Safety and serologic response to a *Haemonchus contortus* vaccine in alpacas

Thesis

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate
School of The Ohio State University

By

Grace Marie VanHoy, DVM

Graduate Program in Comparative and Veterinary Medicine

The Ohio State University

2019

Thesis Committee:

Antoinette Marsh, Advisor

Jeffrey Lakritz, Member

Greg Habing, Member

Andrew J. Niehaus, Member

Copyright by
Grace Marie VanHoy
2019

Abstract

Haemonchosis in camelids remains a challenging disease to treat, and prevention has become increasingly problematic due to widespread anthelmintic resistance. Barbervax[®] is an adjuvanted vaccine containing natural H-11, H-gal-GP antigens obtained from *Haemonchus contortus* adults via a proprietary process and solubilized in Quil A. This vaccine is approved for use in Australia, after demonstrating its safety and efficacy in sheep and goats. There are no published studies evaluating Barbervax in other ruminants/pseudoruminants such as camelids which can be parasitized with *H. contortus*. The vaccine utilizes a mixture of the native parasite gut mucosal membrane enzymes including H-gal-GP and H11 (in a Quil A adjuvant), involved in digesting a blood meal from the host. This study monitored the safety profile of the Barbervax[®] vaccine in a group of adolescent alpacas. Although designed into the original study of vaccine efficacy, the experimental infection with viable *H. contortus* third stage larvae could not be completed due to lack of detectable significant variation of infection following experimental challenge. Twelve alpacas (158 + 15 days) were randomized to vaccination with Barbervax[®] or no treatment. Three doses of Barbervax[®] were administered at 3 week intervals and investigators involved in animal monitoring and sample collection were blinded to the groupings. Clinical pathologic parameters were evaluated 7 days before vaccination, and 1 and 2 months post-vaccination. Daily clinical observations were made and specific observations regarding the injection site and rectal temperatures were monitored in each alpaca twice daily for 1 week following vaccination. Fecal egg counts, packed cell volume, and total protein were monitored following challenge with 1500 *H. contortus* larvae each on days 42, 46, and 50. An increase in rectal temperature for a duration of 2 days (range 2-4 days) was observed post-vaccination. Vaccinated alpacas were lethargic for 2-3 days following vaccination; however, they maintained an appetite and no visible or palpable injection site reactions were observed. Following the

first vaccination, all animals maintained normal clinical pathologic parameters throughout the study period. The vaccinated animals did develop titers to the *H. contortus* antigen as measured by ELISA. In conclusion, the Barbervax ® vaccine demonstrated safety in this small group of young, healthy alpacas, but additional studies are required to evaluate the efficacy of the vaccine under field conditions in protecting alpacas against infection with *H. contortus*.

Dedication

To my family, especially my parents, Theresa and Roger VanHoy, and my brothers Eric and Ben for their unconditional love, encouragement, and good humor. And to Brian Peterson for his strength and love that supported me through this crazy journey.

Acknowledgements

First, I would like to thank my major advisor, Dr. Antoinette Marsh for introducing me to both parasitology and research itself. Without her constant support and direction, I would not have been able to complete this degree, her mentorship was invaluable. I would also like to thank Dr. Jeffrey Lakritz for his support and counsel as well as his rambunctious alpaca-wrangling skills, Dr. Greg Habing for his assistance with the statistics for this project, and Dr. Andrew Niehaus for his support and constantly optimistic encouragement.

I would like to thank Michelle Carman for her assistance with this project. She spent long hours collecting and processing samples with me, and her attention to detail and dedication to the project was honorable.

I would also like to thank Bernie Younkman and Heatherbrook Farms for the donation of the alpacas and support of the project, as well as the team of undergraduates and veterinary students who helped handle the alpacas during the study.

Thank you to Dr. Ray Kaplan and his lab technician Sue Howell at the University of Georgia Parasitology lab for their expertise and the *Haemonchus contortus* larvae. Thank you to Dr. William David Smith and the Moredun Institute for the donation of the Barbervax and antigens for the ELISAs, as well as their advice with the project.

I would also like to thank Dr. Joe Lozier, Sadie Strayer and Megan Lagatta with the Farm Animal section for their assistance with the project and for their moral support.

Vita

- 2007-2011 College of Agriculture, Food and Environmental Sciences (Animal Sciences), California Polytechnic State University, San Luis Obispo, CA
- 2011-2015 College of Veterinary Medicine, The Ohio State University, Columbus, OH
- 2015-2016 Clinical Internship, Veterinary Clinical Sciences, The Ohio State University, Columbus, OH
- 2016-2019 Residency, Farm Animal Internal Medicine, Veterinary Clinical Sciences, The Ohio State University, Columbus, OH

Publications

- VanHoy G, Carman M, Habing, G, Lakritz J, Hinds CA, Niehaus AJ, Kaplan RM, Marsh AE. Safety and serologic response to a *Haemonchus contortus* vaccine in alpacas. *Vet Parasitol.* 2018;252: 180-186.
- Brakel KA, VanHoy G, Hinds CA, Breitbach J, Premanandan C, Kohnken R. Peritoneal and scrotal carcinomas of unknown origin in two bovine calves. *J Vet Diagn Invest.* 2018;30(4): 609-613.

Fields of Study

Major field: Comparative and Veterinary Medicine

Table of Contents

Abstract.....	ii
Dedication.....	iv
Acknowledgements.....	v
Vita.....	vi
List of Tables.....	viii
List of Figures.....	ix
Chapter One	
REVIEW OF LITERATURE	
1.1 <i>Haemonchus contortus</i>	1
1.2 Anthelmintics and resistance.....	10
1.3 Management strategies and alternative control methods.....	13
1.4 Vaccination against <i>Haemonchus contortus</i>	15
Chapter Two	
MATERIALS AND METHODS	
2.1 Alpacas.....	17
2.2 Stalls and diet.....	18
2.3 Experimental design.....	18
2.4 Larval inoculation.....	19
2.5 Sampling.....	19
2.6 Packed cell volume, total protein and serology.....	20
2.7 Fecal egg counts.....	21

2.8 Statistical methods.....	21
Chapter Three	
RESULTS	
3.1 Alpaca health.....	22
3.2 Serology.....	22
3.3 Fecal egg counts and packed cell volume/total protein.....	23
Chapter Four	
DISCUSSION AND CONCLUSIONS.....	23
References.....	28
Appendix A: Tables and Figures.....	37

List of Tables

Table 1. Parameters and fecal flotation results of vaccinate and control alpacas.....	38
Table 2. P-values for Wilcoxon signed rank test of significance of antibody titer results.....	39

List of Figures

Figure 1. Experimental timeline.....	39
Figure 2. Experimental design flow chart.....	40
Figure 3. Mean fecal egg count in vaccinate and control alpacas.....	41
Figure 4. Antibody titer levels against H11/H-gal-GP in vaccinate alpacas.....	42

Chapter One

REVIEW OF LITERATURE

1.1 *Haemonchus contortus*

Parasite biology

Haemonchus contortus is one of the most significant current challenges facing the viability of the camelid and small ruminant industries globally. Known by its common name, “Barber’s pole worm”, this nematode parasite belongs to the order Strongylida and family Trichostrongylidae. Adults feed off blood from the host and reside in the abomasum of small ruminants and the third compartment of camelids, both analogous to the true stomach. Males are 10-20 mm in length and are tapered at the anterior end. Males also have a copulatory bursa and spicules with a barb at the end to hold the female’s genital opening during mating. These features can be uniquely identified from other genera in the family trichostrongylidae. Females are larger at 20-30 mm in length and have a white reproductive tract that is wrapped around the blood-filled intestine, giving it its characteristic common name (Sutherland et al., 2010; Soulsby, 1965; Zajac, 2006). Females may have a prominent vulvar flap tapered at the anterior and posterior ends. Both males and females have a small buccal cavity with a lancet-like tooth made of cuticle which is used to puncture the host in preparation for feeding (Soulsby, 1965, Zajac, 2006).

The life cycle of *H. contortus* is direct and the entire life cycle can take only 18-21 days in the optimal conditions. In sheep, the adults are found exclusively in the posterior portion of the fundus, close to the pylorus (Dash, 1985). Adults are attached to the mucosa of the 3rd compartment in camelids, and lay eggs which pass through the intestines and are deposited by the host in the fecal pellets. Females are fecund and can produce up to 10,000 eggs per day (Zajac, 2006). Heavily infected small ruminants and camelids can often be hosts for hundreds to thousands of adult *Haemonchus contortus*, leading to considerable pasture contamination and morbidity/mortality. The fecundity of *H. contortus* also permits rapid genetic

