Summer 8-2018

The Efficiency of Melatonin in Inhibiting Haemonchus Contortus Development

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THE EFFICIENCY OF MELATONIN IN INHIBITING *HAEMONCHUS CONTORTUS* DEVELOPMENT

by

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A Thesis Submitted in Partial Fulfillment
of the Requirements for a Degree with Honors
Animal and Veterinary Sciences, Pre-Vet Concentration

The Honors College

University of Maine, Orono

June 2018

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ABSTRACT

Haemonchus contortus (HC) is a pathogenic nematode that causes sheep morbidity through anemia, edema, and weight loss. It's the most economically devastating parasite to sheep industries, with increasing global temperatures and anthelmintic resistance increasing HC residing areas and life spans. HC enter hypobiosis, a state comparable to hibernation during the fall when day length decreases. In this state of arrested development, the sheep are not taxed. Hypobiosis occurs in late fall and early winter. Melatonin, with its release having a direct relationship to length of night, is highest during this period. Combining melatonin's relationship to the seasons, along with research demonstrating that melatonin can inhibit locomotion in parasites such as Caenorhabditis elegans, it is predicted that melatonin is the cue for HC to enter hypobiosis.

Effects of melatonin levels on development of HC were determined by manually de-worming and inoculating 16 adult Icelandic ewes, followed by twice-daily doses of 5mg of melatonin for three weeks. Fecal samples for HC egg counts, body condition scores (BCS), and FAMACHA scores were taken every three to four days during the experiment. Analysis of P-Tests and T-Tests showed no detectable differences in egg counts, BCS, or FAMACHA scores between the control and treatment group. Our ELISA assays showed that oral administration of melatonin did increase melatonin blood concentrations above control levels. Due to inadequate parasite colonization of ewes during inoculation, we could not adequately test the hypothesis that melatonin inhibits development of HC. Future experiments using more effective parasite inoculation procedures are necessary to adequately test this hypothesis.

DEDICATION

This project is dedicated to the faculty, professors, and animals associated with Witter Farm. Without the support provided to me, I would not have discovered the love and passion for production animals that I have today.

ACKNOWLEDGMENTS

This project was made possible by University of Maine Associate Professor and veterinarian James Weber, who designed the project and wrote the IACUC Proposal. Ann Bryant was his assistant who helped perform the experiment, and all the laboratory analyses. Anastasia Russo and Kevin Arkin were other summer workers who helped with the experiment and laboratory analyses. The Ewe Maine Icelandic's Club performed daily chores in the morning and afternoon, helping to take care of the ewe's daily health, along with helping to administer melatonin feed to the ewes. This work was supported by the Northeast Sustainable Agriculture, Research, and Education, a part of the Federal USDA.

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INTRODUCTION

Haemonchus contortus (HC) is the one of the most economically significant parasites of the sheep world. Symptoms of HC can be very subtle, causing death of a sheep by HC to take the farmer by surprise (Paddock, 2011). Living in the abomasum, blood-feeding HC adults cause anemia, edema, and weight loss, making the animal become weak and lethargic (Pezzanite et al,. 2009). Even when not causing death, this harmful parasite can still render a sheep to be unproductive and uneconomical by inhibiting growth, compromising reproduction, and decreasing abomasum secretions (Guo et al.,2016). Lambs are not able to build up a high immunity to HC, causing all ages to be susceptible (Haemonchus, 2017).

Haemonchosis can be described as hyper-acute, acute, or chronic. In the hyper-acute version, due to an immediate and high HC infection, along with lack of symptoms, death can occur in as little as a week. With the acute disease, there is extreme anemia that occurs, which is accompanied by general edema. Chronic disease is classified by anemia, but less extreme due to lower worm burdens, and gradual weight loss. Gastric pH also increases in the chronic phase, causing abomasum dysfunction. Chronic cases can lead to heavy morbidity, if not mortality, during lactation. Diarrhea is a result of anemia, and while haemonchosis can lead to diarrhea, the presence of diarrhea in sheep does not always correspond with haemonchosis (Fox, 2016).

HC Ecology on Pastures and the Role of Sheep in Its Development

An individual Haemonchus Contortus can lay up to 5,000 eggs a day, causing a grand total of up to 210,000 in an individual HC's lifespan. The eggs are released in the

morula phase, consisting of 8-16 cells (Angelo-Cubbillan et al., 2007). When temperature reaches 24-30 degrees Celsius, HC will attach to the walls of the abomasum. They will suck out enough blood needed for the development of their eggs, and then release them into the feces of the sheep. Once the feces are expelled from the sheep, and the eggs are exposed to the wet, humid environment outside the sheep, the eggs hatch. First stage larvae (L1) will appear and live for four to six days, feeding on microorganisms found in feces. The L1 larvae will then begin to molt, emerging as second stage larvae (L2). The larvae continue to feed on microorganisms found in the feces, but they also begin to move to be near soil. The L2 exists for around 21 days, when they then molt and become third-stage larvae (L3). This is the active and infective stage.

Again, in warm and humid conditions, the larvae are now mobile enough to move from the feces and soil onto the grass, where they are picked up by sheep who are grazing. Generally, L3 larvae are found on the lower ends of the blades of grass, near the soil. Sheep are considered short grazers, meaning that they eat the grass down to the soil, and that is how they pick up HC. Once ingested, HC enters the abomasum. If the external environment has favorable conditions to promote the external life-cycle, they molt and become fourth stage larvae (L4), and then eventually adults. It is unknown why/how these external conditions affect the life-cycle of HC inside the sheep. Once in an adult stage, they attach to the wall of the abomasum, sucking out blood to nourish the development of their eggs. At high enough HC counts, blood is removed faster than it can be replenished by the sheep. Severe anemia can occur.

If HC enters the abomasum in the early to late fall, conditions are no longer warm and humid, and thus not favorable to begin developing their eggs. Therefore, they will

molt into a late L4 life stage. After molting, HC burrow themselves into the walls of abomasum. Doing so allows the parasites to go into hypobiosis, which is a metabolic state where HC cannot cause harm to the sheep, for HC are in a state of arrested development and movement, and therefore cannot metabolize (feed on blood), grow, or reproduce. Because the parasites cannot tax the sheep, the sheep will not show any signs that they are infested with very high numbers of HC. The winter months are also not a suitable environment for the adult HC larvae, so any that are expelled during this time will not survive the winter. Once humid, warm conditions are present again, the L4 stage HC come out of hypobiosis, molt into their adult stage, and again attach to the walls of the abomasum (Cannon, 2011).

During the late spring, around the middle of May, HC counts increase dramatically, and this is referred to as the spring rise. As stated above, during the winter months, due to a lack of warm and humid conditions to promote growth and reproduction of HC, they enter the metabolic state of hypobiosis. As spring occurs, conditions become more favorable for HC, and thus these arrested larvae leave hypobiosis, and again start to mature and develop. Thus, once sheep are let out into the fields to start grazing again, the feces are highly contaminated with HC. The field then becomes exposed to infected feces, and shortly after the HC hatches in proper climate conditions, HC numbers inside the sheep rise dramatically.

Pregnant and lactating ewes generally display this rise in greater magnitude when compared to non-pregnant, non-lactating sheep. This is a result of their immune systems being more vulnerable due to their high energy-using status, and they cannot combat the dramatic increase as quickly as others. This also coincides with why soon after birth,

adult ewes are let out to pasture in the field, and lambs must stay separated from their mothers. Due to the spring rise, it has been shown that keeping mother and lambs together will increase the risk of the young contracting high numbers of HC. Higher rates of morbidity and mortality have resulted from lack of separation (Corradetti, 1966).

HC Biology and How It Causes Harm to Sheep

The oral cavity in Haemonchus Contortus is poorly developed. It has a tooth or lancelet that allows it to penetrate the gastric mucosa of the abomasum to suck blood, and it does so to produce and release eggs. One HC can absorb .05 milliliters (ml) of blood per day, resulting in a dramatic blood loss when combined with a decrease in packed cell volume. Beginning as early as day 4 when HC exit the mucosa of the sheep in the larval (L4) stage, packed cell volumes begin to drop. During the following three weeks, the female HC attach to the lining to take blood from the sheep to provide for themselves, as well as the developing eggs inside them. During these three weeks, the lancelet becomes the most efficient for sucking blood, resulting in blood loss at an all-time high and packed cell volume at an all-time low (Angelo-Cubbillan et al., 2007).

