold capsule
- Microscope
- Slides
- Disposable pipettes
- 20 µL pipette and tips
- 1000 µL pipette and tips
- Inverted microscope
- Gridded plate
- 50 mL falcon tubes
- Centrifuge

Procedure:

1. Determine the concentration of motile larvae in the flask by pipetting 10 µL droplets of larvae onto a slide and examining them under a microscope.
2. Find average concentration of motile larvae per µL by dividing the total number of motile larvae by the number of droplets examined and dividing by 10.
3. Determine the number of µL needed for 2000 motile larvae by dividing 2000

by the average concentration per µL.

4. Pipette 2000 motile larvae into the capsule to be tested.
5. Seal the capsule and place it in a watertight container.
6. Fill the container with water.
7. Place the container in the Daisy incubator at 37ºC and turn on the rotating function.
8. Leave the capsule in the incubator for a minimum of 12 hours.
9. Remove the container from incubator and turn the incubator off.
10. Remove the capsule from the container.
11. Pour the water from the container into 50 mL falcon tubes.
12. Centrifuge the falcon tubes at 2000 RPM for 3 minutes.
13. Pipette the top water out of the falcon tubes without disturbing the larvae at the bottom.
14. Pour the remaining water from the falcon tubes onto the grid plate and examine using the inverted microscope.
15. Count and record all escaped larvae.

APENDIX 3

Data from Escape Tests:

Date	Capsule Type	Glue	Escapes	Other
12/03/15	A	Silicone (Momentive Performance Materials inc.)	Lots	
12/09/15	A	Silicone (Momentive Performance Materials inc.)	2	
12/09/15	A	Silicone (Momentive Performance Materials inc.)	0	
12/09/15	B		Lots	
12/14/15	A	Silicone (Momentive Performance Materials inc.)	0	
12/14/15	A	Silicone (Momentive Performance Materials inc.)	0	
12/14/15	A	Silicone (Momentive Performance Materials inc.)	1	
12/21/15	A	Stik'N Seal (Loctite®)	Lots	
12/21/15	A	Stik'N Seal (Loctite®)	Several	
12/21/15	A	Stik'N Seal (Loctite®)	Tons	
01/04/16	A	Stik'N Seal (Loctite®)	34	Minimal glue used
01/04/16	A	Stik'N Seal (Loctite®)	15	Minimal glue used
01/06/16	A	Stik'N Seal (Loctite®)	8	

01/06/16	A	Stik'N Seal (Loctite®)	0	
01/11/16	A	Stik'N Seal (Loctite®)	16	
01/11/16	A	Stik'N Seal (Loctite®)	0	
01/11/16	A	Stik'N Seal (Loctite®)	0	
01/11/16	A	Stik'N Seal (Loctite®)	1	Capsule used once before
01/12/16	A	Stik'N Seal (Loctite®)	Tons	
01/12/16	A	Stik'N Seal (Loctite®)	35	
01/12/16	A	Stik'N Seal (Loctite®)	2	
01/14/16	A	Nail Polish (Miracle GEL™)	1	
01/14/16	A	Nail Polish (Miracle GEL™)	0	
01/14/16	C		14	
01/14/16	C		8	
01/14/16	A	Silicone (Momentive Performance Materials inc.)	0	
01/14/16	A	Stik'N Seal (Loctite®)	32	Capsule used before
01/14/16	D		3	
01/18/16	A	Mix (S'NS+Silicon)	7	
01/18/16	A	Mix (S'NS+Silicon)	6	
01/18/16	D	Stik'N Seal (Loctite®)	24	
01/18/16	E	Stik'N Seal (Loctite®)	3	
02/03/16	D		23	Two layers of membrane. Capsule had been previously

				glued.
02/03/16	D		2	
02/03/16	A		0	Were then bubbled in CO_2 (Fell apart)
02/03/16	A		1	Were then bubbled in CO_2 (Fell apart)
02/03/16	A		0	Were then bubbled in CO_2 (Fell apart)
02/03/16	A		0	
02/03/16	A		0	
02/09/16	D		5	
02/09/16	D		15	Had glue previously
02/15/16	D		12	
02/15/16	D		8	
02/18/16	D		4	
02/18/16	D		5	
03/17/16	D		Lots	Used a different brand metal cap
05/24/16	F		2	
05/24/16	F		Most	Cap came off
06/08/16	F		9	
06/08/16	F		0	
06/08/16	F		4	
06/08/16	F		0	Double number of larvae
Capsule type:				
A = thick Tygon® with plastic cap + membrane				
B = Heat sealed membrane				
C = Plastic Container				
D = Metal Hose				
E = Plastic Hose				
F = Nunc™ topped				

APENDIX 4

Rumen Fistula/Cannula Cleaning Procedure:

Supplies:

- Stand
- Halter
- Water source (buckets of warm water during winter, hose during summer)
- Blow drier or towels (cold weather only)
- Gloves
- Dawn soap
- Electric clippers (when needed)
- Bug repellent during fly season such as CLAC (Deo Lotion)

Procedure:

1. Halter ewe and put her on the stand.
2. Put on gloves.
3. Thoroughly soak the dirty area around the cannula (weather permitting).
4. Lift the flap of the cannula and remove the caked-on rumen debris.
5. Place soap on your hand and rub it into the wool to further loosen debris.
6. When all of the rumen debris is loose, rinse off the soap.
7. Wipe excess water off the ewe with gloved hand.
8. During cold weather, dry with blow drier or towel.
9. If needed, clip the wool around and under the cannula flap.
10. During fly season, spread bug repellent on the wool around and under the cannula flap as well as on the cannula.

11. Put ewe away and clean everything up.

APENDIX 5

Data from Metal Capsule Exsheathments:

Date	Date of larvae	Depth in Rumen	Ewe ID	% Change Motility	hours in rumen	% Ex	Feeding	Notes
02/17/16	02/02/16	Not Specified	1314	-2.3	3 hrs	64.7	fed only what stole from goat pen	Note[1,2,10]
02/17/16	02/02/16	Not Specified	1206	0.6	6 hrs	75.4	fed only what stole from goat pen	Note[2,10]
02/24/16	02/02/16	Not Specified	1206	-1.4	3 hrs	35.3	fed after insertion	Note[1,2,3,10]
02/24/16	02/02/16	Not Specified	1314	0.1	6 hrs	34.8	fed after insertion	Note[1,2,3,10]
03/02/16	02/02/16	Not Specified	1301	-4.1	3 hrs	68.4	feed not mentioned	Note[1]
03/02/16	02/02/16	Not Specified	1308	0.9	6 hrs	80.2	feed not mentioned	Note[1]
03/04/16	02/02/16	Not Specified	1314	-1.4	6 hrs	92.5	feed not mentioned	Note[1,2]
03/04/16	02/02/16	Not Specified	1206	-4.9	8 hrs	95.2	feed not mentioned	Note[1,2]
03/09/16	02/02/16	Not Specified	1206	-4.3	3 hrs	67.6	feed not mentioned	
03/09/16	02/02/16	Not Specified	1301	2.2	6 hrs	69.6	feed not mentioned	Note[1]
03/11/16	02/02/16	Not Specified	1314	-5.5	6 hrs	82.7	fed after insertion	Note[1,2]
03/11/16	02/02/16	Not Specified	1301	0.0	9 hrs	85.3	fed after insertion	Note[2]
03/15/16	03/02/16	Not Specified	1308	-0.2	9 hrs	94.5	fed after insertion	
03/15/16	03/02/16	Not Specified	1314		9 hrs	96.6	fed after insertion	Note[3,4]
03/16/16	03/02/16	Not Specified	1308	-2.8	1.5 hrs	26.8	no feed	Note[2]
03/16/16	03/02/16	Not Specified	1301		1.5 hrs	30.9	no feed	Note[2,4]
03/22/16	03/02/16	Not Specified	1206	-2.3	9 hrs	81.8	fed after insertion	Note[10]
03/22/16	03/02/16	Not Specified	1308	-1.9	9 hrs	93.7	fed after insertion	Note[10]
03/22/16	03/02/16	Not Specified	1206		9 hrs	84.9	fed after insertion	Note[4,5,10]

03/22/16	03/02/16	Not Specified	1301		9 hrs	87.2	fed after insertion	Note[4,5,10]
03/22/16	03/02/16	Not Specified	1314		9 hrs	92	fed after insertion	Note[4,5,10]
03/29/16	03/02/16	Not Specified	1206	-14.4	9 hrs	84	fed after insertion	Note[6]
03/29/16	03/02/16	Not Specified	1206	0.7	9 hrs	94.2	fed after insertion	
03/29/16	03/02/16	Not Specified	1301	2.9	9 hrs	59.7	fed after insertion	
03/29/16	03/02/16	Not Specified	1301	1.9	9 hrs	34.2	fed after insertion	
03/29/16	03/02/16	Not Specified	1308	-0.7	9 hrs	82	fed after insertion	
03/29/16	03/02/16	Not Specified	1308	0.3	9 hrs	82.3	fed after insertion	
03/29/16	03/02/16	Not Specified	1314	-1.7	9 hrs	83.5	fed after insertion	
03/29/16	03/02/16	Not Specified	1314	0.4	9 hrs	79.5	fed after insertion	
04/05/16	03/02/16	Not Specified	1206	0.2	12 hrs	81.6	fed prior	
04/05/16	03/02/16	Not Specified	1301	-1.6	12 hrs	99.1	fed prior	
04/05/16	03/02/16	Not Specified	1308	-0.6	12 hrs	90	fed prior	
04/05/16	03/02/16	Not Specified	1314	1.7	12 hrs	88.3	fed prior	
04/12/16	mix	Not Specified	1206	-0.6	12 hrs	76.3	fed after insertion	
04/12/16	mix	Not Specified	1206	-3.6	12 hrs	76	fed after insertion	
04/12/16	mix	Not Specified	1301	0.2	12 hrs	93.3	fed after insertion	
04/12/16	mix	Not Specified	1301	-1.3	12 hrs	82.5	fed after insertion	
04/12/16	mix	Not Specified	1308	-2.9	12 hrs	80.9	fed after insertion	
04/12/16	mix	Not Specified	1308	-4.1	12 hrs	74.3	fed after insertion	
04/12/16	mix	Not Specified	1314	2.3	12 hrs	94.7	fed after insertion	
04/12/16	mix	Not Specified	1314	-2.4	12 hrs	95.1	fed after insertion	
04/18/16	04/07/16	20 cm string	1206	-10.4	12 hrs	91.2	not fed	Note[7]
04/18/16	04/07/16	20 cm string	1206	-15.3	12 hrs	94.1	not fed	Note[7]

04/18/16	04/07/16	20 cm string	1308	-12.5	12 hrs	94.7	not fed	Note[7]
04/18/16	04/07/16	20 cm string	1308	-13.8	12 hrs	96.8	not fed	Note[7]
04/21/16	03/02/16	short string	1206	-6.8	7 hrs	88.3	fed prior to insertion	Note[7,8,10]
04/26/16	02/02/16	10 cm string	1301	-18.3	3 hrs	63	feed not mentioned	Note[9]
04/27/16	03/02/16	10 cm string	1206	-22.3	4 hrs	59.4	fed after insertion	
04/27/16	03/02/16	10 cm string	1301	-14.4	4 hrs	81.6	fed after insertion	
04/27/16	03/02/16	10 cm string	1308	-5.2	6 hrs	92.7	fed after insertion	
04/27/16	03/02/16	10 cm string	1308	-4.6	6 hrs	95.4	fed after insertion	
04/27/16	03/02/16	10 cm string	1314	-5.8	6 hrs	93.5	fed after insertion	
04/27/16	03/02/16	10 cm string	1314	-6.0	6 hrs	94.2	fed after insertion	
05/03/16	mix	Total = 25 cm	1206	-9.0	6 hrs	80.1	fed after insertion	
05/03/16	mix	Total = 25 cm	1206	-13.1	6 hrs	88.1	fed after insertion	
05/03/16	mix	Total = 25 cm	1301	-5.1	6 hrs	77.1	fed after insertion	
05/03/16	mix	Total = 25 cm	1301	-5.0	6 hrs	73.5	fed after insertion	
05/03/16	mix	Total = 25 cm	1308	-12.0	6 hrs	73.4	fed after insertion	
05/03/16	mix	Total = 25 cm	1308	-10.3	6 hrs	85.3	fed after insertion	
05/03/16	mix	Total = 25 cm	1314	-9.3	6 hrs	86.3	fed after insertion	
05/03/16	mix	Total = 25 cm	1314	-16.0	6 hrs	88.1	fed after insertion	
05/05/16	05/02/16	Total = 30 cm	1206	-7.7	6 hrs	88.7	fed after insertion	
05/05/16	05/02/16	Total = 30 cm	1206	-5.0	6 hrs	82.2	fed after insertion	
05/05/16	05/02/16	Total = 30 cm	1301	-9.9	6 hrs	79.3	fed after insertion	
05/05/16	05/02/16	Total = 30 cm	1301	-14.3	6 hrs	70	fed after insertion	
05/05/16	05/02/16	Total = 30 cm	1308	-6.2	6 hrs	63.3	fed after insertion	
05/05/16	05/02/16	Total = 30 cm	1308	-9.5	6 hrs	70.7	fed after insertion	

05/05/16	05/02/16	Total = 30 cm	1314	-4.5	6 hrs	80.1	fed after insertion	
05/05/16	05/02/16	Total = 30 cm	1314	-7.4	6 hrs	67.3	fed after insertion	
	Notes							
1	Exsheathed non-motile was same reported in the same column as ensheathed non-motile							
2	Larvae not set out night before							
3	PVC not used (heat sealed bag)							
4	Killed with Lugol's iodine							
5	Read next day							
6	Started using spacers in PCV							
7	Less than 2000 larvae used							
8	Pre-motility may have been lower (some dried non-motile ones not counted)							
9	More than 2000 larvae used							
10	Less than 100 larvae examined							

APENDIX 6

Procedure for *in vivo* Exsheathment:

Supplies:

- 20,000 *H. contortus* larvae
- Microscope
- Slides
- 20 µL pipette and tips
- 1000 µL pipette and tips
- Eight 3.8 cm pieces of Tygon® tubing; ID: 9.5 mm OD: 14.3 mm (Fisher Scientific, Hampton, NH).
- 16 Nunc™ Cell Culture Inserts (i.e. Nunc™ top)
- 4 Cannula plugs with two 20 cm cords attached to the inner side of each
- 16 small zip ties
- 3 mL syringe and 25 G needle
- Thermometer
- 3 large buckets
- 8 labeled 2mL capsules
- Paper towels
- 8 5x10 cm heat sealed concentrate bags (R510, ANKOM Technology, Macedon, NY)
- Impulse heat sealer
- Shoulder length gloves

- Gloves
- 4 rumen fistulated ewes
- 4 halters
- Movable panel
- Thermo water heater
- Small scissors
- Small labeled cups (labeled with ear tag numbers)
- Tube rocker

Procedure:

Day before Experiment:

1. Set larvae out at room temperature 20-24 hours before start of experiment.
2. Read approximate concentration of motile ensheathed larvae (MEnL).
    - Determine # MEnL per 1µL (divide average MEnL by 10).
    - Determine # µL necessary to = 2000 larvae (divide 2000 by #MEnL per 1 µL).

