Efficacy of Maine Lobster Shell as Treatment for Haemonchus Contortus Parasitism in Sheep

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Efficacy of Maine Lobster Shell as Treatment for Haemonchus Contortus Parasitism in Sheep

by

Morgan H. Gustin

A Thesis Submitted in Partial Fulfillment of the Requirements for a Degree with Honors (Animal and Veterinary Sciences)

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ABSTRACT

Lobster shell, a chitin-based material, was applied to the ova, and to the L3 and L4 larval stages of the ruminant nematode *Haemonchus contortus* to assess its nematicidal effects on the parasite. Methods were developed to more accurately characterize and quantify larvae, including a formalin-kill technique to establish larval viability and a procedure for culturing L3 stage larvae into L4s in AF. Larval cultures containing 80g of fecal matter, 30g vermiculite and 100ml water were treated with various proportions of crushed lobster shell (medium or fine crush) to assess the affect of lobster shell on the maturation of *H. contortus* ova into L3 stage larvae. Fourth stage larvae in AF were treated with lobster shell conditioned media, chitin, chitosan, or Ivermectin and live:dead counts were obtained to compare larval viability.

Larvae incubated in AF developed anatomical characteristics analogous to functional L4 structures, although no consistent larval growth was evident from body length measurements over time. On average, cultures incubated with medium-sized (filtered through sieve No.400, between 38-212µm) lobster shell had total larval counts of 242.5, fine-sized (filtered through sieve No.400, 38µm) lobster shell had 499, and lobster shell-free cultures had 1,253.5 larvae. Overall, lobster shell exhibited nematicidal effects on *H. contortus* development from ova to L3 stage, but no clear comparison of larval viability could be obtained from any of the L4 treatment groups. Further studies may reveal that lobster shell is effective as a supplemental treatment in conjunction with standard anthelmintics, to decrease exposure of sheep to infective larvae on pasture.
DEDICATION

I would like to dedicate this work to my wonderful parents, Randy and Lynn Gustin, who have been by my side through it all.
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INTRODUCTION

Research statement of purpose

This purpose of this study was to assess whether nematicidal effects were exhibited when lobster shell (LS), a chitin-based substance, was applied to different life cycle stages of the ruminant parasitic nematode *H. contortus*. No previous studies exploring the relationship between crustacean shell chitin and mammalian nematode species were found. However, research on the use of LS in other species suggested that LS could be useful in treatment of mammalian GI parasites such as *H. contortus*.

Importance of the research

In the livestock industry, heavy parasite loads have a detrimental effect on the health and production of animals, resulting in lowered efficiency and loss of profit. In sheep, one of the major parasites, *Haemonchus contortus*, has developed resistance to all common antiparasitic drugs, calling for new and innovative solutions to treat the parasite. This research has provided some of the initial groundwork necessary for advancement in production of a potential new small ruminant anthelmintic using lobster shell.

Background

Heavy parasite loads in livestock

Heavy parasite loads have been an area of concern for livestock operations for decades. Why? Heavy parasite loads can negatively impact the health of animals, leading to dramatic loss of production, reduced vitality, and even death of the animals. Losses caused by heavy parasite loads may be direct, in terms of death, poor gains, reproductive
inefficiency, and indirect, a result of susceptibility to secondary infection and greater labor needs (Hale, 2006). From a producer’s standpoint, maintenance of healthy animals is the critical component of managing successful livestock operations. Hence, management of parasites — gastrointestinal worms in particular—is often a key animal health issue on farms and ranches.

_Gastrointestinal parasites in sheep_

More specifically, _internal_ parasite management is a major concern for sheep and goat producers (Hale, 2006; Kaplan, 2005). Internal parasites, also known as gastrointestinal (GI) parasites, are those that reside within the stomach and intestines. Common GI parasites of sheep, such as the _Haemonchus contortus_ worm, are destructive to the health and productivity of sheep populations (Kaplan, 2005). In fact, infections from GI parasites are the most important limiting factor to sheep production around the world (Baloyi et al., 2011).

Among the most common types of GI parasites that target sheep populations are _Teladorsagia (Ostertagia)_ spp., _Trichostrongylus_ spp., _Muellerius capillaris_ (lung worm), and _Haemonchus contortus_ (barber pole worm)(Dikmans et al., 1942; Thomas et al., 1993). _Haemonchus contortus_ were primarily used in this research due to their documented pathogenicity to small ruminants in northern New England (Weber et al., unpublished data).

_Teladorsagia (Ostertagia) and Trichostrongylus_ are stomach worm species commonly found year-round in sheep and goats. The larvae may be distinguished from similar species in a sample by their longer body length (700-830 µm), cone-shaped sheath-tail extension (STE) of 30-35 µm with no filament, and square or rounded head
These intestinal nematodes burrow superficially into the crypts of the mucosa and develop into egg-laying adults within a few weeks. Anorexia, diarrhea and weight loss are the main signs of infection.

Lung worms are considerably smaller than other parasitic nematodes, approximately 300 µm in length. They irritate the bronchioles inside the lung and cause a local reaction with white blood cells and mucus that are trying to expel the parasites (Dikmans et al., 1942). Pain and irritation cause the animal to cough. Lung worms are common year-round. The benzimidazoles and macrocyclic lactones are often used to treat all stages of the nematode.

**H. contortus: characteristics and life cycle**

*Haemonchus contortus* worms, also known as the ‘twisted stomach worm’ or ‘barber pole worm’ due to the spiral striping of their bodies, are bloodsucking stomach worms commonly found in the fourth stomach (abomasum) of their host. *Haemonchus contortus* is considered one of the most important parasites, infecting approximately 60 different ruminant species (Garretson, 2007). Sheep and goats, however, tend to be the preferred host for this parasite. Commonly found from spring to fall seasons in the northeastern part (active all year in the southeast) of the United States, *H. contortus* typically ranges from 650-730 µm in length with an STE of 70-75 µm and ‘bullet-shaped’ head.

The life cycle of *H. contortus* is as follows (Dikmans et al., 1942): Adult worms live in the abomasum of the ruminant host, and eggs are passed out in the feces. Careful study has revealed that the eggshell structure of *H. contortus* appears to be similar to those of other nematodes (Bird et al., 1991). The medial layer is surrounded by a dense
matrix of chitinous fibrils and protein, sensitive to degradation by either proteinase K or chitinase, but not colleganases (Mansfield et al., 1992). Under favorable conditions, eggs develop and molt in the feces over the next few days to progress through first-stage (L1), second-stage (L2) and third-stage (L3) larvae. These third-stage larvae are infective but still enclosed in the skin of the second molt, known as ensheathed larvae. They do not feed, and are significantly resistant to environmental and climactic conditions, due to their protective cuticle. In the adult *H. contortus* nematode cuticle, collagen makes up 80% of the cuticular proteins (Mansfield et al., 1992), largely explaining why the adult outer cuticle is tough and relatively impermeable to factors from the environment (Shamansky et al., 1989).

In warm weather when the grass is wet, the third-stage larvae migrate onto pasture grass blades. Both the grass blades and larvae are ingested by the grazing ruminant host. Larvae soon shed their second-molt skin and reach the abomasum after ingestion. After another molt, they develop into fourth-stage (L4) larvae. The final fourth molt occurs between five to ten days after infection, and the worms reach the adult stage at this point. They reach maturity in 2 to 3 weeks.

As L4 and adults, *H. contortus* larvae are avid blood-feeders, burrowing into the stomach lining of infected individuals to access the blood stream. This can cause abdominal pain, severe anemia, hemorrhagic gastritis, diarrhea, weight loss and acute infections, among other pathologies. High egg counts per day produced by the females allows for population explosions and consequent disease outbreaks that are devastating to sheep populations (Laing et al., 2013). *Haemonchus contortus* has been the widely-studied subject of much GI parasite-based research, even in this local area. Other students
at the University of Maine, for example, continue to explore aspects of parasitology related to *H. contortus*.

*Common anthelmintics used for sheep and goats*

Since the introduction of thiabendazole in 1961, for about 50 years producers have enjoyed the use of safe, affordable, effective anthelmintics for sheep and goats (Shulaw, 2013). Today, avermectins (including macrocyclic lactones such as ivermectin), milbemycins (moxidectin) (Shulaw, 2013), benzimidazoles, and imidazothiazoles (levamisole), are considered main classes of chemical de-wormers used to treat internal parasites in sheep and goats. Each class of anthelmintic has a specific mode of action against parasites.

Macrocyclic lactones, a major type of avermectins, target glutamate-gated chloride channels, causing paralysis of neuromusculature of the parasite. Paralysis of these structures, including the pharynx, prevents the helminth from feeding (Köhler, 2001). Paralyzed helminths are either expelled from the body or they starve. Ivermectin is a widely-known macrocyclic lactone first introduced in 1981 and approved for use in horses. Since then, ivermectin parasitic roundworms in livestock (including horses, sheep, goats), heartworms in pets, and most mites and lice species, among others. Oral administration is commonly used for livestock treatment. Macrocyclic lactones, along with benzimidazoles, have been shown to be effective against adults and immature stages of the nematode (Garretson, 2007).

Moxidectin has more recently been found to work well when ivermectin resistance is present, despite having a chemical structure that is very similar to
ivermectin. It is a very potent drug FDA-approved for use in goats and sheep, and is persistent in tissues. Tissue residues decline over a prolonged period, which provides extended period protection for sheep, but allows exposed worm larvae to select for resistance. It is currently suggested to save moxidectin for when substantial resistance to the other three de-wormer classes is present (Shulaw, 2013).

Benzimidazoles, such as albendazole and fenbendazole, target the tubulin within intestinal cells of the parasite. Tubulin forms into microtubules that are needed for gathering nutrients. Benzimidazoles attach to the beta-tubulin component, preventing it from forming microtubules within the intestinal cells of the nematode. This inhibits the parasite’s ability to uptake nutrients and transport necessary digestive enzymes, resulting in parasite death due to starvation (Köhler, 2001).

Imidazothiazoles, such as levamisole, act as acetylcholine agonists that affect the parasite’s nervous system (Köhler, 2001). These drugs incapacitate the parasite by causing muscle contraction and paralysis, eventually leading to the expulsion of the parasite from the body (Garretson, 2007). They have been shown to be effective against adults and later stages of immature larvae.

*Developing resistance to common chemical de-wormers*

Unfortunately, parasite tolerance to these main classes of chemical de-wormers has been steadily increasing over the decades. The general consensus acknowledges that anthelmintic resistance (Shalaby, 2013) seems to be a ‘pre-adaptive heritable phenomenon’ present within a parasite population even prior to drug exposure. After exposure, only worms that carry the ‘resistance genes’ survive, and these survivors lay eggs. In this way, the gene pool for resistance is increased (Shalaby, 2013). Tolerance,
leading to anthelmintic resistance, may manifest itself in different ways, either as a heritable decline in the efficacy of an anthelmintic against a population of generally-susceptible parasites or as a decrease in the time that a drug exerts its effect on the parasite population (Shalaby, 2013). Recently, ‘resistance’ has been classified as ‘an increase in the proportion of organisms in a population carrying a gene demonstrated to be linked with resistance’ (Shalaby, 2013).