In this period, the sheep will lose on average 5.3-7.7 milligrams of hemoglobin a day (Angelo-Cubbillan et al., 2007). Due to this blood loss, along with an increase in activity of the immune system to fight off the parasites, plasma protein concentrations also decline. The plasma protein levels, however, are difficult to raise again. Sheep with anemia have a reduced food intake, and thus do not receive their necessary daily protein.

They also have an increased pH in their abomasum, and this prevents pepsin synthesis, thus reducing amino acid and small peptide absorption. When HC latches on to

the lining, tissue lesions occur. The lesions cause a decrease in working parietal cells, which are responsible for releasing hydrochloric acid. Hydrochloric acid is responsible for lowering the pH of the abomasum, so a lack of acid results in a higher pH. To further increase pH in the abomasum, HC releases different compounds, such as ammonium salts. The ammonium raises the pH around the parasites, it prevents acids, such as gastric acids, along with enzymes, such as pepsin, from degrading the proteins located in the cuticle of the HC.

Describing and Relating the Melatonin Pathway to Sheep

Melatonin is a modified amino acid that is released from the pineal gland. Melatonin is released in response to day/night cycles; circadian rhythm. The retina of the eye is stimulated through the amount of light placement (Holschbach et al,. 2009). When the retina is stimulated, serotonin is released, causing an inhibitory effect on melatonin. Above a wavelength of 555 nanometers (nm) the amount of stimulation decreases. When there is a lack of stimulation, serotonin undergoes NAT. Nat is the enzyme responsible for this conversion, and this generally tends to be the rate-limiting step in the synthesis of melatonin. O-methylation then occurs, transferring a methyl group, and thus melatonin is synthesized (Ahmed, 2010). Therefore, when the length of darkness increases, the release of melatonin increases accordingly. Melatonin releases increases by up to 10x when comparing concentrations between the day and night (Stellflug et al,. 1988). All of this is controlled by the circadian rhythm clock, located in the suprachiasmatic nucleus in the hypothalamus (Ahmed, 2010). Sheep are “short-day” breeders, which means that they come into estrus when the day length shortens. When light does come through the retina, there is an elevated excitation of retinal neurons in the tonic center of the hypothalamus.

This travels to the superchiasmatic nucleus, where it then travels to the superior cervical ganglion. The presynaptic neurons synapse with inhibitory neurons, converting what was the excitatory response, to one of inhibitory. The post-synaptic adrenergic fibers are then inhibited, and thus reduce secretion of norepinephrine. This results in low levels of melatonin to be released, which further results in excitation of RFamide-related peptide (RFRP) neurons and their neurotransmitter, RFamide-related peptide-3 (RFRP-3). Since sheep are short-day breeders, kisspeptin-10 neurons are inhibited by RFRP-3, and kisspeptin-10 secretion is reduced. Stimulation of gonadotropin-releasing hormone (GnRH) does not occur, and thus luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are not released. This is the cycle of melatonin inhibition in sheep, when the length of day is at its longest. Therefore, if light impacting the retina decreases, the whole process will occur in reverse, and kisspeptin neurons would not be inhibited. With activation of kisspeptin, GnRH can be stimulated, and LH and FSH can be released, causing estrus to soon follow (Senger, 2012). Many experiments have shown that the increase in melatonin due to decreasing day lengths is directly tied to a sheep coming into estrus. Melatonin is used as a hormone to bring sheep into estrus whenever the farmer so desires, help synchronize ewes, and is even proven to increase conception rates within the first 30 days of administration. Generally, oral administration of 10mg of melatonin per day has been deemed effective in initiating estrus (Stellflug et al,. 1988). Ewes are usually given melatonin 6 weeks prior to the joining period, which is two complete cycles of estrous, and is at the end of the anestrous period, which is when administration of melatonin is most effective (Menzies, 2016).

How HC and the Immune System Interact

When HC infects the abomasum, the immune system responds in a step-wise manner. When HC penetrates the abomasum wall, cytokines are induced, for they are associated with the T helper 2 cell response. The different cells that recognize the presence of HC, will send signals to the T helper cells, causing the release of interleukins. These interleukins will stimulate B cells, which produce three specific classes of antibody for this invasion: IgE, IgG, and IgA. IgG is the most abundant antibody in human serum. It is responsible for protecting the newborn during its first few months, and thus is the only antibody that can cross the placenta. It is produced as a secondary immune response to an antigen, and thus has excellent specificity for antigens. IgA is the main defense against local infections, since it is abundant in mucosal secretions such as saliva and tears. IgA prevents passage of foreign substances into the circulatory system. IgE is found in minute quantities in serum yet has a very important rule in defending the body against parasitic invasions. This is the main antibody associated with allergic reactions (Alberts et al., Immunoglobulins). As these antibodies perform their roles and bind to antigens, a complex is formed. This complex is recognized by mastocytes, eosinophils, and neutrophils. These cells release their corresponding granules such as histamine, prostaglandins, and so on. These mediators cause inflammation, increase mucus production, and provoke contractions of the smooth muscle located in the abomasum, thus breaking the HC off, and causing eventual death.

To continue its survival, HC over time has developed strategies to help evade the immune system. Cystatins, which are released by HC, inhibit proteases. Protease is involved in antigen presentation, so with a lack of antigens, the T lymphocytes are not

able to respond. Cystatins also copy cytokine responses, which can lead to a reduction in inflammation, along with reducing the expectancy of immune cells to proliferate. Type C lectin is produced and used by HC to help outcompete leukocyte adhesion. It competes with their cell adhesion molecules and the migration it takes towards the tissue infected with HC, reducing inflammation as well (Angelo-Cubbillan et al., 2007).

Melatonin and the Immune System

Experiments performed with *Caenorhabditis elegans* (CE), a parasite that infests sheep, have shown that melatonin may inhibit homeostasis by arresting the parasite's movement and/or development. Many invertebrates, such as these parasites, have been shown to make their own melatonin, however without displaying some sort of rhythmicity, or circadian rhythm pattern. The role of melatonin in these organisms is still unknown. Research found that orally administered melatonin greatly inhibited *CE* locomotion. When CE was administered melatonin in 15-minute increments, it specifically decreased locomotion rates. Locomotion rates were counted by the number of body bends exhibited by the nematode for 30 seconds, after the 15-minute increment. The CE on the control plate would demonstrate an average of 13.6 bends per 30 seconds. However, when CE was exposed to a 100-picometers of melatonin in the medium, the CE exhibited 7.4 body bends per 30 seconds, and as low as 2.6 body bends per 30 seconds when exposed to 10mM of melatonin (Picture 1). Minute amounts caused a decrease in body movement (Tanaka, et al., 2007).

Research has also been done to test how melatonin effects the longevity of CE worms. It was found that while minute amounts did not affect life expectancy, larger amounts did. The mean life span of control C. elegans is 23.7 days, plus or minus 1.8,

with a maximum life span of 32 days. Experimental CE that were exposed to 10 or 100 mg/L did not have a change in life expectancy, but quantities starting at 1g/L did. Those exposed to 1g/L had their life expectancy drop by 31 percent, and those that were exposed to 100g/l of melatonin daily had their life expectancy drop by 55.7 percent (Anisimov, 2003).

Melatonin inhibits synaptic transmission, by inhibiting the MT1 receptor on motor neurons. The MT1 receptor is inhibited due to the receptor antagonist, luzindole, blocking the effect of locomotion due to the presence of melatonin. This suggests that melatonin is a neuromodulator, and through receptor pathways, is a ligand control. Because the MT1 receptor reacts fast, within a matter of minutes, it shows that this pathway can have a direct regulation of neuronal activities (Tanaka, 2007).