Day of Experiment:

3. Fill one bucket with very warm tap water.
4. Insert one Nunc™ top into one end of each Tygon® tube. Top should be at least 3/4ths covered by tubing.
5. Place all tubes into the bucket filled with very warm tap water to soften tubes.
6. Fill other another bucket with 37ºC tap water (use thermometer).
7. Remove first tube from very warm tap water.
8. Using 1000 µL pipette to pipette the number of µLs necessary to equal 2000

larvae (determined previous day) into softened tube.

9. Pipette 37ºC water into softened tube until approximately 2/3rds full.
10. Carefully insert second Nunc™ top into open end of tube.
11. Place in 37ºC water after seal is made and push Nunc™ top into tube so that 3/4ths is covered by the tubing.
12. Using the syringe and needle, insert the needle into the capsule near the middle of the capsule, but at a nearly parallel angle so that the needle enters the inner part of the tube near one of the Nunc™ tops.
13. Submerge at least one end of the capsule underwater in the 37ºC water and draw back on the syringe to remove the air pocket. The goal is to make the air pocket as small as possible without removing any liquid containing the larvae.
14. Leave the completed capsule in the water and repeat steps 7-13 until all eight capsules are completed.
15. Fill another large bucket half way with 37ºC water.
16. Remove a capsule from the water and dry the outsides using paper towels.
17. Place in one heat seal-able bag and seal end using impulse sealer.
18. Using two zip ties, attach the capsule to one end of a cannula plug string.
    - Wrap one zip tie around the tube and bag and through the loop on the cannula plug string; tighten the zip tie to a snug position.
    - Repeat with the second zip tie.
19. Repeat steps 16-18 until all capsules are attached. When a cannula plug has both capsules attached, submerge in the fresh bucket of 37ºC water.
20. Bring down to fistulated ewes: bucket with cannulas and capsules, shoulder

length gloves, and regular gloves.

21. Use movable panel and halters to catch and secure the four fistulated sheep.
22. Remove cannula plug from sheep with lowest ear-tag number and insert capsules.
    - Using arm with shoulder length glove, cup capsules in hand and insert as deep as possible into rumen.
    - Orient strings to be at the bottom of the U-bolt they are tied to.
    - Insert cannula plug and orient so that the outer U- bolt is parallel to the ground (this makes the inner U-bolt perpendicular to the ground).
23. Repeat for the rest of the fistulated sheep in the order of increasing ear-tag numbers (ex: 1206, 1301, 1308, 1314).
24. Note time of first inserted capsule (Generally 7-8am).
25. Release the ewes and give them their morning feeding.
26. Rinse regular cannula plugs and place somewhere where their smell won't bother others.
27. Read remaining larvae used for set-up to determine pre-experiment motility and exsheathment percentages.
    - Look at a minimum of 150 motile larvae.
    - Be sure to record the age of the larvae and other flask information.
28. Clean-up from set-up.
29. Get together afternoon supplies
    - Fill empty bucket with: more shoulder length gloves, extra regular gloves, 4 halters, small scissors, and labeled cups.

- label the eight 2mL capsules with ear tag numbers (two for each sheep).

30. Wait determined amount of time (8 hours) and remove capsules (3-4pm depending on start time).
31. Approximately 30 minutes before removing capsules:
    - Turn on the Thermo water heater to 37ºC (confirm temp with thermometer).
    - Fill bucket with 37ºC tap water.
32. Bring supplies in bucket from step 29 and the bucket with water to sheep.
33. Dump half the 37ºC water in with the cannula plugs that were removed that morning to soften the plugs.
34. Dump half the remaining water into the labeled cups.
35. Catch the sheep with the movable panel and tie them using the halters.
36. Remove capsules starting with sheep with lowest ear-tag number.
    - When removing capsules avoid pulling out by the strings. Instead reach into rumen with gloved hand, cup capsules, and remove gently.
37. Replace cannula plug with plain plugs from the morning.
38. Cut and discard both zip ties and cut heat sealed bag off of larvae capsule.
39. Rinse capsule in remaining water and place in appropriately labeled cup.
40. Repeat steps 36-40 for all four sheep.
41. Release sheep and clean the dirty cannula plugs and attached strings.
42. Discard rumen-fluid-covered gloves/heatsealed bags/etc in dumpster to avoid attracting flies.
43. Using small scissors, cut several indents in one end of the capsule's tube.

44. Remove Nunc™ top and pour larvae containing fluid into appropriately labeled 2mL capsule.
45. Place capsule into 37ºC water in water heater.
46. Repeat steps 43-46 for all capsules.
47. Place each 2mL capsule on tube rocker when ready to read
48. Read larvae:
    - Using 20uL pipette read 10uL drops of larvae at a time
    - Keep track of exsheathed motile/non-motile and ensheathed motile/non-motile
    - Read until 150 motile larvae or 200 total larvae (whichever comes first).
    - Calculate % Motility and % Exsheathment
    - % Motile = Total motile/Total
    - For exsheathment calculations only motile larvae are included.

% Exsheathment = (#Exsheathed - y)/(Total - y) x 100% where, y = (Pre %Exsheathed) x (Total)

49. Enter information onto online Google document
50. Discard remaining larvae, turn off all equipment, and clean up any other mess.

APENDIX 7

Data from All Nunc™ Top Exsheathments:

Date	Date of larvae	Ewe	% Change Motility	hours in rumen	Post % Ex	Notes
05/26/16	05/17/16	1206	-7.5	3 hrs	26	Note[1,2]
06/01/16	05/17/16	1206	-2.1	6 hrs	83.4	Note[1,2]
06/01/16	05/17/16	1314	-1.8	6 hrs	77.4	Note[1,2]
06/03/16	05/17/16	1206	0.5	6 hrs	71.9	Note[1,2,6]
06/03/16	05/17/16	1314	2.3	6 hrs	83.3	Note[1,2,6]
06/06/16	05/17/16	1206	-12.7	6 hrs	1.6	Note[1,2,3]
06/06/16	05/17/16	1314	-14.3	6 hrs	36.2	Note[1,2,3]
06/09/16	06/06/16	1206	0	6 hrs	66	Note[1,3]
06/09/16	05/17/16	1206	-14.1	6 hrs	24.4	Note[1,2,3]
06/09/16	05/17/16	1314	-4.2	6 hrs	14.6	Note[1,2,3]
06/13/16	05/17/16	1206	-5.2	6 hrs	66.4	Note[1,2,4]
06/13/16	05/17/16	1314	-1	6 hrs	90.6	Note[1,2,4]
06/13/16	06/13/16	1206	-0.6	6 hrs	86.3	Note[1,2,4]
06/13/16	06/13/16	1314	0.6	6 hrs	81.1	Note[1,2,4]
06/16/16	06/13/16	1206	1	6 hrs	78.3	
06/16/16	06/13/16	1206	1	6 hrs	88.7	
06/16/16	06/13/16	1301	0.3	6 hrs	66.9	
06/16/16	06/13/16	1301	-0.3	6 hrs	73.9	
06/16/16	06/13/16	1308	1	6 hrs	77.2	
06/16/16	06/13/16	1308	0.4	6 hrs	75.6	
06/16/16	06/13/16	1314	1	6 hrs	84.9	
06/16/16	06/13/16	1314	-1.5	6 hrs	81.5	
06/21/16	06/15/16	1206	-1.8	6 hrs	88.8	
06/21/16	06/15/16	1206	-1.3	6 hrs	83.8	
06/21/16	06/15/16	1301	-0.6	6 hrs	78.1	
06/21/16	06/15/16	1301	-1.3	6 hrs	80.9	
06/21/16	06/15/16	1308	0	6 hrs	93.6	
06/21/16	06/15/16	1308	-1.5	6 hrs	88.7	
06/21/16	06/15/16	1314	0	6 hrs	93	
06/21/16	06/15/16	1314	-10	6 hrs	86.3	Note[5]
06/28/16	06/13+15/2016	1206	0.7	8 hrs	87.4	
06/28/16	06/13+15/2016	1206	0.1	8 hrs	94.4	

06/28/16	06/13+15/2016	1301	0.2	8 hrs	93.4	
06/28/16	06/13+15/2016	1301	-0.6	8 hrs	92.7	
06/28/16	06/13+15/2016	1308	-1	8 hrs	94.8	
06/28/16	06/13+15/2016	1308	0.1	8 hrs	89	
06/28/16	06/13+15/2016	1314	0.7	8 hrs	90.5	
06/28/16	06/13+15/2016	1314	0.7	8 hrs	94	
07/05/16	06/30/16	1206	1	8 hrs	91.5	Note[2]
07/05/16	06/30/16	1206	-2.6	8 hrs	86.2	Note[2]
07/05/16	06/30/16	1301	-3	8 hrs	97	Note[2]
07/05/16	06/30/16	1301	-2.3	8 hrs	98.7	Note[2]
07/05/16	06/30/16	1308	0.3	8 hrs	93.1	Note[2]
07/05/16	06/30/16	1308	-2.1	8 hrs	97.4	Note[2]
07/05/16	06/30/16	1314	2.2	8 hrs	92.8	Note[2]
07/05/16	06/30/16	1314	0.9	8 hrs	93.4	Note[2]
08/09/16	08/02/16	1206	-3.3	8 hrs	48	Note[7]
08/09/16	08/02/16	1206	-0.7	8 hrs	53.8	Note[7]
08/09/16	08/02/16	1301	-3.6	8 hrs	53.8	Note[7]
08/09/16	08/02/16	1301	-1.9	8 hrs	58.8	Note[7]
08/09/16	08/02/16	1308	0.6	8 hrs	38.7	Note[7]
08/09/16	08/02/16	1308	-0.2	8 hrs	53.5	Note[7]
08/09/16	08/02/16	1314	0.5	8 hrs	45.8	Note[7]
08/09/16	08/02/16	1314	1.8	8 hrs	46	Note[7]
08/10/16	08/02/16	1206	1.9	8 hrs	40.6	Note[8]
08/10/16	08/02/16	1206	0	8 hrs	53	Note[8]
08/11/16	07/25/16	1206	-1.2	8 hrs	68.9	
08/11/16	07/25/16	1206	-1.9	8 hrs	77.7	
08/19/16	June 2016	1206	-0.6	8 hrs	43.9	Note[2,8]
08/19/16	08/18/16	1206	1.3	8 hrs	28.9	Note[8]
08/23/16	08/10/16	1206	5.4	8 hrs	78.2	
08/23/16	08/10/16	1206	-2.8	8 hrs	73.1	Note[9]
08/25/16	08/22/16	1206	-2.4	8 hrs	38.1	
08/25/16	July 2016	1206	0.6	8 hrs	91.1	Note[2]
08/30/16	08/29/16	1206	0.4	8 hrs	30.5	Note[10]
08/30/16	08/26/16	1206	-3.2	8 hrs	64	Note[10]
08/30/16	08/26/16	1206	-4.3	8 hrs	61.1	Note[10]
08/30/16	July 2016	1206	-1.1	8 hrs	90.2	Note[2,10]
08/30/16	08/27/16	1206	1.2	8 hrs	65.5	Note[10]