selection based on selective pressure and contributes to the production of *H. contortus* which are resistant to single or multiple classes of anthelmintics. Adults usually have a short life-span in the host and may persist a few months in the true stomach. Once passed, development of the ova continues in the fecal pellet, and the eggs hatch, molting to first stage larvae (L1). The fecal pellet, which is highly dehydrated in the small ruminant and camelid, provides a protective environment for the eggs to develop. The L1's feed on bacteria from the environment and undergo two additional molts to the third stage larvae (L3) (Zajac, 2006). During each molt, the nematodes will replace its cuticle, the semi-permeable tough outer layer which confers resistance to desiccation under inhospitable environmental conditions. In the L3 the residual L2 cuticle covers the buccal cavity and prevents the L3 from feeding. Thus, the L3 is dependent on metabolic reserves from previous stages. The warmer the environment the larvae are in, the higher their metabolic demand, and they will use the finite reserves more rapidly (2-3 months) (Zajac, 2006). In cooler conditions, the metabolism of the larvae will slow, and reserves may last much longer (6 months). Development of the larvae and migration out of the fecal pellet onto forage is highly dependent on environmental conditions and may take only days in the correct conditions. Egg development on pasture occurs rapidly in warm, wet climates, and at an ambient temperature between 50° F to 96° F. These conditions exist almost year-round in the southeastern United States, and the concurrent existence of a long grazing season increases the exposure of livestock and the risk of infection. Other parts of the United States are more seasonally affected by haemonchosis because the infective season is shorter, but other risk factors in more arid climates, such as the practice of flood irrigation, can contribute to egg development. The presence of high moisture content, or high rainfall, can help release the third stage larvae from the fecal pellet which would otherwise remain trapped in the dry pellet. The length of time required for egg development to the third stage larvae can have an impact on pasture rotation management. In hot, humid conditions where eggs may complete molting more quickly (minimum of 3-4 days), pastures can be rotated to match this minimum life cycle. In cooler environments, it may take months for eggs to

complete molting to third stage larvae (Levine, 1963). Likewise, frequent fecal removal is an excellent control method based on the minimum third-stage larvae appearance of 3-4 days. Once the L3 has migrated out of the fecal pellet, it migrates on herbage preferentially. The larvae's migration ability is again dependent on environmental conditions such as air temperature, soil moisture, and relative humidity, and most L3 are found on grasses no taller than 4-6 inches from the ground. Thus, allowing small ruminants and camelids to graze on grasses taller than 4-6 inches or browse will help to decrease haemonchosis infection. Light and regular rainfall in humid environments resulted in the most rapid emergence of larvae from feces, and a single rainfall event was found to be insufficient for larval emergence (Wang et al., 2014).

Once the L3 is ingested by the host, it must undergo exsheathment in order to molt to blood-feeding stages. This process has been extensively studied and has been found to depend on both changes in carbon dioxide concentration, as well as heat shock (rapid change in temperature) (Bekalaar K et al., 2018). These conditions are achieved in the forestomach of small ruminants, and the first and second compartments of camelids. Once exsheathment is achieved, larvae travel to the abomasum in small ruminants, or the third compartment in camelids, where they attach to the wall of the mucosa. Larvae quickly (1-2 days) molt into the fourth stage (L4) and grow rapidly as they feed on blood in the anterior portion of the fundus (Dash, 1985). Males and females become distinct during this stage; males have recognizable bursa and spicules, and females begin to develop ovaries and uterus. Progesterone produced during pregnancy can arrest larval development by blocking the molt from L3 to L4. This phenomenon may be responsible for the post-parturient rise, in which progesterone declines immediately prior to parturition, and the dam experiences a sharp rise in fecal egg counts due to sudden, massive larval development (Gutierrez-Amezquita et al., 2017). Between 9-12 days after ingestion, L4s undergo a final molt to the fifth stage (L5), otherwise known as the adult stage. The L5 female has a well-developed

uterus and ovary. *H. contortus* are considered adults when spermatozoa are readily visible in the males, and when the uterus becomes entwined around the gastrointestinal tract in females. Patent infection is achieved between 18 to 21 days after ingestion of the L3 from pasture (Soulsby, 1965).

Hypobiosis

Haemonchus contortus undergoes a phenomenon called hypobiosis in the host, like several other trichostrongylid nematodes (Zajac, 2006). This state of arrested development arises when environmental conditions do not favor development and survival of the larvae, such as cold winter months, or cool, dry months. The L4 larvae become metabolically inactive but remain attached or buried in the mucosa of the abomasum or third compartment. In this manner, *H. contortus* may “overwinter” within the host when environmental conditions would prevent development of the eggs and larvae on pasture. When environmental conditions do favor larval development and egg hatching, the arrested L4s continue molting in the host, and begin to produce eggs in the late winter/early spring. This subsequent rise in fecal egg counts generally coincides with parturition and will increase exposure risk of newborn small ruminants and camelids, especially if they are confined for parturition. Hypobiosis is less important in milder climates where warm, humid conditions exist almost year-round. The exact mechanism and triggers for hypobiosis in *H. contortus* is incompletely understood, but may include interactions between the parasite, the host’s immune system, environmental conditions, and genetics (Capitini et al., 1990).

Pathogenesis in the host

Clinical signs of haemonchosis are referable to chronic blood loss and include reduced growth and production, infertility, poor nutrient uptake and utilization, poor fiber quality, pale mucous membranes, and weight loss/ill thrift (Leguia et al., 1999). Animals with severe *H. contortus* infections may also display signs of hypoproteinemia, including edema under the mandible (“bottlejaw”), ventral abdomen, or

brisket area. Signs of acute or severe blood loss may be present in cases of massive infection, or hosts may become acutely decompensated when chronic anemia causes hemoglobinemia and hypoxia. Signs of hypoxia include tachycardia, tachypnea, and death. Low worm burdens may not result in significant clinical signs.

Chronic, low level blood loss is well-tolerated by small ruminants and camelids until hemoglobin levels become critical, and changes are reflected in the blood smear. Regenerative anemia is indicated by the presence of nucleated red blood cells, anisocytosis, basophilic stippling, or increased numbers of red blood cells with Howell-Jolly bodies. Non-regenerative anemia is characterized by microcytosis and hypochromia in the red blood cells and is often due to anemia of chronic disease (parasitism), or iron-deficiency anemia (Albers et al., 1990).

Within the host's gastrointestinal tract, parasite antigen is recognized by macrophages other dendritic cells in the true stomach, and act as antigen-presenting cells to T cells and a predominantly T helper 2 cell response is initiated (Montaner et al., 2014). Activated T helper cells secrete interleukin (IL)-5, which triggers local eosinophilia; both eosinophils and cytokines (IL-5, IL-4, IL-9, and IL-13) activate mast cells and basophils, causing inflammation and gastritis. IL-4 and IL-13 both act on myenteric cells and cause increased smooth-muscle contraction and gastric emptying rate. IL-4 and IL-13 also increase mucosal permeability and increase the stimulus for secretion of mucus by goblet cells. Activation of T helper 2 cells will encourage the development of adaptive immunity and confer at least partial resistance to haemonchosis with continued exposure to the parasite on subsequent grazing seasons (McRae et al., 2015). Breed-dependent differences in the humoral immune response, and heritability of these traits, is well-demonstrated in parasite-resistant sheep breeds (Shepherd et al., 2016; Garza et al., 2017).

Infection with *H. contortus* has a marked effect on microbial communities and diversity within the rumen and the abomasum in sheep, and likely have a similar effect in camelids which also have a complex forestomach. Research has shown that the presence of *H. contortus* rapidly causes necrotizing and inflammatory changes within the abomasum, and increases abomasal gastric pH, as well as increasing the abomasal bacterial load, and decreasing ruminal microbiome in both richness and diversity (El-Ashram et al., 2017). This has serious implications on the resistance of the abomasum to opportunistic secondary infections with bacteria or fungus, and on the interaction of altered microbial populations in the rumen with the animals' feed.

Diagnosis in the host

Diagnosis of *H. contortus* in the host can be achieved several ways. Feces can be collected and processed using centrifugation-flotation techniques, and trichostrongylid ova are most commonly detected using this method. Classification of eggs using the fecal flotation method cannot identify eggs beyond the *trichostrongyle* family level, and advanced staining techniques must be employed in order to identify the genus and species. Staining of eggs with fluorescein-labeled peanut agglutinin has shown that the lectin correctly identifies the percentage of *H. contortus* eggs compared to other trichostrongylid eggs (Palmer et al., 1996). Modified Stoll's or McMaster's egg counting techniques can be used to quantify exact fecal egg counts and monitor improvement after anthelmintic use, or resistance to anthelmintic use. Feces can also be collected and coprocultures can be utilized, then larval stages can be identified using morphologic appearance (Thienpont, 1981). If animals die or are sacrificed, larval and adult stages can be identified on necropsy examination of the abomasum or third compartment.

Distribution and importance

Haemonchus contortus is found throughout the world and causes significant economic losses in climates with high rainfall and warm temperatures (tropics and subtropics) but can also be found in more temperate climates such as the United States. In the southeastern United States, *H. contortus* is a more abundant pathogenic gastrointestinal nematode because of the warm and humid environment (O'Connor et al., 2006). Increases in temperature as a result of global warming will continue to have impacts on the abundance and distribution of gastrointestinal nematodes by creating more favorable environments for development and release of infective stages (Morgan et al., 2009; Jenkins et al., 2006). Climate change will also continue to have effects on the spatio-temporal distribution of *H. contortus* infection risk (Rose et al., 2016) *Haemonchus contortus* thrives best in environmental conditions between 88° F and 93° F (O'Connor et al., 2006), but can be found in temperatures as low as 50° F. Eggs do not hatch below 48° F, and only have a finite amount of metabolic reserves to survive in the environment. As stated before, *H. contortus* prefers humid climates, but can cause problems in drier climates if the pastures are irrigated (western United States).

Production losses, coupled with the cost of diagnosis and treatment, as well as labor involved in the dispensing of treatment easily makes haemonchosis one of the most important diseases in both the small ruminant and camelid industries in the United States (Zajac, 2006). In addition to the disease itself, widespread anthelmintic resistance to multiple classes of anthelmintics is increasing the cost of treatment and diagnosis, as well as increasing morbidity and mortality in livestock populations (Muchiut et al., 2018).