Anthelmintic-resistant nematodes in small ruminants are already a serious issue, and are one of the most pressing problems to be addressed (Aitken, 2007). *H. contortus* parasites in particular have developed drug resistance at alarming speeds (Laing et al., 2013) to all main classes of anthelmintics, suggesting that a common mechanism of resistance may be involved. This mechanism is believed to be an overexpression of P-glycoproteins (Pgp), an efflux transporter that aids in the transport of compounds, including drugs, across membranes (Garretson, 2007). Pgp primarily functions to protect the organism by actively pumping toxic substances out of its cells. P-glycoproteins have been located in the DNA sequence of *H. contortus*. In addition, the drugs ivermectin, benzimidazoles and levamisole have characteristics that are common to Pgp substrates (Garretson, 2007). An overexpression of Pgp in parasites would allow for increased ability to pump toxic substances, including chemical de-wormers that may be ‘recognized’ as Pgp substrates, out of their cells.

**Search for alternative GI parasite controls**

Rapid development of anthelmintic resistance has spurred the search for alternative methods of GI parasite control. Broader techniques such as pasture management, rotational use of the chemical de-wormers, Smart Drenching and selection
of parasite-resistant animals have been utilized (Garretson, 2007; Hale, 2006; Kaplan, 2005). In addition, within the past two decades many studies have focused on the use of various plants and fungal species that may have antiparasitic activity (Shulaw, 2013). Antiparasitic plants or fungi are unlikely to provide the same full benefits that have been enjoyed with traditional anthelmintics, but they certainly may be used in conjunction with traditional de-wormers to maximize parasite control and minimize reliance on chemical de-wormers (Shulaw, 2013).

Plant sources may contain secondary metabolites that negatively affect egg hatching, larvae, or adult worms. The overall concept of nutraceutical plants has emerged in helminthology and veterinary science for improved control of livestock parasites (Hoste et al., 2015). In veterinary science, a nutraceutical is considered to be a livestock feed that has both nutritional value and additional beneficial health effects such as treatment and prevention of a disease (Hoste et al., 2015). The first evidence supporting the use of plants as nutraceutical feeds against GI parasites originated from research in New Zealand that reported substantial GI nematode egg count reductions in sheep when grazing pastures with different legumes, *i.e.* birdsfoot trefoil and big trefoil, sull and sainfoin, which contain condensed tannins (Niezen et al., 1995). Tannin is a bitter plant polyphenolic compound that binds to and precipitates various organic compounds such as amino acids and alkaloids. Two general hypotheses have been proposed to explain the activity of tannin containing feeds against GI parasites: First, a “direct” hypothesis based on various interactions between the polyphenols and different GI parasite stages. Second, an “indirect” hypothesis assuming a possible improvement in host resistance due to the effects of tannin-containing feeds that improve overall host protein nutrition by
increasing by-pass protein (Hoste et al., 2015). This indirectly improves host resistance and resilience to nematode parasite infections (Shalaby, 2013).

Since the original New Zealand study, a vast array of investigations (Hoste et al., 2015; Shalaby, 2013) have analyzed many other plant-based sources for similar GI nematode antiparasitic properties, and have broadened our understanding of their function and application within helminthology and veterinary science. For example, studies have explored diatomaceous earth (DE) with cattle, other ruminants, and beetles (Fernandez et al., 1998; McLean et al., 2005; Shostak et al., 2015), artemisinin and aqueous extracts of plant *Artemisia annua* with sheep (Cala et al., 2014), *Calluna vulgaris* with goats (Moreno-Gonzalo et al., 2012), and *Markhamia obtusifolia* Sprague leaf extracts with ruminant GI parasites in general (Nchu et al., 2011).

Fungi have also been a source of interest for investigations exploring their antiparasitic properties and application. Certain fungi been shown to trap and kill developing larvae in the fecal pat, reducing pasture infectivity in all livestock species. Most notably, *Duddingtonia flagrans* (Shalaby, 2013) have demonstrated such results, and reports of using these fungi have been available for about 20 years (Shulaw, 2013). However, as of 2013, no commercial source was available anywhere worldwide.

*Chitin: another compound with antiparasitic properties*

In addition to research of plant- and fungi-based compounds, chitin and its derivative chitosan have also become important compounds studied for their antiparasitic properties. Chitin is a polymer of unbranched chains of beta-1,4-linked 2-aceta-mido-2-deoxy-D-glucose residues. Its structure is analogous to cellulose found in plant tissues. It is the most common polysaccharide in nature whose basic unit is an amino sugar (known
as an aminopolysaccharide) (Gortari et al., 2008; Spiegel et al., 1986). Known for its rigid, resistant structure, chitin contributes to the mechanical strength of organisms. Chitin is a component of egg shells in parasitic nematodes such as *Haemonchus contortus*, and has been found in other tissues of nematodes such as structures of the feeding apparatus (Veronico et al., 2001). Additionally, it may be derived from the shell exoskeleton of crustaceans such as lobster, crab, krill and shrimp, as well as from insects, yeasts and filamentous fungi (Gortari et al., 2008). Chitosan is a biodegradable, nontoxic, deacetylated form of chitin that can affect microorganisms due to its antimicrobial activity or initiate a series of defense reactions in treated plants (Radwan et al., 2012).

For industrial production, chitin and chitosan are most commonly extracted from crab, shrimp and prawn shell waste (Badawy et al., 2011). Preparation of pure chitin on a large scale requires a series of harsh treatments to separate chitin from other constituents. First, ground shells are treated with either sodium hydroxide or by digestion with proteolytic enzymes such as trypsin, pepsin and pronase to extract proteins. Shell minerals such as calcium phosphate and calcium carbonate are removed with hydrochloric acid. Shell pigments, melanin and carotenoids are extracted with hydrogen peroxide, sodium hypochlorite, or 0.02% potassium permanganate (Badawy et al., 2011). Finally, a deacetylation process, typically achieved by treating chitin with concentrated sodium or potassium hydroxide solution at high temperatures, releases amine groups to form chitosan (Badawy et al., 2011).

Chitin and its derivative chitosan have substantial economic value due to their versatile biological activities and agrochemical applications (Badawy et al., 2011). For instance, chitosan has a potent effect on plant diseases control due to its antimicrobial
properties and plant innate immunity elicited activity (Badawy et al., 2011). Research groups in Europe, Japan, and North America have been investigating chitin as a natural polysaccharide-based material to be used as a reinforcing structure in thermoplastic nanocomposites (Goodrich et al., 2009). Bioplastics reinforced with chitin nanoparticles have recently attracted attention as sustainable and renewable alternatives to current plastic materials (Goodrich et al., 2009).

*Chitin as target for plant and soil nematodes*

Other studies have further explored chitin and chitosan as biological control agents against a variety of plant nematodes. Addition of crustacean chitin to soil was found to be an effective nematicide treatment and fertilizer amendment for bean and tomato plants (Spiegel et al., 1986, 1987). Field trials were conducted with a chitin-urea soil amendment on tomato, potato, walnut and Brussel sprout crop-nematode combinations (Westerdahl et al., 1992). In 2011, chitin was chemically extracted from Egyptian shrimp shell waste and converted into chitosan. Both chitin and chitosan were then incorporated into the soil of tomato plants to assess their nematicidal potential against the root-knot nematode, *Meloidogyne incognita*. Their nematicidal activity was compared with that of oxamyl, a synthetic nematicide. Although neither chitin nor chitosan demonstrated efficacy greater than that of oxamyl, obtained results did show that both reduced second-stage juvenile (J2) nematode counts in the soil in a dose-dependent manner without harming the tomato plants (Radwan et al., 2012). Similarly, use of chitosan as a soil amendment dramatically reduced pine wilt disease (PWD) nematode counts for maritime pine and stone pine plant species (Nunes da Silva et al., 2014). No
research could be found concerning chitin and chitosan as biological agents against *mammalian* nematodes.

**Theorized modes of action of chitin**

Although the mode of action of chitin in controlling *plant*-parasitic nematodes is not fully understood, several mechanisms have been suggested (Radwan et al., 2012). These include: (1) chitin hydrolysis which increases ammonia concentration and consequently nematicidal activity; (2) stimulated growth of antagonistic microorganisms with chitinolytic properties, such as actinomycetes and bacteria; (3) increased microbial chitinase activity which may damage the nematode’s chitin-containing egg shell (Badawy et al., 2011). All three of these mechanisms likely involve chitinolytic enzymes.

Chitinolytic enzymes (chitinases) are part of the cellular system necessary to complete enzymatic hydrolysis and degrade chitin (Gortari et al., 2008). A variety of organisms including insects, plants, and microorganisms produce many types of chitinolytic enzymes, each with different physiological roles in nutrition, defense, and parasitism, among others (Gortari et al., 2008). Microorganisms are the main environmental degraders of chitin, and thus they are an important natural resource of chitinolytic enzymes (Gortari et al., 2008). Chitinases have a variety of industrial and agricultural applications, including biocontrol of plant pathogenic fungi and insects, mosquito control, and single cell protein production (Patil et al., 2000). In addition, research has found that nematode eggs can be disintegrated solely by enzymatic action of chitinases (Gortari et al., 2008).
The presence of chitin in nematodes, and its absence in vertebrate animals and plants allows for selective ‘targeting’ of these chitin-containing pathogenic organisms through the use of chitinolytic enzymes (Gortari et al., 2008; Mansfield et al., 1992; Veronico et al., 2001).

The promise of lobster shell

Lobster shell exoskeleton, as discussed previously, contains a significant amount of chitin as its structural component (Hakim et al., 1998). Crustacean shell waste consists mainly of 20-30% chitin, while lobster species in particular are found to have cuticles consisting of 60-70% chitin (Arbia et al., 2013).

The American lobster (Homarus americanus) has an exoskeleton (cuticle) composed of three main layers: the epicuticle, exocuticle, and endocuticle. The epicuticle is the thin, waxy outermost layer that consists mainly of alcohols, fatty acid esters, and long-chain hydrocarbons (Bobelmann et al., 2007; Raabe et al., 2006). The two innermost layers, the exocuticle and endocuticle, consist mainly of chitin associated with various proteins, hardened by calcium carbonate minerals (Raabe et al., 2006). Chitin occurs in three different polymorphic forms: α-chitin, β-chitin and γ-chitin. α-chitin is the most abundant crystalline form, with its chains arranged in an anti-parallel order (Raabe et al., 2006).

Finally, the seafood industry alone annually generates around $10^5$ metric tons of chitin waste, representing a readily available, underutilized, sustainable resource (Goodrich et al., 2009). In Maine, millions of pounds of lobster shell (LS) from lobster processing facilities are discarded in landfills each year. If successful, this research would
suggest another alternate use for this waste product and potentially benefit the Maine economy.

**Research hypotheses**

We hypothesize that crushed LS will demonstrate nematicidal effects when applied to *H. contortus* eggs and larval stages in a culture environment or live *H. contortus* larvae in a culture environment that mimics the abomasum of a sheep. The following experiments were designed to test this hypothesis.

**Thesis structure**

Overall, this thesis process has been divided into three major sections: (A) Development of preliminary methods, (B) Experimental stage 1, and (C) Experimental stage 2.

The development of preliminary methods encompasses all of the processes and techniques that were developed and/or modified throughout this research. Some of the methods involved establishment of standard operating procedures in order to have a consistent manner of processing samples. Some of the methods involved the development and utilization of new techniques to more accurately measure and quantify data that would need to be obtained.
Experimental stage 1 involved the growth of L3 stage larvae into L4 stage within abomasal fluid. Four major trials were conducted with modifications in an attempt to grow the larvae into more mature stages within a culture setting.