08/30/16	08/26/16	1206	-3.1	8 hrs	52.6	Note[10]
08/30/16	08/27/16	1206	-1.2	8 hrs	55.7	Note[10]
08/30/16	08/27/16	1206	0.6	8 hrs	63.9	Note[10]
08/30/16	July 2016	1314	-0.1	8 hrs	91.3	Note[2,10,11]
09/01/16	July 2016	1206	1.3	8 hrs	97.6	Note[2]
09/01/16	08/31/16	1206	1	8 hrs	83	Note[8]
09/01/16	08/31/16	1206	2.9	8 hrs	86.4	Note[8]
09/06/16	July 2016	1206	0.8	8 hrs	87.2	Note[2,13]
09/06/16	July 2016	1206	-0.8	8 hrs	87.3	Note[2,13]
09/06/16	July 2016	1301	0.1	8 hrs	80.6	Note[2,13]
09/06/16	July 2016	1301	0.3	8 hrs	85.8	Note[2,13]
09/06/16	July 2016	1308	1.7	8 hrs	86.7	Note[2,13]
09/06/16	July 2016	1308	2.4	8 hrs	90.9	Note[2,13]
09/06/16	July 2016	1314	0.9	8 hrs	83.9	Note[2,13]
09/06/16	July 2016	1314	-1.2	8 hrs	81.1	Note[2,13]
09/08/16	July 2016	1206	5	8 hrs	92.7	Note[2,13]
09/08/16	July 2016	1206	1.5	8 hrs	75.9	Note[2,13]
09/08/16	July 2016	1301	1.6	8 hrs	86.2	Note[2,13]
09/08/16	July 2016	1301	-1.1	8 hrs	93.4	Note[2,13]
09/08/16	July 2016	1308	5	8 hrs	91.5	Note[2,13]
09/08/16	July 2016	1308	-2.3	8 hrs	87.6	Note[2,13]
09/08/16	July 2016	1314	5	8 hrs	89.9	Note[2,13]
09/08/16	July 2016	1314	3.8	8 hrs	63.1	Note[2,5,13]
09/13/16	July 2016	1206	3.7	8 hrs	83.4	Note[2,13]
09/13/16	July 2016	1206	-2.8	8 hrs	92.1	Note[2,13]
09/13/16	July 2016	1301	2	8 hrs	92.5	Note[2,13]
09/13/16	July 2016	1301	-0.5	8 hrs	94.6	Note[2,13]
09/13/16	July 2016	1308	0.4	8 hrs	85.3	Note[2,13]
09/13/16	July 2016	1308	1.1	8 hrs	88.1	Note[2,13]
09/13/16	July 2016	1314	-0.5	8 hrs	63.9	Note[2,13]
09/13/16	July 2016	1314	-2.4	8 hrs	78	Note[2,13]
09/22/16	Not Specified	1314	-8	8 hrs	86.1	
09/22/16	Not Specified	1314	-0.2	8 hrs	81.7	
09/29/16	09/26/16	1314	-12.5	8 hrs	61.9	Note[2,14]
09/29/16	09/14/16	1314	-5.5	8 hrs	93.7	Note[5]
09/29/16	09/14/16	1314	1.4	8 hrs	94.1	Note[5]
10/04/16	09/14/16	1206	3.8	8 hrs	84.8	Note[13]

10/04/16	09/14/16	1206	3.8	8 hrs	88.9	Note[13]
10/04/16	09/14/16	1301	0.6	8 hrs	83.4	Note[13]
10/04/16	09/14/16	1301	2.5	8 hrs	93.5	Note[13]
10/04/16	09/14/16	1308	-5.7	8 hrs	90.8	Note[13]
10/04/16	09/14/16	1308	-4.4	8 hrs	84.6	Note[13]
10/04/16	09/14/16	1314	1.1	8 hrs	74.6	Note[13]
10/04/16	09/14/16	1314	-5.1	8 hrs	94.1	Note[13]
10/06/16	09/14/16	1206	1.4	8 hrs	71.7	Note[13]
10/06/16	09/14/16	1206	1.4	8 hrs	88.8	Note[13]
10/06/16	09/14/16	1301	-2.1	8 hrs	83.8	Note[13]
10/06/16	09/14/16	1301	2.7	8 hrs	80.9	Note[13]
10/06/16	09/14/16	1308	-0.1	8 hrs	83	Note[13]
10/06/16	09/14/16	1308	0	8 hrs	84.4	Note[13]
10/06/16	09/14/16	1314	-3.3	8 hrs	79.4	Note[13]
10/06/16	09/14/16	1314	2.1	8 hrs	82.1	Note[13]
10/11/16	09/14/16	1206	-1.9	8 hrs	79.2	Note[13]
10/11/16	09/14/16	1206	-0.7	8 hrs	73.3	Note[13]
10/11/16	09/14/16	1301	0.2	8 hrs	74.5	Note[13]
10/11/16	09/14/16	1301	2.7	8 hrs	76.5	Note[13]
10/11/16	09/14/16	1308	1.5	8 hrs	79.1	Note[13]
10/11/16	09/14/16	1308	1.1	8 hrs	70.3	Note[13]
10/11/16	09/14/16	1314	-1.3	8 hrs	83.3	Note[13]
10/11/16	09/14/16	1314	-4.6	8 hrs	78.6	Note[13]
10/20/16	10/13/16	1308	-51.5	8 hrs	27.9	Note[12]
10/20/16	10/13/16	1308	-49.6	8 hrs	32.5	Note[12]
10/20/16	10/18/16	1308	-23	8 hrs	37.8	Note[12]
10/20/16	10/18/16	1308	-21.1	8 hrs	60.9	Note[12]
10/25/16	10/24/16	1308	-4.8	8 hrs	19.1	
10/25/16	10/24/16	1308	-9.5	8 hrs	60.4	
10/25/16	09/14/16	1308	0.3	8 hrs	75.6	
10/25/16	09/14/16	1308	-3.6	8 hrs	47.6	
10/25/16	10/18/16	1308	-24.3	8 hrs	13.8	
10/27/16	July 2016	1308	-9.1	9 hrs	71.7	Note[2,14]
10/27/16	09/14/16	1308	-1.1	9 hrs	87.9	
10/27/16	09/14/16	1308	-1.2	9 hrs	87.1	
10/27/16	09/29/16	1308	-7.3	9 hrs	80.4	
10/27/16	09/29/16	1308	-5.3	9 hrs	85.2	

11/01/16	09/14/16	1206	-5.4	8 hrs	79.5	Note[13]
11/01/16	09/14/16	1206	-2.9	8 hrs	70.9	Note[13]
11/01/16	09/14/16	1301	-1.7	8 hrs	77.2	Note[13]
11/01/16	09/14/16	1301	0.6	8 hrs	91.7	Note[13]
11/01/16	09/14/16	1308	-2.5	8 hrs	82.4	Note[13]
11/01/16	09/14/16	1308	-1.3	8 hrs	83.7	Note[13]
11/01/16	09/14/16	1314	0.9	8 hrs	68	Note[13]
11/01/16	09/14/16	1314	-3	8 hrs	73.5	Note[13]
11/03/16	09/14/16	1206	-3	8 hrs	84.2	Note[13]
11/03/16	09/14/16	1206	2.8	8 hrs	67.5	Note[13]
11/03/16	09/14/16	1301	4.6	8 hrs	67.7	Note[13]
11/03/16	09/14/16	1301	0.4	8 hrs	82.4	Note[13]
11/03/16	09/14/16	1308	-2.3	8 hrs	80.1	Note[13]
11/03/16	09/14/16	1308	2.1	8 hrs	76.4	Note[13]
11/03/16	09/14/16	1314	0.5	8 hrs	61.7	Note[13]
11/03/16	09/14/16	1314	-0.4	8 hrs	82.2	Note[13]
11/08/16	09/14/16	1206	-1.2	8 hrs	82	Note[13]
11/08/16	09/14/16	1206	0.5	8 hrs	70.4	Note[13]
11/08/16	09/14/16	1301	0.8	8 hrs	69.1	Note[13]
11/08/16	09/14/16	1301	-5.6	8 hrs	85.1	Note[13]
11/08/16	09/14/16	1308	-2.2	8 hrs	80.6	Note[13]
11/08/16	09/14/16	1308	3.1	8 hrs	74.8	Note[13]
11/08/16	09/14/16	1314	0.7	8 hrs	65.2	Note[13]
11/08/16	09/14/16	1314	-3.3	8 hrs	73.6	Note[13]
11/21/16	11/15/16	1301	-1.3	8 hrs	21.2	Note[14]
11/21/16	11/15/16	1301	-2.3	8 hrs	14	Note[14]
11/28/16	09/14/16	1206	0.1	8 hrs	87	Note[13]
11/28/16	09/14/16	1206	-2.3	8 hrs	89.9	Note[13]
11/28/16	09/14/16	1301	1.8	8 hrs	82.5	Note[13]
11/28/16	09/14/16	1301	-3.2	8 hrs	92.6	Note[13]
11/28/16	09/14/16	1308	0.7	8 hrs	91.9	Note[13]
11/28/16	09/14/16	1308	-1.2	8 hrs	73.5	Note[13]
11/28/16	09/14/16	1314	1.2	8 hrs	86.5	Note[13]
11/28/16	09/14/16	1314	-3.1	8 hrs	94.1	Note[13]
12/01/16	09/14/16	1206	2.6	8 hrs	85	Note[13]
12/01/16	09/14/16	1206	5.5	8 hrs	83.8	Note[13]
12/01/16	09/14/16	1301	10.1	8 hrs	78.5	Note[13]

12/01/16	09/14/16	1301	2.7	8 hrs	85.9	Note[13]
12/01/16	09/14/16	1308	4.7	8 hrs	85.9	Note[13]
12/01/16	09/14/16	1308	8.4	8 hrs	85.5	Note[13]
12/01/16	09/14/16	1314	7.6	8 hrs	83.8	Note[13]
12/01/16	09/14/16	1314	-0.7	8 hrs	92.4	Note[13]
12/06/16	09/14/16	1206	-2.5	8 hrs	79.3	Note[13]
12/06/16	09/14/16	1206	-4	8 hrs	88.3	Note[13]
12/06/16	09/14/16	1301	1.4	8 hrs	85.5	Note[13]
12/06/16	09/14/16	1301	-2.7	8 hrs	86.8	Note[13]
12/06/16	09/14/16	1308	-3.2	8 hrs	77.3	Note[13]
12/06/16	09/14/16	1308	-0.6	8 hrs	84.6	Note[13]
12/06/16	09/14/16	1314	-5.7	8 hrs	86.2	Note[13]
12/06/16	09/14/16	1314	-3.9	8 hrs	89.1	Note[13]

	Notes:
Note[1]	Larvae injected into Nunc™ Capsule
Note[2]	Larvae from Dr. Zajac
Note[3]	Capsules placed in PVC: Total length of 30 cm
Note[4]	Larvae left 15 min overtime in Rumen
Note[5]	Mostly air in capsule when removed
Note[6]	25 cm string
Note[7]	Fed about an hour and 15 min later
Note[8]	Larvae set out at room temperature under 20 hrs
Note[9]	Larvae stored in PBS
Note[10]	Testing different larvae growth incubation lengths
Note[11]	Ewe on trefoil diet
Note[12]	May have counted strongyloides
Note[13]	Study Data
Note[14]	Less than 100 larvae examined

APENDIX 8

Fecal Egg Count Data During use of Nunc™ Capsules:

	Ewe 1206 (eggs/gram)	Ewe 1301 (eggs/gram)	Ewe 1308 (eggs/gram)	Ewe 1314 (eggs/gram)
5/23/16	100	0	0	0
5/31/16	0	50	No Sample	0
6/7/16	0	0	0	50
6/14/16	0	50	0	0
6/21/16	0	100	100	200
6/27/16	0	0	0	0
7/5/16	0	0	0	0
7/12/16	0	0	0	0
7/19/16	50	150	0	0
7/26/16	50	150	0	100
8/2/16	200	0	0	50
8/9/16	0	50	50	0
8/16/16	0	100	0	100
8/22/16	50	100	0	0
8/30/16	0	0	50	0
9/6/16	0	150	0	50
9/13/16	0	150	0	0
9/20/16	0	50	0	50
9/27/16	150	0	0	0
10/3/16	0	0	0	0
10/11/16	50	0	150	0
10/18/16	0	0	0	0
10/25/16	0	0	50	0
10/28/16	0	0	0	0
11/1/16	50	0	50	0
11/8/16	0	0	0	0
11/15/16	0	0	0	0
11/22/16	50	0	50	0
11/29/16	0	0	0	0
12/6/16	0	0	0	0
12/13/16	0	0	50	0

APENDIX 9

Hay Growth and Harvesting Procedure:

A 1,200 ft by 140 ft plot was seeded using a Brillion Cultipacker with six cultivars of birdsfoot trefoil on May 27-28, 2014. Previously the field had been used for sod farming and soil was tested and amended prior to planting the birdsfoot trefoil. Inoculated seed was purchased (Pardee, Seedway, Shoreham, VT) (Empire & Leo, Ernst Conservation Seeds, Meadville, PA) (Bruce, Norcen, & Bull, Welter Seed & Honey Co., Onslow, IA) and seeded at a rate of 20 lbs/acre. The six cultivars were planted in parallel rows in the order of Bruce, Bull, Pardee, Empire, Leo, and Norcen with an additional buffer of Bruce at the end. The field was managed organically and the hay was harvested in 2015. Prior to harvesting, the cultivar rows were measured and marked, and the dividing strips between the cultivars were mowed to provide separation between the cultivars. Three random samples were taken of each cultivar. These samples were cut approximately 4 inches above the soil to correspond to the hay harvesting height. The birdsfoot trefoil was separated from the other plants and both were dried. The dried samples were weighed and the % biomass was determined for each cultivar. For hay production, the birdsfoot trefoil was cut, allowed to air dry, sprayed with PRESERVOR^{TM} hay and crop treatment (IBA Inc., Millbury, MA), and baled into large round bales. Each bale was labeled with the cultivar name.