Camelids

South American camelids belong to the order Artiodactyla and the suborder Tylopoda. They are heavily fleeced, herbivorous, even toed ungulates with soft pads on the bottom of the feet, long, slender necks and

legs (Fowler, 1998). They are normally found in the high mountains of the Andes in South America but have been imported into the United States as fiber animals since the 1980s. South American camelids have several physiologic adaptations which allow them to survive in the harsh climate and high altitudes of the Andes mountains. They have a complex forestomach which is comprised of three “compartments”, instead of the four compartments, the rumen, reticulum, omasum, and abomasum, found in true ruminant species such as sheep, goats and cattle. Camelids regurgitate and rechew forages but are more efficient at extracting protein and carbohydrates than ruminants. The first compartment (C1), has glandular mucosa arranged in sacculs which secrete large amounts of bicarbonate, and non-papillated, stratified squamous epithelium in non-glandular regions. The sacculs of C1 undergo regular eversion, which mix and grind the contents into a moist, finely minced roughage. C1 is not generally organized into distinct layers (gas cap, fiber mat, finer particles and fluid) like the rumen. Rather, the camelid C1 has more homogenous contents. The glandular region of C1 is capable of rapid absorption of solutes and water and has a higher pH and slower emptying rate than true ruminants. The second compartment (C2), has sacculs, glandular and stratified squamous epithelium like C1, but the sacculs do not evert, and contents are more liquid. The third compartment (C3) is lined entirely with glandular mucosa and is considered the true stomach, analogous to the abomasum in ruminants. Forestomach motility is continuous (3-4 mixing cycles per minute) and the C3 emptying rate is much slower in camelids than in ruminants. This causes much longer gastrointestinal transit times for camelids compared with true ruminants, allowing for breakdown of poor-quality forages more extensively.

In addition to gastrointestinal tract adaptations, camelids have evolved specialized red blood cells in order to thrive in high altitude environments (Yamaguchi et al., 1987; Jurgens et al., 1988; Fowler, 1998). High altitude camelids (llamas, alpacas, guanacos, vicunas) have smaller, more elliptical erythrocytes which can pass through tighter capillaries more easily and have a higher resistance to oxidative damage. Smaller

red blood cells allow improved loading/unloading of oxygen due to a relatively high oxygen transfer conductance. South American camelids also have increased capillary density as compared to true ruminants and low-altitude camelids (dromedaries), which helps to decrease the diffusion distance in muscle tissue. In addition, the erythrocytes have a much higher blood oxygen affinity and shift their oxygen-hemoglobin dissociation curve more efficiently to the left than low-altitude species, allowing for increased ability to tolerate low oxygen tension environments such as high altitude (Jurgens et al., 1988). Compared with true ruminants and low-altitude camelids, South American camelids tend to have a lower hematocrit, but this is compensated for by all the aforementioned adaptations. These adaptations have major implications for the South American camelids' ability to disguise anemia caused by *H. contortus* until critical hemoglobin levels are reached and decompensation occurs. In addition to their ability to tolerate low oxygen tension (as in the case of anemia due to parasitism), South American camelids are heavily fleeced, and can disguise profound loss of body condition associated with gastrointestinal parasitism (Fowler, 1998). These factors have major implications on the ability of an owner or veterinarian to diagnose anemia based on non-laboratory methods such as the use of the FAMACHA© system (discussed later) and visual body condition scoring, in order to recognize animals who may require treatment for gastrointestinal parasites.

Camelids have evolved an interesting immunoglobulin adaptation which allows for the production of both a canonical antibody, as well as a smaller heavy-chain only antibody (Daley et al., 2005). These heavy-chain antibodies (HCAbs) constitute 50% of the camelids' IgG and are prolate in shape. The antigen-binding fragment of the homodimeric HCAb is a single domain (the variable domain) which has been found to bind tightly and specifically to antigen (Flajnik et al., 2011). The HCAbs possess a wide variety of loop lengths and loop structures which lead to a drastically expanded repertoire of available antigen binding sites that are capable of accessing more complex epitopes, unlike canonical heterodimeric antibodies which are

larger and more structurally complex. The emergence of HCABs in both sharks and camelids is interesting and may suggest that the immune systems of these evolutionarily divergent species were under considerable stress, driving the selection of a more diverse array of antibody structure. The efficiency of HCAB binding to antigen, and their relatively smaller, more physicochemically robust form compared to traditional heterodimeric IgG may give camelids an advantage in their ability to respond to pathogens, as well as to vaccination, and is currently being widely exploited in biomedical engineering for therapeutic applications.

1.2 Anthelmintics and resistance

Anthelmintics

Camelids that are assessed by either the owner or the veterinarian for the clinical signs of haemonchosis and who are deemed in need of treatment, are given anthelmintics. Currently, there are no approved anthelmintics for camelids in the United States and the use of commercial products is considered extra-label use in a minor species. In the late 1950s, early 1960s the first cholinergic agonists were introduced to the market in the United States. They were rapidly effective, relatively safe, and cheap, which led to the beginning of an era in livestock management that relied heavily on a chemo-dominant approach to the management of gastrointestinal parasites (Sangster et al., 1998). There are currently three classes of anthelmintics in use for the control of gastrointestinal nematodes: benzimidazoles, macrocyclic lactones, and cholinergic agonists (Zajac, 2006).

Benzimidazoles

This anthelmintic class, introduced in 1961, includes the drugs albendazole and fenbendazole and are considered broad spectrum (Zajac, 2006). These drugs bind to and interfere with β -tubulin and inhibit the production and assembly of microtubules (Sutherland et al., 2010). As a consequence of the failure of

microtubule formation, the transport of secretory granules and enzyme secretion within the cytoplasm as well as glucose uptake by the parasites' cells is halted. The parasite rapidly exhausts its glycogen stores in this state and becomes paralyzed. Paralysis leads to inability to take up a blood meal and release of the parasite from the host. Benzimidazoles are not easily absorbed by the patient, and are not readily water soluble (Caffrey et al., 2012). In order to slow gastrointestinal transit time and increase exposure of the parasites to the anthelmintic, thus increasing absorption, animals can be held off feed for 12-24 hours before administration of benzimidazoles (Zajac, 2006).

Cholinergic Agonists – Imidazothiazoles and tetrahydropyrimidines

Anthelmintics in this class include levamisole and pyrantel which both act at acetylcholine receptors in the parasite (Sutherland et al., 2010). Binding to nicotinic acetylcholine and opening acetylcholine-gated ion channels in the parasite's muscle produces depolarization and muscle contraction leading to spastic paralysis. Like benzimidazoles, paralysis of the nematode propagates worm detachment from host mucosa.

Macrocyclic lactones

This class of anthelmintics include the drugs ivermectin and moxidectin. Their mode of action centers on the drug's ability to modify chloride channel neurotransmission within the outer membrane layer of glutamate-gated chloride ion channels in the nematode (Zajac, 2006). Modification of neurotransmission is achieved by allosteric modulation that increases the opening of glutamate-gated chloride ion channels in the pharynx and neurons of nematodes like *Haemonchus contortus*. Constant opening of the pharynx inhibits body movement and pharyngeal pumping, effectively starving the worm and causing paralysis. *H. contortus* and ivermectin have been extensively studied as a model for anthelmintic resistance (Gilleard, 2013), and major strides are being made toward the recognition of a locus in the

glutamate-gated chloride channel using gene mapping and whole genome sequencing which may aid in alternative control methods in the future (Doyle et al., 2019).

Anthelmintic resistance

Current treatment protocols rely on repeated use of anthelmintics but widespread, multi-drug resistance to anthelmintics make treatment challenging (Howell et al., 2008; Vidyashankar et al., 2012). Cheap, effective and safe chemotherapeutic options for treatment of trichostongylids in livestock encouraged their heavy use, and especially their use on a herd-wide basis instead of targeting specific animals based on the presence of clinical signs of gastrointestinal nematodiasis. In addition, frequent or chronic under-dosing of livestock has led to the selection of *H. contortus* resistant to the widely used anthelmintics, and often times require 2-10 times the dosage amount in order to be effective. In spite of new drug therapies marketed outside of the USA, resistance to newer drugs, amino-acetonitrile derivative marketed as Monepantel, is now documented (within 2 years of drug approval) (Besier et al., 2012; Van den Brom et al., 2015).

Diagnosis of anthelmintic resistance can be accomplished either *in vivo* or *in vitro*. *In vivo* methods rely on either the differential worm counts in the stomach (abomasum or C3) between treated and non-treated animals at necropsy, or a fecal egg count reduction test which compares pre- and post-treatment samples (Woodgate et al., 2017). *In vitro* methods include the egg hatch assay (specific for benzimidazole resistance), and larval development assay (detects benzimidazole, macrocyclic lactone, amino-acetonitrile derivative, and cholinergic agonists resistance) (Muchiut et al., 2018). *In vitro* methods also include a larval migration inhibition assay and the larval feeding inhibition assay which both detect some, but not all drug class resistance (Muchiut et al., 2018). As mentioned previously, genomic studies targeting specific genes involved in benzimidazole resistance (glutamate-gated chloride ion channels and

cytochrome P450 subtypes), are increasingly being used but are impractical in an on-farm situation (Laing et al. 2015).