Experimental stage 2 involved all trials that used crushed lobster shell to treat various stages of the *Haemonchus contortus* parasite. A “quick and dirty” trial was conducted, to initially combine crushed LS with L3 stage larvae and observe the results. Later on, more established trials were later run to specifically treat the parasite at both juvenile and mature larval stages.

The Methodology, Results, and Discussion categories of this written thesis will each be subdivided into sections dedicated to these three major areas (Development of preliminary methods, Experimental stage 1, Experimental stage 2) of the thesis process.

**METHODOLOGY**

The study was conducted in Maine on *Haemonchus contortus* parasitic nematodes collected from the flock of twenty-six Icelandic sheep at the J. Franklin Witter Teaching and Research Center at the University of Maine in Orono. The research explored the treatment-target relationship between crushed LS and *H. contortus* nematodes. The study revolved around one central question: are nematicidal effects exhibited when LS, a chitin-based substance, is applied to different life cycle stages of the ruminant parasitic nematode *Haemonchus contortus* in vitro?

1. **Development of preliminary methods**

1.1 *Collection and culturing of larvae*
From late August 2015 – mid September 2015 a supply of *H. contortus* eggs were collected, grown to L3-stage, and stored for study. This process involved the collection of fresh fecal samples from the Witter flock, followed by homogenization and culturing of the samples in a 26°C incubator for 8 days to grow the larvae to the L3 stage of development (Appendix I). Pooled samples were homogenized and mixed with vermiculite (autoclaved for 15 minutes at 121°C, 15psi) at proportions of 1:2 (feces: vermiculite) by volume. A volume of tap water equal to the total mass of feces and vermiculite was added and mixed. The mixture was stored in red plastic Solo cups, covered with aluminum foil, labelled and placed in a 26 °C incubator for 8 days.

Next, the Baermann funnel technique (Todd et al., 1970) was used to extract the larvae from the incubated mixture. Each funnel was filled with tap water, 43-45 °C. 10-30 grams of culture was placed in the center of two Kimwipes, folded up to make a pouch and closed with a rubber band. A wooden stick was run through the rubber band to suspend the pouch in the water-filled funnel. After the sample was allowed to sit for 3-7 hours, the water from the bottom of the funnel was drained into a 50 ml centrifuge tube to collect the larvae extract. This Baermann funnel procedure was repeated in order to fully process all of the culture contents.

All centrifuge tubes belonging to a particular batch of cultured larvae were consolidated into a concentrated sample. The concentrated larvae were stored in phosphate-buffered solution (PBS) for later use. See Appendix I for more detailed procedures of the collection, incubation and processing of *H. contortus* larvae. Larvae were stored in batches according to both (1) their date of collection and (2) the group of sheep they were collected from (rams, lambs, ewes).
1.2 Establishing larval counts per microliters (µl) of L3 culture

A repeatability study was conducted on each batch of cultured larvae to establish and verify a consistent measure of larvae count per microliters of L3 culture. This study determined, within a reasonable margin of error, how many larvae were in 50-100 µl of L3 culture. Completion of this repeatability study was necessary to establish the variability of any future measurements. This study was repeated as necessary, after adding PBS to the larval batch over time in storage, or to reestablish a current numerical concentration of larvae.

For this study, 50-100 µl of L3 culture was drawn from the stored centrifuge tube, transferred into a glass tube, and heat-killed at 57-60° C for five minutes. Larvae could then be visualized and counted. This process was repeated at least 3 times and an average of counts were taken to determine the number of larvae present in 50-100 µl of L3 culture.

1.3 Development of formalin-kill alternative technique

A new technique for assessing live:dead larval counts was developed, because the standard Millipore membrane plates for conducting such assessments were back-ordered through September 2015. Millipore membrane plates have plate inserts that contain a nylon screen with 20µm openings (Fisher Scientific, No. MANMN2010). The formalin-kill technique was believed to be a reliable alternative for assessing live:dead larval counts for samples. Formalin used was a 10% buffered formalin (contained 10% of a saturated formaldehyde solution in water).
The purpose of Trial 1 was to assess whether equal parts of L3 culture:formalin would successfully kill all larvae in the culture.

Trial 2 assessed what concentration of L3 culture:formalin most effectively killed the larvae within a 12-hour period of time. Aliquots of cultured L3s were distributed into 5 wells of a 48-well plate, beginning with 10 µl mixture in A1, and increasing by 10 µl for each additional well (10 µl, 20 µl, 30 µl, etc.). Aliquots of 100 µl formalin (Protocol 10% formalin, Patterson Veterinary Supply) were then added to each well. Well contents were thoroughly mixed and set for 12 hours at room temperature (19°C). At the end of 12 hours, well contents were individually heat-killed in glass tubes, to assure that all larvae in the culture were dead for visualization. Total larvae within each well were then counted to compare the number of larvae successfully killed by formalin (would be curled, see Plate 1) and the number of larvae that had still been alive immediately prior to heat-killing (would be straight, see Plate 1).

Trial 3 assessed whether a consistent larval count per 100 µl of L3 culture could be obtained using the formalin-kill technique. L3 culture and formalin aliquots at a 1:10 ratio, respectively, were added to 10 separate wells of a clean 48-well plate and set for 12 hours at room temperature. Larvae in each well were then counted and totaled under the microscope (Olympus CKX41 with Olympus DP71 camera system).

Trial 4 was designed to test if we could correctly identify different percentages of live versus dead larvae using the formalin-kill technique. In a blind study, one student prepared the experimental wells, and another visualized them. L3 larvae aliquots of 600 µl were heat-killed. An additional 600 µl of live L3 larvae were reserved. Six wells in a 48-well plate were each filled with 1000 µl formalin and 100 µl of L3 culture, at various
proportions of live:dead larvae (100 µl live: 0 µl dead, 80 µl live: 20 µl dead, 60 µl live: 40 µl dead, etc.). After the designated 12-hour set time, each well was visualized and live:dead larvae were counted. Trial 4 was run in duplicate.

1.4 Larval migration timing assessment with Millipore membrane plate

To identify live and dead larval counts in any given trial, the Millipore membrane plates were used as the most accurate, up-to-date larval viability test on the market. Healthy, live *Haemonchus* larvae would successfully swim through the 20 micron Millipore membrane that separated the top wells from the bottom ones. Larvae left behind that could not pass through the membranes were counted, and larval viability for that particular trial was calculated. Four different time trials and a control group were set up to (1) confirm that all live L3 larvae would swim through the Millipore membranes of the 96-well assay plate, and (2) establish the appropriate amount of time needed for all larvae to swim through the membrane (6, 12, 24 or 48 hours).

For Trial 1: the bottom of three experimental wells were filled with 300 µl Phosphate-buffered saline (PBS) (Sigma P-5368), the plate top was replaced, and aliquots of 25, 50, and 100 µl of L3 culture were added to the top wells. After 6 hours, 100 µl iodine (Gram stain #2 iodine solution, Jorvet J-323-2) was added to the top of each well to kill the larvae. Top and bottom well contents were individually visualized and all larvae in each were counted.

Trials 2-4 followed the same procedure with varying set time increments: 12, 24, and 48 hours, respectively. Trial 5 was the control group, with aliquots 25, 50 and 100 µl of L3 culture added to the bottom of respective wells and iodine-killed after 48 hours.
1.5 Collection and processing of sheep abomasa

Twelve abomasa were collected from freshly-slaughtered lambs at Herring Brothers Meats (Guilford, ME). Abomasa were cut open lengthwise, contents were poured through a course mesh sieve, and abomasa were lightly rinsed with distilled (DI) water. Abomasal contents were rinsed with the remaining 500 ml DI water and all liquid was squeezed through screen by hand. Solid remnants were discarded. Abomasal fluid (AF) was poured into clean 50 ml centrifuge tubes, and each tube was centrifuged for 5 minutes at 1000 rcf. The supernatant was decanted and filtered with Swinnex filters fitted with 31 µm screens into clean 50 ml centrifuge tubes. The AF was immediately frozen to preserve freshness (see Plate 2).

1.6 Technique for preparing lobster shell-conditioned media (LSCM)

Seven hundred and fifty ml of fresh, filtered, centrifuged AF was mixed with 10 g finely crushed LS and incubated at 37°C for 18-24 hours with constant stirring. The mixture was then transferred to 50 ml centrifuge tubes and centrifuged at 1000 rcf for 5 minutes. The supernatant was decanted into clean tubes and frozen (see Plate 3).

1.7 Exploring possibilities for separating larvae from particles in a mixture

A variety of methods were tested in an attempt to discover an efficient, effective way to separate larvae (both live and dead) from fine particles in a mixture. A need for such a technique arose after conducting initial trials with crushed LS mixed with larvae, because the LS greatly obscured the ability to visualize larvae. Essentially, the presence
of both LS particles and larvae in a mixture rendered assessment of larval viability impossible and inaccurate.

Separation methods tested included: (1) use of 96-well Millipore plate, (2) flushing mixture through a 22 µm mesh membrane in a Swinnex Millipore filter (Fisher SX0001300); (3) sugar solutions with varying degrees of saturation; (4) Ficoll Paque (VWR 17-1440-02, made by GE HealthCare Biosciences) and Hank’s Balanced Salt Solution (HBSS) with centrifuging; (5) filtering with glass wool and a variety of mesh screens (22, 31 and 80µm Sefar Nitix screens); (6) attempts to dissolve LS in AF, to bypass separation technique altogether.

Method #1: 96-well Millipore plate. This process mimicked the one described in section “3.4 larval migration timing assessment,” although this time it was conducted in a much simpler format. Aliquots of the LS mixture were distributed amongst top wells of a 96-well Millipore plate, and the corresponding bottom wells were filled with PBS. After 6 hours, contents of both wells were visualized and larval counts were attempted. The purpose was to see if (A) larvae would be able to still migrate to the bottom of the plate wells, and if (B) dead larvae could still be identified and counted.

Method #2: Swinnex Millipore filter flush. The second technique involved loading a clean 10 ml luer lock syringe with 8 ml of the LS mixture, and flushing the mixture through 22 µm mesh membranes in a Swinnex Millipore filter into a larger well of a 24-well plate. The flushed-out mixture and mesh filter section were both visually assessed.

Method #3: Saturated sugar solutions. The third method involved the preparation of saturated sugar solutions, and the suspension of *H. contortus* larvae in it. In one of the
more formal trials, 20 ml of water was combined with 20g of sugar to make a saturated solution. Four wells of a 24-well plate contained various mixtures: D4 contained 1000 µl sugar solution (SS) plus 200 µl L3 culture; D5 contained 500 µl SS, 500 ml water and 200 µl L3 culture; D2 and D3 contained 1000 µl SS, 200 µl L3 culture and 20mg LS. Mixtures in each well were re-suspended thoroughly, set overnight, and then visualized. To visualize, each well’s contents was carefully drawn off in 200 µl aliquots from the top down, and each of the five aliquots were transferred to separate wells of a clean gridded 24-well plate. Larval counts from each aliquot were then obtained using the microscope.