APENDIX 10

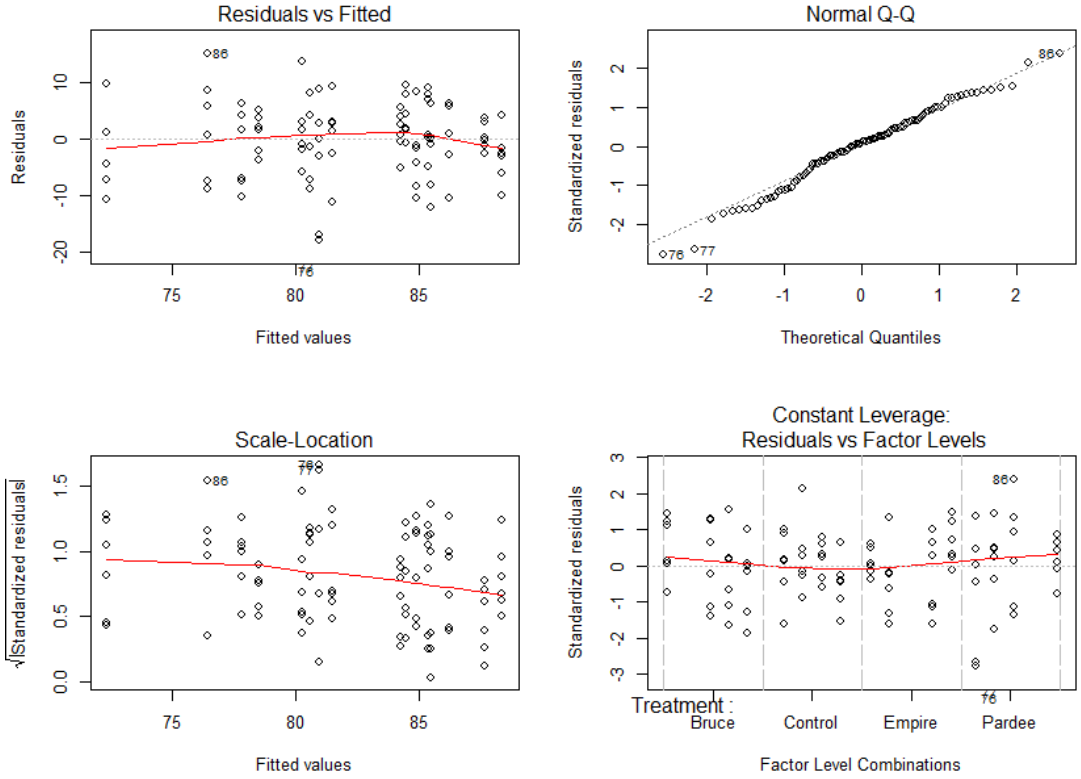
Exsheathment Data from BFT Study:

Date	Control Exsheathment	Bruce Exsheathment	Empire Exsheathment	Pardee Exsheathment
09/06/16	87.2%	80.6%	86.7%	83.9%
09/06/16	87.3%	85.8%	90.9%	81.1%
09/08/16	92.7%	86.2%	91.5%	89.8%
09/08/16	75.9%	93.4%	87.6%	63.1%
09/13/16	83.4%	92.5%	85.3%	63.9%
09/13/16	92.1%	94.6%	88.1%	78.0%
10/04/16	74.6%	84.8%	83.4%	90.8%
10/04/16	94.1%	88.9%	93.5%	84.6%
10/06/16	79.4%	71.7%	83.8%	83.0%
10/06/16	82.1%	88.8%	80.9%	84.4%
10/11/16	83.3%	79.2%	74.5%	79.1%
10/11/16	78.6%	73.3%	76.5%	70.3%
11/01/16	82.4%	68.0%	79.5%	77.2%
11/01/16	83.7%	73.5%	70.9%	91.7%
11/03/16	80.1%	61.7%	84.2%	67.7%
11/03/16	76.4%	82.2%	67.5%	82.4%
11/08/16	80.6%	65.2%	82.0%	69.1%
11/08/16	74.8%	73.6%	70.4%	85.1%
11/29/16	82.5%	91.9%	86.5%	87.0%
11/29/16	92.6%	73.5%	94.1%	89.9%
12/01/16	78.5%	85.9%	83.8%	85.0%
12/01/16	85.9%	85.5%	92.4%	83.8%
12/06/16	85.5%	77.3%	86.2%	79.3%
12/06/16	86.8%	84.6%	89.1%	88.3%
Mean ± SD	83.4 ± 5.7%	80.9 ± 9.2%	83.7 ± 7.4%	80.8 ± 8.4%

APENDIX 11

R Output for Exsheathment Tests

```
> Model1=lm(Exsheathment~Treatment+Cycle+Ewe, data=exsheathmentA)
> anova(Model1)
Analysis of Variance Table
Response: Exsheathment
          Df Sum Sq Mean Sq F value    Pr(>F)
Treatment  3  174.3   58.08  1.2577    0.2941
Cycle      3 1339.3  446.45  9.6669 1.449e-05 ***
Ewe        3  262.3   87.45  1.8935    0.1367
Residuals 86 3971.7   46.18
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
> exsheathmentresidualsA=Model1$residuals
> exsheathmentstdresidualsA=(exsheathmentresidualsA)/sqrt(46.18)
> shapiro.test(exsheathmentstdresidualsA)
        Shapiro-Wilk normality test
data:  exsheathmentstdresidualsA
W = 0.98577, p-value = 0.3895
> par(mfrow=c(2,2))
> plot(Model1)
```




```
> library(DescTools)

> PostHocTest(Model2, method="hsd")

  Posthoc multiple comparisons of means : Tukey HSD

    95% family-wise confidence level

$Treatment

                    diff    lwr.ci    upr.ci    pval

Control-Bruce   2.4083333 -2.731483 7.548150 0.6111

Empire-Bruce    2.7750000 -2.364816 7.914816 0.4938

Pardee-Bruce   -0.1750000 -5.314816 4.964816 0.9997

Empire-Control  0.3666667 -4.773150 5.506483 0.9977

Pardee-Control -2.5833333 -7.723150 2.556483 0.5548
```

```
Pardee-Empire  -2.9500000 -8.089816 2.189816 0.4397

$Cycle

         diff     lwr.ci     upr.ci    pval

B-A -3.2500000  -8.389816  1.8898162 0.35292

C-A -8.8208333 -13.960650 -3.6810172 0.00012 ***

D-A  0.5958333  -4.543983  5.7356495 0.99020

C-B -5.5708333 -10.710650 -0.4310172 0.02830 *

D-B  3.8458333  -1.293983  8.9856495 0.21115

D-C  9.4166667   4.276850 14.5564828 3.9e-05 ***

$Ewe

                diff    lwr.ci    upr.ci   pval

Gertie-Fern   -0.487500 -5.627316 4.6523162 0.9946

Noreen-Fern   -4.270833 -9.410650 0.8689828 0.1379

Spot-Fern     -1.566667 -6.706483 3.5731495 0.8549

Noreen-Gertie -3.783333 -8.923150 1.3564828 0.2239

Spot-Gertie   -1.079167 -6.218983 4.0606495 0.9463

Spot-Noreen    2.704167 -2.435650 7.8439828 0.5162

---

Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

> library("lsmeans")

> lsmeans(Model2,~Treatment)

 Treatment   lsmean       SE df lower.CL upper.CL

 Bruce     80.94583 1.387184 86 78.18820 83.70346
```

Control 83.35417 1.387184 86 80.59654 86.11180

Empire 83.72083 1.387184 86 80.96320 86.47846

Pardee 80.77083 1.387184 86 78.01320 83.52846

Results are averaged over the levels of: Cycle, Ewe

Confidence level used: 0.95

> lsmeans(Model2,~Ewe)

Ewe lsmean SE df lower.CL upper.CL

Fern 83.77917 1.387184 86 81.02154 86.53680

Gertie 83.29167 1.387184 86 80.53404 86.04930

Noreen 79.50833 1.387184 86 76.75070 82.26596

Spot 82.21250 1.387184 86 79.45487 84.97013

Results are averaged over the levels of: Treatment, Cycle

Confidence level used: 0.95

> lsmeans(Model2,~Cycle)

Cycle lsmean SE df lower.CL upper.CL

A 85.06667 1.387184 86 82.30904 87.82430

B 81.81667 1.387184 86 79.05904 84.57430

C 76.24583 1.387184 86 73.48820 79.00346

D 85.66250 1.387184 86 82.90487 88.42013

Results are averaged over the levels of: Treatment, Ewe

Confidence level used: 0.95

APENDIX 12

Motility from Exsheathment Data:

	% Motility Post-Exsheathment				
Date	Control	BFT Bruce	BFT Empire	BFT Pardee	Motile (Pre)
09/06/16	96.5%	95.8%	97.4%	96.6%	95.7%
09/06/16	94.9%	96.0%	98.1%	94.5%	95.7%
09/08/16	100.0%	96.6%	100.0%	100.0%	95.0%
09/08/16	96.5%	93.9%	92.7%	98.8%	95.0%
09/13/16	96.4%	94.7%	93.1%	92.2%	92.7%
09/13/16	89.9%	92.2%	93.8%	90.3%	92.7%
10/04/16	97.3%	100.0%	96.8%	90.5%	96.2%
10/04/16	91.1%	100.0%	98.7%	91.8%	96.2%
10/06/16	92.8%	97.5%	94.0%	96.0%	96.1%
10/06/16	98.2%	97.5%	98.8%	96.1%	96.1%
10/11/16	94.7%	94.1%	96.2%	97.5%	96.0%
10/11/16	91.4%	95.3%	98.7%	97.1%	96.0%
11/01/16	94.4%	97.8%	91.5%	95.2%	96.9%
11/01/16	95.6%	93.9%	94.0%	97.5%	96.9%
11/03/16	93.1%	95.9%	92.4%	100.0%	95.4%
11/03/16	97.5%	95.0%	98.2%	95.8%	95.4%
11/08/16	94.1%	97.0%	95.1%	97.1%	96.3%
11/08/16	99.4%	93.0%	96.8%	90.7%	96.3%
11/29/16	98.7%	97.6%	98.1%	97.0%	96.9%
11/29/16	93.7%	95.7%	93.8%	94.6%	96.9%
12/01/16	93.1%	87.7%	90.6%	85.6%	83.0%
12/01/16	85.7%	91.4%	82.3%	88.5%	83.0%
12/06/16	93.0%	88.4%	85.9%	89.1%	91.6%
12/06/16	88.9%	91.0%	87.7%	87.6%	91.6%
Mean ± SD	94.5 ± 3.5%	94.9 ± 3.1%	94.4 ± 4.4%	94.2 ± 4.1%	94.3 ± 3.8%

APENDIX 13

Procedure from pH Calibration/Measurement:

Supplies:

- shoulder length gloves
- gloves
- pH 7 and 4 buffers
- small cups
- Accumet portable pH meter and electrode (AP115, Fisher Scientific, Hampton, NH)
- large beaker of water
- four small glass beakers
- large syringe with a long piece of Tygon® tubing attached
- record sheet and pen
- distilled water
- halters

Procedure:

1. Attach the electrode to the pH meter (meter should be protected by a gallon zip lock bag).
2. Turn pH meter on by pressing "on" button.
3. With gloves on, remove storage bulb from end of electrode and place the bulb into a cup.
4. Pour approximately 3/4in of each buffer into separate cups.
5. Rinse probe off with distilled water

6. Press standardize button, and when standardize is flashing on screen, insert into pH 7 buffer.
7. When the pH is stable for 30 seconds, press the standardize button again.
8. Repeat steps 5-7 for pH 4 buffer.
9. Rinse probe and place it in the large beaker of water.
10. Halter and tie sheep to pens.
11. Remove rumen cannula plug and insert the free end of the Tygon® tubing deep into the center of the rumen.
12. Draw back on the syringe to pull rumen fluid into the tubing.
13. Cover the end of the tubing with a finger and remove it from the rumen.
14. Place the end of the tube into one of the clean beakers and release the rumen fluid into the beaker.
15. Take a second sample of rumen fluid from the same ewe and add it to the beaker.
16. Rinse the electrode off with distilled water and place it in the beaker with rumen fluid.
17. Thoroughly rinse the syringe and tubing with water.
18. Record the time, the pH, and current temperature of the rumen fluid.
19. Repeat steps 11-18 on the other ewes.
20. Release ewes and clean up any mess.
21. Rinse the electrode, and with gloves on, reinsert probe into the storage bulb.
22. Dispose of pH buffer into waste containers.
23. Put everything away.