1.3 Management strategies and alternative control measures

Management strategies

As a consequence of anthelmintic resistance, parasite control must rely on an integrated management approach. Basic pasture management which utilizes the life cycle and biology of *H. contortus* such as pasture rotation, co-grazing of ruminants and camelids, and managing stocking densities and high-traffic congregation areas such as dung piles, raised feeders, lick tubs, and water sources, helps to decrease the risk of infection by decreasing the presence of eggs and larval stages. Feeding moderate and high levels of dietary protein to at-risk populations can protect against body condition loss as well as provide resilience against the development of haemonchosis (Khan et al., 2017). The development of a National Sheep Improvement program has greatly improved the propagation of heritable traits that may confer resistance to parasite infection. By collecting large amounts of data including fecal egg counts, “estimated breeding values” (EBVs) are generated and allow the industry to measure and track heritable traits. This program has been successful in sheep and may represent another useful tool in the improvement of parasite-resistant traits in camelids as well. Specific knowledge about environmental conditions which favor larval development, and the epidemiology of the disease are crucial to the application of sustainable management strategies which rely on the prediction of infection risk (Besier et al., 2016).

Refugia is another technique used in sustainable parasite control strategies (Muchiut et al., 2018). The concept of refugia centers around two populations of *H. contortus*: that which exists as free-living stages in the environment, and the infrapopulation that exists in the host. If a portion of parasites that are susceptible to anthelmintics are maintained, then anthelmintic resistance will develop more slowly than if

a larger population of resistant *H. contortus* exists (Martin et al., 1981). If the population in refugia is already resistant, as is the case in many herds in the southeastern United States, then susceptible refugia may be recovered by using effective anthelmintics or by pasture resting. Then, the population can be replaced by releasing animals infected with susceptible parasite onto the pasture before levels of pasture infectivity start to rise to maximize establishment of the susceptible refugia (Muchiut et al., 2018).

Although this process is complicated, labor intensive, and involves intimate knowledge of *H. contortus* life cycle patterns for the region as well as comprehensive testing, it may be the only option available for some producers.

Another method used to identify and treat clinical animals in a population is the use of the FAMACHA© system. The FAMACHA© system aims to train producers and veterinarians in identifying the level of anemia primarily in sheep and goats and create cut-off values for treatment. In this way, only animals unable to cope with worm burdens will be treated, and refugia of susceptible worms will be maintained in untreated animals (van Wyk and Barth, 2002). The validity of this system was investigated for camelids specifically, since they possess unique oxygen-hemoglobin dynamics compared to low-altitude species and may compensate for anemia for a longer period of time (Storey et al., 2017). Cut-offs were found to be similar between camelids and small ruminants for detecting anemia and correlated with higher eggs per gram values.

Alternative control methods (non-anthelmintic)

In addition to anthelmintics and management strategies, there has been considerable research into alternative control methods against *H. contortus*. Copper oxide wire particles are currently used in sheep and goats and are administered orally. The copper passes from the rumen to the abomasum and is released free in the abomasum. The increased concentration of copper in the ingesta interferes with the attachment

of *H. contortus* adults and causes detachment (Burke et al., 2004). Several studies show decreased worm burden and decreased fecal egg counts in treated animals, but effects are short-lived and there is a concern for copper toxicity in repeatedly treated animals (Burke et al., 2004). The addition of feed supplements with condensed tannins and flavanoids have shown promise as adjunctive therapies. Tannins and flavanoids have been shown to prevent exsheathment of *H. contortus* L3s (Klongsiriwet et al., 2015). The nematode-trapping fungus, *Duddingtonia flagrans*, has also been studied as a biological control agent (Terrill et al., 2004). This fungus is fed as a supplement and survives the gastrointestinal tract environment well. After being passed in the feces, the fungus traps larval stages within the feces and prevents their dispersion onto vegetation, effectively reducing infective doses to grazing animals on pasture. Current work is also being done to characterize the endosymbiont bacteria that are associated with *H. contortus* eggs, L3s, and adults. The bacteria share a symbiotic relationship (either commensal or mutualistic) with the host parasite, and participate in host energy metabolism (Sinnathamby, 2018). These endosymbionts could represent a potential target for novel control methods against *H. contortus* development and survival.

1.4 Vaccination against *Haemonchus contortus*

It is clear that a holistic approach merging several management factors, alternative control methods and judicious anthelmintic use will be needed in order to decrease the incidence and severity of haemonchosis in the small ruminant and camelid industries, but a novel approach based on the development of a vaccine which supplement's the host's resistance to *H. contortus* shows promise (Bassetto et al., 2014). The exploited vaccine antigens are the H11 and H-gal-GP protein complexes found within the parasite's intestinal mucosa involved in the digestion of blood as taken up by *H. contortus*. These nematode antigens reside on the mucosal membrane of the parasite's gastrointestinal tract and as such, are not available to immunize the host to these antigens

during parasite infection. The vaccine antigen immunizes the host to these worm antigens so when worm blood feeding occurs, the ingested antigen specific antibodies binds to the worm's functional proteins on the brush border of the worm's intestinal cells and compromises its digestive processes, leading to worm starvation, loss of fecundity and eventual detachment (Smith and Zarlenga, 2006). Because these antigens are "hidden" to the host's immune responses, there is no significant anamnestic response achieved in natural infections or exposures (LeJambre et al., 2008).

Sheep and goat antibodies to the H-gal-GP complex neutralize the function of this multi-protease complex and reduce the adult parasite's ability to survive. To date, this vaccine-based technology lacks controlled evaluation in camelids. If this technology meets these two criteria, 1) safe in camelids and 2) stimulates antibodies specific to *H. contortus* H-gal-GP complex which disrupts the parasite's feeding, the vaccine could provide an adjunctive option for the control of haemonchosis, thereby limiting the use of anthelmintics. Animals in prior studies still became infected with *H. contortus*, but the vaccine demonstrated decreased fecal egg shedding, decreased pasture contamination and decreased total worm burdens (Besier et al., 2012; Bassetto et al., 2014; Besier et al., 2012; Meier et al., 2016).

The *Haemonchus* vaccine (Barbervax®) is receiving new attention because of several recent breakthroughs in its research: 1) discovery that native antigen in lower concentrations will stimulate protective immunity and 2) improvements in parasite antigen production, allowing for commercially viable yields for vaccine production (Daley et al., 2010; Bassetto et al., 2014).

Evaluation of the humoral immune response in alpacas vaccinated with other vaccines (West Nile, Bluetongue and *Clostridium perfringens*) suggest that alpacas respond with a predictable humoral response in comparison to other species (Bentancor et al., 2009; Muyldermans et al., 2009; Zanolari et al., 2010). These studies have shown that antibody titers measured post-vaccination are correlated with decreased disease in these populations (Bentancor et al., 2009; Muyldermans et al., 2009; Zanolari et al.,

2010). One of the drawbacks of the Barbervax® vaccine is the potential lack of anamnestic response when the alpaca acquires *H. contortus* parasites through grazing on infected pastures. Our results demonstrated the vaccine's safety in a small number of alpacas, and its ability to cause significant antibody titers in alpacas. However, without the successful establishment of the experimental challenge infection providing measurable differences in eggs per gram (EPG) or clinical parameters, the study results cannot address the vaccine's efficacy to lessen clinical disease in alpacas associated with *H. contortus* infections (Wood et al., 1995).

Chapter Two

MATERIALS AND METHODS

2.1 Alpacas

A randomized, preventive challenge trial was performed with 12 young alpacas (Sargeant et al., 2010). The Ohio State University's Institutional Animal Care and Use Committee approved all animal husbandry and experimentation protocols (2016-5). Fig. 1 shows the timeline of protocol activities and Fig. 2 diagrams the experimental design. Twelve weaned alpacas: seven intact males (5 Suri, 2 Huacaya), five females (4 Huacaya, 1 Suri) between 4–6 months of age (Table 1) were acquired from a local alpaca farm. This farm was chosen as a source of animals for this study since they maintain precise records on their animals, routinely perform fecals on their individual animals and deworm animals based upon the advice of their farm veterinarian. The 12 animals chosen for study were selected based upon FEC prior to onset of study, age, and farm management scheme. The alpacas were housed at the Hospital for Farm Animals at The Ohio State University Veterinary Medical Center. Upon acquisition, physical examination, body weight, fecal, and blood samples were taken to evaluate the health status of each alpaca.

2.2 Stalls and diet

Animals were kept in straw-bedded indoor pens (cleaned daily) with no access to pasture. Alpacas were grouped into 3–4 animals per pen, segregated by gender. The animals were allowed to acclimate to their new environment and diet for a period of one week prior to initiation of the experimental protocol. Grass hay was fed ad libitum in a hay feeder with metal retainer to avoid ground contact of feed. The alpacas were also given approximately 0.225 kg per animal of a commercial, 14% crude protein supplemental alpaca crumble¹ twice daily. The animals had access to free choice minerals, and each alpaca received 240,000 IU vitamin A, 24,000 IU vitamin D and 300 IU vitamin E as 6 g of oral paste² every ten days.

2.3 Experimental design

Alpacas were assigned to vaccinate (n = 7) and non-vaccinate (n = 5) groups based on simple randomization. The lead investigators were blinded to group assignment. Two animals within the group to receive the Barbervax® vaccine were used to test the safety of the vaccine prior to the start of the full study. Each alpaca received 1 mL of Barbervax® subcutaneously in a shaved 6 cm x 6 cm location in the distal cervical region. The alpacas were monitored for elevation in rectal temperature, injection site reaction, and other adverse reactions of vaccine administration for 3 days. Thereafter, the two animals that were vaccinated initially to verify vaccine safety were continued in the study time line and were included in the “vaccine group” with the sampling and immunization dates corresponding with the remaining 10 alpacas enrolled in the study. Five additional alpacas were immunized with 1 mL Barbervax® vaccine subcutaneously in the distal cervical region on day 0 (Fig. 1). All vaccinates were administered a second dose on day 21 (the two safety animals received 24 days after their first immunization), a third dose on day 42 (the two safety animals 45 days after their first immunization). Five non-vaccinated alpacas remained as negative controls.