Method #4: Ficoll Paque and Hank’s Balanced Salt Solution. Ficoll Paque and HBSS were used in a fourth attempt to separate larva from particles in a mixture. Typically, Ficoll Paque is a density gradient solution used to separate out components within blood (plasma, lymphocytes, red blood cells). HBSS is a gentle buffer salt solution with phenol red, a pH indicator, and in this case was intended to mimic the plasma portion of blood. The purpose was to separate out larvae from LS particles according to density. Aliquots of 200 µl larvae and 50 mg of LS to 25 ml of HBSS in a 50 ml centrifuge tube and vortexed thoroughly. In a second tube, 10 ml Ficoll Paque was added, and the HBSS mixture was gently layered on top of the Ficoll Paque. Contents were centrifuged at 900 x g for 30 minutes. After centrifuging, layers were pulled off one at a time, placed in a 6-well plate, and visualized.

Method #5: Glass wool and mesh screens. Next, glass wool and various-sized mesh screens (22, 31 and 80µm) were placed in small plastic funnels suspended above glass beakers. A mixture of 4000 µl phosphate-buffered solution (PBS) and 200mg of crushed LS was slowly poured through each of the funnels. The purpose of these trials
was to assess whether any of the filtering screen materials would trap LS particles while allowing only the fluid to pass through into the beaker.

Method #6: Dissolution in AF. The final technique involved the combination of crushed LS and AF. The purpose of this method was to assess whether or not LS would dissolve in the AF over time. If successful, this would likely allow us to bypass the need for a separation technique altogether. Increasing amounts (1mg, 20mg, 40mg, 80mg) of finely-crushed (filtered through a US-standard testing sieve No.400, 38µm) LS were added to 500 µl of AF in four different wells of a 24-well plate. This set of four wells was deemed “Trial A”. “Trial B” included another set of four wells with the same proportions, except medium-crushed (was caught by a US-standard testing sieve No.400, between 212µm and 38µm) LS was used. “Trial C” was a control set of four wells with only 500 µl of AF in each. All wells were incubated for 9 days. After incubation, well contents were visually assessed for traces of LS.

2. Methods used in experimental stage 1: growth of L3 stage larvae to L4s in abomasal fluid

2.1 Establishing standard for larval size

The next stage of experimentation involved the combination of L3-stage larvae and AF to assess whether L3-stage developed to the L4 stage when incubated in AF. Before beginning initial trials, an average measurement of current L3-stage larvae was obtained. This measurement was used to mark the standard size of the larvae prior to any trials with AF and/or LS. This same larval culture was used throughout the remainder of the experiment.
Images of larvae were taken on 10/9/15 from the L3 culture. An aliquot of 100 µl of larval culture was transferred to a glass tube and heat killed (Appendix I), then transferred to a gridded 6-well plate and photograph using the Olympus microscope and camera system. Twenty individual larvae were randomly selected throughout the sample and photographed at 10x magnification. The head and tail of each larva were also photographed at 40x magnification, for further identification of larvae. All images were labeled and stored on an external hard drive for later measurement. A program called ImageJ (version 1.50c) was used to access these stored images and individually measure each larvae. Data were entered into an Excel spreadsheet for analysis.

2.2 AF + L3 trial 1

Trial 1 with L3 stage larvae in abomasal fluid (AF + L3 Trial 1) was designed to initially observe L3 growth in AF. Each well in a 24-well plate was filled with 50 µl larval culture and 500 µl AF. Starting on October 22, 2015 all well contents were added and the first sample was taken at 0 hours. Samples were then taken from the plate in a clockwise circular fashion, progressing from outer wells to innermost wells. For each sample, the contents of one well were drawn off, heat-killed, rinsed with PBS and stored in the refrigerator. The first 6 samples were taken every 6 hours, followed by 4 samples every 12 hours, and the remaining samples every 24 hours. Two wells were controls: one contained AF only, the other contained PBS and larvae but no AF. Both controls were sampled on the last experimental day. Larval counts and measurements of 10 larva per well were taken and compared.
2.3 AF + L3 trial 2

Trial 2 with L3 stage larvae in abomasal fluid (AF + L3 Trial 2) was designed to establish a clearer point in time at which (1) L3 larvae ex-sheathed and/or died in AF and (2) L3 larvae demonstrated notable growth. Trial 2 was designed similarly to Trial 1, with the following modifications: (1) Only the center wells of separate 24-well plates were used; (2) each well was filled with 100 µl larval culture and 3000 µl AF; (3) samples were taken every 12 hours for 7 days, with the initial sample taken at 0 hours and two control samples taken on day 7; (4) samples were divided and half of the well contents was heat-killed while the other half was formalin-killed; (5) 12 larva were visualized and measured from every heat-killed sample, and total larval counts were taken per sample. In addition, 1500 µl of AF was carefully drawn off the top of every remaining experimental well each morning, and replenished with 1500 µl of fresh AF thawed out from the night before.

2.4 AF + L3 trial 3

Trial 3 with L3 stage larvae in abomasal fluid (AF + L3 Trial 3) was designed to determine whether L3 larvae underwent the same anatomical changes as they had in Trials 1 and 2 if kept in fresh AF (collected November 30, 2015) in a culture environment that more closely mimicked that of a sheep abomasum. Fifty milliliters of fresh AF and 5 ml larval culture were combined in a clean 200 ml Erlenmeyer flask. A small stir bar was placed in the center bottom of the flask and set on low speed. The flask was incubated at 28°C for 6 days, and samples were taken every 24 hours. For each sample, a transfer pipette was used to draw off 2 ml of the mixture from the center of the flask and deposit
it into a clean test tube. One ml of the sample was then transferred into a glass test tube and heat-killed. Five ml formalin was added to the remaining 1 ml of the sample and vortexed for 10 seconds. Formalin- and heat-killed portions were refrigerated separately in labelled test tubes for each sample.

2.5 AF + L3 Trial 4

Trial 4 with L3 stage larvae in abomasal fluid (AF + L3 Trial 4) was designed with the same purpose as Trial 3, with the following modifications to procedure: (1) a 250 ml cell culture flask (VWR Cellstar, No. 658 170) was used rather than the Erlenmeyer flask; (2) the culture flask was secured to a rocker (4651 Ames rocker) instead of using a stir bar; (3) 5 ml were taken every 24 hours for 6 days; (4) flask contents were replenished with 5 ml fresh AF after every sampling.

3. Methods used in experimental stage 2: trials with crushed lobster shell
3.1 Initial trials with crushed lobster shell in a mixture with larvae

An initial “Quick & Dirty” experiment assessed whether or not crushed LS exhibited any kind of effect on live L3 larvae. A 100 µl aliquot of larvae was then added to 2 ml of PBS in each of the wells used on a 24-well plate, and measures of crushed LS (1mg, 20mg, and 80mg, respectively) were added to each corresponding well (other than the initial control). This experiment was run in duplicate. Viability of the larvae was assessed after allowing the plate to incubate for 24 hours on a rocker at 37°C.

3.2 Larval cultures with crushed lobster shell

Fresh fecal samples were collected late September 2015, homogenized, prepared into cultures and divided into six separate culture cups (see Materials and Methods 3.1).
All cultures contained 80g of fecal matter, 30g vermiculite and 100 ml water. Three separate treatment groups, each run in duplicate, were established. Cup cultures 1 and 2 were treated with 5g of fine-crushed LS. Cultures 3 and 4 received 5g of medium-crushed LS. Cultures 5 and 6 were not treated. All were incubated for 8 days, extracted with Baermann funnels, killed and visualized to obtain larval counts.

3.3 L4 stage larvae and assorted treatments (L4 + AT)

This trial with L4 stage larvae in AF with assorted treatment (AT) groups was designed to compare effects of LSCM, chitin, chitosan, and Ivermectin on larval viability. Note: the AF + L3 mixture from Trial 4 will now be referred to as “L4 mixture”. All experimental flasks were secured to a rocker and incubated at 28°C (Plate 8). Samples were taken from each experimental flask every 12 hours for 2 days. Eight experimental flasks were used, containing the following:

- Negative control: 1 flask, 6 ml L4 mixture + 24 ml fresh AF
- Positive control: 1 flask, 6 ml L4 mixture + 23 ml fresh AF + 1 ml Prohibit (30mg per ml solution; Levamisole hydrochloride, AgriLabs, St. Joseph, MO)
- LSCM: 2 flasks, 6 ml L4 mixture + 24 ml LSCM
- Chitin: 2 flasks, 6 ml L4 mixture + 24 ml fresh AF + 150 mg chitin powder
  (Sigma C7170-100G practical grade powder)
- Chitosan: 2 flasks, 6 ml L4 mixture + 24 ml fresh AF + 150 mg chitosan powder
  (Sigma C3644-10G)

For each sample, a transfer pipette was used to draw off 7.5 ml of the mixture from the center of the flask and deposit it into a clean test tube. Flask contents were replenished
with 7.5 ml fresh AF after every sampling. The 7.5 ml of mixture was then divided in half and split between two 50 ml plastic centrifuge tubes. Both tubes were then filled to capacity with formalin (approx. 46.75 ml) and vortexed for 10 seconds to mix thoroughly. Tubes were stored in the refrigerator for a minimum of 24 hours before concentrating. To concentrate, the top 45 ml of fluid from each tube was carefully drawn off and discarded.

RESULTS

1. Results from the development of preliminary methods

1.1 Collection and culturing of larvae

Larvae were stored in batches according to both (1) their date of collection and (2) the group of sheep they were collected from (rams, lambs, ewes). The batch frequently referred to throughout these experimental trials was originally labelled “0301”, collected on 9/3/15 from both ewes and lambs, stored at 20°C. The numerical label is used within the lab merely as a form of identification. This paper will refer to it as “the larval batch,” “L3 larvae”, or other variations of such. Also, note that two other batches of larvae (in addition to 0301) were collected and stored, but were not used in experimental trials. Both larval batches came from a mixture of lamb and ewe fecal samples, and were stored as back-ups to the 0301 batch, in case more larvae were needed.

1.2 Establishing larval counts per microliters (µl) of L3 culture
For the first larval samples measured: On 9/21/15, larval counts were approximately 445 larva per 100 µl of larval culture. This was the first batch collected from Witter sheep in early September (9/3/15). The 0301 batch was significantly diluted at the end of September (9/30/15) so that a more reasonable number of larvae would be present per 100 µl (the ratio was now about 89 larva per 100 µl culture). In addition, this allowed the larval batch to be used more extensively.

Larval counts were then taken mid-October (10/16/15). Counts were moderately high: approximately 250 larva per 100 µl of larval culture (Figure 1). On 10/19/15, the batch was diluted down to a larva:culture ratio of about 130 larva:100 µl, approximately half of the previous concentration. This allowed the larvae to be used more extensively. This batch consisted of 90% *H. contortus*.

![Figure 1. Indicates standard larval counts per 100 µl of culture for batch 0301, throughout the experimental process.](image)

Larval counts were taken on four other occasions (10/21, 10/27, 12/3 and 12/7) throughout the experimental process (Figure 1) to verify consistency. All counts fluctuated minimally within a range of 200-255 larvae per 100 µl.
1.3 Development of formalin-kill alternative technique

Based on observation, when *H. contortus* larvae interact with certain concentrations of formalin, they will contract into a spiral posture upon being chemically-killed with formalin (Plate 1). However, if larvae were previously dead in the culture prior to the formalin-kill procedure, the formalin would not affect their physical attributes and the larvae would be seen laying straight in the liquid (Plate 1). When formalin was used to kill a sample of larvae, the formalin-treated larvae could then be visualized, and live vs. dead counts could be obtained based on the curl-state of the larvae.