APENDIX 14

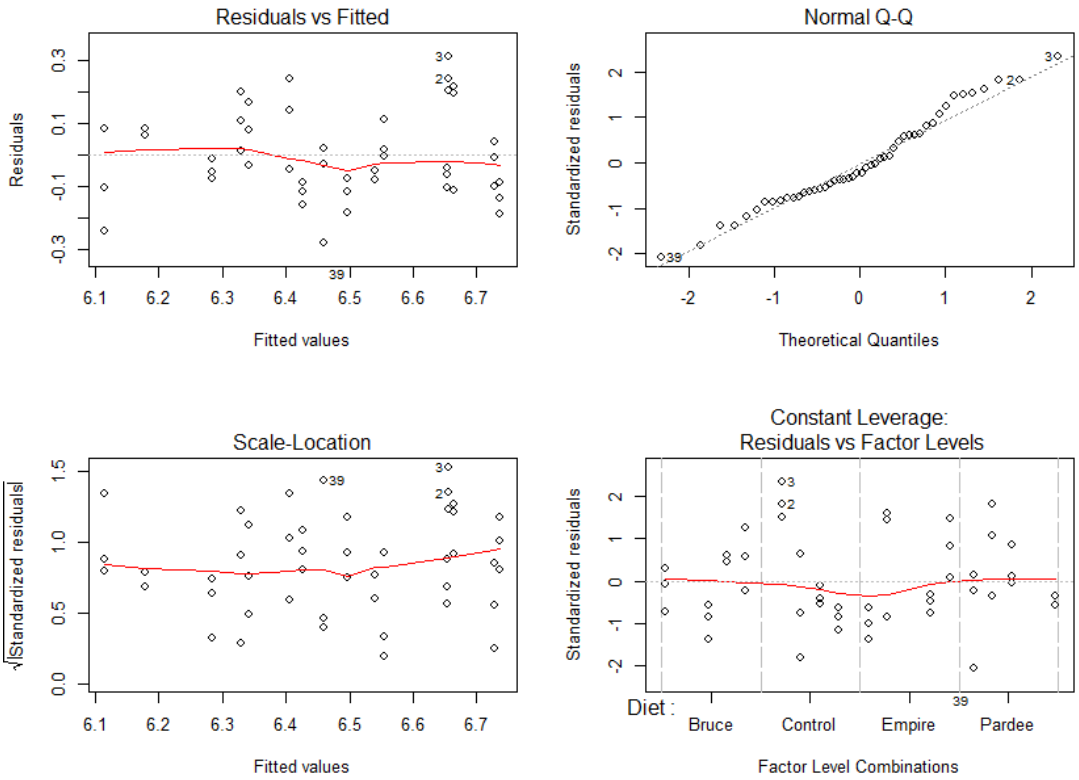
Data from pH Measurements:

Date	Control		Bruce		Empire		Pardee	
	pH	Temp ºC	pH	Temp ºC	pH	Temp ºC	pH	Temp ºC
09/06/16	6.86	30.2	6.72	30.8	6.65	30.8	6.43	30.6
09/08/16	6.90	29.6	6.63	29.5	6.60	29.8	6.48	31.2
09/13/16	6.97	30.8	6.77	31.2	6.55	30.7	6.18	30.3
10/04/16	6.20	29.7	6.38	28.6	6.86	30.4	6.65	29.9
10/06/16	6.01	26.7	6.42	27.1	6.88	27.5	6.55	25.6
10/11/16	5.87	24.6	6.31	25.5	6.55	25.2	6.36	25.8
11/01/16	6.23	20.7	6.26	21.0	6.59	24.9	6.57	22.7
11/03/16	6.21	26.4	6.24	25.2	6.55	26.0	6.55	26.2
11/08/16	6.27	17.5	6.26	19.4	6.61	22.9	6.67	21.4
11/29/16	6.34	25.0	6.42	24.5	6.53	24.1	6.49	26.8
12/01/16	6.31	27.1	6.51	25.3	6.34	27.2	6.49	28.4
12/06/16	6.27	24.1	6.31	21.2	6.44	22.3	6.46	26.6
Mean ± SD	6.37 ± 0.35	26.0 ± 4.0	6.44 ± 0.18	25.8 ± 3.8	6.60 ± 0.15	26.8 ± 3.1	6.49 ± 0.13	27.1 ± 3.1

APENDIX 15

R Output for pH Measurements

```
> Model3=lm(pH~Diet+Cycle+Ewe, data=pHA)

> anova(Model3)

Analysis of Variance Table

Response: pH

          Df  Sum Sq  Mean Sq F value    Pr(>F)

Diet       3 0.32841 0.109469  4.7991 0.0062384 **

Cycle      3 0.47458 0.158192  6.9350 0.0007768 ***

Ewe        3 0.83221 0.277403 12.1612 1.007e-05 ***

Residuals 38 0.86680 0.022811

---

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

> pHresidualsA=Model3$residuals

> pHstdresidualsA=(pHresidualsA)/sqrt(0.0228)

> shapiro.test(pHstdresidualsA)

        Shapiro-Wilk normality test

data:  pHstdresidualsA

W = 0.97235, p-value = 0.312

> par(mfrow=c(2,2))

> plot(Model3)
```



> Model3 = aov(pH~Diet+Cycle+Ewe, data=pHA)

> summary(Model3)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Diet	3	0.3284	0.10947	4.799	0.006238	**
Cycle	3	0.4746	0.15819	6.935	0.000777	***
Ewe	3	0.8322	0.27740	12.161	1.01e-05	***
Residuals	38	0.8668	0.02281			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

> PostHocTest(Model3, method="hsd")

```
  Posthoc multiple comparisons of means : Tukey HSD
    95% family-wise confidence level
$Diet
                    diff       lwr.ci     upr.ci   pval
Control-Bruce  -0.06583333 -0.231477010 0.09981034 0.7110
Empire-Bruce    0.16000000 -0.005643677 0.32564368 0.0616 .
Pardee-Bruce    0.05416667 -0.111477010 0.21981034 0.8159
Empire-Control  0.22583333  0.060189656 0.39147701 0.0040 **
Pardee-Control  0.12000000 -0.045643677 0.28564368 0.2264
Pardee-Empire  -0.10583333 -0.271477010 0.05981034 0.3295
$Cycle
          diff     lwr.ci      upr.ci   pval
B-A -0.225000000 -0.3906437 -0.05935632 0.0042 **
C-A -0.227500000 -0.3931437 -0.06185632 0.0038 **
D-A -0.235833333 -0.4014770 -0.07018966 0.0026 **
C-B -0.002500000 -0.1681437  0.16314368 1.0000
D-B -0.010833333 -0.1764770  0.15481034 0.9980
D-C -0.008333333 -0.1739770  0.15731034 0.9991
$Ewe
                  diff      lwr.ci       upr.ci    pval
Gertie-Fern   -0.1508333 -0.31647701  0.014810344  0.0854 .
Noreen-Fern   -0.3233333 -0.48897701 -0.157689656 3.6e-05 ***
Spot-Fern     -0.0075000 -0.17314368  0.158143677  0.9993
```

```
Noreen-Gertie -0.1725000 -0.33814368 -0.006856323  0.0385 *
Spot-Gertie    0.1433333 -0.02231034  0.308977010  0.1103
Spot-Noreen    0.3158333  0.15018966  0.481477010 5.2e-05 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
> lsmeans(Model3,~Ewe)
 Ewe      lsmean         SE df lower.CL upper.CL
 Fern   6.593333 0.04359905 38 6.505072 6.681595
 Gertie 6.442500 0.04359905 38 6.354238 6.530762
 Noreen 6.270000 0.04359905 38 6.181738 6.358262
 Spot   6.585833 0.04359905 38 6.497572 6.674095
Results are averaged over the levels of: Diet, Cycle
Confidence level used: 0.95
> lsmeans(Model3,~Diet)
 Diet      lsmean         SE df lower.CL upper.CL
 Bruce   6.435833 0.04359905 38 6.347572 6.524095
 Control 6.370000 0.04359905 38 6.281738 6.458262
 Empire  6.595833 0.04359905 38 6.507572 6.684095
 Pardee  6.490000 0.04359905 38 6.401738 6.578262
Results are averaged over the levels of: Cycle, Ewe
Confidence level used: 0.95
> lsmeans(Model3,~Cycle)
 Cycle    lsmean         SE df lower.CL upper.CL
```

A	6.645000	0.04359905	38	6.556738	6.733262
B	6.420000	0.04359905	38	6.331738	6.508262
C	6.417500	0.04359905	38	6.329238	6.505762
D	6.409167	0.04359905	38	6.320905	6.497428

Results are averaged over the levels of: Diet, Ewe

Confidence level used: 0.95

APENDIX 16

Procedure for Hay Sample Collections:

Supplies:

- Extension cord
- Drill
- Corer
- Zip lock bags
- Permanent marker

Procedure (Hay):

Once per week of trial:

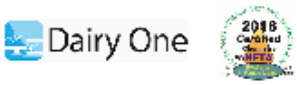
1. Label bags with variety of hay and date.
2. Run extension cord from pig barn to mini-moo.
3. Core each bale several times and empty into appropriate bag.
4. Bring all bags up to the CBLS freezer.
5. Dump into appropriate large composite bag for that cycle of the trial.
    - Large composite bags are labeled with the variety and cycle 1-4 (eg: Bruce 2015 Cycle #1)
6. Return to freezer.

Procedure (Grain):

1. Every morning when feeding grain, dump one scoop into composite container.
2. Once per week bring composite grain up to CBLS freezer.
3. Dump into the appropriate large composite bag for that cycle of the trial.
4. Return to freezer.

APENDIX 17

Data from Dairy One:



FORAGE TESTING LABORATORY
DAIRY ONE, INC.
730 WARREN ROAD
ITHACA, NEW YORK 14850
607-257-1272 (fax 607-257-1350)

Sampled	Recvd	Printed	ST	CO
	01/05/17	01/10/17		

KATHERINE PETERSSON
KATHERINE PETERSSON
UNIV OF RHODE ISLAND
120 FLAGG RD RM 177 CBLS
KINGSTON, RI 02881

ENERGY TABLE - NRC 2001

	Mcal/Lb	Mcal/Kg
DE, 1X	1.55	3.41
ME, 1X	1.36	3.00
NEL, 3X	0.80	1.77
NEM, 3X	0.84	1.86
NEG, 3X	0.56	1.22
TDN1X, %	75	

Sample Description	Farm	Code	Sample
GRAIN MIX, Dry		646	23418900

GRAIN 2016 HAY COMPOSITE CYCLES 1-4 8/10/1

Analysis Results

Components	As Fed	DM
% Moisture	10.8	
% Dry Matter	89.2	
% Crude Protein	16.8	18.9
% Adjusted Crude Protein	16.8	18.9
Soluble Protein % CP		23
% ADF	12.4	13.9
% aNDF	26.9	30.2
% Lignin	2.5	2.8
% NFC	34.3	38.4
% Crude Fat	4.2	4.7
% Ash	6.98	7.83
% TDN	66	74
NEL, (mcal/kg)	1.55	1.74
NEM, (mcal/kg)	1.59	1.79
NEG, (mcal/kg)	1.04	1.16
% Calcium	1.14	1.28
% Phosphorus	.69	.77
% Magnesium	.31	.35
% Potassium	1.01	1.13
% Sodium	.243	.273
PPM Iron	212	237
PPM Zinc	156	175
PPM Copper	12	13
PPM Manganese	107	120
PPM Molybdenum	3.6	4.0
% Sulfur	.29	.33
Horse DE,Mcal/kg	2.63	2.95

Page 1

Dairy One

FORAGE TESTING LABORATORY
DAIRY ONE, INC.
730 WARREN ROAD
ITHACA, NEW YORK 14850
607-257-1272 (fax 607-257-1350)

Sampled	Recvd	Printed	ST	CO
	01/05/17	01/10/17		

KATHERINE PETERSSON
KATHERINE PETERSSON
UNIV OF RHODE ISLAND
120 FLAGG RD RM 177 CBLS
KINGSTON, RI 02881

ENERGY TABLE - NRC 2001

	Mcal/Lb	Mcal/Kg
DE, 1X	1.17	2.59
ME, 1X	0.98	2.16
NEL, 3X	0.55	1.21
NEM, 3X	0.57	1.26
NEG, 3X	0.32	0.69
TDN1X, %	58	

Sample Description	Farm	Code	Sample
MMG HAY		102	23418890

GRASS 2016 HAY COMPOSITE CYCLES 2-4 9/14/1

Analysis Results

Components	As Fed	DM
% Moisture	7.2	
% Dry Matter	92.8	
% Crude Protein	10.1	10.8
% Adjusted Crude Protein	10.1	10.8
Soluble Protein % CP		42
% ADF	37.5	40.4
% aNDF	65.5	70.6
% Lignin	4.5	4.8
% NFC	9.7	10.4
% Crude Fat	2.1	2.2
% Ash	5.49	5.92
% TDN	54	58
NEL, (mcal/kg)	.91	.98
NEM, (mcal/kg)	1.05	1.13
NEG, (mcal/kg)	.53	.57
Relative Feed Value		76
% Calcium	.28	.30
% Phosphorus	.31	.33
% Magnesium	.17	.18
% Potassium	1.88	2.02
% Sodium	.007	.008
PPM Iron	120	129
PPM Zinc	21	23
PPM Copper	6	7
PPM Manganese	55	60
PPM Molybdenum	1.7	1.9
% Sulfur	.16	.17
Horse DE,Mcal/kg	1.78	1.92

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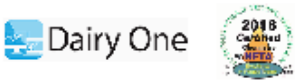

FORAGE TESTING LABORATORY
DAIRY ONE, INC.
730 WARREN ROAD
ITHACA, NEW YORK 14850
607-257-1272 (fax 607-257-1350)

Sampled	Recvd	Printed	ST	CO
	01/05/17	01/10/17		

KATHERINE PETERSSON
KATHERINE PETERSSON
UNIV OF RHODE ISLAND
120 FLAGG RD RM 177 CBLS
KINGSTON, RI 02881

ENERGY TABLE - NRC 2001

	Mcal/Lb	Mcal/Kg
DE, 1X	1.17	2.59
ME, 1X	0.98	2.16
NEL, 3X	0.55	1.21
NEM, 3X	0.57	1.27
NEG, 3X	0.32	0.70
TDN1X, %	56	

Sample Description	Farm	Code	Sample
MMG HAY		102	23418880

ALFALFA/GRASS STANDLESS HAY COMPOSITE CYCLE

Analysis Results

Components	As Fed	DM
% Moisture	9.2	
% Dry Matter	90.8	
% Crude Protein	18.1	19.9
% Adjusted Crude Protein	18.1	19.9
Soluble Protein % CP		39
% ADF	34.4	37.9
% aNDF	40.2	44.3
% Lignin	7.5	8.3
% NFC	21.1	23.2
% Crude Fat	1.7	1.9
% Ash	9.71	10.70
% TDN	51	56
NEL, (mcal/kg)	1.13	1.25
NEM, (mcal/kg)	1.00	1.10
NEG, (mcal/kg)	.50	.55
Relative Feed Value		125
% Calcium	1.31	1.44
% Phosphorus	.24	.27
% Magnesium	.22	.25
% Potassium	2.50	2.76
% Sodium	.074	.082
PPM Iron	137	151
PPM Zinc	14	16
PPM Copper	7	7
PPM Manganese	27	30
PPM Molybdenum	< 1	< 1
% Sulfur	.29	.32
Horse DE,Mcal/kg	2.05	2.26