2.4 Larval inoculation

All vaccinated and non-vaccinated alpacas were inoculated with approximately 1500 viable third stage larvae (L3, UGA 2004 isolate) via orogastric route on days 42, 46, and 50, for a total of approximately 4500 larvae per animal. These larvae were previously harvested from fecal coprocultures using feces from a goat experimentally infected with *H. contortus* L3. Larvae were stored initially at 4°C for 24 hours, and then at 10°C. On the day of inoculation, viability was assessed by larvae motility. To further ensure viability of the *H. contortus* L3 inoculum to cause a patent infection, a single *H. contortus* naïve lamb (28 days old) without prior pasture exposure was used. The lamb received a total of 2500 third-stage larvae divided as two doses 5 days apart. These larvae had been stored at 10°C for an additional 46 days, following the alpaca dosing. Fecal eggs counts were performed weekly until terminated. A necropsy was performed on the lamb to recover but not quantify the adult worms present.

2.5 Sampling

Prior to the start of the experiment, peripheral whole blood was collected from the jugular vein from each alpaca. Packed cell volume (PCV), total serum protein, complete blood count (CBC), and serum biochemistry profiles were performed. Subsequent blood samplings were performed according to the protocol timeline (Fig. 1) following initial vaccination and weekly following the third oral inoculation with *H. contortus* L3 larvae. PCV and total protein (TP) were monitored weekly, CBC and serum biochemistry profiles were performed prior to vaccination, 1 month post-vaccination, and 3 months post-vaccination. Serum was separated and stored at -20°C from all time points for later analysis. Rectal temperatures were monitored on the day of vaccination and daily for 3 days post-vaccination, and the animals were observed daily for clinical and behavioral abnormalities. Individual fecal samples were collected directly from the rectum twice weekly. Body weights were acquired twice weekly throughout the study period.

2.6 Packed cell volume, total protein and serology

Packed cell volume and TP were measured within 30 minutes after blood sampling using a microcentrifuge and refractometer. Samples were collected in an EDTA tube and transferred to a microhematocrit tube. The microhematocrit tubes were spun in the same centrifuge for 5 minutes, then the PCV and TP were evaluated. An indirect ELISA was performed using the Barbervax® intestinal cell native enzymes supplied by Moredun Research Institute, Scotland, UK as the antigen in carbonate-bicarbonate buffer (pH 9.6) (Bassetto et al., 2011; Bassetto et al., 2014). Following overnight plate coating at 4°C, the plates were rinsed with Tris buffered saline with 0.1% Tween 20 (TBS-T). Superblock T20 (TBS) blocking buffer (Thermo Scientific, Waltham, MA, USA) was applied to the wells for 1 hour at room temperature. All sera were diluted in 1% bovine serum albumin in TBS-T (BSA-TBS-T) and applied in triplicate wells for 1 hour at room temperature. A dilution series was prepared for each of the samples by doubling dilution in the BSA-TBS-T from 1:200 to 256,000. Control sheep (pooled vaccinated sera and non-vaccinated sera) were supplied diluted 1:200 as positive and negative controls, respectively. For the assay, 50 µl of each serum dilution was added to the prepared ELISA plate and incubated for 1 hour at room temperature. After the sera incubations, wells were extensively washed with TBS-T followed by the addition of horseradish peroxidase-conjugated secondary antibodies. For the alpaca samples, the assay used anti-llama IgG (H&L) HRP (Bethyl Laboratories Inc., Montgomery, TX, USA) diluted 1:40,000 in BSA-TBS-T. To determine the optimal dilution of the anti-llama-HRP, serial dilutions (1:10,000 to 1:80,000) of the secondary were tested against serial dilutions (1:200 to 1:256,000) of a pre- and post-vaccinated alpaca to establish a linear curve for the sample dilution. For the sheep samples monoclonal anti-goat/sheep IgG-peroxidase conjugated antibody (Sigma-Aldrich, St. Louis, MO, USA), diluted 1:10,000 in BSA TBS-T was used. Secondary antibody applications were incubated for 1 hour at room temperature. All 1-hour incubations were performed on a DPC MicroMix 5 microplate shaker (Euro/DPC, Gwynedd, Wales, UK). Addition of 50 µl of SureBlue TMB Microwell Peroxidate

Substrate 1 Component (KPL, Gaithersburg, MD, USA) was used for color development. The reaction was stopped with the addition of an equal volume of 0.1 N HCl. Absorption was measured at 450 nm. Reagent background control included TBS-T. Titers were calculated as previously described (Bassetto et al., 2011) and compared for Day 0, 21, 42, 56 and 91.

2.7 Monitoring fecal egg counts

Fecal egg counts (FEC) were performed using a variation in the modified Stoll's test with a sensitivity of 5 eggs per gram (EPG) (Zajac and Conboy, 2012). Samples were weighed into 2 gram aliquots, added to 98 ml of water and allowed to disperse overnight at 4°C. Ten ml of the well-mixed suspension was centrifuged, followed by the flotation of the pelleted material in a sugar flotation media (specific gravity 1.2) using centrifugation. A coverslip was placed on the centrifuge tube in contact with the flotation solution for 10 minutes before removing to enumerate the strongyle ova and identify other parasite ova present under the coverslip. The strongyle values are given in EPG.

2.8 Statistical methods

All analysis was undertaken in SAS (SAS v.9.4, Cary, NC). Variation in PCVs and TPs were analyzed using generalized estimating equation, with the individual alpaca included as a repeated effect, and day of trial, treatment (vaccine versus control), and their interaction included as fixed effects. Similarly, weight gains were analyzed using a generalized estimating equation, with the individual alpaca included as a repeated effect, and day of trial, treatment (vaccine versus control), and their interaction included as fixed effects. The FEC was non-normally distributed with many zero values, and so each sample was dichotomized as either positive ($FEC > 0$) or negative ($FEC = 0$), and a logistic regression model was instead used. Alpaca was included within the repeated statement to control for repeated sampling of alpacas over time. Antibody titers were log-2 transformed, but were nonetheless not normally distributed, based on a visual inspection of the frequency distribution. Therefore, differences between vaccinates and controls on individual days was evaluated using a Wilcoxon signed rank test. Likewise,

changes in titers among vaccinates were compared between Day 28, 49, 56 and 91 using the Wilcoxon signed rank test.

Chapter Three

RESULTS

3.1 Alpaca health

Seven days prior to the start of the study fecal samples from 15 cria were transported to OSU CVM and quantitative FECs were < 5 EPG for all cria with 1 cria having 5 EPG. Twelve study cria were provided from this group of 15. On day -1, 1 of the 12 selected cria had 5 EPG and the remainder were < 5 EPG, and on the day of the start of the trial all cria had < 5 EPG. Other parasites seen in the study animals' fecal exams included *Eimeria macusaniensis*, < 10 oocyst/gram of feces in 4/12 cria (2 control, 2 vaccinates), one cria having 35 EPG *Moniezia* on day of first vaccination (vaccinated cria). *Eimeria macusaniensis* was the only coccidia identified using the Modified Stoll's procedure which represented 0.2 g of fecal sample analyzed, and the cria never exhibited clinical signs of coccidiosis. All alpacas remained healthy throughout the study period based on CBC and serum biochemistry, as well as clinical observation. Vaccinates exhibited an increased rectal temperature beginning 8 h post-vaccination which persisted for 2–3 days (up to 41.1°C), then returned to normal over 2–3 days (reference range 37.5°–38.9°C) (Cebra, 2014a). A slight decrease in activity accompanied the pyrexia, but appetite and hydration were unchanged during the febrile episode. No injection site reactions were observed. All alpacas gained weight through the study period, and the weight gain was not different for vaccinates relative to controls ($p = 0.35$).

3.2 Serology

All vaccinates exhibited rising antibody titers out to day 56 and then decreased (Fig. 4). The non-vaccinates remained at baseline levels (titer < 1:400) throughout the time points evaluated. The vaccinated alpaca antibody titers were highest between days 49 and 56. On day 56 titers ranged from

1:51,200 to 1:204,800 and then began to decrease. Of the vaccinated alpacas, one with the highest measured titer on day 28, remained the highest on days 56 and 91. At day 91 all vaccinates retained measurable titers with the mean titer of 1:36,571 (range 1:25,600–1:102,400) and the negative controls remained at background levels ($< 1:400$). With the exception of the titers on day 91, all dates tested were associated with significantly greater titers compared to the previous date (Table 2).

3.3 Fecal egg counts and packed cell volume/total protein

The FECs remained very low (under 70 EPG) in both vaccinates and controls throughout the study period (Table 1), but both groups exhibited increasing FECs over time (Fig. 3). Vaccination was not significantly associated with a lower FEC ($p = 0.1$). There were no significant differences in the PCV between groups or over time ($p = 0.22$). There was a significant decrease in the mean TP over time of both groups ($p = 0.02$); however, the decline in TP was not associated with vaccination status of the alpacas ($p = 0.52$). Additional findings observed on fecals from both groups included other parasite ova such as *Nematodirus* sp., *Trichuris* sp., *Moniezia* sp., and *E. macusaniensis* at low levels. The inoculum challenged lamb began to show positive FECs and by day 27 after receiving the first L3 dose the count was 840 EPG. At necropsy, 43 days after receiving the larvae challenge, the lamb's abomasum contained easily visualized adult *H. contortus*.