Melatonin has an active role in both brain functions and in the efficiency of the immune system. Both TH1 and TH2 cells of the immune system have high affinity for melatonin. Melatonin has demonstrated effects on anti-tumor defenses, cytotoxicity of natural killer cells, and amelioration of breast cancer cells (Rahman, 2005). It also has a positive impact on helping the body protect itself from retroviruses, and chlamydia (Rahman, 2005). When melatonin is administered externally, it can also enhance antibody production (Rahman, 2005). It is a hydroxyl radical scavenger, protecting the body from several radicals that result from oxidative stress (Rahman, 2005). This has been tested in many neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease, demonstrating that it can reduce ischemia-reperfusion injury (Rahman, 2005).

Problems of Anthelmintic Resistance

Haemonchus contortus is easily spread because its eggs are dispelled through the feces, so entire pastures that sheep are grazing can become contaminated (Stubbings, 2015). Anthelmintic resistance, along with global warming, has made it possible for HC to expand their geographical areas to new locations that have never been exposed to HC before (Mavrot et al,. 2015). Throughout the years, as sheep have become resistant to one chemical class of de-wormer, farmers would start to administer other de-wormers, along with the same resistant de-wormer. This, along with farmers administering high doses of the same resistant medications, has selected for strains of resistant HC (Roos, 1970). In as little as 10 years, HC can develop resistance to newly introduced drugs. Resistance has already been documented to benzimidazoles, imidazothiazoles, and macrocyclic lactones, with the beginning of resistance being shown for the new chemical class of anthelmintic called monepantel. Therefore, HC are resistant to the three major types of drugs (Kotze and Pritchard, 2016). While new drugs are still being discovered, their variability from others is not significant, because the new drugs still fall within one of the three existing groups (Vercruysee and Claerebout, 2016).

Due to this drug resistance, and a consumer market that wants inexpensive, organic meat from animals that have not been contaminated with drugs, research has investigated an anthelmintic-independent means of treatment. These include: nutrient supplements, bioactive compounds, and vaccines (Guo et al,. 2016). Developing an effective vaccine that uses melatonin as its base compound would decrease the severity of HC infection. This would increase the overall productivity and economic benefits of farms with sheep, along with improving sheep health by preventing sheep anemia,

edema, and potential death. Since this would be a natural growth remedy, it will help overcome the current high resistance of HC to common day de-wormers. A melatonin-based treatment would prevent the development of larvae to the adult stage, eliminating the spread of eggs through animal feces and thus contamination of pastures. Overall, this could greatly benefit the sheep industry.

The objective of this study is to determine if treating HC-infected sheep with high levels of melatonin (equivalent to dark-period production) would reduce anemia caused by HC, along with egg-laying that commonly takes place in late spring.

It is hypothesized that rising levels of exogenous melatonin that mimic the melatonin response to darkness brought upon by the changing of seasons, will result in HC hypobiosis, causing an arrested development state in the L4 stage of the parasite.

MATERIALS AND METHODS

For this study, 16 adult Icelandic ewes were dewormed, then infected with 1000 HC L3 larvae each. The sheep were then divided randomly into two groups, one representing a control group, the other being treatment. Each group had 8 ewes. Prior to the start of the study in June, eight of the sixteen ewes had been previously dewormed in October. Since the groups were randomly made, consideration for the potential presence of ewes that could have built resistance was not factored. The sheep that were not de-wormed in the past year: numbers 27, 2, 25, 821, 19, 16, 49, and 7, and the sheep that were de-wormed the previous fall were sheep 690, 830, 4, 29, 14, 549, 9, and 21. Analyzation was performed to see if the use of Levamisole as a de-wormer on Witter farm was successful.

The first set of ewes were moved onto pasture on May 22nd, where they remained length of the experiment, which was for the remaining summer months. The sheep were on a rotational grazing system, being moved every four to seven days to a different area of the pasture. The amount of time spent by the ewes on an individual pasture was based on the size of pasture, the length of the grass, the quality of the grass, and the quality of hay that was given to them in feeders. A minimum of three weeks had to pass before returning to the same pasture.

Field Work

Grain was given to the control and treatment sheep daily, at 8am and 4pm. The control sheep received grain that was soaked in ethanol and Phosphate Buffered Saline

(PBS), and the treatment group received grained soaked in a melatonin stock solution, and PBS.

To prepare the control and treatment feed, weekly lab work was done on each Wednesday. PBS was prepared in weekly batches by mixing PBS packets with deionized water. A melatonin stock solution was made, having 10mg per ml of melatonin in ethanol. One gram of melatonin was mixed with 100ml of ethanol, divided into two 50ml tubes, and stored in a freezer. Melatonin is reported soluble at up to 20 mg/ml in 100% ethanol and is a stable mixture at freezer temperatures.

Control and treatment feeds were made daily. Prior to the 8am feeding, 20 doses of 5 mg of melatonin to be soaked in grain were made. For the melatonin feed, 10ml of melatonin stock were mixed with 40ml of PBS and added to five cups of grain (Shepherd 16, Blue Seal Feeds, Lawrence, Massachusetts). For the control feed, 10 ml of ethanol was mixed with 40ml of PBS. This was added to five cups of grain. For each feed, they were divided evenly into two bags: one for the 8am feeding, and one for the 4pm feeding. These bags were put into a cooler and brought out to the pasture for each feeding.

These doses were administered through bowl-feeding of pelletized grain. At the time of each feeding, the sheep were brought in from their grazing pasture, to their sleeping quarters. A mechanism was set up consisting of metal fences, a weight chute, and an enclosed fence area that connected to the feeding pasture. The metal fences were used to isolate the sheep to a corner of the sleeping grounds. After being isolated, they were individually let into a weight chute. A ¼ cup of feed would be put into a Tupperware (Tupperware, Leominster, Massachusetts) container that was labeled for each sheep, and this was given to the sheep inside the weight chute. They were not

allowed to leave until the feed was finished. These containers were washed and dried following every feeding.

After finishing the feed, the ewes were let into an enclosed area where Dr. Weber was waiting to assist with the following procedures. FAMACHA scores were taken twice weekly to monitor anemia. The bottom inner eyelid of the ewe would be popped to show color. This color was then compared to a FAMACHA score card and given a score of a 1-5. Ewes with a FAMACHA score of 4 if seen in the study, were to be immediately removed from data collected and treated with an effective anthelmintic. A sheep showing a five would be euthanized. Body condition scores were also taken during this time. Towards the middle to lower back, the lateral processes of the lumbar vertebrae would be palpated, and the amount of fat that could be felt around the bone would determine the score. Finally, Dr. Weber would remove a minimum of two grams of feces from each sheep, and put into a Ziploc (S.C. Johnson & Son, Racine, Wisconsin) bag labeled for that sheep. After receiving the samples and scores, the sheep was then released into the grazing pasture, and the next sheep was brought into the weight chute.

Lab Work

To test melatonin and its effects on HC, it was decided to de-worm all 16 sheep of any strongyles using Levamisole (WSD Agribusiness Pty. Ltd, South Gilford, Washington) on June 5th, and then to inoculate them with HC grown from a larval culture on June 8th. To produce the HC larval culture, fecal samples were collected from sheep #549 a month before the experiment began. The eggs collected from the feces were cultured to the L3, infective stage in vitro. To culture, equal portions of fecal pellets were weighed out, and broken apart with the bottom of a syringe plunger. Vermiculite was

added in a 1:2 proportion and mixed. Water equal to the mass of feces and vermiculite mix was then added and mixed as well. The culture was then covered with aluminum foil and placed in a 28℃ incubator for 7-8 days.

To extract the larvae, the culture was taken out of the incubator. Between 10-30g of cultured feces was scooped into the center of two Kim wipes, until there was no culture left. The Kim wipes were pulled up to create a pouch, tied shut with a rubber-band, and then wrapped around a wooden stick. A Baermann apparatus was set up for each Kim wipe pouch. A funnel was filled up with tap water at a temperature of 29-32℃. The Kim wipe pouch was then set into the water in the funnel, for 6-8 hours. Then, 50ml of the larvae extract from the funnel was poured into a 50ml beaker and refrigerated overnight. A small portion of the larvae were heat killed at 57-60 degrees Celsius and visualized to evaluate the percentage of HC. The sheep were infected through an oral dose using a clean deworming syringe with 1000 HC L3 larvae, along with other abomasal worm larvae.