Plate 1. Live vs. dead larval counts could be obtained based on the curl-state of the larvae after being treated with formalin. Left: Larva that was alive prior to formalin-kill. Right: Larva dead prior to formalin-kill.

The purpose of Trial 1 was to assess whether equal parts of larval culture:formalin would successfully kill all larvae in the sample. Visual assessment of larvae under a microscope, after combination with formalin in a 50:50 ratio, revealed that fewer than 20% of the larvae died.

Trial 2 assessed what concentration of larval culture:formalin most effectively killed the larvae within a 12-hour period of time. Visual assessment of larvae after
combination with formalin in varying ratios revealed that using a 1:10 ratio of larval culture: formalin was most effective within the 12-hour time designation. Wells containing more diluted concentrations of formalin had increasingly greater numbers of larvae still alive after the 12-hour period. Using a 1:10 ratio successfully killed nearly 100% of larvae. A higher formalin concentration also decreased the allotted time necessary to run each trial, as larvae were killed at a more rapid rate. When visualized after 6 hours and again after 12 hours, no change in the percentage of killed larvae was observed.

Trial 3 assessed whether a consistent larval count per 100 µl of larval culture could be obtained using the formalin-kill technique. Assessment revealed consistent larval counts in each of the 10 experimental wells (Figure 2). Total larval counts per well were plotted along with the mean and +/- 1 standard deviations (SD). All data points fell within two standard deviations of the mean, and the majority fell within one SD of the mean. Well A3 was below 1 SD and B3 was above 1 SD. Larval counts from well B3

**Figure 2.** Formalin-kill technique trial 3 – Comparison of total larval counts per 100 µl of larval culture for each of the 10 experimental wells. A2 – B5 are each of the 10 experimental wells, each containing 1:10 mixtures of 0301 larvae and formalin.
was considered a near-outlier (Figure 2) because it almost falls outside of the 2 SD range. Based on this graph, we can see that larval counts were consistent and within an acceptable margin of variation.

In Trial 4, a blind study was conducted to assess whether the formalin-kill technique could be used to accurately identify different percentages of live versus dead larvae within experimental wells. The percentages of both live and dead larvae counted by the lab assistant in the blind portion of the study closely reflected the percentages of both live and dead larvae that were originally distributed to each well (Table 1).

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<th>Original Percent Dead (%)</th>
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</table>

Table 1. Formalin-kill technique trial 4 – Table of actual larval counts compared with percent live and percent dead larvae in each experimental well. ‘Original’ percentages are the original proportions of live and dead larvae aliquoted into each well. ‘End’ percentages are the calculated percentages based on actual larval counts collected in a blind study.
Figure 3 compared values of the original dead larval percentages distributed to each well with the blind-count dead larval percentages from the blind count. Figure 3 plots the average of each set of duplicate wells for each original concentration. The linear regression line had an $R^2$ value of 0.926, and results of analysis of variance (ANOVA) testing yielded a P-value of 0.942.

The $R^2$ value for a regression line is a statistical measure of how close the data are to the fitted line. An $R^2$ value of 1.00 would indicate that the model (line) accurately explains 100% of the response data. The P-value is a calculated probability used to determine statistical significance in a hypothesis test. A P-value less than 0.05 indicates that there is a significant difference between the two groups tested.

An $R^2$ of 0.926 for Figure 3 indicates that the regression line accurately represents the given data, and a P-value of 0.942 suggests that the plotted percent count values are not statistically different. The blind count percentages yielded results statistically similar to the original percentages of dead larvae in each well. Based on this, using 6-hour time increments during experimentation allowed ample opportunity for all viable larvae to...
migrate through the Millipore membrane plates. In addition, all trials demonstrated consistent ability of the live larvae to navigate through the Millipore membranes, confirming the reliability of the technique.

1.4 Larval migration timing assessment with Millipore membrane plate

The purpose of these trials was to (1) confirm that all live L3 larvae would swim through the Millipore membranes of the 96-well assay plate, and (2) establish the appropriate amount of time needed for all larvae to swim through the membrane. Table 2 provides the actual larval counts taken from each experimental well. The number of larvae remaining in the top of each well were those unable to migrate through the Millipore membrane (Table 2). Figure 4 compares the percentages of larvae that successfully migrated through the plate membranes for each designated time period.

The average percent values from Figure 4 were calculated for all three experimental wells per time increment. Trial 1 average was 95.4% +/- 1.15%. Trial 2 was 94.5% +/- 1.79%. Trial 3 was 94.5% +/- 2.03%. Trial 4 was 99.2% +/- 1.13%. The percent of total larvae that successfully migrated through the Millipore membrane of the plates differs between the highest average (99.2% after 28 hours) and the lowest average (94.5% after both

<table>
<thead>
<tr>
<th>Well</th>
<th>Trial Number</th>
<th>Number of Larvae Remaining in Top of Well</th>
<th>Total Larval Count Per Well</th>
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</thead>
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<tr>
<td>B2</td>
<td>1</td>
<td>1</td>
<td>29</td>
</tr>
<tr>
<td>B3</td>
<td>1</td>
<td>2</td>
<td>35</td>
</tr>
<tr>
<td>B4</td>
<td>1</td>
<td>3</td>
<td>65</td>
</tr>
<tr>
<td>D2</td>
<td>2</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>D3</td>
<td>2</td>
<td>2</td>
<td>39</td>
</tr>
<tr>
<td>D4</td>
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</tr>
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<td>1</td>
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<td>F8</td>
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<td>0</td>
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</tbody>
</table>

Table 2. Results from larval migration timing assessment. Comparison of the total larval counts per well, including the number of individual larva that did not pass through the Millipore membrane (remained at the top of the respective well).
12 and 24 hours) by only 4.7%. Experimental well D6 from Trial 4 (Table 2) yielded a percent value that was significantly different than the other percent values calculated from Trial 4 larval count data. In order to more accurately represent the data, this outlier was excluded from the graph.

In addition, the high success rates of larvae (Figure 4) that were able to navigate through the membranes confirmed that the vast majority (~95%) of live larvae present in solution were able to swim through the membrane. Table 2 lists the actual numbers of larva that did not pass through the Millipore membrane for each experimental well. Fewer than 5% of the total number of larvae per well did not pass through the Millipore membrane.

1.5 Collection and processing of sheep abomasa

See Plate 2 for pictures taken during the process. Two separate batches of sheep abomasa were collected from Herring Brothers Meats. The first batch was collected on 9/23/15, processed, and stored in the refrigerator at 4°C until use. The second batch was collected on 11/30/15 and immediately frozen.

1.6 Technique for preparing lobster shell-conditioned media (LSCM)

See Plate 3 for pictures taken during the process.

Plate 3. Preparation of lobster shell-conditioned media (LSCM).

1.7 Exploring possibilities for separating larvae from particles in a mixture

Method #1: 96-well Millipore plate. Visual assessment under the microscope revealed that the crushed LS particles completely blocked the Millipore mesh openings and prevented any live larvae from navigating through the screen.

Method #2: Swinnex Millipore filter flush. Visual assessment revealed that the
crushed LS particles blocked the Millipore mesh openings. Additionally, larvae were seen to be caught in the mesh and many were not successfully able to be flushed out. This method did not distinguish between live and dead larvae.

Method #3: Saturated sugar solutions. The four different groups for the saturated sugar solution separation method had similar results (Figure 5). The majority of larvae able to be identified and counted within each well was found within the bottom 400 µl of the well’s solution. Very few larvae were seen in the top portions of each experimental well. Well D5 contained a diluted sugar solution and larvae, but no LS. In D5 the majority (87.5%) of larvae were found in the bottom of the well. Figure 5 also shows that few larvae were able to be visualized (the highest count was 14 larvae in D5), despite the expected ratio of 300-500 larvae in 200 µl of larval culture (Figure 1). Reasons for this may include: (1) the sugar solution partially dissolved a number of larva, rendering them unidentifiable; (2) the various particles in a mixture, whether sugar or LS, greatly obscured a large number of larva, and total larval counts were significantly reduced.
Method #4: Ficoll Paque and Hank’s Balanced Salt Solution. After centrifuging, three layers were evident: the top layer was a pink color (indicating pH of 8.0 or greater), followed by a thin clear layer, and a solid pellet of material at the bottom of the centrifuge tube. Layers were pulled off one at a time, placed in a 6-well plate, and visualized to locate larvae. All larvae were found mixed in with the LS particles in the bottom pelleted material.

Method #5: Glass wool and mesh screens. When the mixture was poured through the glass wool and each of the mesh screens, both LS particles and fluid passed through into the beakers indiscriminately.

Method #6: Dissolution in AF. All experimental wells removed from the incubator were completely dried out, leaving LS particles caked on the inside. All liquids from the AF had evaporated.

2. Results from experimental stage 1: growth of L3 stage larvae to L4s in abomasal fluid

2.1 Establishing standard for larval size

Figure 6. Establishing standard for larval size. Comparison of starting larval body lengths (µm) of the 20 larvae imaged and measured, mean length and standard deviations included.
The average length of L3 *H. contortus* larvae prior to the experimental trials was 636.9 µm +/- 28.5 µm. Total larval counts per well were plotted along with the mean and +/- 1 standard deviations. All data points fell within two standard deviations of the mean, and the majority fell within one standard deviation (SD) of the mean. This indicated that body length measurements had little variation from the mean overall. However, body length did differ among individuals to an extent, with the maximum and minimum lengths of 695 µm and 585 µm, respectively (Figure 6). This demonstrated that even larvae grown under the same conditions at the same larval stage can exhibit variation in length. The heads and tail lengths of larvae were also imaged (Plate 4) as additional evidence for qualifying the larvae as *H. contortus* as opposed to other larval species. All twenty larvae were verified as *H. contortus* by visual inspection by an experienced technician.

**Plate 4.** Images taken for each of the L3-stage *H. contortus* larva, after they were extracted from the larval culture. The full body (A), head (B) and tail (C) of each larva was photographed.

2.2 AF + L3 trial 1

Ten larvae from each sample were randomly selected, photographed, and measured. The average body lengths of the ten larvae per sample were used in Figure 7 to plot average body length of larvae over time. Samples were no longer taken after 168
hours (7 days) because on that day remaining well contents were visualized and all larvae seen in the wells were dead by that point in time. Taking further samples would have been futile. Two periods of larval growth were evident between 25-36 hours and 72-96 hours (Figure 7). A regression line fit for data points between 0-100 hours (Figure 8) yielded an $R^2$ value of 0.705. Furthermore, two ANOVA tests were conducted based on
the two overall trends in larval body length changes seen over time. The first ANOVA was between data points 0-100 hours, yielding a P-value of 0.002. The second ANOVA was between data points 100-175 hours, yielding a P-value of 0.30.

According to these results, the means of data points between 0-100 hours are statistically different, indicating a trend in the data. The trend line is a model of the behavior of the system, in this case demonstrating an increase in the system over time. Together, results suggested that there was an upward trend in the data: larval body length has increased over time from 0-100 hours. Results from the second ANOVA test indicated that the null hypothesis, which stated that the means were not statistically different, was true. This conclusion is supported by a P-value that is much greater than the alpha of 0.05 used in the ANOVA calculation.