Chapter Four

DISCUSSION AND CONCLUSIONS

The first aim of the study was to demonstrate the safety of Barbervax® vaccine in young, healthy alpacas. Despite an increase in rectal temperature and decrease in activity level, all vaccinated cria maintained appetite and hydration following vaccination. No adverse injection site reactions were noted. While the vaccine was administered adjacent to the prescapular lymph node in all vaccinated animals, no swelling, heat, pain or discharge was evident at these sites at any time post-vaccination. Furthermore, as the hay provided to these animals during the study was in feed buckets with a metal

retainer and the animals had to ventroflex their cervical spine to intake hay and crumbles, there was no evidence that the vaccination sites were associated with discomfort significant to decrease intake. These findings concur with similar studies performed in goats where vaccine site reactions associated with the Barbervax® vaccine were not observed (Meier et al., 2016). This study and prior studies in goats are in sharp contrast to precautions on the label for Barbervax® usage in sheep. The label approved for this commercial preparation warns that the vaccine may produce a swollen, painful local tissue reaction that persists up to 17 days post-vaccination (Barbervax® label, barbervax.com.au). A lack of local tissue reaction in alpacas may be due to a differential response to the saponin-based adjuvant or to the vaccine antigens (H11/H-gal-GP) themselves. The second aim of the study was to determine whether alpacas responded by producing H-gal-Gp specific antibodies as has been observed in sheep and goats. Alpacas responded to vaccination with increasing antibody titers. In one study where young goats were vaccinated with Barbervax® and experienced natural pasture infection, data indicated that high antibody levels alone were not protective against *H. contortus* infection with the highest antibody titers reaching 1:24,000, after 6 vaccinations, and decreased thereafter (Meier et al., 2016). In another study of naturally challenged and experimentally infected (*H. contortus* L3) dairy goats vaccinated with Barbervax®, antibody titers were correlated with reduced worm burdens (De Matos et al., 2017). The highest antibody titers measuring 1:42,000, occurred after 6 vaccinations, and decreased over time in subsequent samplings (De Matos et al., 2017). The third aim was to correlate the evaluated vaccine-associated antibody titer with a protective effect against *H. contortus* infection and development of abnormal clinical parameters associated with the parasite's presence. Unfortunately, adequate *H. contortus* infection as measured by EPG and PCV was not achieved in our non-vaccinated group and thus we cannot make conclusions about the protective value the H-gal-Gp specific antibodies provide in reducing *H. contortus* infection in alpacas. Several factors may have played into the alpacas' resistance to experimental infection. As this was a challenge trial and not a field trial, the cria were housed inside in stalls with regular removal of feces. This

husbandry mitigated the risk of L3 acquisition from the environment during the study period. Additional natural exposure to L3 acquired through grazing may have been a factor in other studies of goats and sheep when EPG demonstrated significant levels between vaccinates and unvaccinated animals and when the study period included animals kept on pasture (Bassetto et al., 2014; Besier et al., 2012; Meier et al., 2016). Healthy camelids designate a dung pile on which they urinate and defecate, and this is separate from where they eat (Cebra, 2014b). For this reason, the elimination behaviors of camelids provided with adequate physical space may play a protective role in their resistance to *H. contortus* infection.

Hypobiosis may have played a role in the lack of significant *H. contortus* infection in the cria, due either to the inhibition of L3 stages induced during storage or the environmental conditions present in the building these animals were housed in. Hypobiosis is a well-described phenomenon of nematode parasites, allowing persistence of the parasite when environmental conditions (temperature and humidity) are not favorable (Besier et al., 2016; Gibbs, 1986). The cria in the present study were also housed indoors during the months between November and March. Although the cria were in relatively consistent ambient temperature ($21.1^{\circ} \pm 3^{\circ}\text{C}$), environmental conditions may have favored the hypobiosis of *H. contortus* over this time period. Since this was not a terminal trial, we were unable to assess the immature or adult worm burden or the proportion of male to female worms in the alpacas' third compartment (C3), or true stomach. While our L3 were prepared according to published protocols, it is possible that storage may have decreased viability to varying degree. However, in our study, the lamb was challenged with the same L3 inoculum as administered to the alpacas. The lamb demonstrated a fairly predictable rapid increase in FECs and viable adult worms were recovered at autopsy. This suggests the inoculum was viable when administered to the alpacas. Another factor leading to the failure of establishing experimental infection may be the unique resistance of some camelids to *H. contortus* parasites compared with sheep and goats. In a previous unpublished experimental infection trial, sheep and alpacas were administered trickle and bolus dosing of 20,000–50,000 infective *H. contortus* L3/ animal with FEC, PCV, body

weight and condition scores evaluations performed weekly to the end of the 49-day trial (Casey, S.J., “*Haemonchus contortus* infections in alpacas and sheep.” Master’s thesis, Virginia Polytechnic Institute and State University, 2014; available at https://vtechworks.lib.vt.edu/bitstream/handle/10919/48421/Casey_SJ_T_2014.pdf?sequence=1). Total worm burden and pH of the abomasa and C3 were determined in all animals at autopsy. The data collectively show that alpacas were less susceptible than sheep to *H. contortus* experimental infection, with lower FECs and lower total worm burdens within the "true" stomach after euthanasia. This previous study indicated there may be many, as of yet unexplored reasons for this difference. One reason may be physiologic differences in healthy alpaca C3 as compared to the abomasum of sheep. The normal abomasal pH of sheep is 2–3 (Nicholls et al., 1987); whereas, the reported pH of alpaca C3 is between 0.8 and 2.8 (Smith et al., 2010). In contrast, the pH of the first compartment of alpacas is lower than the rumen of sheep (Robinson et al., 2013). This variation in pH may be due to differences in the mucosal lining, proportion of first compartment liquid volume or other still undefined reasons (Fowler, 2010). Altered production of acid may increase the pH of the true stomach, allowing parasites to emerge from the protective mucus layer of the stomach, promoting release and removal of fourth stage larvae and adults (Sutherland and Scott, 2010). This supposition is further supported by the knowledge that experimentally modifying the abomasal pH of ruminants, the worm burden is maintained in a lower pH environment than when the abomasal pH increases > pH 5 (Hall and Oddy, 1984; McKellar et al., 1986). Furthermore, abomasal and C3 motility patterns differ significantly from each other. The present study was not designed to evaluate whether the alpaca’s susceptibility was due to differences in gastrointestinal physiology or other factors. In a study validating the FAMACHA© scoring technique in alpaca and llama herds in the Southeastern United States, it was noted that camelids in the study could compensate for low PCV with little to no clinical signs until reaching life-threatening levels (Storey et al., 2017). Camelids are adapted to lower oxygen environments with uniquely shaped red blood cells and will shift the oxygen-

hemoglobin dissociation curve to the left more efficiently than other species (Storey et al., 2017). Another reason may be the prior low dose natural exposure of the study animals to parasites and therefore the study animals had preexisting immunity to *H. contortus* prior to the start of this study. Based on finding *E. macusaniensis*, these animals did have exposure to environmental parasites. Very low levels of parasites, < 5 EPG, may not have been detected using just the Modified Stoll's since it represents 0.2 grams of feces on the slide as compared to a traditional flotation using 2 grams within centrifuge flotation. While their exposure was limited and fecal examination indicated limited egg counts, potential immunologic responses due to exposure to other nematodes (such as *Nematodirus* and *Trichuris*) may have altered susceptibility. In the Casey study (unpublished, 2014) comparing the infectivity of *H. contortus* in alpacas and rams, the age ranges for the alpacas were 3–16 years of age, whereas the rams were 3–4 years old. The alpacas received 20,000–50,000 *H. contortus* L3, maintained low but detectable EPGs and very low worm recovery at autopsy. In contrast, the rams were susceptible and achieved significantly higher EPG and worm recoveries as compared to the alpacas.

Overall, the vaccine did not harm the alpacas, yet we cannot definitively state if it provides the level of protection against *H. contortus* as reported with goats or sheep. Since alpacas appear to be less susceptible to *H. contortus*, additional studies assessing the ability of the vaccine to reduce worm and egg burdens in naturally infected camelids would provide more information on the ability of this vaccine to reduce losses associated with this parasite.

References

- Albers G.A.A, Gray G.D., LeJambre L.F., Barger I.A., 1990. The effect of *Haemonchus contortus* infection on hematological parameters in young Merino sheep and its significance for productivity. *An. Sci.* 50(1), 99-109.
- Bassetto, C.C., Silva, B.F., Newlands, G.F., Smith, W.D., Amarante, A.F., 2011. Protection of calves against *Haemonchus placei* and *Haemonchus contortus* after immunization with gut membrane proteins from *H. contortus*. *Parasite Immunol.* 33, 377–381.
- Bassetto, C.C., Picharillo, M.E., Newlands, G.F., Smith, W.D., Fernandes, S., Siqueira, E.R., Amarante, A.F., 2014. Attempts to vaccinate ewes and their lambs against natural infection with *Haemonchus contortus* in a tropical environment. *Int. J. Parasitol.* 44, 1049–1054.
- Bekelaar K., Waghorn T., Tavendale M., McKenzie C., Leathwick D., 2018. Heat shock, but not temperature, is a biological trigger for exsheathment of third-stage larvae of *Haemonchus contortus*. *Parasitol. Res.* 117(8), 2395-2402.
- Bentancor, A.B., Halperin, P., Flores, M., Iribarren, F., 2009. Antibody response to the epsilon toxin of *Clostridium perfringens* following vaccination of *Lama glama* crias. *J. Infect. Dev. Ctries.* 3, 624–627.
- Besier, B., Lyon, J., Newlands, G., Smith, D., 2012. Towards a commercial vaccine against *H. contortus*- a field trial in Western Australia. *Australian Sheep Veterinarians.* pp. 14–18.
- Besier, R.B., Kahn, L.P., Sargison, N.D., Van Wyk, J.A., 2016. The pathophysiology, ecology and epidemiology of *Haemonchus contortus* infection in small ruminants. *Adv. Parasitol.* 93, 95–143.