Due to the presence of different types of strongyle larvae in the culture, we used a list of guidelines to help us properly identify what larvae was a L3 HC larva. When looking at the larvae through a microscope on 10x magnification, the larvae should be a length of 650-730 micrometers. The head should have a sheath surrounding it, along with having a bullet shaped appearance. The tail length should range from 65-75 uM, while having 10-15% of that be filamentous, or clear. Other parasite larvae that might also be present in sheep manure would have a longer and more filamentous tail, compared to HC.

The larval culture had to be made twice. The first culture made had double the water administered pre-incubator, compared to the second culture. When the first culture

was taken out and the larvae were heat killed, numbers of only 10 larvae per ml were obtained. A minimum of 50 were needed for there to be enough to inoculate successfully, while the goal of 100 was desired. The culture was soaked in moisture and had a texture like that of slime. Having performed similar cultures before, Ann Bryant knew that this was not a normal texture. After doing additional research, it was found that moisture content played an important role in the hatching of HC eggs. Therefore, the amount of water added to the second culture was halved.

For enumeration of HC egg production, fecal samples were collected twice weekly from each of the 16 ewes in the study and a McMaster Fecal Egg Count was performed in duplicate. To count the eggs, two grams of feces were prepared for examination in a MacMaster slide under a compound microscope at 100x total magnification. After weighing out two grams, 28ml of sodium nitrate solution was added and mixed. This nitrate feces solution was strained through cheesecloth placed in a funnel into a beaker. The cheesecloth was squeezed to allow maximum liquid acquiring. The beaker that the solution funneled into was swirled, and one ml was taken from the top middle of the solution and put into McMaster slides. This was done in duplicate for each sample. An identification chart was used to identify the ova based on the size and appearance of the different eggs that we were quantitating: *Strongyles*, *Strongyloides*, *Coccidia*, *Nematodirus,* Tapeworms, and *Trichuis ovis.* Since HC are one of three species of strongyle, all of which have similar appearing eggs, the strongyle count would include the count for HC as well as for Teladorsagia and Trichostrongylus genera.

To obtain the proportion of HC from within the strongyle population, a fluorescent assay was performed that specifically identified only HC ova. First, isolation

and purification of ova from manure was performed. After adding a measured amount of parasite ova flotation solution (saturated sodium nitrate) to manure obtained from each sheep, the control ewe, had their samples pooled together, and the treatment sheep had their experiments pooled. The tubes were then centrifuged. The supernatant is the top layer of the samples after they were centrifuged, and this is where the eggs lie. Supernatant was obtained by placing a screen over a beaker and pouring out as much supernatant as possible without collecting fecal matter. The screen was then washed with PBS, to allow maximum accumulation of the ova. It was then centrifuged, cleaned with PBS, and centrifuged again.

The fluorescent assay was successfully used for identifying HC due to a peanut agglutinin lectin. This is a protein present only in HC, when compared to the rest of the pathogenic parasites. This is a protein, that when paired to fluorescein isothiocyanate, will cause the HC to glow. This explains why when the fluorescent protein was added to the sample in preparation, it had to sit for at least an hour in a refrigerated place. This allowed the binding between the two proteins to occur. When counting the amount of HC present in the picture, only those emitting a full glow were considered. By having the sample sit with the fluorescent protein, this allowed enough time for the binding to take place, so HC were not showing only low levels of glowing, and thus be overlooked (Bryant, 2017).

To stain the eggs, the supernatant had to be removed, as the eggs sunk to the bottom of the centrifuge tube when suspended in PBS. The remaining solution was mixed with PBS and 100x PNA-FITC concentrate. This was incubated with the eggs for a minimum of one hour, allowing the HC eggs to fluoresce when exposed to ultraviolet

light. To visualize the eggs, the tubes were removed from the fridge with the glow concentrate, and the tube was centrifuged again. The supernatant was removed, and 50 microliters from the top of the sample were deposited into the depression of a glass side. The sample was re-suspended with PBS into the middle of the liquid and placed under a fluorescent lamp at 10x objective. A DP Controller program was used to take photos. As many ova as possible were attempted to fit into the field of view. If more than 50 were in the field, only two or three photos were taken. If only a few could be fit into the field, the whole sample was photographically documented with an incandescent photo and a fluorescent photo taken for each area. The photos were saved on an external hard-drive for later enumeration of %HC in each sample.

HC percentage in samples was estimated by counting eggs in photos that were uploaded onto a computer screen. Only HC ova glowed under the fluorescent light, showing a green ring. After counting the number of eggs in the incandescent photo, and the number of glowing eggs in the fluorescent photo, the number and percentage of HC present was determined by the equation %HC = 100 x (# of incandescent eggs / # of fluorescent eggs).

To determine whether oral administration of melatonin was successful in raising blood melatonin levels in the melatonin-treated ewes, five to ten ml of blood was drawn from each ewe's jugular vein into siliconized Vacutainer tubes once a week from each sheep. An ELISA-based melatonin assay was performed on ethyl acetate-extracted serum samples to determine if oral administration of melatonin influenced melatonin concentrations in the blood serum. The samples were run in duplicate. Prior to running the assay, the wash buffer, standard curve, melatonin tracer, melatonin antibody, spike

samples (one of sheep 549's samples per weekly batch), and a stabilizer were prepared. To prepare samples for melatonin extraction, serum samples were thawed, centrifuged, and had the supernatant removed (which contained solids). A phase dilution was performed to allow the minute volume needed to perform the melatonin assay to contain the correct amount of melatonin. Five milliliters of Ethyl acetate were prepared and cooled and were added to the samples. After vortexing, the top/organic layer was removed and put under a jet of nitrogen gas to allow the solvent to evaporate. To rehydrate each melatonin sample, 1980 ml of 1X Stabilizer was added, and a melatonin ELISA assay was performed using a 96 well plate format. The amount of melatonin in each well plate was analyzed by its absorbance on a plate reader set at a wavelength of 450nm. Standards and blanks were made and used to establish a standard curve for quantitation.

To analyze the obtained results, the egg counts were graphed for both the control and treatment group for each sample set. Each sample set was then averaged for the control and treatment group, and the average of each was graphed to show change over time. The same two graph ideas were also made for FAMACHA scores, along with body condition scores. Finally, a table was made to demonstrate the melatonin blood concentrations for each sample that was taken. A graph was then made to show the average control and treatment group numbers for each date taken, to allow for comparison over time.

A F-Test was performed, to determine if the control and treatment groups were of equal or unequal variances. Based on this answer, a corresponding t-Test would then

be performed. A P-value of .05 was picked, meaning that the results would have to be less than 5 percent to reject the null hypothesis.

Due to the failure to successfully inoculate the sheep with L3 HC larvae, proper data was not able to be obtained. Corresponding graphs and tables were made, showing the number of eggs counted, FAMACHA scores, and body condition scores from each sheep each week. If this research was to be done again and inoculating the sheep was successful, then p-tests would be performed at the end of the research project, comparing each individual sheep from the first week to the last week. A p-test would also be performed comparing treatment groups with control groups over time, and then comparing the two groups against each other.