When measuring the imaged larvae from each sample, the following corrections were made to the original data: (1) After sorting back through the images of all larva measured, any larva measured that still had their sheaths were re-measured without including their sheath to maintain consistency in length measurements; (2) In the C3 control group (contained only PBS and larvae, no AF), the two misshapen larva were removed from the table of data. (3) One larva was removed from the table of data because it was likely not *H. contortus*; (4) Two larvae were in the process of ex-sheathing when the image was taken and thus their original measured length was unnaturally long. They were re-measured without their sheath.

As time progressed, larvae began to develop distinguishable anatomical characteristics not seen in the original L3 stage larvae. Such changes included distinct
increases in width, clusters of globules near the mid-sections of larvae, and loss of outermost cuticles (Plate 5) as well as morphological head changes (Plate 6).

**Plate 5.** Images of selected larvae from abomasal fluid + L3 larvae, trial 1, examples of distinguishable anatomical characteristics seen in developing larvae. Left: Increase in width. Middle: Clusters of globules. Right: Loss of outer cuticle.

**Plate 6.** Images of selected larvae from abomasal fluid + L3 larvae, Trial 1. Arrows indicate close-up of subtle morphological differences in larval head structures, indicating maturation into L4-stage larvae.

2.3 AF + L3 trial 2

When compared to Figure 7, Figure 9 illustrates a more consistent measure of average larval length over time, rather than a pattern of larval growth. According to Figure 9, larvae measured from 0 – 96 hours remained at approximately 640 µm in length. The two particular periods of larval growth evident between 25-36 hours and 72-
96 hours in Figure 7 were not apparent in Figure 9. Similar anatomical changes discussed for AF + L3 Trial 1 were also observed in AF + L3 Trial 2 during corresponding time periods. Also, the same pattern of decreased length and irregularity after 96 hours was evident both Figure 7 and 9.

Figure 10 indicates that the number of live larvae proportionately decreased as the number of dead larvae proportionately increased in each sample over time. The rightmost bar of Figure 10 depicts a control incubated in PBS for 144 hours (entire duration of trial). Even the control demonstrated this pattern of decreased live larval counts. There was a 29.2% decrease in the number of live larvae from the sample taken at zero hours to the control sample taken at 144 hours. There was a 49.6% decrease in the number of live larvae from the sample taken at zero hours to the last experimental sample taken at 144 hours.
A linear regression line ($R^2$ value of 0.611) in Figure 11 notes the overall pattern of decreased total larval counts over time. It also notes the overall pattern of decreased live larval counts over time. The control demonstrated this pattern as well, but the decrease in total larvae in the control well was not as substantial as it was in the experimental wells.
2.4 AF + L3 trial 3

After three days, samples from the third trial of larvae in AF were visualized under the microscope. All larvae appeared battered and broken (Plate 7) and were likely dead prior to use of the formalin- and heat-kill methods. An attempt to count the number of live and dead larvae in one sample taken on Day 3 of AF + L3 Trial 3 (12/6/15) yielded a result of 0 live and 47 dead larvae. Few of the intact larvae appeared to have characteristics qualified as L4-stage larvae (Plate 5, 6), but these results are inconclusive due to poor physical quality of the larvae.

Plate 7. Battered and broken larvae from abomasal fluid + L3 larvae Trial 3, after incubation with a stir bar.

2.5 AF + L3 trial 4

The majority of L3 stage larvae developed new anatomical characteristics within 3-4 days. By Day 6 after initiating Trial 4, the majority of larvae were expected to have had sufficient time to develop into the L4 stage. To maximize remaining resources while generating a batch of matured larvae, AF + L3 Trial 4 was run to completion and its products (more mature larvae) were immediately used in the preparation and initiation of
the final experiment. The final experiment was with L4 stage larvae and assorted anthelmintic treatments (L4 + AT). According to calculations, approximately 1125 larvae would be aliquoted into each of the eight flasks of the last experiment (L4 + AT). In theory, enough larvae were present within each sample to gather sufficient larval counts and images for measurements from each of the eight L4 + AT flasks.

3. Results from experimental stage 2: trails with crushed lobster shell

3.1 Larval cultures with crushed lobster shell

Figure 12. Larval cultures with crushed lobster shell experiment. Total number of larvae counted within each larval culture, after an 8-day incubation period, Baermann funnel extraction and visualization.

LS treatment group was run in duplicate cultures for comparison. As is evident in Figure 12, cultures containing the medium-sized LS particles contained the lowest larval counts. Cultures without LS contained the highest larval counts. On average, the medium-sized LS cultures had a larval count of 242.5, the fine-sized LS of 499, and the LS-free cultures of 1,253.5 larvae total. An ANOVA test run between the “No LS” group
and the “Med LS” group yielded a P-value of 0.013, indicating that there was a significant difference between the two groups. ANOVA tests could not be run using the “Fine LS” group data, because the total larval counts for both duplicates of the fine-sized LS cultures were significantly different from each other, but the counts were not repeated multiple times. Therefore, there are no replicate count values for each culture to compare and sufficiently analyze with statistics. The “Fine LS” group data specifically would need to be repeated to obtain more consistent results before proper statistical analysis could be conducted.

3.2 L4 stage larvae plus assorted treatments (L4 + AT)

**Figure 13.** Results from L4 + AT experiment. Number of live larvae counted in each flask sample taken at designated sampling times (12, 24, 36 hrs, etc.), expressed as a percent of the total larval count per sample.

![Percent of Live Larvae Per Flask Sample Over Time](image)

Figure 13 illustrates the steady decline in percent of live larvae counted per sample over time for each experimental flask. After 24 hours, less than 10% of total larval counts in each sample consisted of live larvae. After 36 hours, less than 5% of all
total larval counts in each sample consisted of live larvae. The one exception—Flask B sample taken at 48 hours—was due to the fact that so few total larvae were counted that 1 larva noted as “live” out of only 11 total larvae resulted in a higher percent of “live larvae” by default. A decrease in live larval percentage occurred between 12-24 hours for all flasks (except Dd, which maintains the same percent).

Duplicate flasks did not exhibit similar patterns when compared with each other, leaving a wide margin of variability and little basis on which to make comparisons. Also, total larval counts for all flask samples varied, not only between different flasks’ samples, but also between samples taken from the same flask over different time increments.

Plate 8. L4 larvae + assorted treatment experimental flasks secured to rocker and incubated at 28°C.

The majority of larvae in solution were deemed ‘dead’ within the first 24 hours of initiating L4 + AF (Figure 13). No particular patterns of nematicidal effects could be observed between the various treatment groups. Even the control groups experienced comparable patterns of decreased percent of live larvae in samples. No significant difference in larval viability between experimental treatments could be determined.
DISCUSSION

1. Preliminary methods development

Before any form of experimentation could begin, the first step was to collect fecal samples and culture the nematode eggs they contained into larvae. Sheep at Witter Farm were chosen as the subjects from whom to take samples, mainly due to easy accessibility. During the time at which all fecal samples were collected—late August through mid-September 2015—high egg counts of *H. contortus* were present (approximately 90% of total parasite population within the sheep). High *H. contortus* counts in these collections are largely attributed to the fact that peak season for parasite infection is late summer and early fall. Furthermore, fecal samples were collected mainly from the ewes and lambs rather than the rams, because ewes and lambs had the highest larval counts compared to the rams (500 larva per 200 µl versus 35 larva per 200 µl, respectively).

These high *H. contortus* influenced the decision to focus the research on *H. contortus* in particular. Throughout the entire experimental process, when assessing larvae under the microscope, any non-*Haemonchus* nematodes were disregarded and not examined. Therefore, data and results only assess *H. contortus* larvae specifically.

The process of taking larval counts for the larval batch was necessary in order to establish a standard, expected larval count per 100 µl for each batch. This was necessary throughout all experimental processes, because they required a reliable base assumption of a consistent larval count in order to more accurately compare results later on.

By the beginning of September, the standard Millipore membrane plates (to be used for conducting larval viability assessments) were back-ordered through the end of the month. However, due to time constraints, experimentation could not be held up, and
therefore a new technique for assessing live:dead larval counts had to be established. This led to the development of the formalin-kill technique. Formalin (a water-based 10% solution of formaldehyde) has been widely used to kill bacteria, fungi, spores and viruses. It is also often used as a preservative, among other things. As a fungicide or bacteriocide, formaldehyde compromises the integrity of cellular structures by severe dehydration and consequent lysis (Denyer et al., 1998). As an embalming agent, formaldehyde fixes cells or tissues (irreversibly, when used in conjunction with higher temperatures) by cross-linking primary amino groups in proteins with other nearby nitrogen atoms (Denyer et al., 1998). More specific reasons for its effects on the physical attributes of *H. contortus* larvae when combined are yet unclear.

Results from the development of the formalin-kill technique determined that using a 1:10 ratio (larval culture:formalin) for a 6-hour time period was most effective and efficient at killing larvae. Further study confirmed that the technique could be used to generate consistent larval counts, and that even in a blind study (the individual gathering the data and counting larvae was not aware of the original percentages of live and dead larvae in each well), count values were consistent. Well B3 (Figure 2) was the only exception, and was considered a near-outlier (outside 2 SD from the mean). Reasons for this inconsistency are not well understood, but are likely due to human error in either the aliquoting procedure or larval counting. Overall, results from the blind assessment revealed that the formalin-kill technique is an accurate, consistent, viable method that can be used to identify ratios of live and dead larvae in a mixture.

Once the Millipore membrane plates arrived, they too could be used to measure larval viability, along with the recently-developed formalin-kill technique. Before using,
the plates had to be tested in order to ensure that live larvae would, indeed, migrate through the membrane as expected. In addition, this assessment tested the appropriate length of time necessary to allow all live larvae to swim through the membrane. Results indicated that only a slightly greater percentage (4.7% more larvae) of larvae migrated through the Millipore membrane after 48 hours compared to 6 hours. This was not a significant difference. Therefore, using 6-hour time increments during experimentation allowed ample opportunity for all viable larvae to migrate through the Millipore membrane plates. In addition, all trials demonstrated consistent ability of the live larvae to navigate through the Millipore membranes, confirming the reliability of the technique. Unfortunately, Millipore membranes were not used again for the remainder of experimental trials. Millipore membrane plates could not be used as a larval viability test when testing mixtures of larvae and crushed LS, because the LS severely clogged the membranes and prevented migration of larvae.

Abomasal fluid, collected directly from the abomasas of freshly-slaughtered lambs, was used throughout experimental trials in an attempt to more accurately re-create a culture environment for the larvae that mimicked the natural environment in vivo. Ultimately, it been theorized that lobster shell, when undergoing the process of digestion within the animal, would be degraded into its chitin-based components by the natural flora and fauna of the animal’s digestive system. We know that chitin and chitosan are most commonly extracted from crustacean shell waste during industrial production, using large-scale chemical processes (Badawy et al., 2011). Preparation of pure chitin at this large scale requires a series of harsh treatments to separate chitin from other constituents in the shell. First, ground shells are treated with either sodium hydroxide or proteolytic
enzymes such as trypsin, pepsin and pronase to extract proteins. Shell minerals are then removed with hydrochloric acid. Lastly, adeacetylation process is typically achieved by treating the newly-extracted chitin with concentrated sodium or potassium hydroxide solution, releasing amine groups to form chitosan (Badawy et al., 2011). This industrial process of treatment with enzymes and strong acidic followed by alkali chemicals is very similar to the chemical processes that occur within the gastrointestinal tract of sheep from the abomasum through the intestines.