- Burke J.M., Miller J.E., Olcott D.D., Olcott B.M., Terrill T.M., 2004. Effect of copper oxide wire particles dosage and feed supplement level on *Haemonchus contortus* infection in lambs. Vet. Parasitol. 123, 235-243.
- Caffrey C.R. (Ed.), 2012. Parasitic Helminths: Targets, screens, drugs and vaccines volume 3. Wiley-VCH Verlag GmbH and Co. KGaA, pp 233-249.
- Capitini L.A., McClure K.E., Herd R.P., 1990. Effect of environmental stimuli on pre-infective and infective stages of *Haemonchus contortus* in the northern United States for the induction of hypobiosis. Vet. Parasitol. 35, 281-293.
- Cebra, C., 2014a. Physical exam and conformation. In: Cebra, C. (Ed.), Llama and Alpaca Care: Medicine, Surgery, Reproduction, Nutrition, and Herd Health. Elsevier, St. Louis, pp. 328–337.
- Cebra, C., 2014b. Endoparasite control. In: Cebra, C. (Ed.), Llama and Alpaca Care: Medicine, Surgery, Reproduction, Nutrition, and Herd Health. Elsevier, St. Louis, pp. 12–16.
- Daley L.P., Gagliardo L.F., Duffy M.S., Smith M.C., Appleton, J.A., 2005. Application of monoclonal antibodies in functional and comparative investigations of heavy-chain immunoglobulins in new world camelids. Clin. Diagn. Lab. Immunol. 12(3), 380-386.
- Daley, L., Kutzler, M., Bennett, B., Smith, M., Glaser, A., Appleton, J., 2010. Effector functions of camelids heavy-chain antibodies in immunity to West Nile virus. Clin. Vaccine Immunol. 17, 239–246.
- Dash K.M., 1985. Distribution of trichostrongylid nematodes in the abomasum of sheep. Int. J. Parasitol. 15, 505-510.

- De Matos, A., Nobre, C., Monteiro, J., Bevilaqua, C., Smith, D., Teixeira, M., 2017. Attempts to control *Haemonchus contortus* in dairy goats with Barbervax®, a vaccine derived from the nematode gut membrane glycoproteins. *Small Rumin Res.* 151, 1–4.
- Doyle S.R., Illingworth C.J.R., Laing R., Bartley D.J., Redman E., Martinelli A., Holroyd N., Morrison A.A., Rezansoff A., Tracey A., Devaney E., Berriman M., Sargison N., Cotton J.A., Gilleard J.S., 2019. Population genomic and evolutionary modelling analyses reveal a single major QTL for ivermectin drug resistance in the parasitic nematode, *Haemonchus contortus*. *BMC Genomics* 20(1), 218.
- El-Ashram S., Al Nasar I., Abouhajer F., El-Kemary M., Huang G., Dincel G., Mehmood R., Hu M., Suo X., 2017. Microbial community and ovine host response varies with early and late stages of *Haemonchus contortus* infection. *Vet. Res. Commun.* 41(4), 263-277.
- Flajnik M.F., Deschacht N., Muyldermans S., 2011. A case of convergence: why did a simple alternative to canonical antibodies arise in sharks and camels? *PLoS Biol.* 9(8), e1001120.
- Fowler, M.E., 2010. *Medicine and Surgery of Camelids*, 3rd edition. Blackwell Publishing, Ames, Iowa.
- Garner-Paulin, E., 2007. Worms and drench resistance. *Alpacas Australia* 52, 18–21.
- Garza J.J., Greiner S.P., Bowdridge S.A., 2017. Serum-mediated *Haemonchus contortus* larval aggregation differs by larval stage and is enhanced by complement. *Parasite Immunol.* 39(3).
- Gibbs, H.C., 1986. Hypobiosis and the periparturient rise in sheep. *Vet. Clin. North. Am. Food Anim Pract.* 2, 345–353.

- Gilleard J.S., 2013. *Haemonchus contortus* as a paradigm and model to study anthelmintic drug resistance. *Parasitology* 140(12), 1506-1522.
- Gutierrez-Amezquita R.A., Morales-Montor J., Munoz-Guzman M.A., Nava-Castro K.E., Ramirez-Alvarez H., Cuenca-Verde C., Moreno-Mendoza N.A., Cuellar-Ordaz J.A., Alba-Hurtado, F., 2017. Progesterone inhibits the *in vitro* L3/L4 molting process in *Haemonchus contortus*. *Vet. Parasitol.* 248, 48-53.
- Hall, C.A., Oddy, V.H., 1984. Effect of cimetidine on abomasal pH and *Haemonchus* and *Ostertagia* species in sheep. *Res. Vet. Sci.* 36, 316–319.
- Howell, S.B., Burke, J.M., Miller, J.E., Terrill, T.H., Valencia, E., Williams, M.J., Williamson, L.H., Zajac, A.M., Kaplan, R.M., 2008. Prevalence of anthelmintic resistance on sheep and goat farms in the southeastern United States. *J. Am. Vet. Med. Assoc.* 233, 1913–1919.
- Jabbar, A., Campbell, A.J., Charles, J.A., Gasser, R.B., 2013. First report of anthelmintic resistance in *Haemonchus contortus* in alpacas in Australia. *Parasit. Vectors* 6, 243.
- Jenkins E.J., Veitch A.M., Kutz S.J., Hoberg E.P., Polley L. Climate change and the epidemiology of protostrongylid nematodes in northern ecosystems: *Parelaphostrongylus odocoilei* and *Protostrongylus stilesi* in Dall's sheep (*Ovis d. dalli*). *Parasitology* 132(3), 387-401.
- Jurgens K.D., Pietschmann M., Yamaguchi K., Kleinschmidt T, 1988. Oxygen binding properties, capillary densities and heart weights in high altitude camelids. *J. Comp. Physiol. B.* 158(4), 469-477.
- Khan F.A., Sahoo A., Karim S.A., 2017. Moderate and high levels of dietary protein on clinico-biochemical and production responses of lambs to repeated *Haemonchus contortus* infection. *Small Rumin. Res.* 150, 52-59.

- Klongsiriwet C., Quijada J., Williams A.R., Mueller-Harvey I., Williamson E.M., Hoste H., 2015. Synergistic inhibition of *Haemonchus contortus* exsheathment by flavanoid monomers and condensed tannins. *Int. J. Parasitol.* 5, 127-134.
- Laing, R., Bartley D.J., Morrison A.A., Rezansoff A., Martinelli A., Laing S.T., Gilleard J.S., 2015. The cytochrome P450 family in the parasitic nematode *Haemonchus contortus*. *Int. J. Parasitol.* 45(4), 243-251.
- Leguia, G., 1991. The epidemiology and economic impact of llama parasites. *Parasitol. Today* 7, 54–56.
- LeJambre, L.F., Windon, R.G., Smith, W.D., 2008. Vaccination against *Haemonchus contortus*: performance of native parasite gut membrane glycoproteins in Merino lambs grazing contaminated pasture. *Vet. Parasitol.* 153, 302–312.
- Levine N.D., 1963. Weather, climate and the bionomics of ruminant nematode larvae. *Adv. Vet. Sci.* 8, 215-261.
- Martin P.J., Le Jambre L.F., Claxton J.H., 1981. The impact of refugia on the development of thiabendazole resistance in *Haemonchus contortus*. *Int. J. Parasitol.* 11, 35-41.
- McKellar, Q., Duncan, J.L., Armour, J.P.M., 1986. Response to transplanted *Ostertagia ostertagi* in calves. *Res. Vet. Sci.* 40, 367–371.
- McRae K.M., Stear M.J., Good B., Keane O.M., 2015. The host immune response to gastrointestinal nematode infection in sheep. *Parasite Immunol.* 37(12), 605-613.