RESULTS

De-worming

The results showed that using Levamisole as a de-wormer on Witter farm reduced fecal egg counts to nearly undetectable levels. The results were formulated from using the two groups that where formed, based on those that have and have not been de-wormed in the past year. Fecal samples that were taken 16 days pre-inoculation, showed that the non-de-wormed group had an average of 156.25 +/- 132.12 strongyle eggs per gram (EPG), with an average of 50 +/- 42.28 EPG being HC. The previously de-wormed group had an average of 37.5 +/- 16.48 strongyle EPG, and an average of 7.5 +/- 16.48 HC EPG. Three days pre-inoculation, fecal samples were taken again. The fecal samples showed no HC or strongyle EPG for the sheep that had no recent de-worming. The group that was de-wormed the previous fall showed smaller, but still existing egg counts. There was an average of 12.5 +/-35.35 strongyle EPG, and an average of .25 +/- .707 HC EPG. Both 16 days pre-inoculation, and three days pre-inoculation, the previously dewormed group had an outlier that was greater than two standard deviations away from the mean. While the previously de-wormed group alone did not show a significant change, when put into the hypothetical situation that all the strongyle and HC counts were 0, there still was no significant difference. Therefore, there was not a high enough worm count from the group pre-inoculation to show a large enough difference. (Figure and Table 1).

Strongyle Counts

Three days pre-inoculation is when the first set of fecal samples were recorded. The control group had an average of 0 +/- 15.03 strongyle EPG, and the treatment group had

an average of 12.5 +/- 15.71 strongyle EPG. Throughout the length of the experiment, both groups fluctuated but had an overall increasing number. On the last day of the experiment, 21 days post-inoculation, the control group ended with an average of 37.5 +/- 15.03 strongyle EPG, and the treatment group ended with an average of 43.75 +/- strongyle EPG. The control group increased by an average of 37.5 strongyle EPG, and the treatment group increased by 31.25 strongyle EPG. The overall experimental average was 12.5 strongly EPG for the control group, and 21.205 strongyle EPG for the experimental. The treatment ended with a larger average strongyle EPG, and a larger overall strongyle EPG, being larger by 8.705. The treatment group had a larger increase of strongyle EPG, however (Figure and Table 2).

HC Egg Counts

Inoculating with only half of the desired number of HC L3 larvae, combined with not seeing the presence of HC eggs until nine days post-inoculation, leads to the conclusion that HC inoculation was not successful. The treatment group displayed 2 +/- 7.34HC EPG, or 1% of the strongyle count, 9 days post-inoculation. At the end of the experiment, the treatment group had increased the number of HC present by 17 EPG, ending with 20 +/- 7.34 HC EPG, or 6% of the total strongyle count. This day was also the first and only day of the experiment that the control group displayed numbers of HC as well. The control group had an average of 4.5, +/- 1.7 HC EPG, 1.5% of the total strongyle count. Overall, the control group had an average of .64 +/- 1.7, HC EPG, and the treatment group had an overall average of 3.57 +/-7.34. These results showed that the treatment group had more 3 HC EPG more than the control group and consisted of 4.5% more of the total strongyle counts compared to the control group. (Table 3).

FAMACHA Scores

Three days pre-inoculation is when the FAMACHA score recording began. The control group started off with an average score of 2.25 +/- .28, and the control group started with an average of 2.125 +/- .189. While both groups had fluctuating scores throughout the length of the experiment, they both had an overall increasing trend. On the last day of the experiment, 21 days post-inoculation, both groups had increased average scores since the first recording from three days post-inoculation. The control group had an average score of 2.5 +/- .28. This is an increase of .25 since the first recording. The treatment group had an ending average score of 2.25 +/- .189. This is an increase of .10 since the first recording. Overall, the control group had not only the larger score, but also the larger increase. The overall average for the control was 2.1875 +/- .28, and 2.125 +/- .189 for the treatment group. This is a difference of .0625, further supporting the control group having a larger score. (Figure 5 and Table 4).

Body Condition Scores

FAMACHA scores and body condition scores (BCS) were recorded at the same time. (Figure 6 and Table 5). The control group started with an average score of 2.75 +/- .2, and the treatment group started with an average score of 3 +/- .148. While the scores for both groups fluctuated throughout the length of the experiment, decreasing in score was the overall trend. When 26 days post-inoculation occurred, both the control and treatment group had a decreased score of .25 from the first recorded score. The control group ended with an average score of 2.5 +/- .2, and the treatment group ended with an average score of 2.75 +/- .148. The overall average score of the control group throughout the length of

the experiment was 2.6875 +/-.2, and 2.797 +/- .148 for the treatment group. Therefore, the treatment group ended with a higher score that was .1095 higher than the control.

Melatonin Blood Concentration

The first melatonin blood concentration sample was taken one day post-inoculation. The control group had an average melatonin blood concentration (MBC) of .21 +/-.0347 ng/ml, and the treatment group had an average MBC of .237 +/- .17 ng/ml. Both groups followed the same trend line. However, 14 days post-inoculation, there was a large increase in the MBC for the treatment group. Where the control group had an increase from .0177 +/- .034 MBC ng/ml between eight and 14 days post-inoculation, the treatment group increased by .371 +/- .17 MBC ng/ml. This is a difference of .3533 MBC ng/ml. This large difference continued until the end of the experiment. On the last day of the experiment, the control group ended with an MBC of .12763 +/- .034 ng/ml, and the treatment group ended with .54524 +/- .17 ng/ml. This comes to be a difference of .41761 MBC ng/ml, the largest difference during the experiment. Due to the large increase eight days post-inoculation in the treatment group, the treatment group increased by .30824 +/- .17 MBC ng/ml, compared to an overall decrease for the control group by .08251 +/- .034 MBC ng/ml. Where the overall average for the control group was .1652 +/- .034 MBC ng/ml, the overall average for the treatment group was .40263 +/- .17 MBC ng/ml, which is a difference of .28391 MBC ng/ml. Therefore, the treatment group had a higher finishing MBC, increase, and average amount (Figure 7 and Table 6).

DISCUSSION

Discussing Results

The results did not support the hypothesis that melatonin would decrease the concentration of HC eggs present in the treatment group, compared to the control group. After the experiment ended, multiple statistical tests were performed. The tests showed there was not a significant difference between the control and treatment group. Therefore, we cannot assume that melatonin caused a lack of HC eggs in ewes.

The oral administration of melatonin did increase blood melatonin concentration levels. However, due to potential failures in the administration of the larval culture, there was no original rise in egg count concentrations. Therefore, we were unable to detect a treatment-related difference between the control and treatment groups in egg counts, FAMACHA scores, or body condition scores.

Melatonin Administration

We could see through the different statistical tests, that our melatonin administration was successful. Oral administration of 5mg of melatonin at 8am and 4pm increased melatonin concentration present in blood. Melatonin is used to help bring sheep into estrus, to allow for manipulated breeding. The administration of exogeneous melatonin in the late afternoon in the mid-summer causes the onset of short photoperiodic conditions for ewes, allowing the early onset of breeding activity to occur. Not only does exogeneous melatonin lead to early estrus, it also will help maintain behavioral and ovulatory activities for a longer time (Poulton, 1988). Exogenous melatonin has shown to be more effective when administration was given starting only slightly before the summer

solstice and continuing into the summer. This is due to an increasing resistance to melatonin that ewes gain as the winter months proceed and giving the endocrine system time to lose this resistance, by readjusting to shorter nights starting in the spring. This study, which was done during the last three weeks of June, was done at what studies show to be optimal administration time, due to lower melatonin resistance. By being able to manipulate the ewe to come into an early onset estrus, this will allow the farmer to coordinate estrus, mating, and birth to correspond to the time of year where lamb sales are the highest. Exogeneous melatonin administration in other studies have shown an increase in melatonin blood concentrations in as little as seven to ten days. This study further supports this timeline, having a dramatic increase of melatonin concentration between the timeline taken of seven to fourteen days after the melatonin treatment began. While this experiment was not testing for early estrus or increased ovarian activity, it did support other data and methods used for increasing blood melatonin concentration to demonstrate these later effects (Stellflug et al., 1987).

Potential Larval Culture Problems

One factor that may have influenced our results was an inadequate dosing of the ewes with HC larvae prior to the start of the melatonin dosing. The original date for when the experiment was supposed to begin had to be pushed back, due to a lack of larvae in the original culture. The number that we were attempting to produce was 2000 larvae per ml of culture. However, when the first culture was counted, the yield was lower than the amount that we required. We attributed our culture failure to excessive moisture content and humidity of the incubator. Moisture content was not taken into consideration when the first culture was made, and after seeing such low results, further research showed that

moisture content can have an impact on developing HC. Therefore, since we did not reach the minimum number of larvae for a successful inoculation, a second culture was made. Even when the second culture was made, we only counted around 2500 HC L3 larvae to be present in its entirety. We decided to proceed with this culture, even though we had half of the count that we desired.