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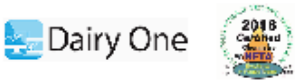

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KATHERINE PETERSSON
KATHERINE PETERSSON
UNIV OF RHODE ISLAND
120 FLAGG RD RM 177 CBLS
KINGSTON, RI 02881

ENERGY TABLE - NRC 2001

	Mcal/Lb	Mcal/Kg
DE, 1X	1.02	2.25
ME, 1X	0.83	1.82
NEL, 3X	0.45	0.99
NEM, 3X	0.46	1.00
NEG, 3X	0.21	0.45
TDN1X, %	50	

Sample Description	Farm	Code	Sample
MMG HAY		102	23418860

BFT 2015 EMPIRE HAY COMPOSITE CYCLES 1-4 8

Analysis Results

Components	As Fed	DM
% Moisture	8.1	
% Dry Matter	91.9	
% Crude Protein	10.4	11.4
% Adjusted Crude Protein	10.4	11.4
Soluble Protein % CP		42
% ADF	45.8	49.8
% aNDF	57.2	62.3
% Lignin	10.3	11.2
% NFC	16.3	17.7
% Crude Fat	1.7	1.9
% Ash	6.24	6.80
% TDN	46	50
NEL, (mcal/kg)	.89	.97
NEM, (mcal/kg)	.81	.88
NEG, (mcal/kg)	.31	.33
Relative Feed Value		75
% Calcium	.53	.58
% Phosphorus	.24	.26
% Magnesium	.24	.26
% Potassium	2.13	2.32
% Sodium	.020	.022
PPM Iron	72	79
PPM Zinc	14	15
PPM Copper	5	5
PPM Manganese	10	11
PPM Molybdenum	2.4	2.7
% Sulfur	.11	.12
Horse DE,Mcal/kg	1.85	2.01

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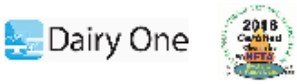

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	01/05/17	01/10/17		

KATHERINE PETERSSON
KATHERINE PETERSSON
UNIV OF RHODE ISLAND
120 FLAGG RD RM 177 CBLS
KINGSTON, RI 02881

ENERGY TABLE - NRC 2001

	Mcal/Lb	Mcal/Kg
DE, 1X	1.03	2.28
ME, 1X	0.84	1.85
NEL, 3X	0.46	1.01
NEM, 3X	0.47	1.03
NEG, 3X	0.22	0.47
TDN1X, %	51	

Sample Description	Farm Code	Sample
MMG HAY	102	23418850

BFT 2015 PARDEE HAY COMPOSITE CYCLES 1-4 8

Analysis Results

Components	As Fed	DM
% Moisture	8.9	
% Dry Matter	91.1	
% Crude Protein	11.3	12.4
% Adjusted Crude Protein	11.3	12.4
Soluble Protein % CP		39
% ADF	44.3	48.6
% aNDF	56.9	62.4
% Lignin	9.3	10.2
% NFC	14.3	15.7
% Crude Fat	1.7	1.9
% Ash	6.94	7.62
% TDN	46	51
NEL, (mcal/kg)	.89	.98
NEM, (mcal/kg)	.81	.89
NEG, (mcal/kg)	.31	.34
Relative Feed Value		76
% Calcium	.52	.57
% Phosphorus	.25	.28
% Magnesium	.26	.28
% Potassium	2.56	2.81
% Sodium	.019	.021
PPM Iron	71	78
PPM Zinc	16	18
PPM Copper	6	6
PPM Manganese	13	14
PPM Molybdenum	2.5	2.7
% Sulfur	.13	.14
Horse DE,Mcal/kg	1.79	1.97

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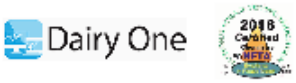

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Sampled	Recvd	Printed	ST	CO
	01/05/17	01/11/17		

KATHERINE PETERSSON
KATHERINE PETERSSON
UNIV OF RHODE ISLAND
120 FLAGG RD RM 177 CBLS
KINGSTON, RI 02881

ENERGY TABLE - NRC 2001

	Mcal/Lb	Mcal/Kg
DE, 1X	1.04	2.30
ME, 1X	0.85	1.87
NEL, 3X	0.46	1.02
NEM, 3X	0.47	1.04
NEG, 3X	0.22	0.49
TDN1X, %	51	

COMMENTS:
1. THIS SAMPLE WAS TESTED TWICE FOR CRUDE FAT TO CONFIRM THE VALUE LISTED.

Sample Description	Farm	Code	Sample
MMG HAY		102	23418870
BFT 2015 BRUCE HAY COMPOSITE CYCLES 1-4 8/			

Analysis Results

Components	As Fed	DM
% Moisture	8.0	
% Dry Matter	92.0	
% Crude Protein	11.3	12.3
% Adjusted Crude Protein	11.3	12.3
Soluble Protein % CP		40
% ADF	46.7	50.8
% aNDF	57.2	62.1
% Lignin	10.4	11.3
% NFC	16.7	18.2
% Crude Fat	1.6	1.7
% Ash	5.25	5.71
% TDN	47	51
NEL, (mcal/kg)	.91	.99
NEM, (mcal/kg)	.83	.90
NEG, (mcal/kg)	.33	.36
Relative Feed Value		74
% Calcium	.49	.53
% Phosphorus	.25	.27
% Magnesium	.20	.22
% Potassium	1.77	1.93
% Sodium	.027	.030
PPM Iron	70	76
PPM Zinc	16	17
PPM Copper	6	7
PPM Manganese	11	12
PPM Molybdenum	2.2	2.4
% Sulfur	.12	.13
Horse DE,Mcal/kg	1.89	2.05

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APENDIX 18

Hay Consumption Data:

Date	Hay Recovered (grams)				Hay Fed (grams)			
	Ewe	Ewe	Ewe	Ewe	Ewe	Ewe	Ewe	Ewe
	1206	1301	1308	1314	1206	1301	1308	1314
08/17/16	5.7	0.0	21.5	12.8	1814	1814	1814	1814
08/18/16	44.2	71.8	27.6	20.2	1814	1814	1814	1814
08/19/16	20.2	6.8	36.7	20.5	1814	1814	1814	1814
08/20/16	0.0	0.0	4.5	0.0	1814	1814	1814	1814
08/21/16	3.1	30.7	45.4	6.5	1814	1814	1814	1814
08/22/16	2.2	16.8	39.1	9.4	1814	1814	1814	1814
08/23/16	0.0	0.0	11.3	3.1	1814	1814	1814	1814
T. Hay Fed	12701	12701	12701	12701	Week #1 Totals			
T. Recovered	75.4	126.1	186.1	72.5				
T. Consumed	12625.2	12574.5	12514.5	12628.1				
% Consumed	99.4%	99.0%	98.5%	99.4%				
08/24/16	1.1	10.1	51.5	9.8	1814	1814	1814	1814
08/25/16	2.3	3.3	38.3	3.6	1814	1814	1814	1814
08/26/16	3.3	0.9	31.9	21.1	1814	1814	1814	1814
08/27/16	4.2	1.8	44.0	34.8	1814	1814	1814	1814
08/28/16	2.9	0.0	20.6	9.9	1814	1814	1814	1814
08/29/16	5.1	4.6	11.6	8.0	1814	1814	1814	1814
08/30/16	1.1	0.9	18.1	27.8	1814	1814	1814	1814
T. Hay Fed	12701	12701	12701	12701	Week #2 Totals			
T. Recovered	20	21.6	216.0	115.0				
T. Consumed	12680.6	12679.0	12484.6	12585.6				
% Consumed	99.8%	99.8%	98.3%	99.1%				
08/31/16	10.0	3.1	7.7	6.8	1814	1814	1814	1814
09/01/16	18.7	2.7	14.3	0.3	1814	1814	1814	1814
09/02/16	9.2	2.8	1.9	4.3	1814	1814	1814	1814
09/03/16	0.2	0.2	5.8	0.5	1814	1814	1814	1814
09/04/16	1.7	6.5	7.7	0.6	1814	1814	1814	1814
09/05/16	0.7	2.0	10.7	13.7	1814	1814	1814	1814
09/06/16	0.0	3.0	7.7	18.2	1814	1814	1814	1814
T. Hay Fed	12701	12701	12701	12701	Week #3 Totals			

T. Recovered	40.5	20.3	55.8	44.4				
T. Consumed	12660.1	12680.3	12644.8	12656.2				
% Consumed	99.7%	99.8%	99.6%	99.7%				
09/07/16	0.8	1.0	4.0	12.2	1814	1814	1814	1814
09/08/16	0.5	10.3	12.9	66.9	1814	1814	1814	1814
09/09/16	0.9	6.2	3.3	454.4	1814	1814	1814	1814
09/10/16	7.7	4.4	20.0	452.0	1814	1814	1814	1814
09/11/16	12.5	8.9	11.2	695.9	1814	1814	1814	1814
09/12/16	0.0	0.0	0.0	338.4	1814	1814	1814	1814
09/13/16	5.0	16.2	8.1	592.0	1814	1814	1814	1814
T. Hay Fed	12701	12701	12701	12701	Week #4 Totals			
T. Recovered	27.4	47.0	59.5	2611.8				
T. Consumed	12673.2	12653.6	12641.1	10088.8				
% Consumed	99.8%	99.6%	99.5%	79.4%				
09/14/16	0.0	0.0	0.0	500.0	1814	1814	1814	1814
09/15/16	4.9	8.4	42.7	52.4	1814	1814	1814	1814
09/16/16	4.4	8.3	6.5	12.7	1814	1814	1814	1814
09/17/16	0.0	0.0	0.6	0.0	1814	1814	1814	1814
09/18/16	74.0	0.0	0.0	0.0	1814	1814	1814	1814
09/19/16	548.0	8.2	35.8	13.9	1814	1814	1814	1814
09/20/16	1080.0	30.2	35.6	13.1	1814	1814	1814	1814
T. Hay Fed	12701	12701	12701	12701	Week #5 Totals			
T. Recovered	1711.3	55.1	121.2	592.1				
T. Consumed	10989.3	12645.5	12579.4	12108.5				
% Consumed	86.5%	99.6%	99.0%	95.3%				
09/21/16	758.0	0.0	8.0	0.0	1814	1814	1814	1814
09/22/16	484.0	8.9	70.4	16.1	1814	1814	1814	1814
09/23/16	100.4	20.4	24.7	5.7	1814	1814	1814	1814
09/24/16	12.0	0.0	0.0	6.0	1814	1814	1814	1814
09/25/16	16.0	1.0	12.0	6.0	1814	1814	1814	1814
09/26/16	12.7	7.3	48.1	7.3	1814	1814	1814	1814
09/27/16	46.1	7.9	43.0	14.3	1814	1814	1814	1814
T. Hay Fed	12701	12701	12701	12701	Week #6 Totals			
T. Recovered	1429.2	45.5	206.2	55.4				
T. Consumed	11271.4	12655.1	12494.4	12645.2				
% Consumed	88.7%	99.6%	98.4%	99.6%				

09/28/16	73.5	0.0	24.8	24.8	1814	1814	1814	1814
09/29/16	236.0	7.9	24.1	11.6	1814	1814	1814	1814
09/30/16	71.8	2.8	17.3	4.2	1814	1814	1814	1814
10/01/16	252.0	2.0	0.0	6.0	1814	1814	1814	1814
10/02/16	610.0	20.0	50.0	22.0	1814	1814	1814	1814
10/03/16	436.0	8.8	20.3	29.0	1814	1814	1814	1814
10/04/16	102.5	3.5	28.2	11.7	1814	1814	1814	1814
T. Hay Fed	12701	12701	12701	12701	Week #7 Totals			
T. Recovered	1781.8	45.0	164.7	109.3				
T. Consumed	10918.8	12655.6	12535.9	12591.3				
% Consumed	86.0%	99.6%	98.7%	99.1%				
10/05/16	20.2	0.0	0.0	0.0	1814	1814	1814	1814
10/06/16	154.0	6.5	13.1	7.4	1814	1814	1814	1814
10/07/16	154.0	0.5	1.7	1.1	1814	1814	1814	1814
10/08/16	21.0	2.0	19.0	6.0	1814	1814	1814	1814
10/09/16	146.0	8.0	28.0	6.0	1814	1814	1814	1814
10/10/16	228.0	6.8	44.5	7.5	1814	1814	1814	1814
10/11/16	420.0	7.7	27.0	6.7	1814	1814	1814	1814
T. Hay Fed	12701	12701	12701	12701	Week #8 Totals			
T. Recovered	1143.2	31.5	133.3	34.7				
T. Consumed	11557.4	12669.1	12567.3	12665.9				
% Consumed	91.0%	99.8%	99.0%	99.7%				
10/12/16	574	10	294	314	2722	3175	3175	2722
10/13/16	1070	156	574	226	2722	3175	2722	2722
10/14/16	530	484	682	124	2722	3175	2722	2722
10/15/16	660	509	877	898	2722	2722	2722	2722
10/16/16	950	526	862	1030	2722	2722	2722	2722
10/17/16	710	530	1012	518	2722	2722	2722	2722
10/18/16	874	428	1140	688	2722	2722	2722	2722
T. Hay Fed	19051	20412	19505	19051	Week #9 Totals			
T. Recovered	5368	2643	5441	3798				
T. Consumed	13683	17769	14064	15253				
% Consumed	71.8%	87.1%	72.1%	80.1%				
10/19/16	994	598	1040	670	2722	3629	2722	2722
10/20/16	1050	966	964	470	3175	3629	2722	2722
10/21/16	338	690	988	426	2722	3175	2722	2722