- Meier, L., Torgerson, P.R., Hertzberg, H., 2016. Vaccination of goats against *Haemonchus contortus* with the gut membrane proteins H11/H-gal-GP. *Vet. Parasitol.* 229, 15–21.
- Montaner S., Galiano A., Trelis M., Martin-Jaular L., Del Portillo H.A., Bernal D., Marcillo A., 2014. The role of extracellular vesicles in modulation of the host immune response during parasitic infections. *Front. Immunol.* 8(5), 433.
- Morgan E.R., Wall R., 2009. Climate change and parasitic disease: farmer mitigation? *Trends Parasitol.* 25(7), 308-313.
- Muchiut S.M., Fernandez A.S., Steffan P.E., Riva E., Fiel C.A., 2018. Anthelmintic resistance: Management of parasite refugia for *Haemonchus contortus* through the replacement of resistant with susceptible populations. *Vet. Parasitol.* 254, 43-48.
- Muyldermans, S., Baral, T.N., Retamozzo, V.C., De Baetselier, P., De Genst, E., Kinne, J., Leonhardt, H., Magez, S., Nguyen, V.K., Revets, H., Rothbauer, U., Stijlemans, B., Tillib, S., Wernery, U., Wyns, L., Hassanzadeh-Ghassabeh, G., Saerens, D., 2009. Camelid immunoglobulins and nanobody technology. *Vet. Immunol. Immunopathol.* 128, 178–183.
- Nicholls, C.D., Hayes, P.R., Lee, D.L., 1987. Physiological and microbiological changes in the abomasum of sheep infected with large doses of *Haemonchus contortus*. *J. Comp. Pathol.* 97, 299–308.
- O'Connor L.J., Walkden-Brown S.W., Kahn L.P., 2006. Ecology of the free-living stages of major trichostrongylid parasites of sheep. *Vet. Parasitol.* 142, 1-15.
- Palmer D.G., McCombe I.L., 1996. Lectin staining of trichostrongylid nematode eggs of sheep: rapid identification of *Haemonchus contortus* eggs with peanut agglutinin. *Int. J. Parasitol.* 26(4), 447-450.

- Robinson, T.F., Harris, B.W., Johnston, N.P., 2013. Initial compartment 1 pH response to grain supplementation in alpacas (*Vicugna pacos*) fed alfalfa and grass hay. *J. Anim. Sci. Adv.* 3, 354–360.
- Rose H., Caminade C., Bolajoko M.B., Phelan P., van Dijk J., Baylis M., Williams D., Morgan E.R., 2016. Climate-driven change in the spatio-temporal distribution of the parasitic nematode, *Haemonchus contortus*, in sheep in Europe. *Glob. Chang. Biol.* 22(3), 1271-1285.
- Sangster, N.C., 1998. Anthelmintic resistance: Past, present and future. *Int. J. Parasitol.* 29(1), 137-138.
- Sargeant, J.M., O'Connor, A.M., Gardner, I.A., Dickson, J.S., Torrence, M.E., Consensus Meeting Participants, Dohoo, I.R., Lefebvre, S.L., Morley, P.S., Ramirez, A., Snedeker, K., 2010. *J. Food Protect.* 73, 579–603.
- Shepherd E.A., Garza J.J., Greiner S.P., Bowdridge S.A., 2016. The effect of ovine peripheral blood mononuclear cells on *Haemonchus contortus* larval morbidity in vitro. *Parasite Immunol.* 39(4).
- Sinnathamby G., Henderson G., Umair S., Janssen P., Bland R., Simpson H., 2018. The bacterial community associated with the sheep gastrointestinal nematode parasite *Haemonchus contortus*. *PLoS One.* 13(2), e0192164.
- Smith, W.D., Zarlenga, D.S., 2006. Developments and hurdles in generating vaccines for controlling helminth parasites of grazing ruminants. *Vet. Parasitol.* 139, 347–359.
- Smith, G.W., Davis, J.L., Smith, S.M., Gerard, M.P., Campbell, N.B., Foster, D.M., 2010. Efficacy and pharmacokinetics of pantoprazole in alpacas. *J. Vet. Intern. Med.* 24, 949–955.

- Soulsby E.J.L., 1965. Textbook of Veterinary Clinical Parasitology Volume 1: Helminths. Blackwell Scientific Publications, Great Britain, 1120 pp.
- Storey, B.E., Williamson, L.H., Howell, S.B., Terrill, T.H., Berghaus, R., Vidyashankar, A.N., Kaplan, R.M., 2017. Validation of the FAMACHA system in South American camelids. Vet. Parasitol. 243, 85–91.
- Sutherland, I., Scott, I., 2010. Pathophysiology of nematode infections. Gastrointestinal nematodes of Sheep and Cattle. Wiley-Blackwell, West Sussex, UK.
- Terrill T.H., Larsen M., Samples O., Husted S., Miller J.E., Kaplan R.M., Gelaye S., 2004. Capability of the nematode-trapping fungus *Duddingtonia flagrans* to reduce infective larvae of gastrointestinal nematodes in goat feces in southeastern United States: dose titration and dose time interval studies. Vet Parasitol. 120, 285-296.
- Thienpont D., Rochette F., Vanparijs O.F.J., 1981. Diagnosing helminthiasis through coprological examination. Vet. Parasitol. 8, 341-342.
- Van den Brom, R., Moll, L., Kappert, C., Vellema, P., 2015. *Haemonchus contortus* resistance to monepantel in sheep. Vet. Parasitol. 209, 278–280.
- van Wyk J.A., Barth G.F., 2002. The FAMACHA© system for managing haemonchosis in sheep and goats by clinically identifying individual animals for treatment. Vet. Res. 33, 509-529.
- Vidyashankar, A.N., Hanlon, B.M., Kaplan, R.M., 2012. Statistical and biological considerations in evaluating drug efficacy in equine strongyle parasites using fecal egg count data. Vet. Parasitol. 185, 45–56.
- Wang T., van Wyk J.A., Morrison A., Morgan E.R., 2014. Moisture requirements for the migration of *Haemonchus contortus* third stage larvae out of faeces. Vet. Parasitol. 204, 258-264.

- Wood I.B., Amaral N.K., Bairden K., Duncan J.L., Kassai T., Malone J.B., Pankavich J.A., Reinecke R.K., Slocombe O., Taylor S.M., Vercruysse J., 1995. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) second edition of guidelines for evaluating the efficacy of anthelmintics in ruminants (bovine, ovine, caprine). *Vet. Parasitol.* 58, 181-213.
- Woodgate R.G., Cornell A.J., Sangster N.C., 2017. Occurrence, measurement and clinical perspectives of drug resistance in important parasitic helminths of livestock. Springer, Cham, pp 1305-136.
- Yamaguchi K., Jurgens K.D., Bartels H., Piiper J, 1987. Oxygen transfer properties and dimensions of red blood cells in high-altitude camelids, dromedary camel and goat. *J. Comp. Physiol. B.* 157(1), 1-9.
- Zajac A.M., 2006. Gastrointestinal nematodes of Small Ruminants: life cycle, anthelmintics, and diagnosis. *Vet. Clin. Food Anim.* 22, 529-541.
- Zajac, A.M., Conboy, G.A., 2012. *Veterinary Clinical Parasitology*, 8th edition. Wiley Blackwell, Ames, Iowa.
- Zanolari, P., Bruckner, L., Fricker, R., Kaufmann, C., Mudry, M., Griot, C., Meylan, M., 2010. Humoral responses to 2 inactivated Bluetongue virus serotype-8 vaccines in South American Camelids. *J. Vet. Intern. Med.* 24, 956–959.

Appendix A: Tables and Figures

Table 1

Parameters and fecal flotation results of vaccinate alpacas 1-7 and control alpacas 8-12.

Alpaca	Sex	Age ^a	Gain kg ^b	21 DPI ^c	42 DPI	60 DPI	114 DPI	Other Parasites ^d
1	M	171	3.99	0	0	0	5	Few E ^e , few N ^f , consistent M ^g
2	M	168	4.35	0	0	0	0	Few T ^h
3	M	148	3.71	0	0	0	0	Few E
4	F	149	6.80	0	0	0	0	Few E
5	F	167	10.43	5	0	0	5	Few T, consistent E, consistent M
6	F	150	8.62	0	0	0	0	Few T
7	F	141	8.16	0	10	5	0	Few T
8	M	165	6.53	0	0	0	0	Few M, few T, consistent E
9	M	160	5.71	0	0	0	30	Few E, few M, few T
10	M	144	7.89	0	0	0	0	Few E, few N, few T
11	M	194	6.53	0	0	0	0	Few E, few M
12	F	140	8.43	0	0	0	10	Few E

^aDay 0, first immunization, age in days.^bWeight gain from Day 0 to end of study.^cDays post-inoculation after last dose of larvae.^dFew = 1-3, Occasional = 4-10, Consistent = >10 parasites detected on different fecal examination during the study.^e*Eimeria macusaniensis*^f*Nematodirus*^g*Moniezia*^h*Trichuris*

Table 2.

P-values for Wilcoxon signed rank tests of significance. Each tests the hypothesis that the titers are different between days.

	Day 28	Day 49	Day 56	Day 91
Day 28	---	---	---	---
Day 49	0.0156	---	---	---
Day 56	0.0156	0.0156	---	---
Day 91	0.0626	0.0156	0.0156	---

Figure 1.

Alpaca experiment timeline. Alpacas were monitored for evidence of serum antibody detection by obtaining blood samples at various time points during the course of the study (■). For alpacas that received the vaccine (n=7), the vaccine dose was re-administered 21 days, 42 days, and 114 days (▲) after initial vaccination on day 0 (●). All alpacas were inoculated via nasogastric route with viable third stage larvae (L3) obtained from an experimentally infected *Haemonchus contortus* goat on days 42, 46, and 50 (*). Blood samples occurred every week and fecal sampling biweekly for all alpacas following the final larval inoculation until day 147 of the study.

Figure 2.

Experimental design flow chart.

Figure 3.

Mean fecal egg counts in vaccinates and controls. Vaccinates received Barbervax® on Day 0, 21, 42, and 114. Controls remained unvaccinated. All alpacas received 1500 viable third stage larvae (L3) of *H. contortus* via orogastric tube on Days 42, 46, and 50.

Figure 4.

Antibody titer levels against H11/H-gal-GP in vaccinate alpacas. Alpacas were vaccinated on Day 0, 21, and 42.