The AF would still contain its original mixture of fluids and enzymes after AF was processed in order to filter out large particles, indigested materials, and potential ova. Two separate batches of AF were collected and stored because the first batch was left in the refrigerator for a substantial period of time between use, and was believed to have eventually ‘gone bad’ (evidenced by its putrid smell, appearance, and high larval death rates when kept in the fluid). In an attempt to combat its degradation, a second batch of AF was collected, processed within a few hours of collection, and immediately frozen into 50 ml centrifuge tubes. Samples used were thawed a few hours prior to use to minimize exposure time. Some of the AF from the second batch was used to create the lobster shell conditioned media (LSCM). For later experimental trials, LSCM was used to test chemical properties of LS in the absence of physical properties.

The first “quick and dirty” run with the LS and larvae mixture was set up on September 30, 2015. However, shortly thereafter progress with assessing the samples was arrested again. When visualized under the microscope, even the fine-crushed LS particles obscured vision of the larvae in the mixture. A method was needed to allow the careful separation of LS particles and larvae in a mixture, so that larval viability could be
accurately assessed. Knowing larval viability within a sample was a critical factor that would be used to determine any potential effects the LS may have exhibited on the larvae.

After conducting multiple trials for six different methods in an attempt to discover an efficient, effective way to separate larvae (both live and dead) from fine LS particles in a mixture, no method was found to be sufficient. In light of this, a new direction of study was taken: attempted maturation of L3 larvae into L4-stage larvae within AF. If L4 larvae could be grown, LS could then be used to treat L4- rather than L3-stage larvae. The loss of an outer cuticle during this maturation process (Veglia, 1915) leaves larvae more vulnerable to antiparasitic treatments, and LS may exhibit a more potent effect on the larvae.

2. Experimental stage 1: growth of L3 stage larvae to L4s in abomasal fluid

Before beginning experimental trials with larvae and AF, an average measurement of L3-stage larvae had to be obtained. This measurement marked the standard size of the larvae prior to any trials with AF and/or LS. Results demonstrated that even same-stage larvae kept under identical conditions may vary in length, even though *H. contortus* are typically 700-830 µm in length (van Wyk et al., 2013).

No consistent pattern of larval growth was evident from the first trial with AF and larvae (Figure 7). However, two particular periods of larval growth were evident between 25-36 hours and 72-96 hours. Knowledge of the *H. contortus* life cycle (Dikmans et al., 1942; Veglia, 1915) indicates that the first period of growth could be likened to the maturing of L3 stage larvae into L4 stage larvae, as the transition generally occurs within 48 hours from time of ingestion in live sheep. The second period of larval growth
corresponds more accurately with the time frame that one would expect L4 larvae to mature into adults (Veglia, 1915). Because of this, perhaps these results indicate progression of L3 larvae through multiple stages into mature adults.

Anatomical characteristics seen in the larvae after several days of interaction with AF (Plate 5) were consistent with the expected development of certain features characterizing L4 and possibly even adult-stage worms. Appearance of a thicker, clear outer encasing (Plate 5) was analogous to images depicting L3 stage larvae in the process of shedding the outer cuticle and developing into the first parasitic (fourth larval) stage. Clusters of globules and wrinkled outer edges (Plate 5) in our images were analogous to the development of granulations, appearance of vacuoles in intestinal cells and contraction of the larvae as illustrated by Veglia (1915) in figures describing matured adult larvae. Also, changes in the morphological shape of larvae head structures were seen (Plate 6) which resembles depictions of the anterior end of larvae just before initiation of the L4 stage larval development (Veglia, 1915). Based on rudimentary larval growth patterns and anatomical observations, we concluded that some of the larvae throughout AF + L3 Trial 1 may have actually progressed past the L4 stage into adult form. Some of these characteristics are distinctively adult but not L4 stage traits.

The inconsistent, irregular pattern of larval growth from a section of Figure 7 could be due to a combination of various factors. First, individual larvae can be variable in body length (based on results from Figure 6), and therefore some measure of variability in larval body length despite additional growth and maturation is to be expected. Second, the steady decline in length after 96 hours (see Figure 7) likely resulted from either degradation of older larvae in the mixture as time progressed, or
poorer image quality of the larvae in more concentrated AF of the later wells resulting in inaccurate body length measurements. Third, samples may not have been taken in short enough time increments to enable careful monitoring of specific periods of larval growth, and thus a more consistent curve of results was not obtained. Fourth, imaged larvae from each sample were selected at random from different sections of the plate when being visualized. There may have been an element of optical distortion due to a variety of microscope-based factors: meniscus alterations from fluid in wells, use of different contrast or phase settings on the scope, etc. Such optical distortion could influence the perception and measurement of correct larval lengths.

In an attempt to resolve some of these inconsistencies and pinpoint a more precise point in time at which L3 stage larvae may have transitioned into L4 stage larvae, Trial 2 was conducted (AF + L3 Trial 2). Changes made to the experimental method included: (1) filling wells with more AF in order to avoid excessive evaporation and thus improving visualization of samples taken at later periods of time; (2) sampling at more frequent, regular intervals; (3) only imaging larvae from the center of plate wells to minimize optical distortion; (4) each sample was split in half to formalin-kill a portion in order to gather larval viability data throughout the experiment; and (5) twelve larvae were imaged and measured from each sample to improve representation within the sample.

The second experimental trial with larvae and AF (Figure 9) did not resolve the inconsistencies see in Figure 7. Sampling at a consistent pattern of every 12 hours was perhaps not a small enough increment to accurately capture these supposed periods of larval growth. Or sampling more larvae in a shorter period of time (as was done in this second trial) may have indicated that these periods of growth were not as distinct as
previously thought. Anatomical evidence and developing structures seen in AF + L3 Trial 2 larvae, however, still supported the idea that L3 larvae are maturing into L4 larvae in the AF. Also, the same pattern of decreased length and irregularity after 96 hours (see Figure 7, 9) occurred in both trials. This likely suggested that larvae had undergone substantial degradation, and visibility of well contents was poor, resulting in inconsistent, inaccurate measurements of larval length. Growth patterns of larvae after 96 hours were considered unreliable.

A distinct decrease in total larval counts was notable in this trial (Figure 10, 11). Overall, this was expected. Each day, the concentration of larvae in each sample decreased slightly due to the removal and replenishment of old AF, which contained some larvae. This process was done in order to keep the AF as fresh as possible and prevent the formation of a problematic top biofilm layer over the wells, as had been observed from AF + L3 Trial 1. This partially accounted for the relatively consistent decline in total larval concentrations for each sample over time.

Another factor likely influencing this decline in total larval counts in each sample was the AF itself. Visualization of larvae within AF (all experimental wells) was substantially more difficult than visualization of larvae within PBS without AF (such as the control) due to the viscosity and properties of each medium. Therefore, as the concentration of larvae decreased and concentration of AF increased over time due to the replenishment of AF, larvae became more difficult to identify, image and measure, resulting in lower total larval counts per sample. This could also help account for the inconsistent body length measures of larvae in later stages of experimentation, as illustrated in Figure 7 and 9.
Results further suggested that larvae in AF experienced greater death rates than those not kept in AF over the same amount of time (compare Figure 10 experimental wells with control). Perhaps properties of the AF itself were negatively affecting the larvae. This led to the idea of using fresh AF for the next round of experimentation. This first batch of AF (collected 10/8/15) used for AF + L3 Trial 1 and Trial 2 had been processed and left refrigerated for an extended period of time (weeks) before being frozen. The AF, when not frozen immediately after processing, likely ‘spoiled’ over time, resulting in more negative consequences to larvae when combined in a mixture. The second batch of AF (collected and processed 11/30/15) was immediately frozen. Aliquots were thawed directly prior to experimental use to ensure that the AF would be as fresh and unspoiled as possible throughout the duration of the experiments (specifically AF + L3 Trial 3, AF + L3 Trial 4, and L4 + AT).

Trial 3 with L3 stage larvae in AF was designed to only assess any anatomical changes of larvae in *fresh* AF and see whether any difference was evident compared to the use of ‘old’ AF. Larval growth was not tracked, and no measurements of larvae were taken. Use of the larger sample in a flask with a stir bar was intended to create an environment that more closely mimicked that of the abomasum of a live sheep, compared to tiny individual 24-well plates that could not generate mixing capacity or gas exchange. Despite sound reasoning for using the stir bar, stir bar-related damage was the most likely factor responsible for the broken and battered larvae (Plate 7), even when used at low speed. One final trial of larvae in AF (AF + L3 Trial 4) was prepared, omitting use of the stir bar. Instead, a culture flask was filled with a new mixture and laid broadside on the Ames rocker in order to accomplish more gently, regular rocking and mixing of contents.
Trial 4 was the last AF + L3 trial conducted, due to both time and resource restraints. The main influencing factor was the resources: only enough AF and larvae remained to successfully run this final trial. Time remaining to conduct further experimental trials was also limited to a few more weeks. In Trial 4, the goal was to see the majority of L3 stage larvae develop new anatomical characteristics (signaling larval maturity into L4 stage larvae and possibly adults) within 3-4 days. By 6 days after initiating Trial 4, the majority of larvae were expected to have had sufficient time to develop into the L4 stage. AF + L3 Trial 4 was run to completion and its products (more mature larvae) immediately used in the preparation and initiation of the final experiment with L4 stage larvae and assorted anthelmintic treatments (L4 + assorted treatments). This was the only effective way supposed to maximize remaining resources while generating a batch of matured larvae.

3. Experimental stage 2: trials with crushed lobster shell

The first experiment that involved mixing LS in with larval cultures (Figure 12) had initially been designed and implemented to discover whether crushed LS altered the maturation of *H. contortus* ova into L3-stage larvae in vitro.

Overall, these results suggested that crushed LS may have a toxic effect on *H. contortus* in sheep. Perhaps LS could be used to reduce larval counts by targeting the transitional stage from *H. contortus* ova into L3 stage larvae. Careful study of *H. contortus* eggshell structure (Bird et al., 1991) has revealed that its medial layer is sensitive to degradation by chitinase (Mansfield et al., 1992) because the eggshell contains a large proportion of chitin components. Furthermore, chitin components have been found in the egg stages, but *not* larval and adult stages, of parasitic nematodes.
(Gortari et al., 2008, Veronico et al., 2001). This could help explain why chitin-based LS treatments only seem to have an effect on ova stages of *H. contortus* rather than larval stages.

Our preliminary results did not demonstrate 100% nematicidal effects on the part of the LS (see Figure 12). However, this does not necessarily render LS an ineffective treatment of *H. contortus*. Other alternative antiparasitic treatments used in the past, such as antiparasitic plants and fungi (Shulaw, 2013), are known to not provide the same full benefits as have been enjoyed with traditional anthelmintics, but rather are used in conjunction with traditional de-wormers to maximize parasite control and minimize reliance on chemical de-wormers. Further exploration of LS in this context could suggest similar potential.

Further speculation suggests that medium-sized LS (compared to fine-sized LS) would cause more substantial damage to the ova simply through greater amounts of abrasion from the larger-sized particles. Adding larger proportions of chitin (in this case, from LS) to the soil may stimulate the production and function of chitinase, resulting in an abundance of the enzyme. The chitinases then will target chitin-containing materials in the surrounding area, including the ova, because nematode egg shells contain a substantial proportion of chitin.