10/22/16	402	240	601	974	3175	3629	2722	3175
10/23/16	536	600	774	712	2722	3175	2722	2722
10/24/16	388	800	787	376	2722	3629	2722	2722
10/25/16	544	366	682	516	2722	3175	2722	2722
T. Hay Fed	19958	24040	19051	19505	Week #10 Totals			
T. Recovered	4252	4260	5836	4144				
T. Consumed	15706	19780	13215	15361				
% Consumed	78.7%	82.3%	69.4%	78.8%				
10/26/16	356	400	344	256	2722	3629	2722	2722
10/27/16	1132	1158	1048	928	3175	3629	2722	3175
10/28/16	850	1202	596	618	2722	3629	2722	3175
10/29/16	570	524	470	324	2722	3856	2722	2722
10/30/16	884	954	730	680	2722	3629	2722	2722
10/31/16	1090	846	558	580	2722	3629	2722	2722
11/01/16	1244	1034	794	566	2722	3855	2722	3175
T. Hay Fed	19504	25855	19051	20412	Week #11 Totals			
T. Recovered	6126	6118	4540	3952				
T. Consumed	13378	19737	14511	16460				
% Consumed	68.6%	76.3%	76.2%	80.6%				
11/02/16	796	1000	684	620	2722	3629	2722	2722
11/03/16	646	1300	742	908	2722	4082	2722	2722
11/04/16	878	1070	490	304	2722	4082	2722	2722
11/05/16	512	508	580	290	2722	3629	2722	2722
11/06/16	330	680	410	560	2722	3629	2722	2722
11/07/16	970	1438	462	172	2722	4082	2722	2722
11/08/16	1154	862	512	330	3175	4082	2722	2722
T. Hay Fed	19504	27216	19051	19051	Week #12 Totals			
T. Recovered	5286	6858	3880	3184				
T. Consumed	14218	20358	15171	15867				
% Consumed	72.9%	74.8%	79.6%	83.3%				
11/09/16	6	476	84	228	2722	4082	2722	2722
11/10/16	406	812	444	246	2722	3629	2722	3175
11/11/16	468	686	484	1044	2722	4082	2722	3175
11/12/16	314	1090	478	290	2722	3629	2722	2722
11/13/16	404	1250	696	386	2722	3629	2722	2722
11/14/16	1068	1644	802	470	3175	3629	3175	3175

11/15/16	742	2300	486	492	2722	3629	2722	3175
T. Hay Fed	19504	26308	19505	20865	Week #13 Totals			
T. Recovered	3408	8258	3474	3156				
T. Consumed	16096	18050	16031	17709				
% Consumed	82.5%	68.6%	82.2%	84.9%				
11/16/16	1160	1680	784	410	2722	3629	2722	2722
11/17/16	516	1625	726	408	2722	3629	2722	3629
11/18/16	606	1736	632	266	2722	3629	2722	2722
11/19/16	430	1084	410	352	2722	3629	2722	2722
11/20/16	528	1300	364	72	2722	3629	2722	2722
11/21/16	692	1160	630	532	2722	3629	2722	3629
11/22/16	436	1258	582	360	2722	3629	2722	3629
T. Hay Fed	19051	25401	19051	21772	Week #14 Totals			
T. Recovered	4368	9843	4128	2400				
T. Consumed	14683	15558	14923	19372				
% Consumed	77.1%	61.2%	78.3%	89.0%				
11/23/16	644	1230	306	204	2722	3629	2722	2722
11/24/16	1178	1416	1218	972	2722	3629	3175	3629
11/25/16	672	1306	780	974	2722	3629	2722	3629
11/26/16	866	1494	762	890	3175	3629	2722	3629
11/27/16	290	962	482	1080	2722	3629	2722	3629
11/28/16	806	1380	690	1040	3175	3629	2722	3175
11/29/16	1090	1280	744	650	3175	3629	3175	3175
T. Hay Fed	20412	25401	19958	23587	Week #15 Totals			
T. Recovered	5546	9068	4982	5810				
T. Consumed	14866	16333	14976	17777				
% Consumed	72.8%	64.3%	75.0%	75.4%				
11/30/16	884	1380	1310	690	2722	3629	2722	3175
12/01/16	960	876	908	574	3175	3629	2722	2722
12/02/16	444	1134	278	210	2722	3629	2722	3175
12/03/16	92	852	232	88	2722	3629	2722	2722
12/04/16	440	1412	306	360	2722	3629	2722	2722
12/05/16	728	948	1030	462	3175	3629	3175	3175
12/06/16	358	840	490	342	2722	3629	2722	2722
T. Hay Fed	19958	25401	19505	20412	Week #16 Totals			
T. Recovered	3906	7442	4554	2726				

T. Consumed	16052	17959	14951	17686				
% Consumed	80.4%	70.7%	76.7%	86.6%				

APENDIX 19

R Output for Hay Consumption:

Ewe Body Weight Data: > ORTS = read.csv("C:/Users/goatdorothy/Documents/schoo

l/Thesis/ortsR.csv")

> Modelorts=lm(Dry.Matter~CULTIVAR+CYCLE+EWE, data=ORTS)

> anova(Modelorts)

Analysis of Variance Table

Response: Dry.Matter

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
CULTIVAR	3	1052721	350907	6.0693	0.0004701	***
CYCLE	3	28950900	9650300	166.9131	< 2.2e-16	***
EWE	3	5219461	1739820	30.0922	< 2.2e-16	***
Residuals	438	25323538	57816			

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

> ORTS = read.csv("C:/Users/goatdorothy/Documents/school/Thesis/ortsR.csv")

> Modelortsb = aov(Dry.Matter~CYCLE+CULTIVAR+EWE, data=ORTS)

> summary(Modelortsb)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
CYCLE	3	28950900	9650300	166.913	< 2e-16	***
CULTIVAR	3	1052721	350907	6.069	0.00047	***
EWE	3	5219461	1739820	30.092	< 2e-16	***
Residuals	438	25323538	57816			

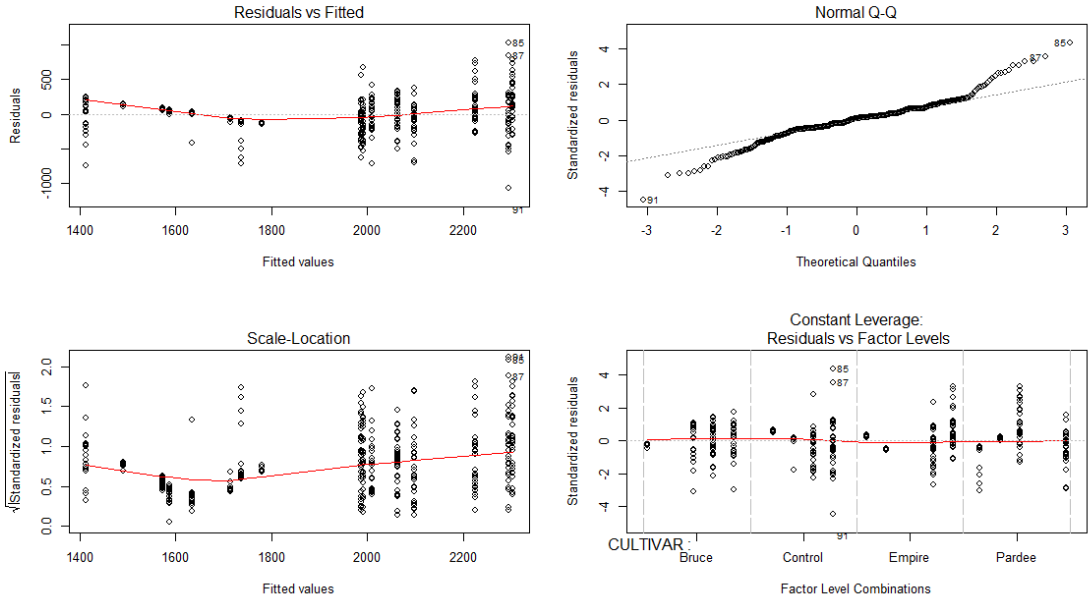
Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

> ortsresiduals=Modelorts$residuals

> ortsstdresiduals=(ortsresiduals)/sqrt(57816)

> par(mfrow=c(2,2))

> plot(Modelo



> par(mfrow=c(1,1))

> shapiro.test(ortsstdresiduals)

	Shapiro-Wilk normality test

data: ortsstdresiduals

W = 0.94683, p-value = 1.35e-11

> library(DescTools)

> PostHocTest(Modelortsb, method="hsd")

 Posthoc multiple comparisons of means : Tukey HSD

95% family-wise confidence level

$CYCLE

	diff	lwr.ci	upr.ci	pval	
B-A	-25.38482	-108.24943	57.47979	0.8590	
C-A	457.25446	374.38985	540.11907	<2e-16	***
D-A	528.55089	445.68628	611.41550	<2e-16	***
C-B	482.63929	399.77468	565.50390	<2e-16	***
D-B	553.93571	471.07110	636.80032	<2e-16	***
D-C	71.29643	-11.56818	154.16104	0.1197	

$CULTIVAR

	diff	lwr.ci	upr.ci	pval	
Control-Bruce	52.91518	-29.949432	135.7798	0.3535	
Empire-Bruce	90.74196	7.877354	173.6066	0.0255	*
Pardee-Bruce	131.50625	48.641640	214.3709	0.0003	***
Empire-Control	37.82679	-45.037825	120.6914	0.6416	
Pardee-Control	78.59107	-4.273539	161.4557	0.0702	.
Pardee-Empire	40.76429	-42.100325	123.6289	0.5834	

$EWE

	diff	lwr.ci	upr.ci	pval	
GERTIE-FERN	-231.91696	-314.78157	-149.05235	< 2e-16	***
NOREEN-FERN	-107.94018	-190.80479	-25.07557	0.00470	**
SPOT-FERN	-275.21875	-358.08336	-192.35414	< 2e-16	***
NOREEN-GERTIE	123.97679	41.11218	206.84140	0.00076	***

```
SPOT-GERTIE    -43.30179 -126.16640   39.56282 0.53306
SPOT-NOREEN   -167.27857 -250.14318  -84.41396 1.8e-06 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
> library("lsmeans")
> lsmeans(Modelortsb,~CULTIVAR)
 CULTIVAR   lsmean       SE  df lower.CL upper.CL
 Bruce    1800.116 22.72041 438 1755.461 1844.771
 Control  1853.031 22.72041 438 1808.377 1897.686
 Empire   1890.858 22.72041 438 1846.203 1935.513
 Pardee   1931.622 22.72041 438 1886.968 1976.277
Results are averaged over the levels of: CYCLE, EWE
Confidence level used: 0.95
> lsmeans(Modelortsb,~EWE)
 EWE      lsmean       SE  df lower.CL upper.CL
 FERN   2022.676 22.72041 438 1978.021 2067.330
 GERTIE 1790.759 22.72041 438 1746.104 1835.414
 NOREEN 1914.736 22.72041 438 1870.081 1959.390
 SPOT   1747.457 22.72041 438 1702.803 1792.112
Results are averaged over the levels of: CYCLE, CULTIVAR
Confidence level used: 0.95
> lsmeans(Modelortsb,~CYCLE)
 CYCLE   lsmean       SE  df lower.CL upper.CL
```

A 1628.802 22.72041 438 1584.147 1673.456

B 1603.417 22.72041 438 1558.762 1648.072

C 2086.056 22.72041 438 2041.402 2130.711

D 2157.353 22.72041 438 2112.698 2202.007

Results are averaged over the levels of: CULTIVAR, EWE

Confidence level used: 0.95

APENDIX 20

Ewe Body Weight Data:

Weight (lbs)

	Ewe	Ewe	Ewe	Ewe
Date:	1206	1301	1308	1314
08/08/16	196	188	194	224
08/16/16	205	198	196	223
08/23/16	197	195	192	225
08/30/16	189	193	192	225
09/05/16	189	186	187	224
09/12/16	192	187	189	238
09/20/16	199	191	189	222
09/28/16	195	191	186	224
10/04/16	192	186	187	223
10/11/16	196	186	183	226
10/18/16	200	191	187	228
10/25/16	200	191	192	232
11/01/16	202	196	194	232
11/08/16	202	198	198	234
11/15/16	203	197	198	241
11/22/16	205	207	197	239
11/29/16	206	205	196	238
12/06/16	210	206	202	241
Mean ± SD	199 ± 6	194 ± 7	192 ± 5	230 ± 7

APENDIX 21

R Output of Percent Bodyweight Change:

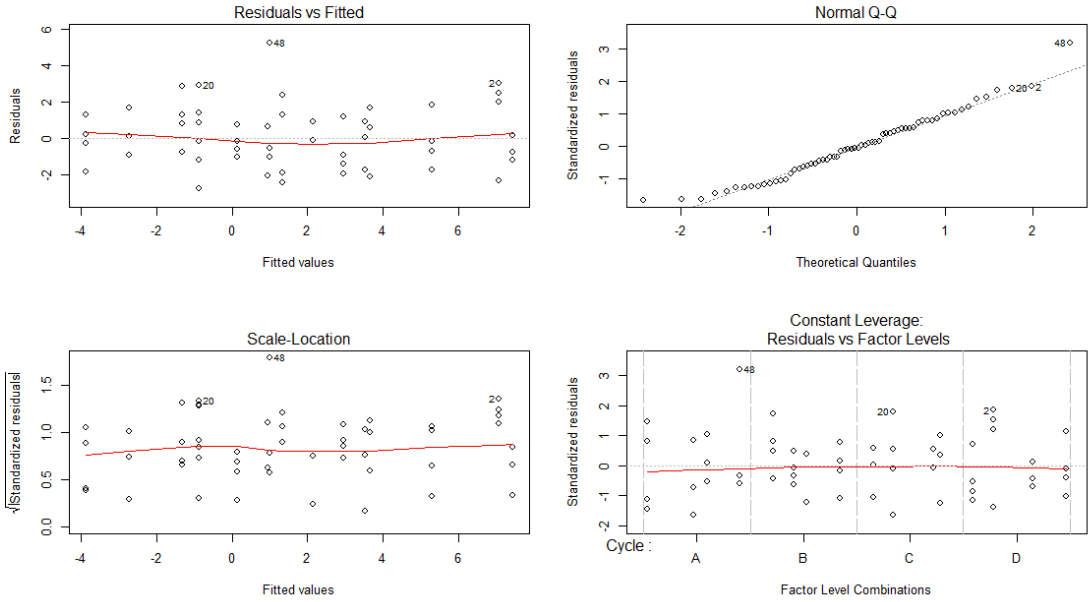
> Weight = read.csv("C:/Users/goatdorothy/Documents/school/Thesis/pweightc.csv")

> Modelweight = aov(pweightc~Cycle+Diet+Ewe, data=Weight)

> summary(Modelweight)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Cycle	3	438.8	146.27	45.741	7.55e-15 ***
Diet	3	2.9	0.98	0.307	0.82
Ewe	3	196.2	65.39	20.449	5.55e-09 ***
Residuals	54	172.7	3.20		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1



> weightresiduals=Modelweight$residuals

> weightstdresiduals=(weightresiduals)/sqrt(3.198)

```
> par(mfrow=c(2,2))
> plot(Modelweight)
> par(mfrow=c(1,1))
> shapiro.test(weightstdresiduals)

        Shapiro-Wilk normality test

data:  weightstdresiduals
W = 0.9727, p-value = 0.1669

> library(DescTools)
> PostHocTest(Modelweight, method="hsd")
  Posthoc multiple comparisons of means : Tukey HSD
    95% family-wise confidence level
$Cycle
       diff     lwr.ci    upr.ci    pval
B-A -0.712500 -2.3884716 0.9634716  0.6747
C-A  2.424375  0.7484034 4.1003466  0.0018 **
D-A  5.988750  4.3127784 7.6647216 3.2e-12 ***
C-B  3.136875  1.4609034 4.8128466 4.3e-05 ***
D-B  6.701250  5.0252784 8.3772216 5.1e-13 ***
D-C  3.564375  1.8884034 5.2403466 3.8e-06 ***
$Diet
                diff    lwr.ci   upr.ci   pval
```

```
Control-Bruce  -0.255625 -1.931597 1.420347 0.9774

Empire-Bruce    0.330000 -1.345972 2.005972 0.9534

Pardee-Bruce   -0.098750 -1.774722 1.577222 0.9986

Empire-Control  0.585625 -1.090347 2.261597 0.7909

Pardee-Control  0.156875 -1.519097 1.832847 0.9946

Pardee-Empire  -0.428750 -2.104722 1.247222 0.9049

$Ewe

                diff     lwr.ci      upr.ci    pval

Gertie-Fern   -4.379375 -6.0553466 -2.70340335 3.2e-08 ***

Noreen-Fern   -0.233750 -1.9097216  1.44222165  0.9826

Spot-Fern     -1.938750 -3.6147216 -0.26277835  0.0173 *

Noreen-Gertie  4.145625  2.4696534  5.82159665 1.3e-07 ***

Spot-Gertie    2.440625  0.7646534  4.11659665  0.0017 **

Spot-Noreen   -1.705000 -3.3809716 -0.02902835  0.0447 *

---

Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

> library("lsmeans")

> lsmeans(Modelweight,~Ewe)

 Ewe       lsmean        SE df   lower.CL   upper.CL

 Fern    3.250625 0.4470564 54  2.3543308  4.1469192

 Gertie -1.128750 0.4470564 54 -2.0250442 -0.2324558

 Noreen  3.016875 0.4470564 54  2.1205808  3.9131692
```

```
 Spot    1.311875 0.4470564 54  0.4155808  2.2081692

Results are averaged over the levels of: Cycle, Diet

Confidence level used: 0.95

> lsmeans(Modelweight,~Diet)

 Diet      lsmean        SE df  lower.CL upper.CL

 Bruce   1.618750 0.4470564 54 0.7224558 2.515044

 Control 1.363125 0.4470564 54 0.4668308 2.259419

 Empire  1.948750 0.4470564 54 1.0524558 2.845044

 Pardee  1.520000 0.4470564 54 0.6237058 2.416294

Results are averaged over the levels of: Cycle, Ewe

Confidence level used: 0.95

> lsmeans(Modelweight,~Cycle)

 Cycle    lsmean        SE df  lower.CL    upper.CL

 A     -0.312500 0.4470564 54 -1.208794  0.5837942

 B     -1.025000 0.4470564 54 -1.921294 -0.1287058

 C      2.111875 0.4470564 54  1.215581  3.0081692

 D      5.676250 0.4470564 54  4.779956  6.5725442

Results are averaged over the levels of: Diet, Ewe

Confidence level used: 0.95
```

APENDIX 22

Fecal Egg Count Procedure:

Supplies:

- gloves
- small plastic cups
- cheese cloth
- disposable pipette
- fecasol (1.2 Standard Specific Gravity, Fisher Scientific, Hampton, NH)
- McMaster slides
- water-based lubricant
- scale that weighs in grams
- tongue depressors
- microscope

Procedure (Whitlock, 1948; Zajac & Conboy, 2012):

1. Restrain the sheep.
2. Place a small amount of lubricant onto two fingers of a gloved hand.
3. Insert the lubricated fingers into the rectum of the sheep and scoop out a fecal sample.
4. Remove the glove by inverting it so that the fecal sample ends up on the inside of the glove.
5. Label the glove with the date and the ear tag number of the sheep.
6. Store the sample in the refrigerator until ready to run the sample.
7. Squeeze or knead the glove so that the fecal sample is thoroughly mixed.

8. Weigh out 2 grams of the fecal sample into a small plastic cup.
9. Add 28mL of fecasol and mix with a tongue depressor.
10. Allow the sample to sit for a minimum of 5 minutes.
11. Pour the sample through two layers of cheese cloth into a clean cup.
12. Thoroughly mix the sample with a pipette and using the pipette fill both sides of a McMaster slide.
13. Allow the slide to sit for a minimum of 5 minutes.
14. Place the slide on a microscope and examine using the 10x objective.
15. Count all of the strongylid eggs within the grid lines on both sides of the slide.
16. Multiply the total number of eggs by 50 to determine the eggs per gram of feces.
17. Record the egg count, sample date, and sheep ear tag number.
18. Clean up any mess and put supplies away.

APENDIX 23

Ewe Fecal Egg Count Data During BFT Study:

	Fecal Egg Counts (eggs/gram)			
	Ewe	Ewe	Ewe	Ewe
Date:	1206	1301	1308	1314
08/09/16	0	50	50	0
08/16/16	0	100	0	100
08/22/16	50	100	0	0
08/30/16	0	0	50	0
09/06/16	0	150	0	50
09/13/16	0	150	0	0
09/20/16	0	50	0	50
09/27/16	150	0	0	0
10/04/16	0	0	0	0
10/11/16	50	0	150	0
10/18/16	0	0	0	0
10/25/16	0	0	50	0
11/01/16	50	0	50	0
11/08/16	0	0	0	0
11/15/16	0	0	0	0
11/22/16	50	0	50	0
11/29/16	0	0	0	0
12/06/16	0	0	0	0

APENDIX 24

Ewe Body Condition Score Data During BFT Study:

	Body Condition Score			
	Ewe	Ewe	Ewe	Ewe
Date:	1206	1301	1308	1314
08/09/16	3.0	3.0	3.5	3.5
08/16/16	3.5	3.0	3.5	3.0
08/22/16	3.0	2.5	3.0	3.5
08/30/16	3.0	3.0	3.0	3.5
09/06/16	3.0	2.5	3.0	3.5
09/13/16	3.0	2.5	3.0	3.5
09/20/16	3.5	2.5	3.0	3.0
09/27/16	3.0	2.5	3.0	3.0
10/04/16	3.0	2.5	3.0	3.0
10/11/16	3.0	2.5	3.0	3.5
10/18/16	3.0	2.5	3.0	3.5
10/25/16	3.0	2.5	3.0	3.5
11/01/16	3.0	2.5	3.0	3.5
11/08/16	3.0	3.0	3.0	3.5
11/15/16	3.0	2.5	3.0	3.5
11/22/16	3.0	3.0	3.0	3.5
11/29/16	3.5	3.0	3.0	3.5
12/06/16	3.0	3.0	3.5	3.5

APENDIX 25

Packed Cell Volume Procedure:

Supplies:

- glass blood collection tube w/ K3 EDTA (Vacutainer™)
- blood draw tube holder (Vacutainer™)
- 20G 1.5 inch blood draw needles (Fisher Scientific, Hampton, NH)
- microhematocrit capillary tubes (Fisherbrand™)
- sealant pad (StatSpin™)
- tube rocker
- Kimwipes™ (Fisher Scientific, Hampton, NH)
- microhematocrit rotor
- centrifuge
- circular hematocrit reader
- gloves

Procedure:

1. Attach a new needle to the blood draw tube holder.
2. Have a helper restrain the animal against a fence (preferably a corner).
3. Hold off the jugular vein just above the point of shoulder on the animal.
4. Locate the vein, remove the cap from the needle, and insert the needle in an upward direction nearly parallel to the vein.
5. Continue to hold off the vein and insert a blood collection tube into the holder.
6. If blood does not enter the tube, gently re-position the needle, holder, and tube as a unit until blood enters the tube.

7. Once blood has filled the tube release the vein and remove the blood tube from the holder.
8. While inverting the tube to thoroughly mix the blood with the anticoagulant, remove the needle and holder from the vein.
9. Discard the used needle in a sharps container.
10. Record the animal's ear tag number on the blood tube.
11. Store the blood tube in the refrigerator until ready to measure the packed cell volume (must be done the same day blood was drawn).
12. Place the blood tubes on a tube rocker.
13. For each capillary tube record the ear tag number and the location in the rotor.
14. While wearing gloves, remove the rubber plug from the top of a blood tube and insert a capillary tube.
15. Tip the blood tube so that the blood begins to rise in the capillary tube.
16. When 9/10 full, hold off the end of the capillary tube and remove it from the blood tube.
17. Insert the end of the capillary tube into the sealant pad.
18. Clean off the capillary tube with a Kimwipe™ and place in the appropriate location in the rotor (sealant facing outward).
19. Repeat the process for the same blood tube so that there are duplicates of each sample.
20. When all samples are ready, place the cover on the rotor and attach the rotor to the centrifuge.
21. Spin the samples at 15,000 RPM for 3 minutes.

22. Remove rotor from the centrifuge and take the cover off.
23. Remove a capillary tube and place it on the circular hematocrit reader.
24. Line the beginning of the blood up with the start line and adjust the reader so that the spiral line is under the end of the plasma.
25. Spin the reader so that the spiral line moves to the beginning of the plasma and read percentage displayed.
26. Record the result and repeat with the duplicate capillary tube.
27. Discard the blood tubes and capillary tubes and clean up any mess.

APENDIX 26

Ewe Packed Cell Volume Data:

	% Red Blood Cells			
	Ewe	Ewe	Ewe	Ewe
Date:	1206	1301	1308	1314
08/09/16	30/29	27/27	30/31	31/30
08/16/16	27/28	30/30	31/31	31/30
08/22/16	31/30	30/30	31/31	31/32
08/30/16	33/32	31/30	33/32	31/30
09/06/16	29/28	30/29	31/31	31/30
09/13/16	30/29	28/29	30/29	32/33
09/20/16	29/30	29/29	30/31	31/31
09/27/16	30/30	27/27	31/31	29/29
10/04/16	30/31	30/30	30/30	30/30
10/11/16	31/32	27/27	29/29	29/30
10/18/16	32/31	27/28	28/28	30/30
10/25/16	27/27	25/26	29/29	31/31
11/01/16	27/27	25/25	27/26	32/32
11/08/16	29/29	24/24	28/29	29/28
11/15/16	28/29	26/26	31/30	31/31
11/22/16	30/31	27/26	32/32	30/30
11/29/16	27/27	24/24	31/30	30/30
12/06/16	29/28	26/26	32/33	30/31

APENDIX 27

FAMACHA© Scores:

	FAMACHA© Score			
	Ewe	Ewe	Ewe	Ewe
Date:	1206	1301	1308	1314
08/09/16	2	1	1	2
08/16/16	1	1	1	1
08/22/16	1	2	1	1
08/30/16	1	1	1	1
09/06/16	1	1	1	1
09/13/16	1	2	1	2
09/20/16	2	2	2	1
09/27/16	1	1	2	1
10/04/16	2	1	1	1
10/11/16	2	2	2	2
10/18/16	1	1	1	2
10/25/16	2	1	1	2
11/01/16	2	1	1	1
11/08/16	1	1	1	1
11/15/16	2	2	1	1
11/22/16	1	1	2	2
11/29/16	1	1	1	1
12/06/16	1	1	1	2

Appendices References:

Whitlock, H.V., 1948. Some modifications of the McMaster helminth egg-counting technique and apparatus. J. Counc. Sci. Res. 21, 177–180.

Zajac, A.M., Conboy, G.A., 2012. Veterinary Clinical Parasitology, 8th ed. Wiley-Blackwell, Ames, Iowa.