Therefore, we believe that due to low numbers obtained from the culture, and due to relatively low numbers in both the control and treatment groups throughout the experiment, that there was an issue administering the HC larvae. Only three sheep were consistent in maintaining levels of HC in the control group, and only two in the treatment group. The amount of HC that causes levels of concern is around 1,250-1500 eggs, and during the actual experiment, the highest number observed was 250. A control group that had infectious larvae administered to them should have had levels shown in most, if not all the sheep, along with higher levels throughout the four weeks of the experiment.

As stated in the Methods and Materials section, to properly design a HC culture for this experiment, we incubated the culture in an incubator for seven to eight days, at a temperature of 28 degrees Celsius. This was done to hatch HC eggs, and grow them to the L3 larvae stage. Sheep number 549 was chosen to have her fecals used as the way to gain the HC eggs, since she had the largest counts of HC present on the farm. Therefore, even when adjusting for moisture, still receiving only half of the desired amount of HC L3 larvae is puzzling. Other procedures used for HC larvae culture preparation have led to cultures containing 4,000, up to 8,000 HC L3 larvae per ml of culture, and 8000,000 in its entirety. The culture that was prepared for this experiment was seeking 2,000 HC L3 larvae per ml of culture, yet only showed an entirety yield of 2,500. Other procedures

used a shorter incubation time of only five to seven days. The incubation temperature used was also lower, having a range of 25-27 degrees Celsius.

Comparing the two procedures, there are quite a few differences. Our culture came from a sheep that had a much lower number of HC per gram, it was prepared in a hotter temperature, along with being incubated for a longer amount of time. Being exposed to high temperatures for an extended period can lead to inactivity of certain enzymes and ribosomes in a species. Also, research has shown that there are at least three to four different physical shapes of HC. These shapes cause the HC to prefer slightly different temperatures, regarding how they transition between each stage of their development. Therefore, a degree or two difference in temperature can have a huge effect on how a specific physical shape will perform (Lamb, 2016). There are two possible assumptions that can be made from this. If the temperature being too high is an issue, this can be because either there are too many various phenotypes present in the culture, or due to constantly being exposed to the same few pastures year to year, all the HC are the same shape, and the shape does not perform well at this higher temperature (Lamb, 2016).

Other research has been done to find optimum temperature levels for HC culture incubation. Temperatures were tested as low as five degrees Celsius, and up to 35 degrees. Temperature and humidity levels were kept constant during the entire incubation period. There was no larvae development at temperatures as low as 5 degrees, and the research showed that the optimum temperature was around 20 degrees Celsius. Larval mortality was shown to be independent of age. This is another research article that

demonstrates lower temperatures than the one that we used for the actual procedure (Coyne and Smith, 1992).

CONCLUSION

While oral administration of melatonin was successful in raising blood melatonin levels in treated ewes, our data did not support the hypothesis that melatonin causes an inhibition of development in HC. Melatonin administration did not have a detectable effect on fecal Egg counts, body condition scores, or FAMACHA scores compared to the untreated controls. An adjusted procedure and continued research should be done to definitively rule out this possibility, however. Due to the variability of HC L3 larvae produced from culturing, along with multiple environmental factors that can limit and/or add to a HC population in sheep, performing a similar study in the lab to directly see how HC and increase blood melatonin concentrations interact, could lead to a more conclusive answer.

IMPLICATIONS

Populations of HC around the world have unfortunately developed strong resistance to the three main brands of de-wormers that are available for use. This is becoming an imperative issue, due to HC being a major cause of sheep death by bringing on anemia and other diseases. An effective method that uses melatonin as a base would firstly, be a safe regulator of HC, that would cause little to no detrimental effects to the sheep. Treatment would decrease severity of HC parasitism, which would then increase the health and efficiency of these animals. This would cause a rise in the profitability of sheep production, being overall very beneficial to the sheep industry.

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APPENDIX A

Graphs

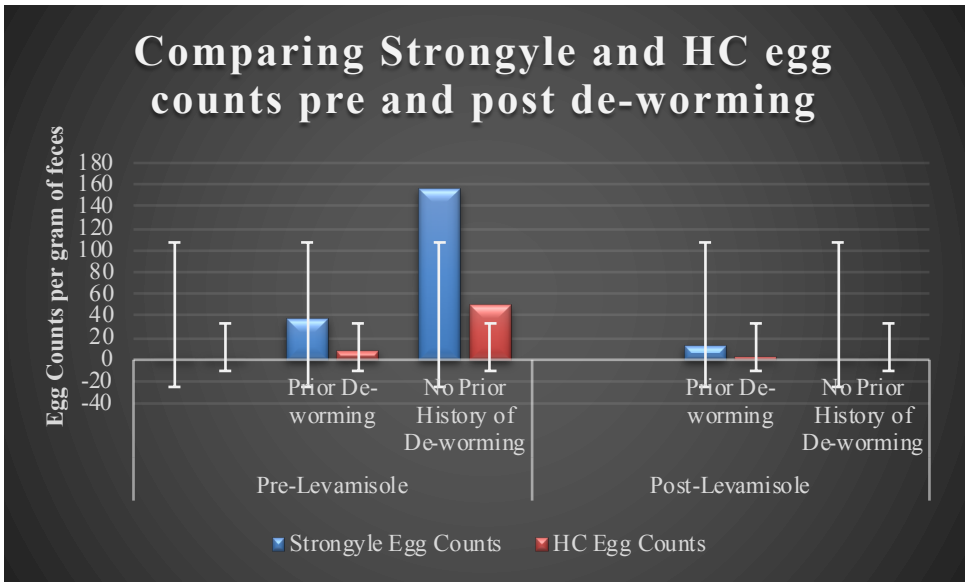


Figure 1: Comparing the Strongyle and HC egg counts before and after de-worming took place on June 2nd.