Since this experiment (Figure 12) was exploratory with an emphasis of methods development rather than comparison of treatments, no substantial conclusions could be drawn from its results. More detailed experiments are needed in order to more fully explore and understand how crushed LS could be used to reduce larval counts from egg and early larval stages of *H. contortus*. Suggestions for future studies include: (1) running
more elaborate tests that focus on LS application on the ova-to-L3 stage larvae transition; (2) including other experimental groups that contain similarly-textured materials resembling LS, but with different compositions (i.e. sand, fine gravel, etc.); (3) incorporating other treatments into the larval cultures, much like those used in L4 + AT, to compare results; (4) growing up samples of pasture grass in buckets, infecting them with larvae, and treating each sample with LS to mimic normal parasite development on pasture.

The final experiment involved combining L4 stage larvae (supposedly grown from the fourth trial with L3-stage larvae in AF) and assorted anthelmintic treatments (Figure 13). This experiment was referred to as L4 + AT. The purpose of experiment L4 + AT was to demonstrate, based on larval viability counts, whether or not LS extractions (lobster shell conditioned media, chitin or chitosan) had a nematicidal effect on L4 stage H. contortus larvae. A standard chemical de-wormer, Prohibin, was used as a positive control in order to compare its larval viability rates with those of LS and the negative control (no additional treatments). All samples were only killed with formalin to maximize the number of larvae per sample that would be counted for viability assessment. The heat-kill procedure was unnecessary for this stage of experimentation because larval viability would be assessed with the formalin-kill technique, and larval growth was not monitored.

Overall, results from L4 + AT were inconsistent and inconclusive (Figure 13). No clear basis for comparison existed between results. First, total larval counts for all flask samples varied dramatically. These inconsistencies were likely due to a combination of the following factors: (1) poor visibility of larvae in AF, especially with particles (chitin
or chitosan) present; (2) human error during visualization, as frequent prolonged periods of study were tiring and taxing on the eyes; (3) such low numbers of larvae present in the mixture made them difficult to locate and identify; and (4) any tightly-curled larvae (ones live prior to formalin-kill) were extremely difficult to notice and recognize in the low-visibility samples.

Furthermore, a few other factors must be considered. First, larval counts were not substantial enough at any point in time from which reliable larval viability counts could be generated. If this experiment were to be repeated, at least 50-100 larvae per flask sample would need to be visible in order to more accurately represent larval viability as a whole. Second, it was not clear whether enough of the larvae grown in AF + L3 Trial 4 were consistently matured into L4 stage larvae. Some of them may have still been in the L3 stage, some may have been L3 stage larvae that died and arrested growth prior to initiation of the L4 + AT experiment, and some may have matured to the adult stage. Due to both time and resource restraints, all larvae from AF + L3 Trial 4 were used in L4 + AT Trials. Had there been more time and/or larvae available, more careful assessment of the Trial 4 larvae could have been conducted prior to using them in the L4 + AT experiment. As it was, we had decided to move forward with the process to at least try the various treatments on larvae.

**CONCLUSIONS**

From this research, a variety of preliminary conclusions may be drawn. First and foremost, LS does exhibit nematicidal effects on *H. contortus* development from ova to L3 stage, although further testing must be conducted to substantiate this claim. Thus, crushed lobster shell shows initial potential as an antiparasitic treatment of *H. contortus*
nematode in early stages of development. Secondly, in order to fully assess the effects that LS may exhibit on more advanced stages of larvae such as L3, L4 or adult larvae when combined in a mixture, all larvae—live and dead—must be able to be separated from the particles in solution and tested for viability. This was a major obstacle during the experimental process, and a solution has yet to be found. According to these trials, no evidence for LS exhibiting nematicidal effects on L4 or adult stage larvae was found.

Furthermore, larval development and maturation cannot be accurately determined based only on body length measurements. Both anatomical characteristics and body length measures must be assessed in order to qualify matured larvae. L4 larvae may be identified from a variety of characteristics including a distinct head morphology, loss of outer cuticle, formation of enlarged internal globules, and overall increase in size.

**IMPLICATIONS**

This research may have provided some of the initial groundwork necessary for advancement in production of a new small ruminant anthelmintic. Specifically, further studies may reveal that LS is effective as a supplemental treatment in conjunction with standard anthelmintics, to decrease exposure of sheep to infective larvae on pasture.

After further research, should it prove to be useful, production and utilization of chitin and chitosan from lobster shell could constitute an economically attractive mean of lobster shell waste disposal.

**Next steps: suggestions for future research**

Based on this research, the most promising next step would be to further investigate the exposure of infective larvae to LS on pasture. A new set of experiments
could involve growth of pasture grass in bucket samples that were “infected” with *H. contortus* ova and treated with various proportions of LS. Ova could also be extracted and treated *in vitro* with more elaborate testing. In regards to further testing of LS effects on L4 and adult larvae, a growth medium for the larvae could be introduced in order to maintain higher larval viability percentages while treating experimental groups.
LITERATURE CITED


Niezen J. H., T. S. Waghorn, W.A. G. Charleston and G. C. Waghorn. 1995. Growth and gastrointestinal nematode parasitism in lambs grazing either lucerne (Medicago sativa) or
sulla (*Hedysarum coronarium*) which contains condensed tannins. Journal of Agricultural Science **125**: 281-289.


APPENDIX


Purpose: Culturing fecal samples to analyze percentage of Haemonchus contortus L3 Larvae present.

Materials:
- Disposable gloves
- Balance with sensitivity of 0.1 mg
- Thermometer (Celsius)
- Worksheets: Culture Prep, Larval Tally, Larval summary
- Step 1: Culturing
  - Autoclaved Vermiculite
  - Syringe plunger
  - Scoopula or wooden stick
  - Tap Water
  - Graduated cylinder
  - Aluminum foil
  - 28°C Incubator
- Step 2: Extracting Larvae
  - Baermann setup: Short Stem Funnel, Tubing, Clamp and Rack
  - Kimwipes
  - Rubber Bands
- Wooden Craft Stick
- Label tape

- **Step 3:**
  - Heating block
  - Pyrex tubes (same size as 15 mL centrifuge tubes)
  - Transfer Pipette

**Notes:**
1. A fresh fecal sample should be collected. For pooled samples, take small amounts from 10 animals, if possible. Keep sample tightly wrapped in plastic to exclude air.
2. Fecals should never be cooled prior to culturing. Temperatures below 4°C will severely impact *Haemonchus contortus* recovery.

**Procedure:**

**Step 1: Culturing**
1. Tare labeled plastic cup and weigh out fecal sample. If pooling, add approximately equal portions of fecal pellets from each animal. Record date and mass on Larval Culture Worksheet. Mash pellets with the bottom of a syringe plunger.
2. Add vermiculite at proportions of 1:2 (feces:vermiculite) by volume and record vermiculite mass. Mix well with scoopula.
3. Add volume of water equal to total mass of feces and vermiculite and mix. E.g. 28 mL water to 28 g. feces and vermiculite (Add about 10% more water to dry samples and 10% less to wet samples.)
4. Cover cup with aluminum foil, tighten down well around lip and write sample #, date, initials and sample type on foil.
5. Place in 28°C incubator for 7-8 days.

**Step 2: Extracting Larvae:**
1. Set up Baermann apparatus. (Short stemmed funnel with tubing fitted on the end, clamped with a tube clamp.) Support apparatus with a ring stand or rack with at least 4 inches under the bottom of the tubing. Label each funnel with sample number, date and initials.
2. Fill a beaker with tap water 30°C. Close tubing clamp and fill funnel with water. Place beaker underneath apparatus and open clamp to release enough water to fill stem and tube. Close clamp tightly and check for leaks.
3. Remove culture from incubator and stir with scoopula.
4. Place two Kimwipes flat on counter in an X shape.
5. Scoop out 10-30 g. culture into the center of the Kimwipes.
6. Pull up the edges of the Kimwipes to make a pouch and close with a rubber band. Record the date and mass on the Larval Culture Worksheet. (Subtract about 1 gram for the rubber bands and kimwipe)
7. Run a wooden stick through the rubber band to suspend the pouch in the funnel. Ideally it should be centered and suspended above the bottom of the funnel. Add water if necessary to cover the pouch.
8. Allow sample to sit for 7 hours. The larvae will travel out of the pouch and sink into the stem and tubing concentrating in the bottom of the apparatus.
9. Remove the pouch and dispose in trash (reserving the rubber band for future use.)
10. Place a clean 50 mL centrifuge tube under the apparatus. Carefully open the
clamp and fill the tube with the larvae extract. Transfer the label to the centrifuge tube, cap and refrigerate overnight.

**Step 3: Heat Kill Larvae**

1. Turn on the heat block to high.
2. Remove the larvae extract from the refrigerator, decant and dispose the top 46-48 mL water from the centrifuge tube taking care not to disturb the larvae sitting on the bottom cone of the tube.
3. Using a transfer pipette, resuspend and transfer the larvae to a glass tube. Rinse the 50 mL centrifuge tube at least once with tap water and add to the larvae concentrate.
4. Place tube(s) in heating block and suspend thermometer in one tube to monitor temperature.
5. Heat to 57-60°C and quickly remove from the heat block. Place in Styrofoam rack and allow samples to sit at least 5 minutes.
6. Larvae may be visualized immediately or stored in refrigerator. Long-term storage should be in 10% formalin.

**Hints:**

- Larvae may be stored in refrigerator for an extended time in water, but the longer they sit, the more physical degradation may occur making it difficult to identify them via morphology. Refrigerate larva live then kill before visualizing if they will be sitting for an extended time.
- Larval culturing is not a quantitative procedure and care should be taken when interpreting results qualitatively. Treatment of fecal samples prior to culturing and variables within culturing (such as mold and humidity) can affect larval growth and recovery.

This procedure is optimized for *Haemonchus contortus* L3 growth and recovery possibly biasing percent recovery results. Each species has different conditions and timing under which they respond to when being cultured. By being consistent within the Weber lab, results generated may be compared internally, but may not be directly comparable between different labs.
BIOGRAPHY OF THE AUTHOR

Morgan will be graduating in May 2016 with a Bachelor of Science in Animal and Veterinary Sciences and an Equine Studies minor. She will also be graduating with Highest Honors, as a member of the University of Maine Honors College. She hopes to pursue an internship with the Kentucky Equine Management Internship (KEMI) program in Lexington, Kentucky, and then aspires to have a career working within the field of animal science.

At the University of Maine, Morgan has worked as a member of the AHL Weber laboratory team with lab supervisor Ann Bryant, researching parasitology under the direction of Dr. James Weber. During her time at UM, Morgan has appreciated the endless opportunities to travel and adventure, particularly with her semester abroad in Ireland, missions trips to Haiti and Chile, a Cru summer project in Yellowstone National Park, and major-related jobs taking her around the country. She has also found great joy in the diversity of campus life, getting involved with many programs and organizations including CrossFit Black Bear, Cru UMaine, All Maine Women, Witter Farm cooperative, Ewe-Maine Icelandics club, the Tutor Program, Honors College Student Advisory Board, and an assortment of other clubs. Morgan is an outdoors enthusiast who loves hiking mountains and backpacking, road trips, horseback riding, playing the piano, learning the guitar, and building up her basis of intellectual knowledge. Her dedication and commitment is not only evidenced by her 3.99 GPA, but also by her work ethic, lasting relationships, and diligence to seek out new opportunities.