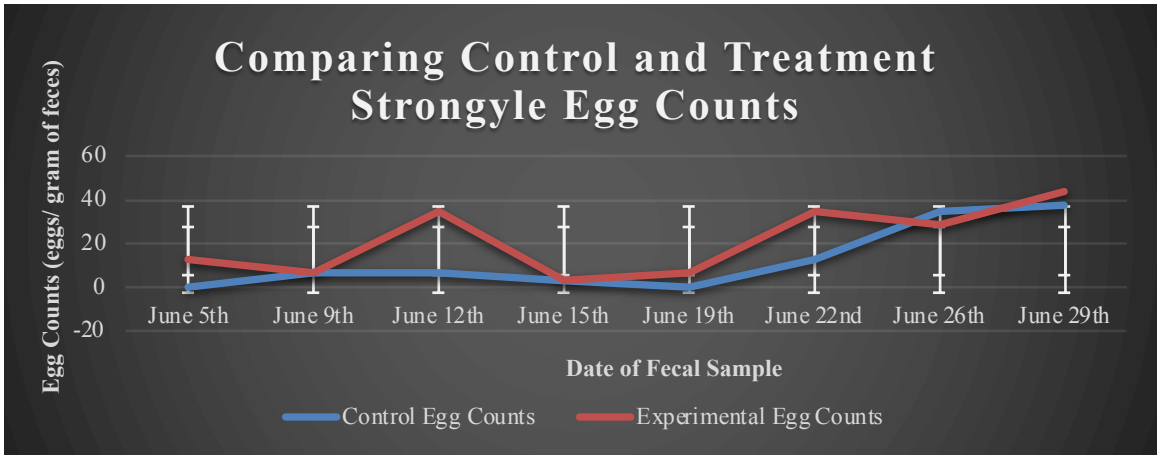


Figure 2: Comparing Strongyle Egg Counts between the control and treatment sheep

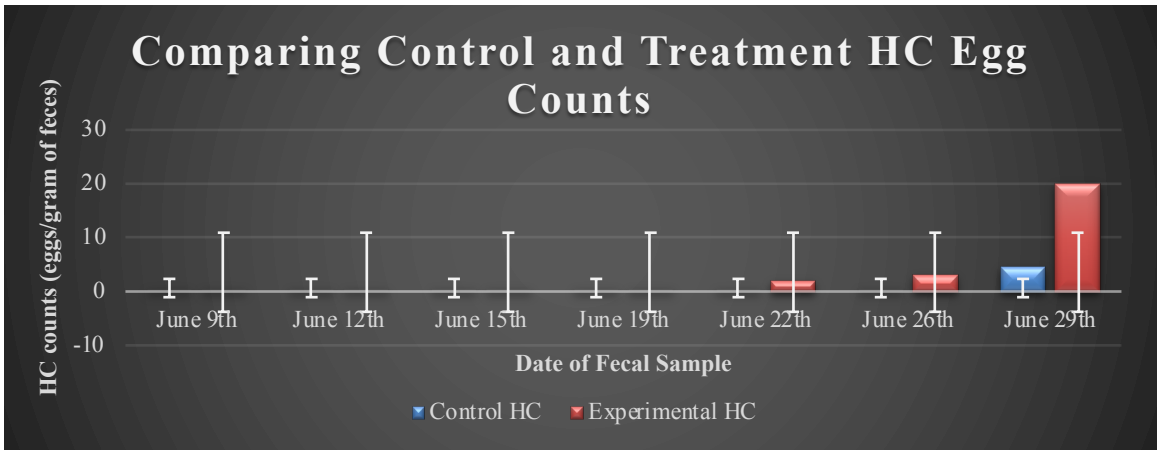


Figure 3: Comparing the number of HC present between the control and treatment sheep

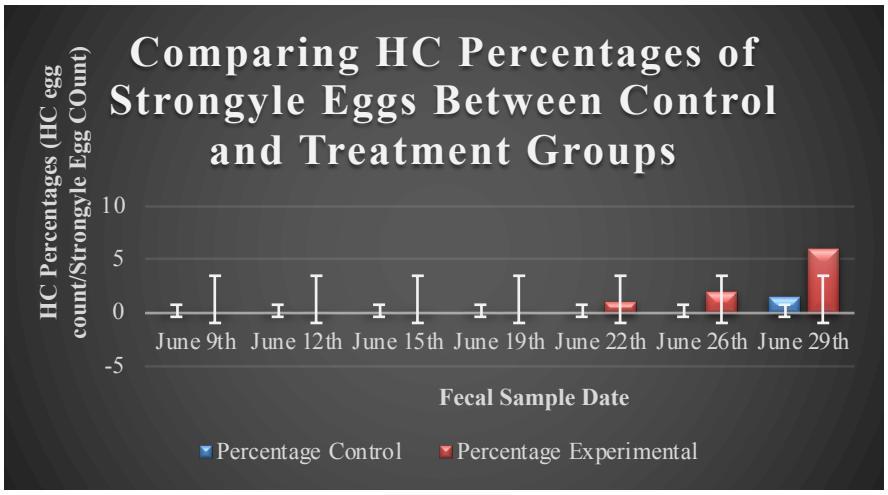


Figure 4: Comparing the percentage of HC present in the amount of Strongyle eggs between the control and treatment sheep

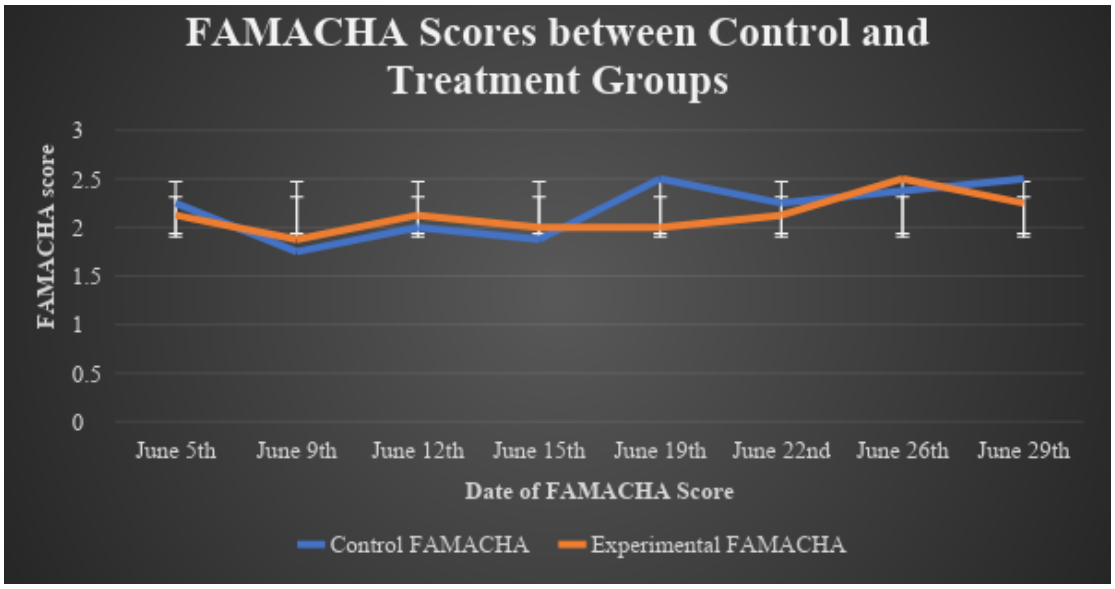


Figure 5: Comparing the FAMACHA scores over the length of the experiment between the control and treatment sheep

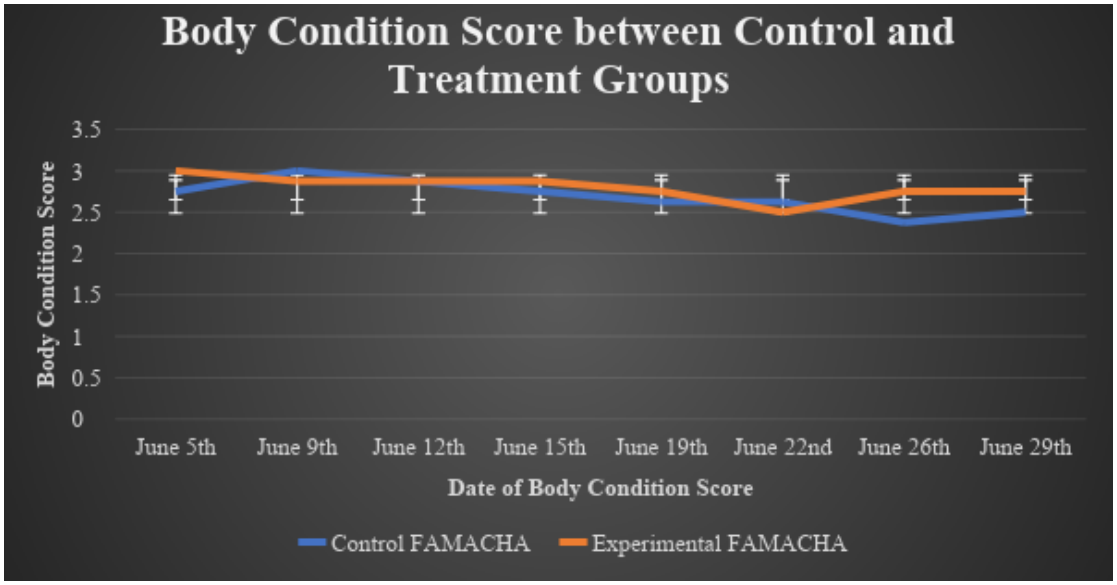


Figure 6: Comparing body condition scores over the length of the experiment between the control and treatment sheep

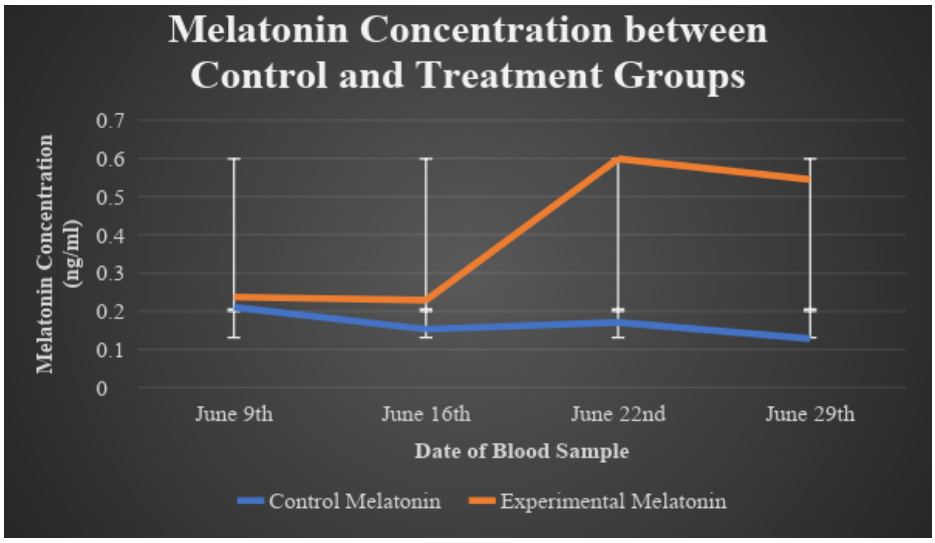


Figure 7: Comparing the blood melatonin concentration over time between the control and treatment group

Tables

Sheep Number	5/23 Egg Counts	5/23 HC Counts	6/5 Egg Counts	6/5 HC counts
Previously de-wormed sheep				
690	50	4	0	0
830	150	48	0	0
4	50	4	100	2
29	0	0	0	0
14	50	4	0	0
549	0	0	0	0
9	0	0	0	0
21	0	0	0	0
Non-dewormed sheep				
27	0	0	0	0
2	150	48	0	0
25	400	128	0	0
821	300	96	0	0
19	150	48	0	0
16	100	32	0	0
49	100	32	0	0
7	50	16	0	0

Table 1: Table showing the egg counts before and after de-worming occurred on June 2nd

Date of fecal sample	Control Egg Counts	Treatment Egg Counts
June 5th	0	12.5
June 9th	6.25	6.25
June 12th	6.25	34.375
June 15th	3.125	3.125
June 19th	0	7.14
June 22nd	12.5	34.375
June 26th	34.375	28.125
June 29th	37.5	43.75
Average	12.5	21.205
STD	15.03344486	15.7080548

Table 2: Table showing amount of strongyle egg counts present in each fecal sample for the control and treatment groups

Date	Control HC	Treatment HC	Percentage Control	Percentage Treatment
June 9th	0	0	0	0
June 12th	0	0	0	0
June 15th	0	0	0	0
June 19th	0	0	0	0
June 22th	0	2	0	1
June 26th	0	3	0	2
June 29th	4.5	20	1.5	6
Average	0.642857143	3.571428571	0.214285714	1.285714286
STD	1.700840129	7.345228448	0.56694671	2.214669706

Table 3: Table showing that amount of HC present in each fecal sample of the control and treatment sheep, and the percentage of HC out of all Strongyles present

Date	Control FAMACHA	Treatment FAMACHA	Total
June 5th	2.25	2.125	
June 9th	1.75	1.875	
June 12th	2	2.125	
June 15th	1.875	2	
June 19th	2.5	2	
June 22nd	2.25	2.125	
June 26th	2.375	2.5	
June 29th	2.5	2.25	
Average	2.1875	2.125	2.15625
Standard Deviation	0.283473355	0.188982237	0.220970869

Table 4: Table showing the FAMACHA scores taken for the control and treatment groups

Date	Control BCS	Treatment BCS	Total
June 5th	2.75	3	
June 9th	3	2.875	
June 12th	2.875	2.875	
June 15th	2.75	2.875	
June 19th	2.625	2.75	
June 22nd	2.625	2.5	
June 26th	2.375	2.75	
June 29th	2.5	2.75	
Average	2.6875	2.796875	2.7421875
STD	0.200445931	0.148466867	0.179517583

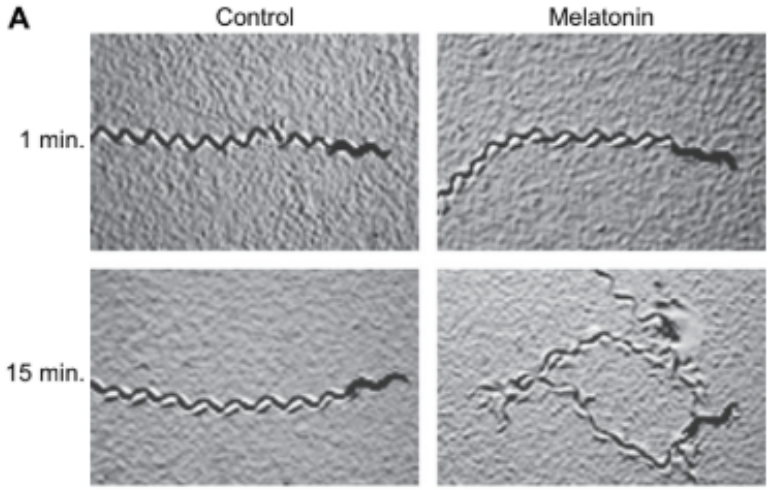
Table 5: Table showing the BCS taken for the control and treatment groups

Date	Control Melatonin	Treatment Melatonin
June 9th	0.210142857	0.237
June 16th	0.152651136	0.228768898
June 22nd	0.170375	0.5995
June 29th	0.127630051	0.545237494
Average	0.165199761	0.402626598
STD	0.034716068	0.170847647

Control Sheep Number	June 9th	June 16th	June 22nd	June 29th
2	0.17	0.05525	0.132	0.124
549	0.172	0.138636364	0.176	0.279
9		0.137004545	0.129	0.054
821	0.264	0.240681818	0.247	0.004040404
16	0.181	0.053863636	0.117	0.16
19	0.241	0.367204545	0.3	0.164
25	0.244	0.14	0.093	0.145
27	0.199	0.088568182	0.169	0.091
Treatment sheep number	June 9th	June 16th	June 22nd	June 29th
49	0.166	0.235681818	0.422	0.413
830	0.189	0.251272727	0.399	0.33961039
7	0.14	0.1701	0.541	0.287
4	0.234	0.16885	0.461	0.563
14	0.114	0.359181818	1.062	0.991
21	0.277	0.339681818	0.845	0.921
29	0.165	0.239465909	0.619	0.285
690	0.611	0.065909091	0.447	0.562289562

Table 6: Table showing individual melatonin blood concentration levels for each sheep over the four samples, then showing the average of all control and all treatment for each date

Pictures



Picture 1: Wild-type C. Elegans were placed on a plate containing melatonin. The control exhibits a natural sinusoidal curve, while the melatonin affected C. Elegans shows a decrease in the amplitude of the curve over time exposed to melatonin.

AUTHOR'S BIOGRAPHY

Stephanie Marie McAvoy graduated from the University of Maine with a Bachelor of Science in Animal and Veterinary Sciences, with a Pre-Vet Concentration, and a minor in zoology. Originally from Rochester, NY, she attended The University of Maine in hopes to move on to a Veterinary school. Being exposed to Witter Farm, and having ample opportunities through class and extra-curricular activities has led her to gain experience dealing with cows, horses, sheep, and pigs. Her involvement in Ewe-Maine Icelandic's Sheep Club led to her participating in this research.

She will be attending Cornell's School of Veterinary Medicine in the fall. There, she will follow a large-animal track, to become specialized in dairy cattle production. She hopes to have her own practice, along with owning cows, sheep, and horses for pleasure. Working with ruminants through research has introduced Stephanie to an interest in research. She hopes to become involved in more research at Cornell, where she would like to embark on her own idea, of making a non-surgical ring that can be non-surgically be put into cows, to reduce the emission of methane.